BIO-MODULATORY PROPERTIES OF:
(i) C-PHYCOCYANIN, A BILIPROTEIN FROM *SPIRULINA PLATENSIS*
(ii) NOVEL ANALOGUES OF URIC ACID

A Thesis

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By

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To

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Dedicated to
My Parents

CONTENTS

DECLARATION

I hereby declare that the entire work embodied in this thesis, entitled "**Bio-modulatory properties of (i) C-phycocyanin, a biliprotein from Spirulina platensis, and (ii) novel analogues of uric acid**" is the result of the investigations carried out by me at the Chemical Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore-560 064, India, under the supervision of Prof. K. M. Madyastha and that it has not been submitted elsewhere for the award of degree, diploma, associateship etc.

In keeping with the general practice in reporting scientific observations, due acknowledgements are made wherever the work described is based on the findings of other investigators.

Vadiraja Bhat

[VADIRAJA BHAT B.]

CERTIFICATE

I hereby certify that the entire work embodied in this thesis entitled "**Bio-modulatory properties of (i) C-phycocyanin, a biliprotein from *Spirulina platensis* and (ii) novel analogues of uric acid**" has been carried out by **Mr. Vadiraja Bhat B.** at Chemical Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore-560 064, India, under my supervision and that it has not been submitted elsewhere, for the award of degree, diploma, associateship, etc.

K. M. Madyastha.

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Research Supervisor

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Vadiraja Bhat B.

LIST OF ABBREVIATIONS

AAPH	: 2,2'-azobis(2-amidinopropane) hydrochloride
ADP	: Adenosine diphosphate
ALT	: Alanine transaminase
BHT	: Butylated hydroxytoluene
BSA	: Bovine Serum Albumin
BR	: Bilirubin
BV	: Biliverdin
CAT	: Catalase
$CDCl_3$	: Deuterated Chloroform
CPCs	: C-Phycocyanins
CPC_SP	: C-Phycocyanin _ *Spirulina platensis*
COX	: cyclooxygenase
d	: doublet
dd	: doublet of a doublet
D_2O	: Deutereated water
DCPIP	: Dichlorophenol indophenol
EDTA	: Ethylenediaminetetraacetic acid (disodium salt)
Fig.	: Figure
h	: Hour
hr(s)	: Hour(s)
HRMS	: High resolution mass spectrum
ESI-MS	: Electrospray ionization Mass Spectrum
FPLC	: Fast Performance Liquid Chromatography
GC	: Gas Chromatography
GPx	: Glutathione Peroxidase
GSH	: Glutathione
GSSG	: Glutathione (oxidized)
HPLC	: High Performance Liquid Chromatography
HRMS	: High Resolution Mass Spectrum
i.p	: Intra peritoneal
IR	: Infrared
J	: Coupling constant
kDa	: kilo Dalton
LDL	: Low Density Lipoprotein
M	: molar
m	: multiplet
MALDI-TOF	: Matrix Assisted Laser Desorption / Ionization-Time Of Flight
MDA	: Malondialdehyde
MHz	: Mega Hertz
min	: minute(s)
mol	: mole(s)

mM	: millimolar
µM	: micromolar
MP	: Melting point
MS	: Mass Spectrum
NAD^+	: Nicotinamide Adenine Dinucleotide (oxidized form)
NADH	: Nicotinamide Adenine Dinucleotide (reduced form)
$NADP^+$	: Nicotinamide Adenine Dinucleotide Phosphate (oxidized form)
NADPH	: Nicotinamide Adenine Dinucleotide Phosphate (Reduced form)
NBT	: Nitroblue tetrazolium
NEM	: N-ethylmaleimide
NMM	: N-methylmaleimide
NMR	: Nuclear magnetic resonance
NSAID	: Non-steroidal anti-inflammatory drugs
PAGE	: Polyacrylamide gel electrophoresis
PC	: Phosphotidyl choline
PCB	: Phycocyanobilin
PES	: Phenazine ethosulfate
PG	: Prostaglandin
PMS	: Phenazine methosulfate
PR	: Pyrogallol Red
PUFA	: Poly unsaturated fatty acid
q	: quartet
R_t	: Retention time
RNS	: Reactive nitrogen species
rpm	: Revolutions per minute
ROS	: reactive oxygen species
s	: singlet
SDS	: Sodium dodecyl sulfate
SGPT	: Serum glutamate pyruvate transaminase
SOD	: Superoxide dismutase
t	: triplet
TBA	: Thiobarbituric acid
TBARS	: Thiobarbituric acid reactive substance
TEMED	: N,N,N',N'-Tetramethylethylenediamine
Tris	: Tris(hydroxymethyl)aminomethane
TLC	: Thin Layer Chromatography
UV	: Ultraviolet

Synopsis of the Thesis

Bio-modulatory properties of:
(i) C-phycocyanin, a biliprotein from *Spirulina platensis*, a blue-green algae
(ii) Novel analogues of uric acid

Spirulina platensis, a blue-green algae is very well known for its nutritional and therapeutic properties. This algae has preventive effect on the fatty liver induced by a fructose-rich diet in the rat. C-phycocyanin, a water soluble protein pigment is one of the major constituents of *Spirulina platensis*. The chromophore of phycocyanin is phycocyanobilin (PCB), a linear tetrapyrrole which is structurally similar to the natural antioxidant bilirubin, a heme degradation product. Although anti-cancer and anti-inflammatory properties of phycocyanin have been reported, its mechanism of action is not clearly understood. In the present study we have made an attempt to understand the biochemical basis for some of the observed pharmacological properties of phycocyanin.

C-phycocyanin was isolated from fresh and spray dried spirulina, purified to homogeneity ($A_{max}/A_{280} > 4.0$) and fully characterized (ESI-MS, 37,468 Da, monomer, $\alpha\beta$ subunits). Detailed studies on the effect of phycocyanin on CCl_4 and R-(+)-pulegone-induced hepatotoxicity in rats were carried out. These studies have established: (a) phycocyanin significantly prevents CCl_4 and R-(+)-pulegone-mediated hepatotoxicity in rats, (b) phycocyanin inhibits the formation of reactive metabolites from R-(+)-pulegone responsible for the observed hepatotoxicity, (c) phycocyanin effectively inhibits CCl_4-induced lipid peroxidation in rat liver *in vivo*.

We have also studied the radical scavenging property of phycocyanin both in vivo and in vitro. Native and reduced phycocyanin significantly inhibits peroxyl radical-induced lipid peroxidation in rat liver microsomes with an IC_{50} of 11.35 and 12.7 μM, respectively. The radical scavenging property of phycocyanin was established by studying its reactivity with peroxyl and hydroxyl radicals and also by competition kinetics of crocin bleaching. Our studies suggest that the chromophore, phycocyanobilin (PCB) is involved in the antioxidant and radical scavenging activity and provides an explanation for the anti-inflammatory property of phycocyanin. We have also demonstrated that phycocyanin is a selective inhibitor of cyclooxygenase-2 activity (COX-2) with potency comparable to celecoxib and rofecoxib, the known selective COX-2 inhibitors. Phycocyanin also has the

ability to efficiently scavenge peroxynitrite ($ONNO^-$), a potent physiological toxin and its chromophore, PCB significantly inhibits the $ONOO^-$-mediated single strand breaks in supercoiled plasmid DNA in a dose dependent manner with an IC_{50} value of 2.9 ± 0.6 μM. This suggests that phycocyanin inhibits the ONOO-mediated deleterious biological effects. Studies have also been carried out on the antioxidant, radical scavenging and iron chelating properties of PCB. Our studies suggest that phycocyanin has the potential to be used as a therapeutic agent.

The second part of the thesis deals with the preparation and bio-modulatory properties of 8-oxo derivatives of various xanthine analogues and xanthine drugs. We have isolated a bacterial consortium consisting of strains belonging to the genus Klebsiella and Rhodococcus which quantitatively converts various analogues of 1,3,7-trimethylxanthine with N-1 methyl groups replaced by alkyl, hydroxethyl, benzyl, 2-oxopropyl, allyl, propargyl and butenyl groups to their corresponding 8-oxo compounds. The enzyme responsible for this novel transformation, caffeine oxidase has been purified to homogeneity. Many of the uric acids prepared by this method are hitherto not known. These uric acids were used as test compounds to evaluate their protective potential against lipid peroxidation and ability to scavenge oxygen free radicals. These properties are compared with that observed from known methyluric acids and thus these studies provide information on structure-activity relationship. Our studies clearly indicated that 8-oxopentoxifylline and 8-oxolisofylline are significantly better hydroxyl, peroxyl and superoxide radical scavengers and more potent inhibitors of lipid peroxidation than the parent drugs. These results indicated that anti-inflammatory property of pentoxifylline and lisofylline is exerted more through their 8-oxo derivatives than the parent drugs. We have also demonstrated that the 8-oxo derivatives of pentoxifylline and lisofylline specifically inhibit rabbit reticulocyte 15-lipoxygenase whereas the parent drugs failed to do that suggesting that the anti-atherogenic property of pentoxifylline and lisofylline is exerted through their 8-oxo derivatives. All these studies will be discussed in detail.

LIST OF PUBLICATIONS

1. **Bhat B. Vadiraja**, Nilesh. W. Gaikwad, and K. M. Madyastha. Hepatoprotective Effect of C-Phycocyanin: Protection for Carbon Tetrachloride and R-(+)-Pulegone-Mediated Hepatotoxicty in Rats. *Biochemical And Biophysical Research Communications* **249,** 428–431 (1998)

2. K. M. Madyastha, G. R. Sridhar, **Bhat B. Vadiraja**, and Y. Sudha Madhavi. Purification and Partial Characterization of Caffeine Oxidase—A Novel Enzyme from a Mixed Culture Consortium. *Biochemical and Biophysical Research Communications* **263,** 460–464 (1999)

3. **Vadiraja B. Bhat** and K. M. Madyastha. C-phycocyanin: a potent peroxyl radical scavenger *in vivo* and *in vitro*. *Biochemical and Biophysical Research Communications* **275,** 20–25 (2000).

4. C. Madhava Reddy, **Vadiraja B. Bhat**, G. Kiranmai, M. Narsa Reddy, P. Reddanna, and K. M. Madyastha. Selective inhibition of cyclooxygenase-2 by C-phycocyanin, a biliprotein from *Spirulina platensis*. *Biochemical and Biophysical Research Communications* **277**, 599-603 (2000)

5. Anil K. Padyana, **Vadiraja B. Bhat**, K. M. Madyastha, K. R. Rajashankar, and S. Ramakumar. Crystal Structure of a Light-Harvesting Protein C-Phycocyanin from *Spirulina platensis*. *Biochemical and Biophysical Research Communications* 282, 893–898 (2001).

6. **Vadiraja B. Bhat**, G. R. Sridhar, and K. M. Madyastha. Efficient Scavenging of Hydroxyl Radicals and Inhibition of Lipid Peroxidation by Novel Analogues of 1,3,7-Trimethyluric acid. *Life Sciences* 2001 (In Press)

7. **Vadiraja B. Bhat**, and K. M. Madyastha. Scavenging of Peroxynitrite by Phycocyanin and Phycocyanobilin from *Spirulina platensis:* Protection against Oxidative Damage to DNA. *Biochemical and Biophysical Research Communications* 285, 262-266 (2001).

8. **Vadiraja B. Bhat**, and K. M. Madyastha. Antioxidant and radical scavenging properties of 8-oxo derivatives of xanthine drugs, Pentoxifylline and Lisofylline. 2001. (Submitted for publication).

9. **Vadiraja B. Bhat**, and K. M. Madyastha. Antioxidant, radical scavenging and iron chelating properties of Phycocyanobilin from *Spirulina platensis*. 2001. (Submitted for publication).

10. **Vadiraja B. Bhat**, C. Madhava Reddy, P. Reddanna, and K. M. Madyastha. Selective inhibition of Reticulocyte 15-Lipoxygenase by 8-Oxo derivatives of Xanthine Drugs, Pentoxifylline and Lisofylline. (Manuscript under preparation).

CHAPTER 1

General Introduction

Oxygen is an essential element for aerobes, as it is the terminal acceptor of electrons during respiration, which is the main source of energy in these organisms. However, oxygen is toxic when supplied at concentrations greater than those in air. Some experiments show harmful effects of this element even at a concentration of 21% (1-3). It was initially suggested that oxygen inactivated cellular enzymes. However, *in vitro* experiments showed that enzyme inactivation by oxygen was too slow to account for the rate at which its toxic effects developed. In 1954, Gershman and Gilbert proposed that, known harmful effects of oxygen were due to the formation of free radicals derived from it (4) and in 1956, Denham Harman suggested that free radicals produced during aerobic respiration cause cumulative oxidative damage, resulting in aging and death. He noted parallels between the effects of aging and of ionizing radiation, including mutagenesis, cancer, and gross cellular damage (5). At the same time, it had been discovered that radiolysis of water generates hydroxyl radical ($^{\bullet}OH$) (6), and early experiments using paramagnetic resonance spectroscopy had identified the presence of $^{\bullet}OH$ in living matter (7). Harman (5) therefore hypothesized that endogenous oxygen radical generation occurs *in vivo* as a by-product of enzymatic redox chemistry. He ventured that the enzymes involved would be those "involved in the direct utilization of molecular oxygen, particularly those containing iron." Finally, he hypothesized that traces of iron and other metals would catalyze oxidative reactions *in vivo* and that peroxidative chain reactions were possible, by analogy to the principles of *in vitro* polymer chemistry. However, this idea was not completely accepted until the superoxide dismutase (SOD), an enzyme that catalyzes the superoxide radical dismutation to hydrogen peroxide, was discovered in 1969 (8-10) which provided the first compelling evidence for *in vivo* generation of superoxide

$$O_2^{\bullet-} + O_2^{\bullet-} 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

anion ($O_2^{\bullet-}$). By using SOD as a tool to locate subcellular sites of $O_2^{\bullet-}$ generation, it was found that mitochondria are a principal source of endogenous oxidants ($O_2^{\bullet-}$) (10a). Since then, the pioneering work of McCord and Fridovich has captured the interest of many researchers investigating the role of oxygen free radicals in different pathologies.

Characteristics of Free Radicals

Free radicals are chemical species that have one or more unpaired electrons; the terms radical and free radical can be used interchangeably. Free radicals are extremely

Table 1: Examples of Free Radicals

Name	Formula	Comments/examples
Hydrogen atom	$H^{\bullet}$	The simplest free radical
Trichloromethyl	$CCl_3^{\bullet}$	A carbon-centred radical (i.e. the unpaired electron resides on carbon). $CCl_3^{\bullet}$ is formed during metabolism of CCl_4 in the liver and contributes to the toxic effects of this solvent. Carbon radicals usually react rapidly with O_2 to make peroxyl radicals e.g. $CCl_3^{\bullet} + O_2 \rightarrow CCl_3O_2^{\bullet}$
Superoxide	$O_2^{\bullet-}$	An oxygen-centred radical
Hydroxyl	$OH^{\bullet}$	A highly reactive oxygen-centred radical; attacks all biomolecules
Thiyl/perthiyl	$RS^{\bullet}$/$RSS^{\bullet}$	A group of radicals that have unpaired electrons residing on sulphur
Peroxyl,alkoxyl	$RO_2^{\bullet}$, $RO^{\bullet}$	Oxygen-centred radicals formed (among other routes) during the breakdown of organic peroxides and reaction of carbon radicals with O_2 ($RO_2^{\bullet}$)
Oxides of nitrogen	$NO^{\bullet}$, $NO_2^{\bullet}$	Nitric oxide is formed *in vivo* from the amino acid L-arginine; nitrogen dioxide is made when $NO^{\bullet}$ reacts with O2; both are found in polluted air and smoke from burning organic materials; e.g. cigarette smoke
Nitrogen-centred radicals	$C_6H_5N{=}N^{\bullet}$	Formed during oxidation of phenylhydrazine by erythrocytes; e.g. phenyldiazine radical
Transition-metal ions	Fe, Cu, etc	Ability to change oxidation numbers by one allows them to accept/donate single electrons; hence they are often powerful catalysts of free-radical reactions

reactive, with a very short half-life and low steady-state concentration (11-13). **Table 1** gives some examples of free radicals. Free radicals can be mono-atomic such as the halogen atoms Cl• or Br•, alkali metal atoms such as Na· or K· or transition metal atoms such as Cu^{2+} or Ce^{3+} whose reactions indicate that they have radical character. Larger groupings of inorganic atoms yield more complex radicals, for example, ClO•, $Cl_2^{\bullet-}$, or $O_2^{\bullet-}$, that are important in atmospheric chemistry and biochemistry. Some combinations of atoms are classified as radicals due to electron book-keeping that leaves a single electron in an orbital. For example, ˙NO, which may function as an endothelium-derived relaxing factor, and dioxygen (O_2) are radicals of this type. The electronic structures of these radicals are shown below. Dioxygen (molecular oxygen) is in fact a diradical, having one electron associated with each of the two oxygen atoms (one unpaired electron in each of its two π^* outer antibonding orbitals). However, due to the parallel directions of spin of these electrons, the reactivity of molecular oxygen is very low. Dioxygen is the terminal electron acceptor in the important free-radical process called respiration.

•N::O O::N::O •O::O•

Because of its radical nature, dioxygen is involved in many biologically important free-radical processes. Collections of atoms that carry a single unpaired electron are the familiar types of free radicals. Several examples are shown below.

$CH_3\bullet$ $\bullet CH_3CHOH$ $(CH_3)_3CO\bullet$ $(CH_3)_3COO\bullet$

The most common mechanisms for generating radicals are homolytic bond cleavage:

$$A - B \rightarrow A\bullet + B\bullet$$

and the transfer of an electron from one molecule to another:

$$A + B \rightarrow A^{+\bullet} + B^{-\bullet}$$

Since the extra electron in a free radical is not spin paired with a second electron in a chemical bond, free radicals are usually found to be extremely reactive, unless the extra electron is delocalized over several atoms. Therefore, one of the main characteristics of free radicals is that, they react readily with other molecules by several different mechanisms. The reactions between two radicals create a neutral species:

$$CH_3\bullet + CH_3\bullet \rightarrow CH_3CH_3$$

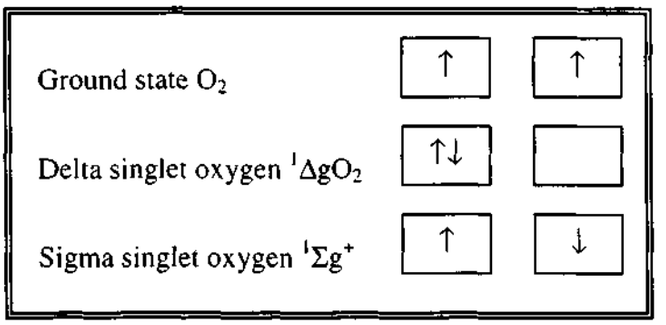


Fig. 1: Arrangement of electrons in π^* antibonding orbitals

However, the most common reactions that occur between radicals and neutral molecules are atom abstraction and addition:

$$ROO\bullet + RH_2 \rightarrow ROOH + RH\bullet \quad \text{Abstraction}$$

$$HO\bullet + \text{aromatic} \rightarrow \text{HO-aromatic}\bullet \quad \text{Addition}$$

Singlet Oxygen

Singlet oxygen ($^1O_2$) is not a true radical because it does not contain unpaired electrons. A way to increase the reactivity of oxygen is, to turn the two parallel spinning electrons into antiparallel by means of an input of energy. This process produces singlet oxygen, which is highly reactive because spin restriction has been removed. There are two forms of singlet oxygen: delta singlet oxygen ($^1\Delta gO_2$) and sigma singlet oxygen ($^1\Sigma g^+O_2$) (**Fig. 1**). The former is more important biologically due to its longer life (the outer two electrons occupy the same orbital and have opposite directions). Sigma singlet oxygen, on the other hand, has electrons of antiparallel spins occupying different orbitals. This species has very high reactivity but a short half-life because, it decays immediately after being formed to the delta singlet oxygen state (8,11,12). Singlet oxygen is highly reactive with membrane lipids to produce peroxides, but information about its role in tissue damage is limited (2).

Singlet oxygen is most often generated by photosensitization reactions (2). If certain molecules are illuminated with light of appropriate wavelength, they absorb it and the energy raises the molecule to an excited state. The excitation energy can then be transferred to an adjacent O_2 molecule, converting it to the singlet state whilst the photosensitizer molecule returns to its ground state. Popular sensitizers of singlet oxygen formation in the laboratory include the dye acridine orange, methylene blue, rose bengal and toluidine blue; but many biological compounds are also effective *in vitro*, such as riboflavin and its derivatives (FMN and FAD). Also effective are chlorophylls a and b, bilirubin, retinal and various porphyrins, both free and protein bound forms.

Several diseases can lead to excessive singlet O_2 formation (2). For example, the porphyrias are diseases caused by defects in the heme biosynthesis. In some of these diseases, porphyrins accumulate in the skin, exposure of which to light causes damage leading to unpleasant eruptions, scarring and thickening. The severity of the damage

depends upon which porphyrin is accumulated, and thus differs in different types of porphyria.

Superoxide Radical

The superoxide anion ($O_2^{\bullet-}$) is formed chemically by the addition of one electron to ground state dioxygen ($O_2 + e^- \rightarrow O_2^{\bullet-}$) (reduction of oxygen by one electron). This reaction may be accidental, when electrons leak from their carriers within the respiratory chain of mitochondria and pass directly on to oxygen (14,15). This very reactive chemical species is unstable in aqueous solutions due to its ability to react spontaneously with itself producing hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (dismutation reaction) (Eq. 1) (10,11). The overall rate of dismutation at pH 7.0 is about 5×10^5 M^{-1} sec^{-1}, and any other reaction involving $O_2^{\bullet-}$ in aqueous solution will be in competition with this dismutation reaction (16).

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \longrightarrow \quad \text{[Eq.1]}$$

In organic solvents, $O_2^{\bullet-}$ is a strong base and nucleophile: for example, it can displace Cl^- from such unreactive chlorinated hydrocarbons as CCl_4 (17). In aqueous solution, the superoxide anion can be in its protonated form as perhydroxyl radical ($HO_2^{\bullet}$), which exhibits even higher reactivity, acting as a reducing agent {e.g., it will reduce cytochrome c or nitroblue tetrazolium (NBT^{2+}) [yellow NBT^{2+} to blue colored formazan]} and as a weak oxidizing agent to such molecules as adrenaline and ascorbic acid. However, at physiological pH, the unprotonated form predominates (8,18). Ability to reduce cytochrome c and NBT^{2+} is used in assays of SOD activity (2).

$$\text{cyt c (Fe(III)} + O_2^{\bullet-} \rightarrow O_2 + \text{cyt c } (Fe^{+2})$$

However, certain other chemical properties of $O_2^{\bullet-}$ are important in a biological context. These include (1) its participation in the so-called Haber-Weiss reaction to generate ·OH, and in a closely related reaction to generate alkoxyl radical (RO·), the reactions being catalyzed by transition metals such as iron or copper (19,20):

$$O_2^{\bullet-} + Fe^{+3} \rightarrow O_2 + Fe^{+2}$$

$$Fe^{+2} + H_2O_2 \text{ (or ROOH)} \rightarrow Fe^{+3} + OH^- + \cdot OH \text{ (or RO}\cdot)$$

(2) Its ability to obtain Fe^{2+} needed for the Haber-Weiss reaction by liberating it from the iron storage protein ferritin and from iron-sulfur proteins such as aconitase (21);

(3) Its reaction with ·OH to form singlet oxygen (22):

$$O_2^{\bullet-} + \cdot OH \rightarrow {}^1O_2 + OH^-$$

and (4) Its reaction with nitric oxide to form peroxynitrite, a highly reactive oxidant that breaks down to produce a nitrating agent (23):

$$O_2^{\bullet-} + NO^{\bullet} \rightarrow OONO^- + H \rightarrow ? NO_2^+ + OH^-$$

In addition, the H_2O_2 produced by the dismutation of $O_2^{\bullet-}$ is used by phagocytes to oxidize halide ions to the level of hypohalous acids (e.g., HOCl) (24), a group of highly reactive compounds, which in turn react with amines to produce halamines (e.g., NH_2Cl), some of which are even more reactive than the hypohalous acids (25). In turn, the hypohalous acids can react with H_2O_2 to generate singlet oxygen (26). For example,

$$OBr^- + H_2O_2 \rightarrow {}^1O_2 + H_2O + Br^-$$

All this was of little interest to biologists, however, until 1969, when McCord and Fridovich (8) discovered superoxide dismutase. This enzyme destroys $O_2^{\bullet-}$ by catalyzing the dismutation reaction described above. The ubiquitous occurrence of an enzyme that catalyzes the destruction of $O_2^{\bullet-}$ implied that $O_2^{\bullet-}$ had to be participating in an important way in the biological economy (2).

Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is a pale-blue viscous liquid, widely regarded as a cytotoxic agent, whose levels must be minimized by the action of antioxidant defence enzymes. In fact, H_2O_2 is poorly reactive in the absence of transition metal ions. In chemical terms, H_2O_2 is poorly reactive: it can act as a mild oxidizing or reducing agent, but it does not oxidize most biological molecules readily, including lipids, DNA and proteins unless the latter have hyper-reactive thiol groups or methionine residues (2,27,28). Indeed, the weak antiseptic activity of honey, which has been used in wound treatment since ancient times, is partly due to the formation of H_2O_2 by enzymes present in it (29).

A system generating superoxide would be expected to produce H_2O_2 by non-enzymatic or SOD-catalyzed dismutation [Eq. (1)]. The reduction by two electrons of molecular oxygen produces the peroxide ion (O_2^{2-}), of which the protonated form is hydrogen peroxide. Hydrogen peroxide, although not a free radical by itself, is not especially toxic unless it is in high concentrations within the cells. However, it is very

harmful and hazardous to cells because it may cross biological membranes and can thereby distribute to sites distant from where it was generated. The danger of H_2O_2 largely comes from its ready conversion to the indiscriminately reactive hydroxyl radical ($OH^{\bullet}$), either by exposure to ultraviolet light (30) or by interaction with a range of transition metal ions, of which the most important one is probably iron *in vivo* (2,31).

$$Fe^{2+} + H_2O_2 \rightarrow \text{intermediate complexes (ferryl??)} \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

In the presence of transition metals, most often Fe^{+2} but also Cu^{+1}, H_2O_2 is reduced to highly reactive hydroxyl radical ($OH^{\bullet}$) via either the Haber-Weiss or Fenton reactions (13,31-34) and results in subsequent tissue damage. It has been proposed that hydrogen peroxide may act as a metabolic signal by oxidizing protein thiol groups and triggering intracellular events (2). An example would be the oxidation of an important nuclear regulatory protein NF-κB (nuclear factor κB), a process responsible for the transcription of several pro-inflammatory cytokines of importance to pathogenesis (35), including interleukin-2 (IL-2), IL-6, IL-8, β-interferon and TNF-α (36).

The ultimate fate of H_2O_2, however, is not always the $OH^{\bullet}$. In most cells, H_2O_2 is converted to innocuous products by the actions of two important antioxidative enzymes, catalase and selenium-dependent glutathione peroxidases (GPx). In the brain, the GPx is more important than catalase because of the low activity of the latter enzyme in most parts of the CNS (37). GPx utilizes H_2O_2 and hydroperoxides as substrates during the conversion of reduced glutathione (GSH) to its disulfide (GSSG) (38).

Hydroxyl Radical

The tri-electron reduction product of molecular oxygen is the hydroxyl radical. This is an extremely reactive chemical species, which can react with any biological molecule. As a result, its half-life is very short (its estimated half-life at 37° C is of the order of 1×10^{-9} sec) as is its radius of action (30 Å) and within this radius, it rapidly reacts with any molecule. However, the type of damage would depend on its site of formation; for instance, production of $OH^{\bullet}$ close to DNA could lead to modification of purines or pyrimidines or strand breakage (32), whereas production of $OH^{\bullet}$ close to an enzyme molecule present in excess in the cell, such as lactate dehydrogenase, might have biological consequences (39). $O_2^{\bullet-}$ and H_2O_2 are less reactive oxidants than $OH^{\bullet}$, but they

have a longer life time, which allows them to react with molecules in locations far from the site where the free radical is produced (13). The $OH^{\bullet}$ readily damages nuclear and mitochondrial DNA, membrane lipids, proteins and carbohydrates.

The main source of hydroxyl radicals is the metal-catalyzed Haber-Weiss reaction, the second of which is the Fenton-type reaction (reaction 3). An intermediate in the reaction of H_2O_2 with Fe^{2+} may be the iron-oxygen complex referred to as ferryl which itself is highly oxidizing and degrades to form the $OH^{\bullet}$. These reactions, involving transition metals, occur *in vivo* and because of that it has been suggested that chelant compounds could be suitable for treating diseases in which oxygen free radical production is involved (2,40,41).

$$O_2^{\bullet-} + Fe^{+3} \rightarrow O_2 + Fe^{+2} \quad (2)$$

$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^- + OH^{\bullet} \quad (3)$$

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^- + OH^{\bullet} \quad (4)$$

Transition metal-catalyzed oxidations and hydroxylations

A growing body of evidence indicates that transition metals act as catalysts in the oxidative deterioration of biological macromolecules, and therefore, the toxicities associated with these metals may be due, at least in part, to oxidative tissue damage. Recent studies have shown that metals such as iron, copper, cadmium, chromium, lead, mercury, nickel, and vanadium exhibit the ability to produce reactive oxygen species, resulting in lipid peroxidation, DNA damage, depletion of sulfhydryls, and altered calcium homeostasis.

The toxicities produced by the transition metals generally involve neurotoxicity, hepatotoxicity, and nephrotoxicity. The specific differences in the toxicities of metal ions may be related to differences in solubilities, absorbability, transport, chemical reactivity, and the complexes that are formed within the body. In spite of these factors, the basic mechanisms involving production of reactive oxygen species are the same for these transition metal ions. Furthermore, the basic mechanism of reactive oxygen species production and ultimate toxicity produced by metal ions may involve those which are common to redox cycling organic xenobiotics, as for example, the quinones.

The two most commonly studied transition metals are the cations of iron and copper. A variety of studies have demonstrated the ability of iron chelates or complexes to catalyze the formation of reactive oxygen species and stimulate lipid peroxidation. Aust (42) has reviewed the relationship between metal ions, oxygen radicals, and tissue damage. The role of iron in the initiation of lipid peroxidation has also been reviewed by Minotti and Aust (43) and Alleman et al (44). These investigators have presented evidence for the fact that, lipid peroxidation requires both Fe^{+3} and Fe^{+2}, probably as a dioxygen-iron complex. Iron is capable of catalyzing redox reactions between oxygen and biological macromolecules that would not occur if catalytically activated iron was not present. Iron complexed with ADP, histidine, EDTA, citrate, and other chelators has been shown to facilitate the formation of reactive oxygen species and enhance lipid peroxidation (42).

The abundance of evidence indicates that chelated iron acts as a catalyst for the Fenton reaction, facilitating the conversion of superoxide anion and hydrogen peroxide to hydroxyl radical, a species frequently proposed to initiate lipid peroxidation (19) and DNA damage (2). However, some investigators have suggested that Fenton reagent oxidizes substrates through a mechanism involving iron-oxo species without the involvement of free radicals (45).

Iron is in Group VII of the periodic table and has two main valency states, the divalent ferrous (Fe^{+2}) and the trivalent ferric (Fe^{+3}) forms. Transition metals such as iron have an incomplete outer shell of d electrons, and are thus able to undergo changes in oxidation states involving one electron. The easy access to two oxidation states allows iron to participate in redox processes making it an essential biological catalyst (46). The Fenton reaction may be generalized as $[ML_m^n + H_2O_2 \rightarrow ML_m^{n+1} + {}^{\bullet}OH + OH^-]$, where the low valent transition metal ion is depicted as M, while L corresponds to any ligands that are complexed to the metal, and n refers to the charge on the metal ion. A modification of the Haber-Weiss reaction, the Fenton reaction, utilizes the redox cycling ability of iron to increase the rate of reaction, and is more feasible *in vivo* (2). Here, the $O_2^{\bullet -}$ radical both dismutates to form H_2O_2 and reduces any iron complexes present to the ferrous state (Fe^{+2}). This reduction proceeds with the intermediate formation of perferryl, an iron-oxygen complex, with a resonance structure intermediate between that of Fe^{+3}-$O_2^{\cdot}$ and Fe^{+2}-$O_2^{\cdot}$:

$O_2^{\cdot} + Fe^{+3} \leftrightarrow (Fe^{+3}\text{-}O_2^{\cdot} / Fe^{+2}\text{-}O_2^{\cdot}) \leftrightarrow O_2 + Fe^{+2}$

perferryl species

Fe^{+2} ions and H_2O_2 then react to generate the $^{\bullet}OH$:

$Fe^{+2} + H_2O_2 \rightarrow$ intermediate complex(es) $\rightarrow Fe^{+3} + {}^{\bullet}OH + OH^{-}$.

It is generally recognized that, small organic radicals are reactive and therefore, short-lived. However, some radicals are sufficiently stable to diffuse a certain distance in the cell, whereas others such as ·OH are highly reactive that they react within 1-5 molecular diameters of the site of formation (13), with a rate constant which is almost diffusion controlled (10^{-9} mol/s) (47,48). The hydroxyl radical reacts with almost every type of molecule found in living cells, but its production is dependent on the presence of iron capable of catalyzing the Fenton reaction. Therefore, a major determinant of the cell toxicity of $O_2^{\bullet -}$ and H_2O_2 is the availability and location of iron to catalyze ·OH production. The subsequent site of attack of ·OH will be determined by the site of the bound iron, so called "site specific attack" (49). For example, if iron is bound to membrane lipids, introduction of $O_2^{\bullet -}$ and H_2O_2 may then peroxidize the membrane lipids.

The Fenton reaction involving Fe^{+2}-EDTA is probably the most thoroughly investigated example of this class of reaction. A number of studies employing substrate trapping and / or direct spectroscopic examination (pulse radiolysis) have demonstrated that, the reaction of this complex with H_2O_2 produces a radical with the kinetic and chemical properties of ·OH (34,50). The majority of these studies have used non-catalytic conditions, i.e., iron in large excess over substrate. The relative reactivities for oxidation of a variety of organic substrates by the iron Fenton reagent were directly compared to those obtained by independent generation of ·OH via ionizing radiation, and found to be in good general agreement (51). The exact nature of the ligand (L) in ML^{n}_{m} can affect the product distribution, presumably by altering the yields of oxidizing radicals produced in the Fenton reaction. Thus, when Fe^{+2}-NTA (nitrilotriacetate) is used in place of Fe^{+2}-EDTA, ·OH is no longer the main reactive intermediate produced (52). In the case where L is a functionalized porphyrin molecule, hydroxyl radicals may not be produced at all. For example, P-450 enzymes of the monooxygenase family use iron to add "OH" equivalents to saturated and aromatic substrates by a mechanism which does not involve the production of freely diffusing hydroxyl radicals (53). These reactions most likely proceed

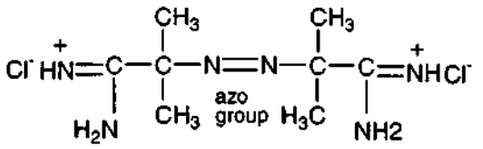
via high valent iron oxo [Fe^{+4}=O] intermediates. The biomimetic modelling of these reactions using transition metal ions and small organic ligands is an extremely active area of chemical research (54).

Peroxyl Radical

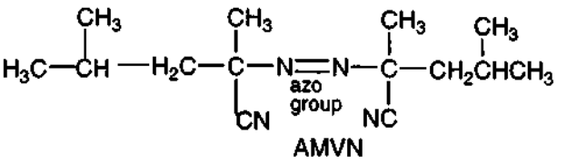
Peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals are important reactive intermediates in organic and biological oxidations, and in atmospheric chemistry, they are formed by the addition of oxygen to alkyl radicals, and both are good oxidizing agents, although $RO^{\bullet}$ radicals formed in biological systems often undergo rapid molecular rearrangement to other radical species. One-electron reduction of molecular oxygen produces the superoxide anion (52). This is a facile process that occurs widely in aerobic organisms. Protonation of superoxide produces the perhydroxyl radical, H-O-$O^{\bullet}$, which is a more reactive oxidant than superoxide and more lipophilic. Analogs of the perhydroxyl radical in which the H-atom is replaced by an organic group are called peroxyl radicals ($ROO^{\bullet}$).

$O\text{-}O^{\bullet}$	$H\text{-}O\text{-}O^{\bullet}$	$R\text{-}O\text{-}O^{\bullet}$
superoxide	perhydroxyl	organic peroxyl

Azo initiators can be used in the laboratory to generate $RO_2^{\bullet}$ radicals (and $O_2^{\bullet-}$ radicals) for studies on lipid peroxidation and antioxidant activities. AAPH is water-soluble whereas AMVN is hydrophobic. Both decompose at a temperature-controlled rate to give carbon-centered radicals.



AAPH



AMVN

Structures of the azo initiators, 2,2'-azobis(2-amidinopropane)hydrochloride (AAPH) and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN).

Decomposition of organic peroxides (ROOH) can generate $ROO^{\bullet}$ and $RO^{\bullet}$. Peroxyl radicals are much more stable than other oxygen radicals (53), and have the ability to diffuse relatively far from the site of their generation before they react with a target molecule. Pryor has estimated that, the half-lives of peroxyl radicals are of the order of seconds (13). Changing the organic group attached to the oxygen does not significantly

alter the reactivity of peroxyl radicals, but it can have a dramatic effect on the physical properties of the radicals. Most biological peroxyl radicals are neutral and highly lipophilic, which endows them with the ability to penetrate cells or subcellular organelles. Furthermore, unlike other oxygen-derived free radicals, they have the ability to insert oxygen atoms into non-aromatic C-C double bonds to produce epoxides (54). Many organic epoxides react with macromolecular nucleophiles to form stable adducts that alter the structure and function of the adducted macromolecule (55). Epoxide adducts to DNA frequently induce mutations, which can contribute to the multi-stage process of carcinogenesis (56).

Peroxyl radicals can oxidize other substrates via H-atom abstraction, producing alkyl hydroperoxides and an oxidized radical intermediate. These alkyl hydroperoxides, ROOH, undergo many of the same reactions with metal ions as does hydrogen peroxide. In the special case of lipid hydroperoxides, additional structural considerations govern the distribution of reactive radical intermediates produced, and these are likely to have implications on mutagenesis. However, much of the chemistry of the peroxyl radical has been studied within the context of polyunsaturated lipid oxidation, where, peroxyl radical abstracts hydrogen atom (57,58).

Alkyl and Alkoxyl Radicals

In a manner entirely analogous to the Fenton-catalyzed decomposition of hydrogen peroxide, alkyl hydroperoxides are reduced to alkoxyl radicals by iron complexes. (ML_m^n + ROOH $\rightarrow$ ML_m^{n+1} + $RO^{\bullet}$ + $^{\cdot}OH$). Alkoxyl radicals can be generated from peroxyl radicals via the fragmentation of alkyl tetroxide (reaction a and b), or one-electron reduction (reaction c) of alkyl hydroperoxides (generated via d and e) or dialkylperoxides (RO-OR; from reaction f). Alkoxyl radicals undergo rapid addition and hydrogen abstraction reactions, as well as facile unimolecular fragmentation and rearrangement reactions (59,60). Alkoxyl radicals are good hydrogen atom abstractors, and have been shown to attack both the bases and the carbohydrate backbone of DNA (61). In view of their brief half-life, they would be predicted to be less selective chemically than peroxyl radicals, and to possess a much shorter mean diffusion path. Alkoxyl radicals formed from polyunsaturated lipid hydroperoxides undergo a rapid intra-molecular rearrangement to

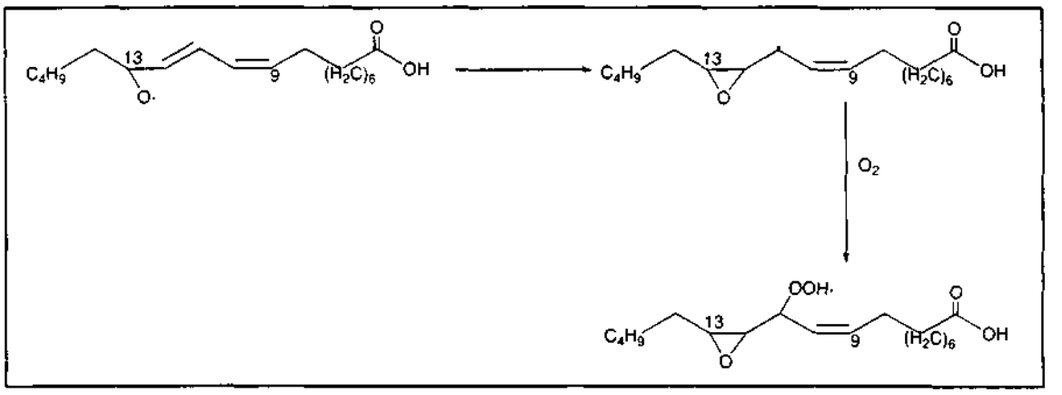
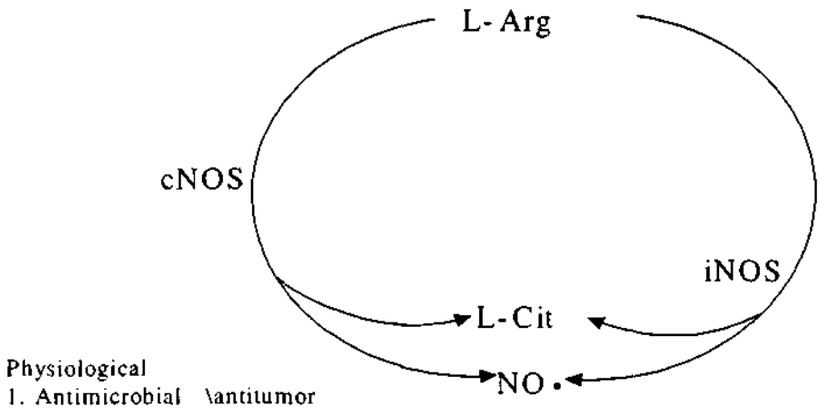


Fig. 2: Intramolecular rearrangement of lipid alkoxyl radicals and subsequent oxygen trapping to generate peroxyl radicals.



Physiological
1. Antimicrobial \antitumor
2. Vasodilation
3. Neurotransmission
4. Regulation of Platelet
5. Penil Erection
6. Bronchodialation

Pathological
1. Hypotension
2. Septic Shock
3. Nitrosation
4. DNA deamination
5. Oxidation
6. Neural Damage

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Fig. 3: Synthesis, physiology and pathology of nitric oxide in vivo

form an allylic epoxide carbon radical, which in the presence of O_2, is rapidly quenched to form a new peroxyl radical (62).

$$2ROO^{\bullet} \rightarrow ROO\text{-}OOR \quad (a)$$

$$ROO\text{-}OOR \rightarrow 2RO^{\bullet} + O_2 \quad (b)$$

$$ROOH + e^{-} \rightarrow RO^{\bullet} + HO^{-} \quad (c)$$

$$ROO^{\bullet} + R'\text{-}H \rightarrow ROOH + R'^{\bullet} \quad (d)$$

$$ROO^{\bullet} + e^{-} \rightarrow ROO^{-} + H^{+} \rightarrow ROOH \quad (e)$$

$$ROO\text{-}OOR \rightarrow RO\text{-}OR + O_2 \quad (f)$$

This reaction, depicted in **Fig. 2**, takes place much faster than inter-molecular reactions of the polyunsaturated lipid alkoxyl radical. For these reasons, allylic alkoxyl radicals generated in the course of lipid peroxidation are not predicted to be a significant oxidative intermediate.

Nitric Oxide and Peroxynitrite Anion

Since its initial discovery, nitric oxide ($NO^{\bullet}$) has become one of the most highly studied and important biological molecules, and it is often characterized as a double-edged sword. Along with nitric oxide's many essential roles *in vivo*, such as neurotransmission, vasodilation, and immune response, it can also be involved in reactions that may produce cell damage (**Fig. 3**). Nitric oxide (colorless gas) is a relatively unstable molecule that is potentially toxic due, in part, to the high reactivity of its unpaired electron. It has long been studied as an environmental pollutant because it is produced by the internal combustion engines and contributes to the formation of photochemical smog, acid rain and is also involved in the destruction of the ozone layer (63). Due to nitric oxide's short lifetime in air (~5-10 s), it was surprising when it was found to play an important role in mammalian physiology.

$NO^{\bullet}$ consists of seven electrons from the nitrogen atom and eight electrons from the oxygen atom. Therefore, $NO^{\bullet}$ is paramagnetic with one unpaired electron in a π^*2p antibonding orbital, hence it is a free radical and is highly reactive. $NO^{\bullet}$ can be produced by oxidation of atmospheric nitrogen ($N_2 + O_2 \rightarrow 2NO^{\bullet}$) but this reaction normally takes place at temperatures of 1200-1750° C. Further oxidation of $NO^{\bullet}$ (e.g. $2\ NO^{\bullet} + O_2 \rightarrow 2NO_2^{\bullet}$) occurs in the presence of O_2. Almost 90% of the pollutant $NO^{\bullet}$ in the air is

produced by the fuel combustion in motor vehicles and stationary sources. The combined forms of oxides of nitrogen (NO_x), sunlight, and hydrocarbons are the essential ingredients of photochemical smog. Cigarette smoke contains as much as 100 ppm NO_x (64).

Since $NO^{\bullet}$ has an unpaired electron, it is highly reactive in the gas phase with oxygen to form nitrosating agents. $NO^{\bullet}$ reacts with molecular oxygen (O_2) to produce $NO_2^{\bullet}$ (nitrogen dioxide), in combination with radical-radical combination products, dinitrogen trioxide (N_2O_3) and dinitrogen tetroxide (N_2O_4) (65). It has been known for some time that, nitrogen dioxide is able to initiate lipid peroxidation (66). Both N_2O_3 and N_2O_4 are capable of reacting with water to yield NO_2^- and NO_3^-. NO_2^- is oxidized to NO_3^- in the presence of hemoglobin, and is the urinary excretory end product of $NO^{\bullet}$. The intracellular half-life of $NO^{\bullet}$ is inversely proportional to its concentration (67), and also may be affected by reacting with other compounds such as heme-Fe^{+2}. $NO^{\bullet}$ is also likely to react rapidly with superoxide ($O_2^{\bullet-}$), copper and manganese in addition to O_2. The half-life of $NO^{\bullet}$ in biological systems is estimated to be 4-50 s (68). $NO^{\bullet}$ can migrate freely in and out of the cells similar to oxygen (69).

Peroxynitrite

Nitric oxide also reacts to form additional reactive species that can participate in other types of chemistry. Peroxynitrite [$ONOO^-$, technically called as oxoperoxynitrite (1-)], and hydroxyl radicals are potent oxidizing agents produced by redox reactions of $NO^{\bullet}$ (70,71). $NO^{\bullet}$ rapidly reacts with superoxide anion radical to form peroxynitrite anion in high yield. This is an extremely fast reaction due to the fact that, both species are radicals. The half-life of $NO^{\bullet}$ is approximately doubled by SOD, suggesting that, superoxide is involved in this reaction (72). The rate of the nitric oxide/superoxide reaction, with a rate constant of $6.7 \times 10^9\ M^{-1}\ s^{-1}$, is near the diffusion limit (70,73,74).

$$NO^{\bullet} + O_2^{\bullet-} \rightarrow ONOO^- \quad (k \approx 7 \times 10^9\ M^{-1}\ s^{-1})$$

This rate constant is approximately 3.5 times larger than that for the superoxide dismutase (SOD)-catalyzed decomposition of $O_2^{\bullet-}$ indicating that the nitric oxide/superoxide reaction may predominate over the superoxide/SOD reaction. The formation of both nitric oxide and superoxide indeed occur simultaneously in cells such as macrophages, neutrophils, Kupffer cells, and endothelial cells (75). In the vicinity of these cells, peroxynitrite may be

present at high concentrations, although the mechanism and extent of $ONOO^-$ formation are strongly influenced by the relative fluxes of $O_2^{\bullet-}$ and $NO^\bullet$ (23,76,77).

$ONOO^-$ has a pK_a of 6.8 at 37° C (23) and is stable in alkaline solutions (stable for weeks if kept frozen), but unstable at physiological pH. Peroxynitrite is usually synthesized in the laboratory by reacting nitrite with H_2O_2 at low pH,

$$NO_2^- + H^+ \rightarrow HONO \text{ (nitrous acid)}$$

$$HONO + H_2O_2 \rightarrow HOONO + H_2O$$

and immediately stabilizing the $ONOO^-$ by adding excess sodium hydroxide, and it was estimated by using the absorbance of $ONOO^-$ at 302 nm in alkali (ε = 1670 M^{-1} cm^{-1}). It is protonated in acidic solution to form peroxynitrous acid (ONOOH) with a half-life below 1 s at pH 7.4, which then decays rapidly to form predominantly nitrate. Peroxynitrite is a potent one-electron and two-electron oxidant and can therefore oxidize protein and non-protein sulfhydryls (23,78). Peroxynitrite is also a nitrating agent; this is supported by the observations that both 3-nitrotyrosine and 8-nitroguanine are found in cells that had been exposed to peroxynitrite (74,79). The most important modulator of peroxynitrite, both *in vitro* and *in vivo*, is carbon dioxide (CO_2) (70). The rate constant for reaction of peroxynitrite anion with carbon dioxide is 3×10^4 M^{-1} s^{-1}; this indicates that CO_2 greatly accelerates the decomposition of $ONOO^-$ (78). This is one of the fastest reactions known for peroxynitrite. Given the high concentrations of carbon dioxide (up to 1 mM) in biological fluids, it is believed that, the reaction of peroxynitrite with carbon dioxide will be the predominant pathway for decay of peroxynitrite *in vivo* (78).

Hypochlorous acid

Hypochlorous acid is produced by the enzyme myeloperoxidase (MPO) in the activated neutrophils. Stimulated neutrophils generate superoxide and its dismutation product, hydrogen peroxide, and release the heme enzyme myeloperoxidase (81,82).

$$H_2O_2 + Cl^- \xrightarrow{\text{Myeloperoxidase}} OH^- + HOCl$$

Myeloperoxidase (MPO), in addition to oxidizing classical peroxidase substrates to radical intermediates, has the unique property of converting chloride to hypochlorous acid (HOCl) (83) or its conjugate base, hypochlorite (OCl^-; pK_a 7.4) (83). In addition to Cl^-, MPO also oxidizes Br^- to hypobromous acid (HOBr) (82).

$$HOCl \rightleftharpoons H^+ + OCl^-$$

HOCl is a highly reactive species that participates in both oxidation and chlorination reactions. It has many biological targets for oxidation and attacks a wide range of physiologically relevant molecules, such as thiols and thioethers. However, other compounds, including ascorbate, urate, pyridine nucleotides, and tryptophan, are also oxidized by HOCl, although not so rapidly (24,84). HOCl can generate very reactive oxidizing species, like singlet oxygen and hydroxyl radical, via its reaction with H_2O_2 and superoxide anion, respectively (85). The high reactivity of HOCl could suggest that its toxicity might be somehow dissipated within a short distance from the generation sites. MPO, however, is a charged (i.e. cationic) protein at physiological pH values, which enables the enzyme to bind to biological anionic structures, such as the negatively charged phospholipids of membranes, followed by HOCl generation and cell injury (85).

Sources of Oxidants (Free radical production by biological systems)

The presence of free radicals, at times, can be advantageous for cells. In fact, they are being continuously produced in organisms and many of them are necessary to carry out certain biological reactions. However, when there is a free radical overproduction or the antioxidant defence systems are weakened for any reason, cellular damage can appear (2,10,13,86).

Ground-state diatomic oxygen ($^3\Sigma g^- O_2$ or more commonly, O_2), despite being a radical species and the most important oxidant in aerobic organisms, is only sparingly reactive by itself due to the fact that, its two unpaired electrons are located in different molecular orbitals and possess "parallel spins". As a consequence, if O_2 has to simultaneously accept two electrons, both of these must possess antiparallel spins relative to the unpaired electrons in O_2, a criterion which is not satisfied by a typical pair of electrons in atomic or molecular orbitals (which have opposite spins according to the Pauli's exclusion principle). As a result, O_2 preferentially accepts electrons, one at a time, from other radicals (such as transition metals in certain valences). Thus, in vivo, typical two- or four-electron reduction of O_2 relies on coordinated, serial, enzyme-catalyzed one-electron reductions, and the enzymes that carry out these reactions typically possess active-site radical species such as iron. One- and two-electron reduction of O_2 generates $O_2^{\bullet -}$ and hydrogen peroxide (H_2O_2), respectively, both of which are generated by numerous routes

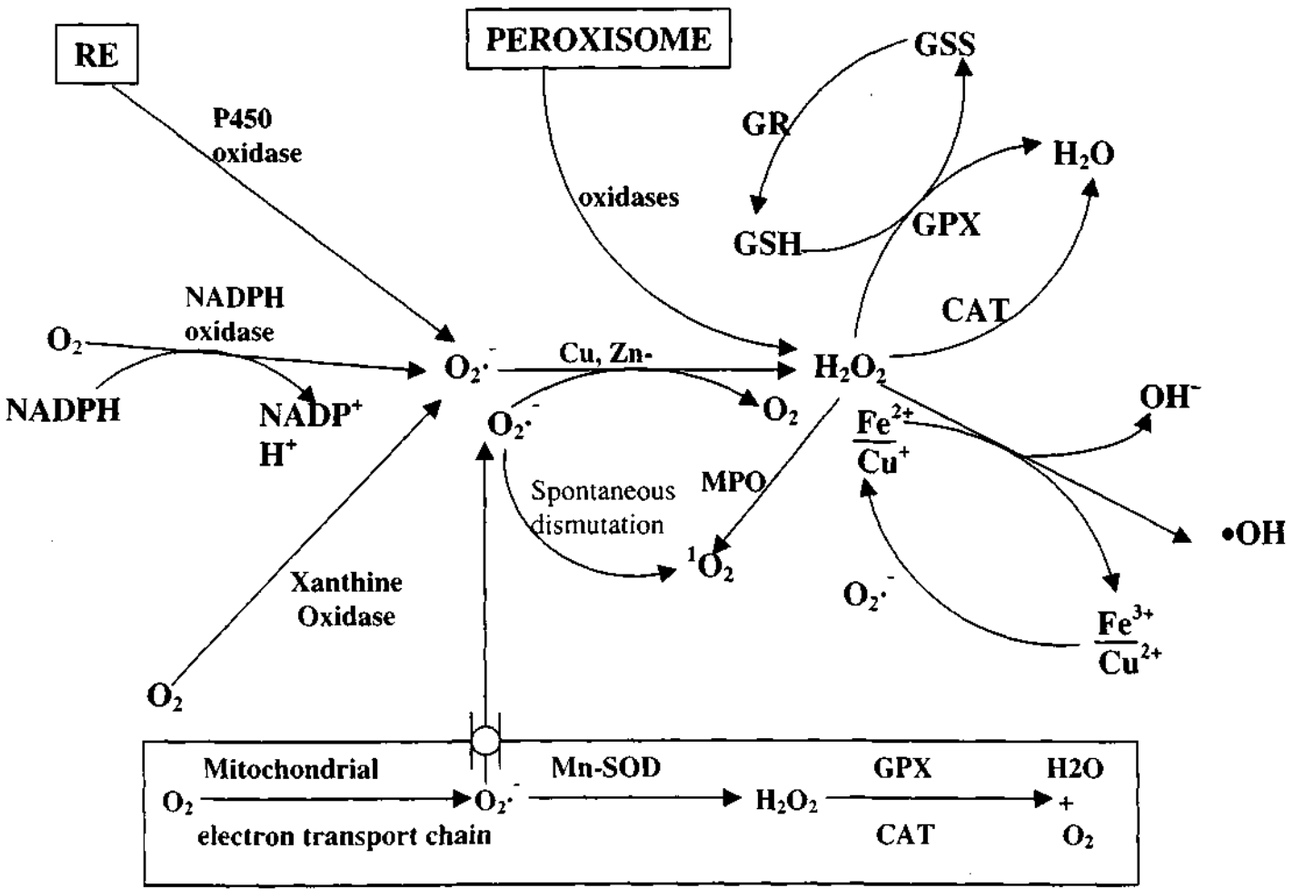


Fig. 4: Generation of ROS and main defence mechanisms against damage produced by reactive oxygen species

in vivo, as discussed below. In the presence of free transition metals (in particular iron and copper), $O_2^{\bullet-}$ and H_2O_2 together generate the extremely reactive hydroxyl radical ($^{\bullet}OH$). Ultimately, $^{\bullet}OH$ is assumed to be the species responsible for initiating the oxidative destruction of biomolecules. In addition to these radicals, two energetically excited species of O_2, termed "singlet oxygen", can result from the absorption of energy (for instance, from ultraviolet light). The chemistry of oxygen and its derivatives has been extensively discussed elsewhere (2,87). All of these species ($O_2^{\bullet-}$, H_2O_2, $^{\bullet}OH$, $^1\Delta gO_2$, and $^1\Sigma g^+O_2$), formed by different routes, are involved in oxygen's toxicity, collectively referred as "oxidants."

It is now beyond doubt that oxidants are generated *in vivo* and can cause significant harm (9,11,88.89). There are numerous sites of oxidant generation (**Fig. 4**), four of which have attracted much attention: (i) mitochondrial electron transport, (ii) peroxisomal fatty acid metabolism, (iii) cytochrome *P*-450 reactions, and (iv) phagocytic cells (the "respiratory burst").

In the textbook scheme of mitochondrial respiration, electron transport involves a coordinated four-electron reduction of O_2 to H_2O, the electrons being donated by NADH or succinate to complexes I and II, respectively, of the mitochondrial electron transport chain (ETC). Ubiquinone (coenzyme Q, or UQ), which accepts electrons from complexes I (NADH dehydrogenase) and II (succinate dehydrogenase), undergoes two sequential one-electron reductions to ubisemiquinone and ubiquinol (the Q cycle), ultimately transferring reducing equivalents to the remainder of the electron transport chain: complex III (UQ-cytochrome *c* reductase), cytochrome *c*, complex IV (cytochrome-*c* oxidase), and finally, O_2 (2). However, it appears that mitochondrial electron transport is imperfect, and one-electron reduction of O_2 to form $O_2^{\bullet-}$occurs. The spontaneous and enzymatic dismutation of $O_2^{\bullet-}$yields H_2O_2 , so a significant by-product of the actual sequence of oxidation-reduction reactions may be the generation of $O_2^{\bullet-}$and H_2O_2 .

A second source of oxygen radicals is peroxisomal β-oxidation of fatty acids, which generates H_2O_2 as a by-product. Peroxisomes also contain high concentration of glycollate oxidase or D-amino acid oxidase, which catalyze the divalent reduction of molecular oxygen to superoxide (90). Peroxisomes possess high concentrations of catalase,

but it is unclear whether or not leakage of H_2O_2 from peroxisomes contributes significantly to cytosolic oxidative stress under normal circumstances. However, a class of nonmutagenic carcinogens, the peroxisome proliferators, which increase the number of hepatocellular peroxisomes and result in liver cancer in rodents, also cause oxidative stress and damage (91-93). Interestingly, during the regeneration of the liver after partial hepatectomy, there exists peroxisomes that do not stain for catalase activity (94), hinting that during rapid cell proliferation, oxidant leakage from peroxisomes may be enhanced.

Microsomal cytochrome P450 enzymes metabolize various types of xenobiotic compounds, many of them are of plant origin. Although these reactions typically involve NADPH and an organic substrate, some of the numerous cytochrome P450 isozymes directly reduce O_2 to $O_2^{\bullet-}$ (95,96) and may cause oxidative stress. An alternative route for cytochrome P450-mediated oxidation involves redox cycling in which, substrates accept single electrons from cytochrome P450 and transfer them to oxygen. This generates $O_2^{\bullet-}$ and simultaneously regenerates the substrate, allowing subsequent rounds of $O_2^{\bullet-}$ generation (2). Although it is unclear to what extent cytochrome P450 side reactions proceed under normal conditions, it is possible that such chronic $O_2^{\bullet-}$ generation by cytochrome P450 is the price animals pay for their ability to detoxify acute doses of toxins (97). Cytochrome reductases involved in redox reactions of cytochrome P450 and b_5 can also produce superoxide radicals and hydrogen peroxide when they undergo autoxidation (98).

Finally, phagocytic cells attack pathogens with a mixture of oxidants and free radicals, including $O_2^{\bullet-}$, H_2O_2, $NO^{\bullet}$, and hypochlorite (99,100). Although the massive generation of oxidants by immune cells differs from the above three sources of free radicals to the extent that it is the result of pathogenesis, it is nevertheless a normal and unavoidable consequence of innate immunity. This process is due to an enzyme named NADPH oxidase, which is located on the external surface of the plasmic membrane (2).

$$NADPH + 2O_2 \xrightarrow{\text{NADPH oxidase}} NADP^+ + H^+ + O_2^{\bullet-}$$

Chronic inflammation is therefore unique among the endogenous sources of oxidants because, it is mostly preventable (101,102). It has been estimated that, approximately one-third of the world's cancers are due to the effects of chronic

inflammation (103). Support for this hypothesis comes from the observations that, antibody titers to oxidized DNA are significantly higher in individuals with chronic inflammatory dermatoses or histories of cancer (104). Examples of cancers arising from chronic inflammation include mesothelioma after asbestos exposures and cancer after chronic ulcerative colitis (105,106). In addition to these four sources of oxidants, there exist numerous other enzymes capable of generating oxidants under normal or pathological conditions, often in a tissue-specific manner (2). To give a single relevant example, the deamination of dopamine by monoamine oxidase (flavoprotein enzyme located in the outer mitochondrial membrane) generates H_2O_2 in some neurons, and has been implicated in the etiology of Parkinson's disease (107).

Ionizing radiation (IR) was first demonstrated to be mutagenic in 1927 by Muller, who found that IR induced mutations in *Drosophila* species (108). Since then, IR has been found to cause mutations and cancer in a large number of systems (109-112). IR damages all cellular constituents and produces more than 100 different DNA lesions (111). Although IR damages DNA through the direct effects of radiation energy on DNA, it also damages DNA indirectly through the production of ROS (109,113,114). Water is the predominant cellular constituent and approximately 80% of the radiation energy deposited within cells results in the extraction of electrons from water. After this event, ROS species including H_2O_2, $^{\bullet}OH$, H_2, e^-, H., and $O_2^{\bullet-}$ are formed, all of which can damage DNA (115).

Ischaemia-reperfusion: Lack of blood supply or ischaemia underlies many of the important diseases faced by physicians and surgeons in their daily practice, including myocardial infarction, thrombotic stroke, embolic vascular occlusions, angina pectoris, peripheral vascular insufficiency, cardiac surgery, many types of liver surgery and organ transplantation. Now it is well known that free radical-mediates the reperfusion injury (2).

A potential source of reactive oxygen-derived free radicals in ischaemic and reperfused tissues is the enzyme xanthine dehydrogenase/oxidase. The enzyme is synthesized as xanthine dehydrogenase (type D) which accounts for 90% of the enzyme in healthy tissues (116). This form uses nicotinamide adenine dinucleotide (NAD) as an electron acceptor during oxidation of xanthine:

$$\text{Xanthine} + H_2O + NAD^+ \rightarrow \text{Uric acid} + NADH + H^+$$

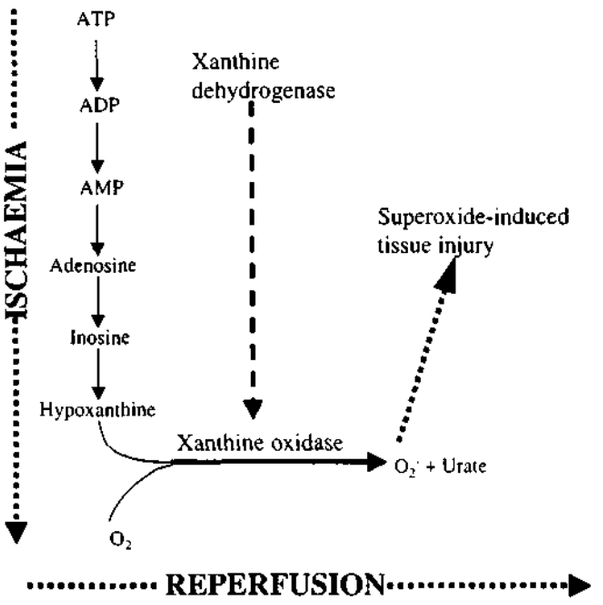


Fig.5. The simultaneous conversion of the enzyme xanthine dehydrogenase to its oxidase form and the breakdown of purine nucleotides to hypoxanthine create an ideal environment for the formation of the superoxide radical when oxygen is readmitted at reperfusion.

Alternatively, the enzyme can exist as the oxidase (type O), using molecular oxygen as an electron acceptor, to produce the superoxide radical:

$$\text{Xanthine} + H_2O + 2O_2 \rightarrow \text{Uric acid} + 2\,O_2^{\bullet -} + 2H^+$$

It has recently been established that, conversion of the enzyme from the D-form to the O-form occurs as a result of tissue ischaemia (117,118). This may be precipitated by depletion of ATP and subsequent loss of control over membrane calcium gradients. Increased cytosolic calcium concentration activates a calcium-dependent protease, which in turn converts the dehydrogenase D-form to an oxidase (119). The failure to resynthesize ATP due to interruption of oxidative phosphorylation promotes the breakdown of adenine nucleotides such as AMP, and subsequently to adenosine, inosine, hypoxanthine and xanthine. The latter two substances are oxidizable substrates for xanthine oxidase and build up in ischaemic tissues (120). Only when the other substrate, molecular oxygen, is readmitted at the time of reperfusion, rapid production of superoxide radicals occur (**Fig. 5**).

Although this sequence of events is plausible and has been substantiated in some animal models, doubt has been cast about its relevance in man. It has been difficult to demonstrate significant quantities of xanthine oxidase in human myocardium, although it is well supplied in the endothelial cells. Low xanthine oxidase activity may also result in a high level of myocardial adenosine accumulation during ischaemia, and has been postulated as a natural defence mechanism in the ischemic/reperfused rabbit heart (121).

Cytotoxicity of Oxygen Free Radicals

The high reactivity of free radicals results in their having a short half-life as well as a short radius for action. However, when these molecules react with non-radical compounds, new free radicals can be formed which again react. In this way, long chains of propagation are established causing biological effects far from the system which produced the first radical. A clear example is lipid peroxidation where secondary radicals and degradation products may exert extremely injurious effects distant from the initial site of radical production

Targets of Oxidants

The three main classes of biological macromolecules (lipids, nucleic acids and proteins) are susceptible to free radical attack, and there is plentiful evidence that all suffer

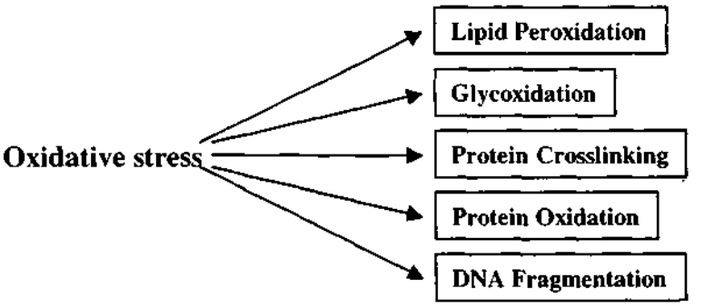


Fig. 6: Oxidative stress results in a wide array of deleterious cellular consequences that affect all of the major cellular macromolecules

oxidative damage *in vivo* (**Fig. 6**). A synopsis of the better known pathways of oxidative damage are described here.

A) Lipid Peroxidation

Lipid peroxidation has been defined by A. L. Tappel as 'the oxidative deterioration of polyunsaturated lipids'. Polyunsaturated fatty acids (PUFA) are those that contain two or more C-C double bonds, (>C=C<).

The earliest research on the destruction of biological molecules by oxidants involved lipids (122). Lipids have a critical structural and functional role in membranes. Any disruption of this role can lead to cell death. The double bonds found in polyunsaturated fatty acids are ready targets for free radical attack. Hydroxyl and hydroperoxyl radicals, but not superoxide and H_2O_2, are able to attack unsaturated fatty acids of phospholipids and other membrane lipid compounds in order to initiate the lipid peroxidation. The abstraction of a hydrogen atom from one of these double bonds, which can be mediated by free radicals, yields a new radical species that can readily interact with molecular oxygen, which is a diradical. The resultant lipid peroxyl radical can abstract a hydrogen atom from another fatty acid yielding yet another radical and a lipid hydroperoxide thereby establishing a chain reaction. The lipid hydroperoxide formed is unstable and can decompose to various species including malondialdehyde (MDA), or it can be reduced to the more stable alcohol form. Alcohols, aldehydes, volatile hydrocarbons and hydroperoxides, the final products of peroxidation, inhibit protein synthesis and are also able to alter vascular permeability, the inflammatory response and chemotactic activity (123,124). In addition, MDA, a main indicator of lipid peroxidation, has also been found to cause cross-linking and polymerization of membrane components as well as to react with nitrogenous bases of DNA (125). In addition, some of the oxidized fatty acid species that are formed, such as the isoprostanes (126) or the hydroperoxides (127,128), have biological activity in terms of an ability to affect signalling pathways including those that regulate the apoptotic form of cell death.

Food chemists known the fact that the rancidity of fats results from peroxidative chain reactions in lipids ("autoxidation"): a lipid hydroperoxyl radical abstracts a hydrogen atom from the double bond of a neighboring unsaturated lipid, forming a hydroperoxide and an alkyl radical, the latter of which combines with O_2 to regenerate a lipid

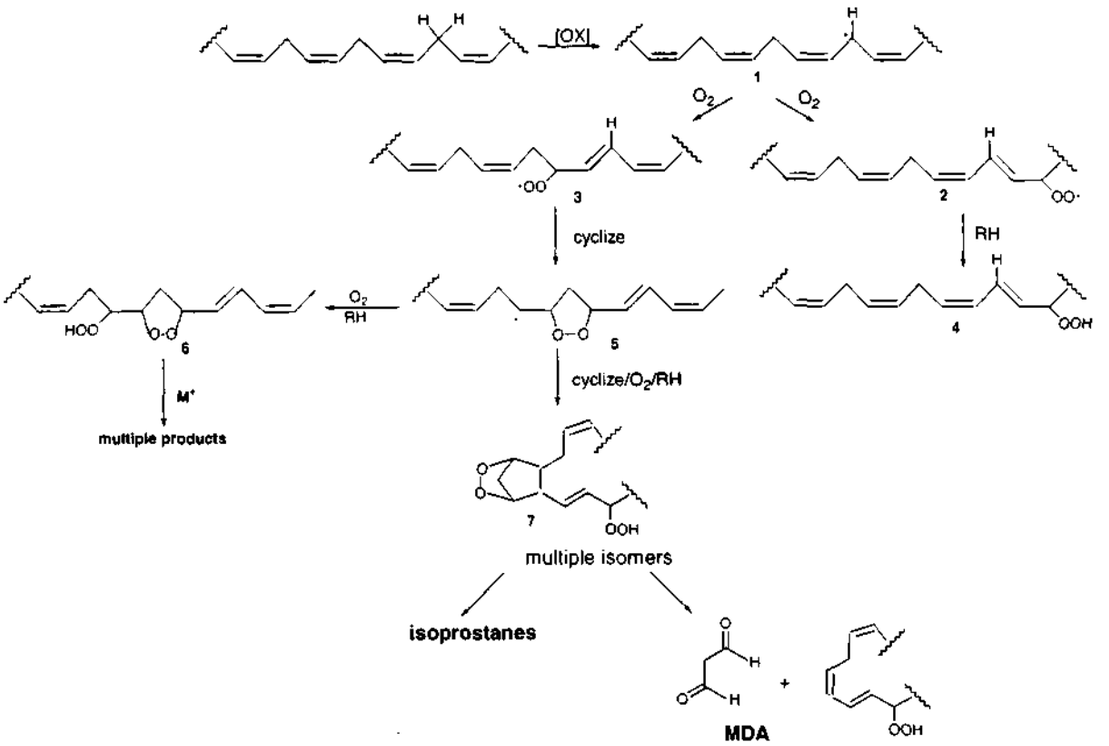


Fig. 7: Pathways of lipid peroxidation

hydroperoxyl radical capable of initiating another round of oxidation. The possible importance of lipid peroxidation in biology as a damaging process for cellular membranes was first suggested by the studies of Tappel (129). However, at that time, any possible implication in biopathology appeared restricted to conditions of deficiencies of vitamin E or other antioxidants, which are mainly limited to animals maintained on particular diets. The knowledge that lipid peroxidation can be linked to the electron transport chain of drug metabolism, the recognition that the metabolism of CCl_4 yields haloalkane free radicals, and the observation that CCl_4 greatly stimulated the peroxidation of liver microsomal lipids, led to the assumption that lipid peroxidation could be a basic mechanism of toxicity for a wide variety of chemicals. Now it is well established that, lipid peroxidation is one of the reactions set into motion as a consequence of the formation of free radicals in cells and tissues.

Lipid peroxidation is a complex process, broadly defined as the oxidative deterioration of polyunsaturated fatty acids (2), and is commonly divided into three phases, namely, initiation, propagation and termination (**Fig. 7**). Lipid peroxidation process is initiated by the abstraction of a hydrogen atom from a methylene group located between two isolated double bonds of the fatty acid. A carbon-centered radical (e.g. **1**) is formed that reacts after rearrangement with molecular oxygen to yield a peroxyl radical (e.g. **2** and **3**). Thus, the initial products of polyunsaturated fatty acid oxidation are peroxyl radicals. The fate of the peroxyl radical depends on its position in the carbon chain of the fatty acid. If the peroxyl radical exists at one of the two ends of the double bond system (e.g., **2**), it is reduced to a hydroperoxide. Conjugated diene hydroperoxides, such as **4**, are the simplest products of lipid peroxidation and are relatively stable in the absence of metals. However, metal complexes and metalloproteins are abundant in cells and they rapidly reduce all fatty acid hydroperoxides by one electron to alkoxyl radicals, which undergo multiple chemical reactions to generate a broad range of products (130,131). Thus, even the simplest initial lipid peroxidation products generate a complex range of epoxides, hydroperoxides, and carbonyl compounds, inter alia.

If the peroxyl radical is at an internal position in the fatty acid chain (e.g., **3**), cyclization to an adjacent double bond will compete with reduction to a hydroperoxide. The cyclization product (**5**) is a cyclic peroxide adjacent to a carbon-centered radical. This

Table 2: Oxidative DNA lesions

Lesion	Examples
Small oxidative base lesions	Thymine glycol, FaPy lesions, 8-oxodG, 8-oxodA
Strand breaks	Single strand breaks, double strand breaks
Crosslinks	DNA-protein, DNA intrastrand, DNA interstrand
Exocyclic adducts	εdA, εdC, 1, N^2-dεG, N^2 ,3-dεG, AdG, CdG
Intracyclic adducts	8,5′ -cyclopurine 2′ -deoxyribonucleoside
Alkylated adducts	N^7 – (2-hydroxyethyl) -2′ -deoxyguanine, M_1dA, M_1dC
I-compounds	Unknown

carbon-centered radical has two fates. The radical can couple with O_2 to form a peroxyl radical, which is reduced to a hydroperoxide (**6**) as described above. Alternatively, carbon-centered radical (**5**) can undergo a second cyclization to form a bicyclic peroxide, which after coupling to O_2 and reduction, yields a molecule (**7**) structurally analogous to the prostaglandin endoperoxide, PGG_2, albeit without the stereochemical control exhibited in the enzymatic reaction (132). Compound **7** serves as a common intermediate for the production of isoprostanes and malondialdehyde (MDA) through chemical conversion of the bicyclic peroxide group (132,133). The fragmentation that produces MDA generates a 17-carbon fatty acid as the other product (134). MDA has been used for many years as a convenient biomarker for lipid peroxidation because of its facile reaction with thiobarbituric acid to form an intensely colored chromogen (135). However, the thiobarbituric acid reaction is notoriously non-specific, which has led to substantial controversy over its use for quantitation of MDA from *in vivo* samples. Recently, isoprostanes have become popular biomarkers of lipid peroxidation and their use has been validated in several *in vivo* models (133).

The decomposed products of lipid hydroperoxides and lipid peroxyl radicals including cyclic endoperoxides, malondialdehyde (unsaturated aldehydes) and various hydroxyalkenals, may be biologically active, toxic, or mutagenic; they may further react with functional groups of proteins (inactivate enzymes (136)) or DNA (act as mutagens) (137), thus modifying such macromolecules (138,139). Moreover, a primary effect of lipid peroxidation is decreased membrane fluidity, which alters membrane properties and can significantly disrupt membrane-bound proteins (140).

2) Nucleic Acids

A major development over the past 20 years has been the realization that DNA damage and mutation arise from endogenous products of cellular metabolism (141). Types of oxidative DNA damage are given in **Table 2**. Oxygen radicals generated during reduction of O_2 can attack DNA bases or deoxyribose residues to produce damaged bases or strand breaks (142). Alternatively, oxygen radicals can oxidize lipid or protein molecules to generate intermediates that react with DNA to form adducts (143). Attempted replication of this damage leads to mutation or apoptosis (144). It appears that, cancer is linked to oxidative damage of DNA caused by a variety of chemical and physical agents.

The identity of the oxidants responsible for the production of oxidized DNA bases is still the focus of study. The hydroxyl radical ($^{\bullet}OH$) is an obvious candidate because it is extremely reactive and adds to DNA bases or abstracts hydrogen atoms to produce many of the products that occur in the genomic DNA (114). It is likely that, $^{\bullet}OH$ plays a role in the endogenous oxidation of DNA, but it is certainly not free $^{\bullet}OH$ generated in one component of the cell and diffusing to the nucleus. The reactivity of $^{\bullet}OH$ is so high that it does not diffuse more than one or two molecular diameters before reacting with a cellular component (13). To the extent that $^{\bullet}OH$ oxidizes DNA, it must be generated immediately adjacent to the nucleic acid molecule. It is likely that H_2O_2 serves as a diffusible source, latent form of $^{\bullet}OH$, that reacts with a metal ion in the vicinity of a DNA molecule to generate the oxidant (145,146).

Another oxidant that can generate many of the products observed with $^{\bullet}OH$ is peroxynitrite ($ONOO^-$) (70). Peroxynitrite is the coupling product of nitric oxide and superoxide, and is an extremely strong oxidant. Although $ONOO^-$ does generate small quantities of $^{\bullet}OH$, the protonated form of $ONOO^-$ (peroxynitrous acid, ONOOH; pK_a = 6.8) is an extremely reactive oxidant capable of oxidizing DNA, independent of its ability to generate $^{\bullet}OH$ (147). The pattern of products generated by DNA oxidation by $ONOO^-$ is complex and mirrors the diversity of oxidized DNA detected in tissues (148). Interestingly, $ONOO^-$ is more reactive towards 8-oxo-dG than to unmodified DNA bases. So, as 8-oxo-dG levels build up, they can compete with unmodified bases present in much higher concentrations for reaction with $ONOO^-$. Since nitric oxide and superoxide are produced simultaneously in macrophages, one anticipates that, elevated levels of $ONOO^-$ would be produced in activated inflammatory cells. In contrast to $^{\bullet}OH$, $ONOO^-$ has the ability to diffuse within cells and may be taken up by some cells via anion transporters (149). This may provide a link between inflammation and the induction of mutation by virtue of the ability of $ONOO^-$ to oxidize DNA. In fact, Ambs *et al.* recently demonstrated an association between the occurrence of G $\rightarrow$T transversions in the *p53* gene in human colorectal cancers and the level of expression of the inducible form of nitric oxide synthase (150).

Lipid peroxidation generates a variety of complex products, many of which are reactive electrophiles. Some of these react with protein and DNA, as a result are toxic and mutagenic. The major aldehyde products of lipid peroxidation are malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (151). MDA is mutagenic in bacterial and mammalian cells and carcinogenic in rats (152,153). HNE is weakly mutagenic, but appears to be the major toxic product of lipid peroxidation (154). MDA reacts with DNA to form multiple adducts to dG, dA and dC (139).

Oxidative radical damage occurs to a large extent in nucleic acids. Strand scission in DNA preparations exposed to high concentrations of oxygen radicals has been demonstrated (155,156). Oxidative damage to nucleic acids includes adducts of base and sugar groups, single- and double-strand breaks in the backbone, and cross-links to other molecules (**Table 2**). The components of DNA most susceptible to the free radical action are the thymine and cytosine bases, followed by adenine and guanine and finally the deoxyribose sugar. However, for double-stranded DNA, the deoxyribose moiety is modified more frequently than the bases, due to its external location in the helix (157). The spectrum of adducts in mammalian chromatin oxidized *in vitro* and *in vivo* consists of more than 20 known products, including damage to all four bases as well as thymine-tyrosine cross-links (**Table 2**) (158,159). The presence of oxidized DNA bases is often used as a marker for ROS-mediated DNA damage (160), and particularly for more sensitive guanine base. The electrochemical properties of the adduct 8-oxo-guanine (oxo8gua) and the deoxynucleoside 8-oxo-2,7-dihydro-2'-deoxyguanosine (oxo8dG), which have permitted the coupling of extremely sensitive electrochemical detection to high-performance liquid chromatography (HPLC), have resulted in hundreds of studies of its formation, accumulation, and excretion (161). The identification of specific enzymatic repair of oxidative lesions has recently provided proof for the significance of oxidative DNA damage as well as tools to manipulate the load of damage *in vivo* by genetic knockout (161-163).

Oxidized DNA bases will impair DNA function; such bases always exist at some basal level, and cells have numerous repair systems to remove such species (164). However, if they occur at critical sites, or are not quickly repaired, oxidized purines or

pyrimidines can cause functional problems. As a result, oxidation of DNA bases is considered an important event in chemical carcinogenesis (165).

DNA damage associated with oxidative stress/inflammation

Many types of cancer can be considered as degenerative diseases of old age and it is frequently suggested that, this is related to the effects of continuous damage over a life span by ROS and RNS (166,167). Malins and Haimanot first reported an association of oxidized DNA bases with breast cancer, demonstrating a 9-fold elevation in the levels of 8-oxo-G, 8-oxo-A and a formamidopyrimidine in tumor tissue compared with surrounding normal tissue (168). Subsequently, Shimoda *et al.* reported that the levels of 8-oxo-dG in liver tissue from individuals with chronic inflammatory diseases (hepatitis, cirrhosis) are elevated compared with control liver (169). Similar findings were reported in a transgenic mouse model that expressed the hepatitis B virus large envelope protein in the liver (170). Elevated 8-oxo-dG was detected in the early period of life and increased progressively with the progression of disease. The levels of 8-oxo-dG in the DNA from gastric tissue of patients infected with *Helicobacter pylori* are elevated relative to the levels in uninfected individuals (171). The levels are highest in patients with chronic atrophic gastritis, and reduced in patients with chronic non-atrophic gastritis as well as patients with gastric cancer. Individuals with hyperplastic diseases of the stomach exhibit higher levels of 8-oxo-dG than healthy controls (171). However, the link between cancer and inflammation is, by no means, a simple one. One chronic inflammatory disease in which patients suffer oxidative stress is rheumatoid arthritis. There is increased damage by ROS (172) [and probably also by RNS (173)] to lipids, proteins and DNA. Rheumatoid arthritis patients also show increased urinary excretion of 8-OHdG (174). Increased DNA damage, and increased susceptibility to killing by H_2O_2, have also been reported in lymphocytes from patients with autoimmune diseases: lymphocyte DNA from patients with rheumatoid arthritis, systemic lupus erythematosus, vasculitis and Behcet's disease contained significantly more 8-OHdG than that from healthy controls (175).

There is no clear evidence that rheumatoid arthritis patients develop cancer at an increased rate, certainly not at the most intense site of oxidative stress, the inflamed joint. Despite the apparent anomaly of rheumatoid arthritis, there is considerable evidence that ROS/RNS are somehow involved in the link between chronic inflammation and cancer

Table 3: Amino acids most susceptible to oxidation

Amino acids	Oxidation products
Cysteine	Disulfides, cysteic acid
Methionine	Methionine sulfoxide, methionine sulfone
Tryptophan	2-, 4-, 5-, 6-, and 7-Hydroxytryptophan, nitrotryptophan, kynurenine, 3-hydroxykynurinine, formylkynurinine
Phenylalanine	2,3-Dihydroxyphenylalanine, 2-, 3-, and 4-hydroxyphenylalanine
Tyrosine	3,4-Dihydroxyphenylalanine, tyrosine-tyrosine cross-linkages, Tyr-*O*-Tyr, cross-linked nitrotyro
Histidine	2-Oxohistidine, asparagine, aspartic acid
Arginine	Glutamic semialdehyde
Lysine	α-Aminoadipic semialdehyde
Proline	2-Pyrrolidone, 4- and 5-hydroxyproline pyroglutamic acid, glutamic semialdehyde
Threonine	2-Amino-3-ketobutyric acid
Glutamyl	Oxalic acid, pyruvic acid

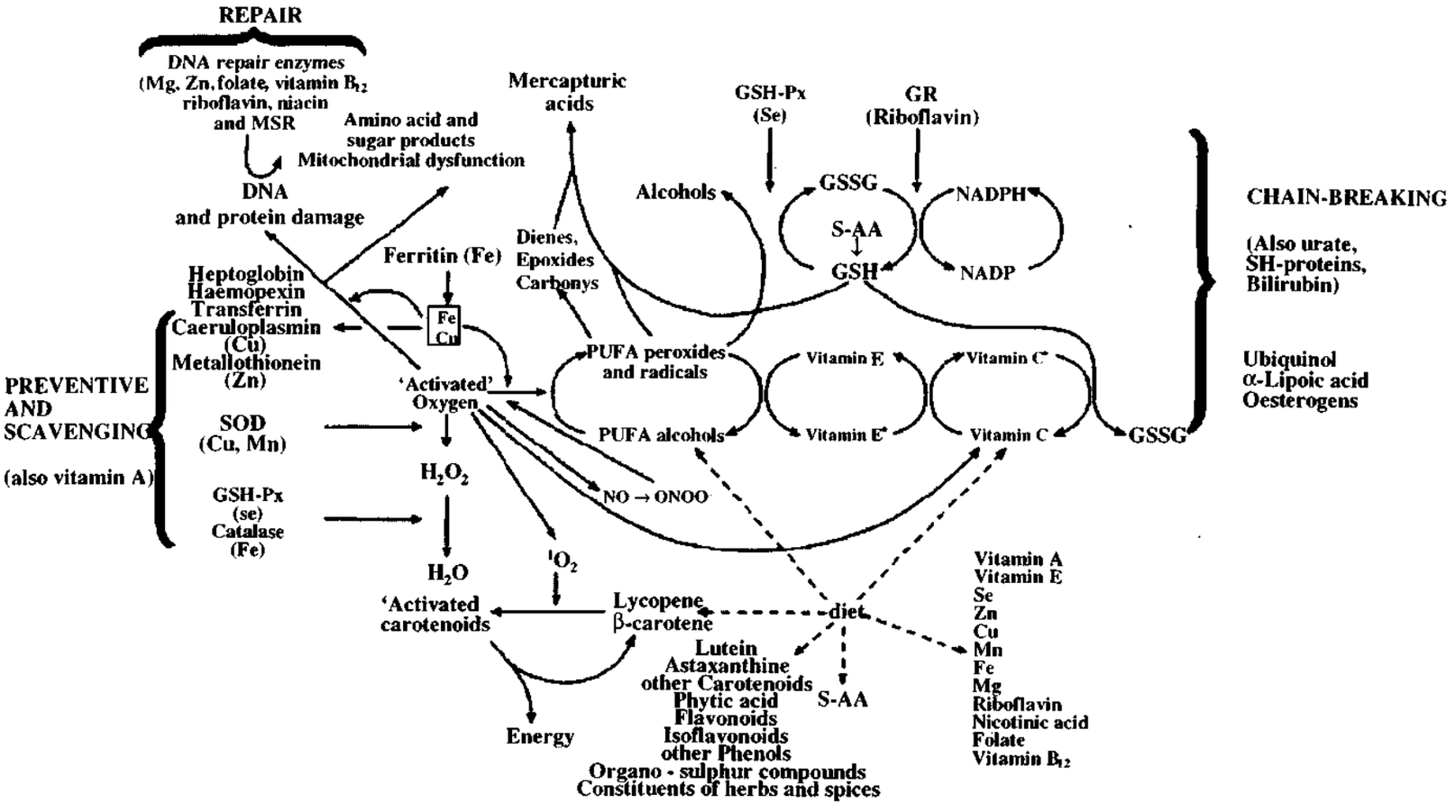
(170,176,177). A notable activity of tumor promoters is their ability to recruit inflammatory cells and to stimulate them to generate ROS/RNS. Indeed, there is a strong relationship between the capacity of tumor promoters to stimulate inflammatory cells to release ROS/RNS, and their capacity to promote tumors (177-179). Genetic damage and neoplastic transformation have been demonstrated in cells co-cultured *in vitro* with activated phagocytes (179). The genotoxic effects observed in these cells include the formation of DNA strand breaks (179), sister chromatid exchange (180) and mutations (181).

3) Protein Damage

The biological importance of oxidative damage to proteins has only recently been considered in detail. The delay in focusing attention on proteins perhaps reflects the considerable damage many proteins can sustain without impairment of their observed functions. Damage to proteins can occur by direct attack of ROS/RNS upon them, or by 'secondary damage', involving attack by end products of lipid peroxidation, such as MDA and HNE. The oxidation of proteins by ROS can generate a range of stable as well as reactive products (**Table 3**). However, the oxidation of proteins is less characterized. Several classes of damage have been documented, including oxidation of sulfhydryl groups, reduction of disulfides, oxidative adduction of amino acid residues close to metal-binding sites via metal-catalyzed oxidation, reactions with aldehydes, protein-protein cross-linking, and peptide fragmentation (182,183). Oxidized protein and amino acid species found in biological systems are listed in **Table 3**. A particularly intriguing recent development has been the realization that a number of enzymes possessing active-site iron-sulfur clusters are acutely sensitive to inactivation by $O_2^{\bullet-}$ (184,185). For example, *E. coli* aconitase is inactivated by $O_2^{\bullet-}$ with a rate constant of 10^9 $M^{-1}\cdot s^{-1}$ (186,187). Mammalian mitochondrial aconitase is inactivated *in vitro* and *in vivo* by treatments that increased mitochondrial $O_2^{\bullet-}$ generation, such as growth under hyperbaric conditions (21,188). Since aconitase participates in the citric acid cycle, its inhibition would be expected to have pleiotropic effects. Moreover, the mechanism of aconitase inhibition by $O_2^{\bullet-}$ has been demonstrated to involve the release of free iron from the enzyme (184). Free iron atoms catalytically exacerbate oxygen stress, and it has been proposed that superoxide's genotoxicity is a function of its ability to liberate protein-bound iron (189,190).

Table 4: Some antioxidants and proposed mechanism of action

Compounds	**Comment/mechanism (s) of Actions**
Transferrin, ferritin, caeruloplasmin, albumin	Protein binding/ inactivation of metal ions
Vitamin C (ascorbic acid)	Water-soluble chain-breaking antioxidant
Vitamin E (alpha-tocopherol)	Lipid-soluble chain-breaking antioxidant
n-Acetylcysteine	Glutathione precursor; scavenges hydroxyl radicals
21-Aminosteroids (e.g., tirilazad)	Scavenges lipid peroxyl and hydroxyl radicals
Catalase	Converts hydrogen peroxide to hydrogen and water
Co-enzyme Q10 (ubiquinone)	Lipid soluble mitochondrial antioxidant
Ebselen	Glutathione peroxidase-like action
Flavonoids	Inhibit lipid peroxidation; decrease LDL oxidation *in vitro*
Idebenone	Co-enzyme Q10 analogue
Lycopene	Non–provitamin A carotenoid; scavenges peroxyl radicals
Melatonin	Scavenges lipid peroxyl and hydroxyl radicals
Pyrrolopyrimidines	Similar to 21-aminosteroids; greater BBB permeability
Selegiline	Scavenges hydroxyl radicals; upregulates SOD and catalase
Selenium	Co-factor for glutathione peroxidase
Superoxide dismutase	Converts superoxide anion to hydrogen peroxide
Terpenoids	Inhibit iron-induced mitochondrial lipid peroxidation
Uric acid	Reduces peroxynitrite formation



Antioxidant defence system (after Strain, 1991).

***Proceedings of the Nutrition Society* 50:591-604.**

Fig. 8: Antioxidant defence system in vivo.

4) Carbohydrates

Carbohydrates are also targets of oxygen free radicals. Consequently, glycosylated proteins are more sensitive to oxidative damage (191,192). Radicals may oxidize monosaccharides, but they can also react with polysaccharides inducing their depolymerization. Patients with rheumatoid arthritis suffer from neutrophil accumulation in their joints (193,194). It has been demonstrated that these neutrophils overproduce reactive oxygen species responsible for depolymerization of hyaluronic acid, a glycosaminoglycan, necessary for maintaining synovial fluid viscosity in joints (195-197). As mentioned earlier, deoxyribose in nucleic acids is prone to oxidative degradation. This property has been exploited for the development of an assay for the formation of free radicals. In the TBA assay, deoxyribose yields a degradation product that is almost identical to the product obtained with MDA (50).

Ascorbic acid (vitamin C), a potent natural antioxidant, protects biomolecules against some highly carcinogenic radicals (198). During radical scavenging process, it gets oxidized easily to the product, dehydroascorbic acid (199-200).

Cellular Defenses against Oxygen Free Radicals

Our bodies are protected against free radical induced oxidative damage by various antioxidants with different functions, which constitute a defense system either independently, cooperatively, or even synergistically (**Fig. 8**). The functions and actions of the antioxidant defense have been studied extensively both *in vitro* and *in vivo*. The epidemiological studies imply the involvement of free radical-mediated oxidative damage in various diseases and also the protective role of various antioxidants. The therapeutic applications of natural and synthetic antioxidants have been explored.

There are enzymatic systems and chemical scavengers, which are able to remove oxygen free radicals formed in cells (**Fig. 8**). So-called primary or preventive defenses diminish the initiation rate of radical reactions by decreasing free radical concentration. Secondary or chain-breaking defenses, on the other hand, trap propagator radicals, stopping their harmful effects in early stages (11,201-204). Some antioxidants and their proposed mechanism of action are given in **Table 4**.

Table 5: Decomposition of hydroperoxides and hydrogen peroxide by enzymes.

Enzyme	Function
Glutathione peroxidase	Reduction of fatty acid hydroperoxides and hydrogen peroxide $LOOH + 2GSH \rightarrow LOH + H_2O + GSSG$ $H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$
Glutathione-S-Transferase	Reduction of fatty acid hydroperoxide
Phospholoipid hydroperoxide glutathione peroxidase	Reduction of phospholipid hydroperoxide $PLOOH + 2GSH \rightarrow PLOH + H_2O + GSSG$
Peroxidase	Reduction of fatty acid hydroperoxide and hydrogen peroxide $H_2O_2 + AH_2 \rightarrow 2H_2O + A$
Catalase	Reduction of hydrogen peroxide $2H_2O_2 \rightarrow 2H_2O + O_2$

Preventive Antioxidants

The first line of defense is the preventive antioxidants, which suppress the formation of free radicals. Although the precise mechanism and site of radical formation *in vivo* are not well elucidated yet, the metal-induced decompositions of hydroperoxides and hydrogen peroxide must be one of the important sources. To suppress such reactions, some antioxidants reduce hydroperoxides and hydrogen peroxide beforehand to alcohols and water, respectively without generation of free radicals, and some proteins sequester metal ions.

Table 5 summarizes the enzymes, which reduce hydroperoxides and hydrogen peroxide. Glutathione peroxidase, glutathione-S-transferase, phospholipid hydroperoxide glutathione peroxidase (PHGPX) and peroxidase are known to decompose lipid hydroperoxides to corresponding alcohols. PHGPX is unique in that, it can reduce hydroperoxides of phospholipids integrated into biomembranes (2). Glutathione peroxidase, peroxidase, and catalase reduce hydrogen peroxide to water.

Enzymes

Superoxide Dismutase: These are a family of metalloenzymes with different prosthetic groups, variable intracellular location and great tissue heterogeneity. The prevalent isozymatic form CuZnSOD has been found in almost all eukaryotic cells. Marklund and Marklund *et al.* have also found an extracellular high-molecular mass superoxide dismutase. Superoxide dismutase (EC 1.15.1.1) is the antioxidant enzyme that catalyses the dismutation of the highly reactive superoxide anion to O_2 and to the less reactive species H_2O_2 at a rate 104 times higher than spontaneous dismutation at physiological pH (reaction 1). Peroxide can be destroyed by CAT or GPX reactions (9,205,206).

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad [1]$$

In humans, there are three forms of SOD: cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD, and extra-cellular SOD (EC-SOD) (207,208). SOD destroys $O_2^{\bullet-}$ by successive oxidation and reduction of the transition metal ion at the active site in a Ping-Pong type mechanism with remarkably high reaction rates (209).

Catalase: Catalase (EC 1.11.1.6) is a tetrameric enzyme consisting of four identical tetrahedrally arranged subunits of 60 kDa each, that contain a single ferriprotoporphyrin group per subunit, and has a molecular mass of about 240 kDa (210). This hemoprotein is

located within the peroxisomes. Like SOD, CAT is widely distributed in tissues and removes H_2O_2 from the cell when the H_2O_2 is at high concentration (12,123,208,211). It reacts very efficiently with H_2O_2 to form water and molecular oxygen; and with H-donors (methanol, ethanol, formic acid, or phenols), possess peroxidase activity:

$$2\,H_2O_2 \xrightarrow{CAT} 2\,H_2O + O_2$$

$$ROOH + AH_2 \xrightarrow{CAT} H_2O + ROH + A$$

In animals, hydrogen peroxide is detoxified by CAT and GPX. Catalase protects cells from hydrogen peroxide generated within them. Even though CAT is not essential for some cell types under normal conditions, it plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of cells.

Glutathione Peroxidase: The selenium-containing peroxidase, glutathione peroxidase (EC 1.11.1.19), contains a single seleno-cysteine (Sec) residue in each of the four identical subunits, which is essential for enzyme activity (212). It is located in the cytoplasm of eukaryotic cells, though also found within mitochondria and in many kinds of tissues (213). GPX (80 kDa) catalyses the reduction of hydroperoxides using GSH as a co-substrate, thereby protecting mammalian cells against oxidative damage. In fact, glutathione metabolism is one of the most essential antioxidative defense mechanisms (214-216).

$$ROOH + 2GSH \xrightarrow{GPX} ROH + GSSH + H_2O$$

$$H_2O_2 + 2GSH \xrightarrow{GPX} GSSH + 2H_2O$$

There are five GPX isoenzymes found in mammals. However, their expression is ubiquitous, the levels of each isoform vary depending on the tissue type. Although GPX shares the substrate H_2O_2 with CAT, it alone can react effectively with lipid and other organic hydroperoxides, being the major source of protection against low levels of oxidant stress.

Glutathione Reductase: It is a cytosolic flavoprotein with a tissue distribution similar to that of glutathione peroxidase. The enzyme reduces oxidized glutathione utilizing NADPH generated by various systems (12,217) to keep the glutathione pool in the cell in a very reduced state. Even when large amounts of hydrogen peroxide are present, this enzyme is

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very effective at reducing the cellular glutathione pool. The net result of this cycle is to use NADPH to reduce hydrogen peroxide to water, a process that requires two electrons.

$$GSSG + NADPH \Rightarrow 2\ GSH + NADP^{+}$$

Other enzymes: Enzymes such as DT-diaphorase or epoxide hydrolase are also considered to be primary antioxidant defenses (218-220).

Other proteins: The transition metal ions such as iron and copper are also accepted to play a vital role in the formation of free radicals *in vivo* through Fenton-type reactions that they catalyze. Nevertheless, when these metals are linked to proteins, they are unable to carry out this catalysis. Transferrin, ferritin, and lactoferrin sequester iron, while albumin and ceruloplasmin sequester copper (49,221,222). Heme and heme proteins can act as pro-oxidants, but they must often be released from damaged cells. Plasma contains hemoglobin-binding haptoglobin, as well as heme-binding hemopexin. Binding of hemoglobin to haptoglobin, or heme to hemopexin, decreases the effectiveness of these compounds in stimulating lipid peroxidation. Ceruloplasmin is an important copper chelating protein present in plasma; it contains six tightly-bound copper ions and often a seventh, which is less tightly bound. Wilson's disease, an inherited metabolic defect characterized by low concentration of ceruloplasmin in the blood, is accepted to be induced by copper-stimulated free radical oxidations. The chelating agents such as penicillamine, which promote copper excretion, are used for treatment of Wilson's disease.

Small molecules: There are a certain number of small molecules, widely distributed in biological systems, which can scavenge oxygen free radicals non-enzymatically and are thought to be important in antioxidant defence. These can be divided into compounds made *in vivo* and compounds obtained from the diet (**Table 4 and Fig. 8**). Glutathione (GSH), vitamin C, uric acid, bilirubin, carotenoids such as β-carotene and lycopene, taurine and hypotaurine are some of these small molecules.

GSH can either act by reducing peroxides to H_2O and oxidized glutathione (GSSG) by means of glutathione peroxidases or react directly with oxygen radicals, first forming the thiyl radicals and later GSSG (11,223). Like GSH, vitamin C may reduce oxygen free radicals. Dehydroascorbate formed in this reaction may be reduced by GSH (224). Uric acid and bilirubin are antioxidants, which trap free radicals very efficiently (2). This compound is an important protector against oxidation in plasma (12,225,226). Carotenoids

quench singlet oxygen quite rapidly and inhibit the hydroperoxide formation (singlet oxygen oxidizes unsaturated lipids rapidly to give lipid hydroperoxides which are important precursors of alkoxyl and peroxyl radicals). It has been demonstrated that taurine and hypotaurine also have a protective function preventing damage by free radicals. These compounds are present in many biological fluids (227).

Radical-Scavenging Antioxidants

Although the generation of free radicals *in vivo* is minimized by the above-mentioned preventive primary antioxidants, small amounts of free radicals may be formed *in vivo* or taken into body exogenously. The second line of defence is the antioxidants, which scavenge the active radicals to suppress chain initiation and / or break the chain propagation reactions. Various endogenous radical-scavenging antioxidants are known; some are hydrophilic while others are lipophilic. Vitamin C, uric acid, albumin and thiols are hydrophilic radical-scavenging antioxidants, while vitamin E, bilirubin, ubiquinol are lipophilic radical-scavenging antioxidants. The function of carotenoids as a radical-scavenging antioxidant has also been reported (228) in addition to the quenching of singlet oxygen.

The antioxidant activities of radical-scavenging antioxidants are determined not only by their inherent chemical reactivities and concentrations but also by the location and its mobility in the microenvironment. Vitamin E is accepted as the most potent radical-scavenging lipophilic antioxidant, but it has been shown that its mobility, especially vertical mobility, in the membranes is very low and hence its efficiency for radical scavenging is reduced in the membranes (229).

Other important factors which determine the overall antioxidant activities are the fate of the radicals derived from the antioxidants and the sites of their formation. In general, the radical-scavenging antioxidant donates hydrogen to active radicals and a new radical is formed from the antioxidant. This antioxidant-derived radical may undergo various reactions. It may scavenge another radical to give non-radical product, react with reducing agent to regenerate vitamin E, or under some circumstances, it may react with lipids or lipid hydroperoxides to give active radicals, which initiate another chain reaction. The relative importance of these reactions, and hence the overall antioxidant activity, depend on various conditions such as concentration of radicals and reducing agents.

The site of free radical formation is also important in determining the antioxidant activity. The hydrophilic antioxidants such as vitamin C effectively scavenge aqueous radicals. but they cannot scavenge radicals within the membranes and hence not a chain-breaking antioxidant. Even a small fraction of radicals formed initially in the aqueous phase, but penetrated into the membrane, induce the chain reaction and amplify the damage. The lipophilic antioxidants are responsible for scavenging such lipophilic, chain-carrying radicals within the membranes.

Vitamin E (α-tocopherol): α-Tocopherol, the major constituent of the fat-soluble vitamin known as vitamin E, is the most important (230) [but by no means the only (231)] free radical scavenger within membranes and lipoproteins, and is a major chain breaking antioxidant (232), but has limited mobility which restricts its efficacy (233). It protects the PUFA in the membranes against peroxidation by scavenging peroxyl radicals, which are intermediates in chain reaction: $\alpha TH + LOO^{\bullet} \rightarrow \alpha T^{\bullet} + LOOH$

Ascorbic acid: Ascorbic acid (vitamin C), is the most abundant water-soluble antioxidant in the body, which plays several essential metabolic roles *in vivo*, such as, in the synthesis of collagen. Ascorbic acid is indeed a good scavenger of several ROS (234). One of its major antioxidant activities is its ability to regenerate α-tocopherol from the tocopherol radical that forms at membrane surfaces. It is a powerful scavenger of hypochlorous acid, superoxide, singlet oxygen and hydroxyl radicals and protects against oxidants present in cigarette smoke (234). However, in the presence of transition metal ions (iron and copper), ascorbate can become pro-oxidant, acting as a reducing agent and generating superoxide, H_2O_2 and hydroxyl radical. Normally, since such metal ions are available in very limited amounts *in vivo*, the antioxidant properties of ascorbate predominates (235). However, ascorbate can be toxic if given to iron-overloaded patients without iron chelators (236). In disease and tissue injury, transition metal ions do sometimes become more available, and the possibility that pro-oxidant actions of ascorbate might occur should not be ignored (31,237).

Uric acid: Uric acid is primarily considered as the waste product of purine metabolism by the action of xanthine oxidase and dehydrogenase enzymes from hypoxanthine and xanthine. Urate accumulates in human plasma to concentrations normally in the range of 0.2-0.5 mM, and is excreted in the urine. It is also present intracellularly and in all other

body fluids, usually at somewhat lower levels. It is a relatively powerful, circulating, scavenging antioxidant of water-soluble radicals. It scavenges hydroxyl radical, hypochlorous acid, peroxynitrite and singlet oxygen and it protects red cells from peroxidation lysis. It can also bind copper and iron and act as a transition metal ion chelator. It has recently been reported that uric acid is the major (< 70%) antioxidant in saliva.

Carotenoids (vitamin A): Carotenoids are a set of several hundreds of fat-soluble pigments present in yellow and green fruits and vegetables. In addition to being precursors of vitamin A, these pigments are also excellent antioxidants and radical-trapping agents, especially for peroxyl and hydroxyl radicals (228,238), and also good singlet oxygen quenchers (239). Carotenoids like β-carotene have conjugated double bonds to attack and quench radicals (240), but currently little is known about the interactions of carotenoids and ROS. Carotenoids serve as another dietary source of lipid-soluble antioxidants, which are important in protecting lipid membranes against oxidation.

Flavonoids: Flavonoids are a large group of polyphenolic antioxidants, that occur naturally in fruits and vegetables and in beverages such as tea and wine. The most important flavonoids are anthocyanins, flavonols and flavones. Flavonoids are water-soluble scavengers of singlet oxygen, superoxide, peroxyl, and lipid peroxyl radicals. Flavonoids are good metal ion chelators, and they are site specific scavengers too. Hence they are powerful antioxidants (241). However, some flavonoids such as quercetin have been reported to be mutagenic (242), co-carcinogenic (243) and some are prooxidants, which in presence of metal ions generate radicals (244).

Ubiquinone (coenzyme Q): Ubiquinone is a vital component of mammalian cell mitochondria and performs an important function in the hydrogen-electron transfer. It has also strong antioxidant properties in its reduced form (UQH_2). The recent discovery of another powerful antioxidant coenzyme, pyrroloquinolinequinone (PQQ), a neighbour of Q_{10} in the mitochondrial electron transfer chain, may prove to be more effective at inhibiting superoxide release by PMNLs (245).

Bilirubin: Bilirubin and biliverdin are heme degradation products in mammals. Biliverdin is the precursor of bilirubin. Bilirubin tightly binds to albumin in a 1:1 ratio. It has been recently suggested that both bilirubin and biliverdin break the chain of damage propagation

by reacting with the oxygen free radicals. Bilirubin is an efficient scavenger of peroxyl radical and singlet oxygen. It protects both protein and albumin bound fatty acids. However, there is little direct evidence that bilirubin is an important antioxidant *in vivo* (246,247).

Repair and *de novo* antioxidants

The third line of defense is the repair and *de novo* antioxidants (248,249). Various phospholipases repair the oxidatively damaged phospholipids and their activities are known to be stimulated by oxidation. Among others, phospholipase A_2 has received much attention since this enzyme is assumed to selectively cleave peroxidized lipids from the membranes, thus preventing the accumulation of toxic products. Upon action of phospholipase A_2 on peroxidized phospholipid, lysophospholipid and free fatty acid hydroperoxide are formed. The released fatty acid hydroperoxide is reduced by peroxidases to the corresponding alcohol and detoxicated. The lysophospholipid is reacylated by a fatty-acyl-coenzyme A to complete the repair.

Different oxidoreductases that catalyze reduction reactions of thiol and other protein groups when these are oxidatively damaged, are protective enzymes against oxygen free radicals. Oxidatively modified proteins can be involved in reactions harmful to cells. As a result, the degradation of irrepairably damaged protein contributes to the host defense. Proteasomes and other mammalian proteolytic enzymes have been demonstrated to be responsible for this process (11,250-252).

Nuclear enzymes for DNA repair may be considered to be involved in the defense against oxidative injury by oxygen free radicals. For example, when an apurinic or apyrimidinic site is formed by oxidative damage and the DNA replication is stopped, DNA polymerase I and DNA ligase act repairing the break. There are other enzymes that have a critical role in protecting the cellular DNA and the flux of genetic information, as in the case of endonucleases and glycosylases. Activities of these enzymes have been described in human as well as in other eukaryotic organisms (251,253). However, the specific role of these proteins in repairing DNA is still unclear. As with proteins, when the DNA damage is too extensive to be repaired, cells have to be eliminated. Cytotoxicity can be initiated by the poly(ADP-ribose)synthetase when this lowers NAD^+ levels. The enzyme, which is stimulated by DNA strand breaks, transfers ADP-ribose groups of NAD^+ to amino acids or

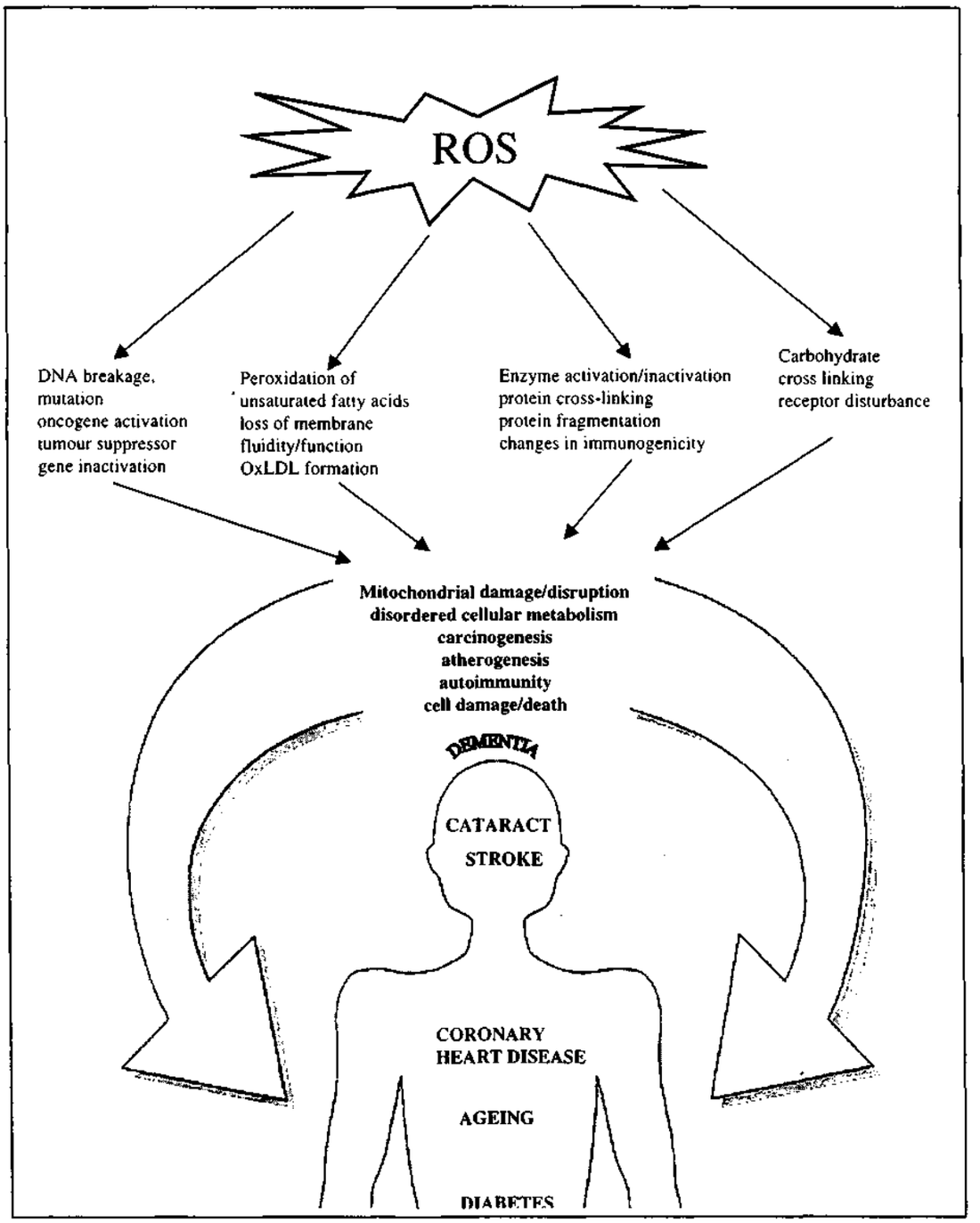

Fig. 9: The possible role of reactive oxygen species (ROS) in inducing disease

Table 6: Reactive Oxygen Species and Human Diseases

Human Disease	
Allergy	**Genetic and metabolic disorders**
Bronchial asthma	
Intolerance to aspirin	Chronic granulomatous
Intolerance to foods	disease
Response to mercury	Diabetes
Response to other drugs	Down's syndrome
Response to other oxidants	
	Infectious diseases
Cancer	*Helicobacter pylori*
	Hepatitis
Bladder	HIV
Bowel	*Influenza* virus
Breast	Pneumonia
Colorectal	Rheumatoid arthritis
Esophageal	
Kidney	**Neurodegenerative diseases**
Leukemia	
Liver	Allergic encephalomyelitis
Lung	Alzheimer's disease
Prostate	Amyotrophic lateral sclerosis
Skin	Huntington's disease
Cardiac and vessels injuries	Parkinson's disease
	Prion disease
Atherosclerosis	
Ischemia	**Ophthalmologic problems**
	Cataract
	Glaucoma

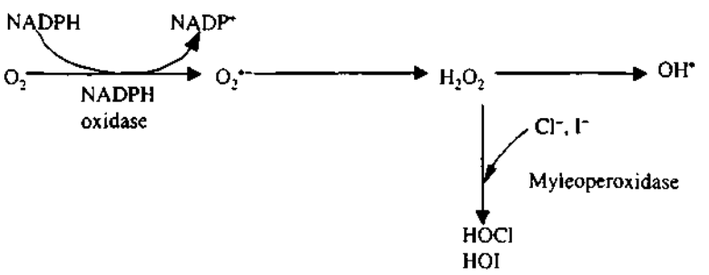

Fig. 10: Production of reactive oxygen species during the 'respiratory burst'

to other ADP-ribose groups previously linked to the protein. In this way, the DNA unpacking is allowed (251-254).

Pathological processes that involve oxygen free radical reactions (Fig. 9)

There is evidence to implicate oxygen free radicals in the development of a number of tissue injuries. Although a detailed discussion of each of these is not possible here, several representative examples with clinical relevance will be considered. **Table 6** presents a list of diseases that involve radical reactions in mammalian systems.

Inflammatory Diseases: Free radicals play a major role in inflammation (39,47,123). This process, which is the response of the host organism to injury, involves enhanced vascular permeability with edema formation and leukocyte infiltration into the damaged area. When polymorphonuclear leukocytes and macrophages are activated by contact with a foreign substance, they increase their consumption of oxygen, which is transformed into the superoxide radical. This is then converted into H_2O_2 by a dismutation reaction. Finally, H_2O_2 is converted into a hydroxyl radical. This 'respiratory burst' is due to the enzyme complex NADPH oxidase, which is associated with the plasma membrane (**Fig. 10**). As each foreign particle is engulfed by a phagocytic vacuole, it is exposed to the toxicity of oxygen free radicals. In addition, lysosomal myeloperoxidase, which is released into the vacuole, can oxidize halides at the expense of H_2O_2 to form hypohalous acid, a strong oxidant capable of reacting with many biological molecules (**Fig. 10**).

Rheumatoid arthritis, characterized by chronic joint inflammation, is a disease frequently studied as an example of many inflammatory states. Synovial fluid of rheumatoid arthritis patients contains abundant neutrophils as well as other kinds of inflammatory cells (lymphocytes, activated macrophages, etc). The presence of neutrophils and macrophages in the inflamed joint raises the possibility that reactive oxidants may be, at least partly, responsible for the damage sustained in rheumatoid arthritis. Neutrophils and macrophages from synovial fluids of patients with rheumatoid arthritis has shown enhanced $O_2^{\bullet -}$production, possibly because of their exposure to cytokines present in the synovial fluids (255). Iron, a catalyst for hydroxyl radical production from H_2O_2, is present in greater amounts in synovial fluids and tissue from patients with rheumatoid arthritis than in controls (256). Between them, the studies of oxidant damage and phagocyte function strongly suggest that reactive oxidants are important mediators in the pathogenesis of

rheumatoid arthritis. The radicals depolymerizes the hyaluronic acid, as a result it loses its lubricant properties. The cartilage matrix is also susceptible to free radical damage, especially the polypeptide part of the proteoglycan. The enzyme elastase, released by neutrophils, plays a major function in the deterioration of rheumatoid joints.

There is enough evidence to suggest that oxidative damage occurs during inflammation and iron plays an important role during inflammation, for example, the effectiveness of free radical scavengers in ameliorating symptoms of inflammatory diseases (257,258). However, it is still not clear why, in specific circumstances, the inflammatory response is overactivated.

Retrolental fibroplasia: The eye is not a very susceptible organ to free radical action because it possesses a great quantity of antioxidant systems. However, a large number of ocular complaints are associated with oxidative damage. The importance of oxygen free radicals in the pathogenesis of disease processes in the cornea and lens has been demonstrated. But, amongst the components of the eye, the retina is the most sensitive to free radical oxidation, due to fundamentally its high polyunsaturated fatty acid content (2,259-261).

Lung injury: The lung is an organ greatly affected by free radical production. Long periods of exposure to high oxygen pressures damage the lung of different animal species, causing many diseases (pulmonary emphysema, bronchopulmonary dysplasia, adult respiratory distress syndrome, etc) and even death (2,262). Oxygen free radicals and other toxic products formed by the lung cells themselves and by activated neutrophils that accumulate in the lung when pure oxygen is breathed, may possibly contribute to the hyperoxidant damage. Two hypotheses have been proposed to explain recruitment and activation of neutrophils into lungs as a result of high oxygen tension breathing: lung hyperoxia-damaged macrophages release chemotaxins that attract and activate neutrophils, or neutrophils adhere directly to the lung endothelial cells damaged by hyperoxia (263-265).

Tobacco has been suggested as being a contributory factor in the appearance of lung pathogenesis. Cigarette smoking is associated, for example, with pulmonary emphysema. There is an association between this illness and α_1-protease inhibitor deficiency (264-266). α_1-Protease inhibitor is the main serum antiprotease and is

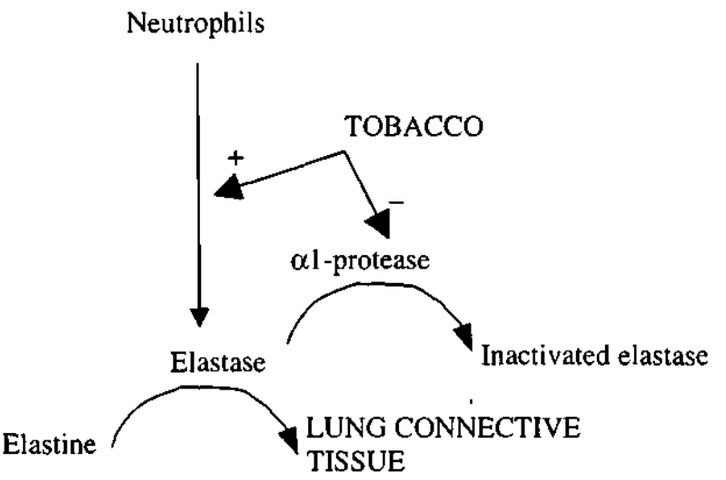

Fig. 11: Involvement of tobacco in pulmonary emphysema

responsible for the majority of antielastase activity. Smoking impairs the ability of this antiprotease to protect lung elastin from neutrophil proteases because free radicals contained in cigarette smoke inactivate this protein. In addition, these free radicals also increase accumulation of neutrophils in the lung. The recruited and activated neutrophils can cause further damage to the lung through oxy-radical generation. The final result is the destruction of lung connective tissue elastin (**Fig. 11**) (267,268).

Atherosclerosis: Recent studies suggest that oxygen free radicals might also be involved in the development of atherosclerosis. It has been demonstrated that, one of the earliest events which occur in atheroma formation is the accumulation of cholesterol-laden foam cells in the subendothelial space. Most of the cholesterol deposited in these cells is derived from LDL. However, macrophages, the main precursors of the foam cells, do not take up low density lipoproteins at a rate rapid enough to cause lipid loading (269-271). The removal of LDL from plasma has long been known to be mediated by the LDL receptors. Nevertheless, there is evidence to suggest that arterial uptake of LDL to give rise to foam cells is a long pathway independent of LDL receptors (272,273). Goldstein *et al.* (269) have shown that certain chemical modifications of LDL result in their rapid uptake in macrophages. Modified LDLs are taken up by a specific receptor named 'scavenger receptor' or 'acetyl LDL receptor', which is not regulated by cellular cholesterol. As a result, massive accumulation of cholesterol esters occurs in macrophages *in vitro* (192,269,270,273,274).

Free radical oxidation of LDL is one of the biological modifications occurring *in vivo,* that increases the rate at which LDLs are taken up by macrophages (192,274,275). Human LDL is not only rich in cholesterol, but also in PUFA which are susceptible to lipid peroxidation. As a consequence of the peroxidation process, fatty acid chains can be fragmented, leading to the formation of very reactive shorter chain metabolites. These lipid peroxidation products may bind to apoprotein B by covalent linkages to ε-amino groups of lysine residues, giving rise to modified apoprotein B, which is recognized by 'scavenger receptor' (276,277).

SOD has been found to inhibit the oxidation of LDL, suggesting that the superoxide radical is responsible for the process, however, metal ion chelators and other general free radical scavengers can also prevent this oxidation (278,279). Superoxide has

been demonstrated to be necessary for the initiation of oxidative modification of LDL. However, other lipid oxyradicals, formed especially in the presence of metal ions, are involved in the propagation of the oxidative modification (115,271,273,280).

Aging: A number of theories have been proposed to explain the nature of aging and one such is the free-radical theory (281-283). According to the free-radical theory of aging, these very reactive species produced continuously during normal metabolism eventually accumulate, damaging DNA and other macromolecules. This is due to progressive defects in the defense systems against reactions that generate free radicals. The result is the appearance of degenerative lesions and cellular death. Thus the organism ages and finally, dies.

Cancer: Carcinogenesis is the malignant transformation of a cell or group of cells. This multi-step process can be divided into two main stages: initiation and propagation. The initiation phase is caused by a single exposure to a carcinogenic agent and involves an irreversible modification of the genetic material of the cells. On the other hand, promotion requires multiple exposures to the promoters to alter gene expression and produce a tumor. In contrast to initiation, the promotion stage is reversible (225,254,281,284).

Although there are some oxygen free radicals, which are complete carcinogens, these very reactive metabolites mainly play an important role in the promotion phase of carcinogenesis. Cerutti has proposed a model to explain the oxygen free radical mediated alteration of gene expression (254). Free radicals would cause breaking of DNA, which would stimulate the poly (ADPribose) synthetase to act. Simultaneously, oxidative stress produced by oxygen free radicals would give rise to increased levels of NAD^+ which would be a substrate for the enzyme. As a result, the poly ADP-ribosylation of the chromosomal material would rise and, consequently, gene expression would be modified.

Neurological Disorders: Neural tissue may be particularly susceptible to oxidative damage because it receives a disproportionately large percentage of oxygen and has a high concentration of polyunsaturated fatty acids which are highly prone to oxidation (2,285,). Moreover, brain is not particularly enriched in antioxidant defenses (286). In fact, brain is lower in catalase activity about 10% as compared to that of liver (2,287). Additionally, human brain has higher levels of iron (Fe) in certain regions and in general, has high levels of ascorbate. Thus, if tissue organizational disruption occurs, the Fe/ascorbate mixture is

Table 7: Xenobiotics whose toxicity can be attributed to free radical generation.

Xenobiotic	Use
Adrimycin (doxorubicin)	Anticancer drug
Bleomycin	Anticancer drug
Carbon tetrachloride	Organic solvent
Chloroform	Organic solvent
Cigarette smoke	Air pollutant
Daunomycin A (daunorubicin)	Anticancer drug
Ethanol	Alcoholic drink
Halothane	Anaesthesic
Hydralazine	Antihypertensive drug
Iproniazid	Antidepresent drug
Isoniazid	Antimycobacterial drug
Metronidazole	Antimicrobial drug
Mitomycin C	Anticancer drug
Nitrofurantoin	Antimicrobial drug
Nitrogen dioxide	Air contaminant
Paracetamol (acetaminophen)	Analgesic and antipyretic drug
Paraquat (methylviologen)	Herbicide
Sulfonamides	Antibacterial drug

expected to be an abnormally potent pro-oxidant for brain membranes (2,288). In addition, many neurotransmitters are autoxidizable molecules, which react with O_2 to generate superoxide, H_2O_2 and reactive quinones/semiquinones that can deplete GSH and bind to protein –SH groups.

The imbalance between cellular production of ROS and ability of cells to defend against them is referred to as oxidative stress. Oxidative stress can cause cellular damage and subsequent cell death because of ROS, oxidize critical cellular components, such as lipids, proteins, and DNA. Neural cells such as substantia nigra would undergo degeneration and death and hence responsible for neurodegenerative disorders like Parkinson's disease, Alzheimer's disease, Huntington's disease, multiple sclerosis, amylotrophic lateral sclerosis, etc (2,289,290)(**Table 6**).

Drug Toxicity: Toxicity of certain drugs has been associated with oxidative stress (2,12,291-294). Many xenobiotics can be reduced by one electron producing species that react with oxygen to produce the superoxide radical and regenerate the original molecule. The one electron reduction reaction is normally catalyzed by a flavoprotein such as the NADPH-cyt P450 reductase. This process, named 'redox cycling', involves disproportionate consumption of molecular oxygen and redox equivalents. In this way, active oxygen species are formed producing a continuous oxidative stress in the cell, which give rise to devastating consequences. **Table 7** shows chemicals whose toxicity can be attributed to oxygen free radicals.

Xenobiotic Metabolism

Living systems are constantly being exposed to various types of chemicals, which are termed as "**xenobiotics**". These chemicals, either present in the food or in the environment when ingested, get metabolized predominantly in the liver. These organic molecules are metabolized in the liver essentially by a group of enzymes known as cytochrome P450 system. (2). The metabolism of xenobiotics takes place in two phases, phase I and phase II. Phase I reactions introduce (or sometimes unmask) a polar functional group within the molecule, often by oxidations involving cytochromes P450. Other enzymes, such as esterases, monoamine oxidases and alcohol dehydrogenases, can sometimes do the same thing. Concentrations of 'total P450' vary widely between tissues,

the liver having the highest. Some of the cytochromes P450s are inducible; e.g. barbiturates and ethanol can raise levels of certain cytochrome P450.

Phase II reactions are conjugation reactions: an endogenous molecule is added to the phase I reaction product, or sometimes directly to the xenobiotic. Some of the enzymes involved in phase II reactions are; glutathione transferases, glucuronyl transferases, sulphotransferases, methyltransferases, N-acetyltransferases, etc.

Carbon Tetrachloride (CCl_4) and Liver Injury

CCl_4 was the first toxin for which it was showed that the liver injury it produces is largely or entirely mediated by a free-radical mechanism (2,295,296). CCl_4 is a colorless liquid, immiscible with water, used in industry as a 'degreaser' and organic solvent. CCl_4 was formerly employed as a dry-cleaning agent, although the latter use was banned in the USA in 1970 in favour of less toxic agents. When introduced into medical practice in 1847, CCl_4 was enthusiastically promoted as an anaesthetic (soon phased out in favour of chloroform after several deaths due to liver failure) and anti-liver fluke agent.

The lipid-solubility of CCl_4 allows it to cross cell membranes, and any CCl_4 ingested is distributed to all organs. However, its main toxic effects are shown on the liver (and it is much more toxic than chloroform) although there is some injury to other tissues. Toxic levels of administered CCl_4 to animals produce fat accumulation in the liver due to a blockage in synthesis of the lipoproteins that carry triglyceride away from this organ. The structure of the liver cell endoplasmic reticulum becomes distorted, hepatic protein synthesis slows down, and the activity of enzymes located in the endoplasmic reticulum, such as glucose-6-phosphatase and cytochrome P450, rapidly declines, as does the ability of the endoplasmic reticulum to sequester Ca^{+2} ions by the Ca^{+2}-ATPase. Hence rise in intracellular Ca^{+2} concentrations occur. All these events eventually lead to necrosis of liver in the central areas of the lobes.

Despite this evidence that the toxicity of CCl_4 involves the endoplasmic reticulum, incubation of microsomal fraction with CCl_4 *in vitro* does not cause damage. However, in the presence of NADPH, the microsomal fraction which contains both cytochrome P450 and cytochrome P450 reductase generates a more damaging product from CCl_4. This event results in a rapid peroxidation of microsomal lipids accompanied by the inactivation of the above enzymes, and destruction of cytochromes P450. Microsomal fractions isolated from

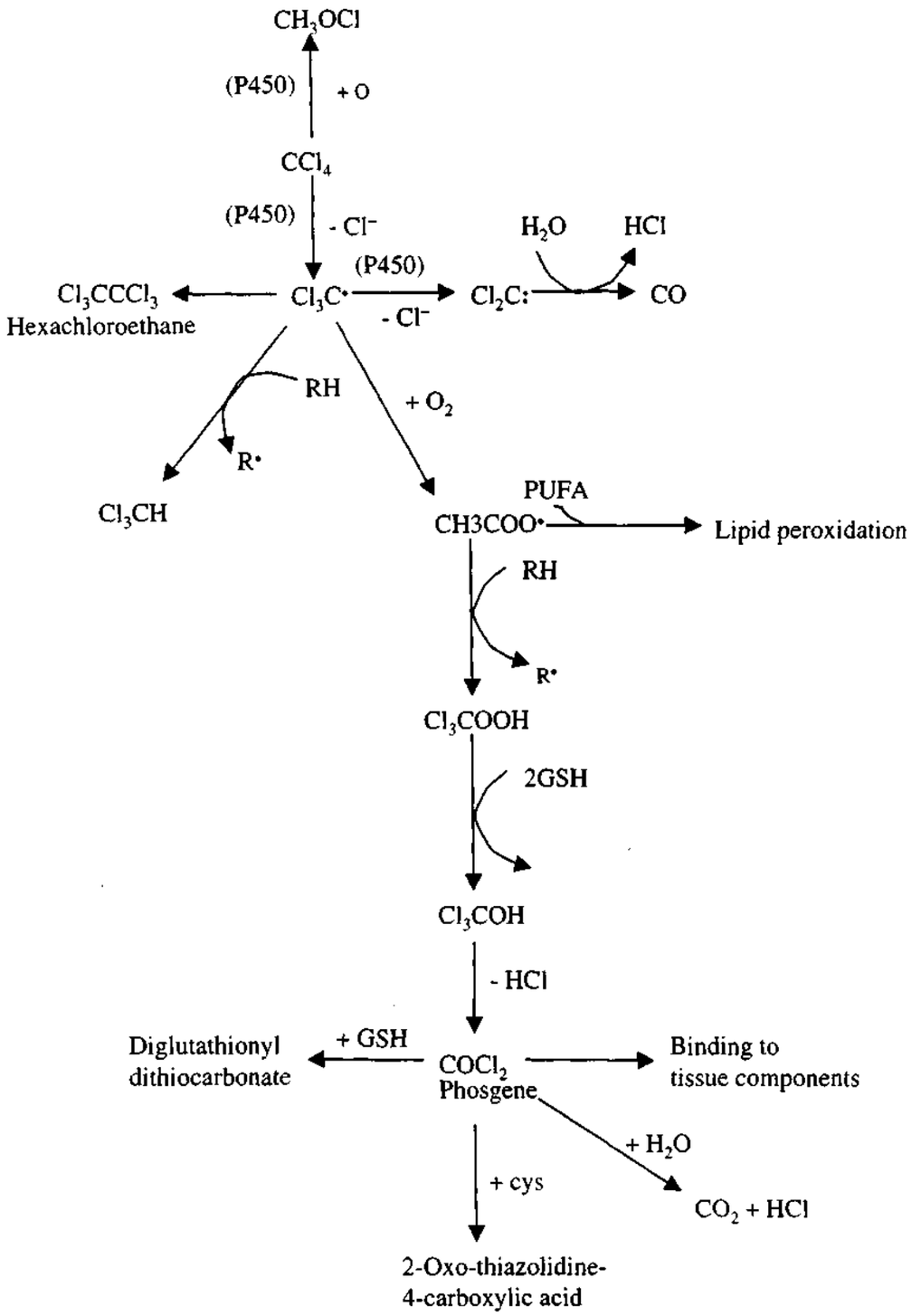

Fig. 12: Metabolic pathway of carbon tetrachloride

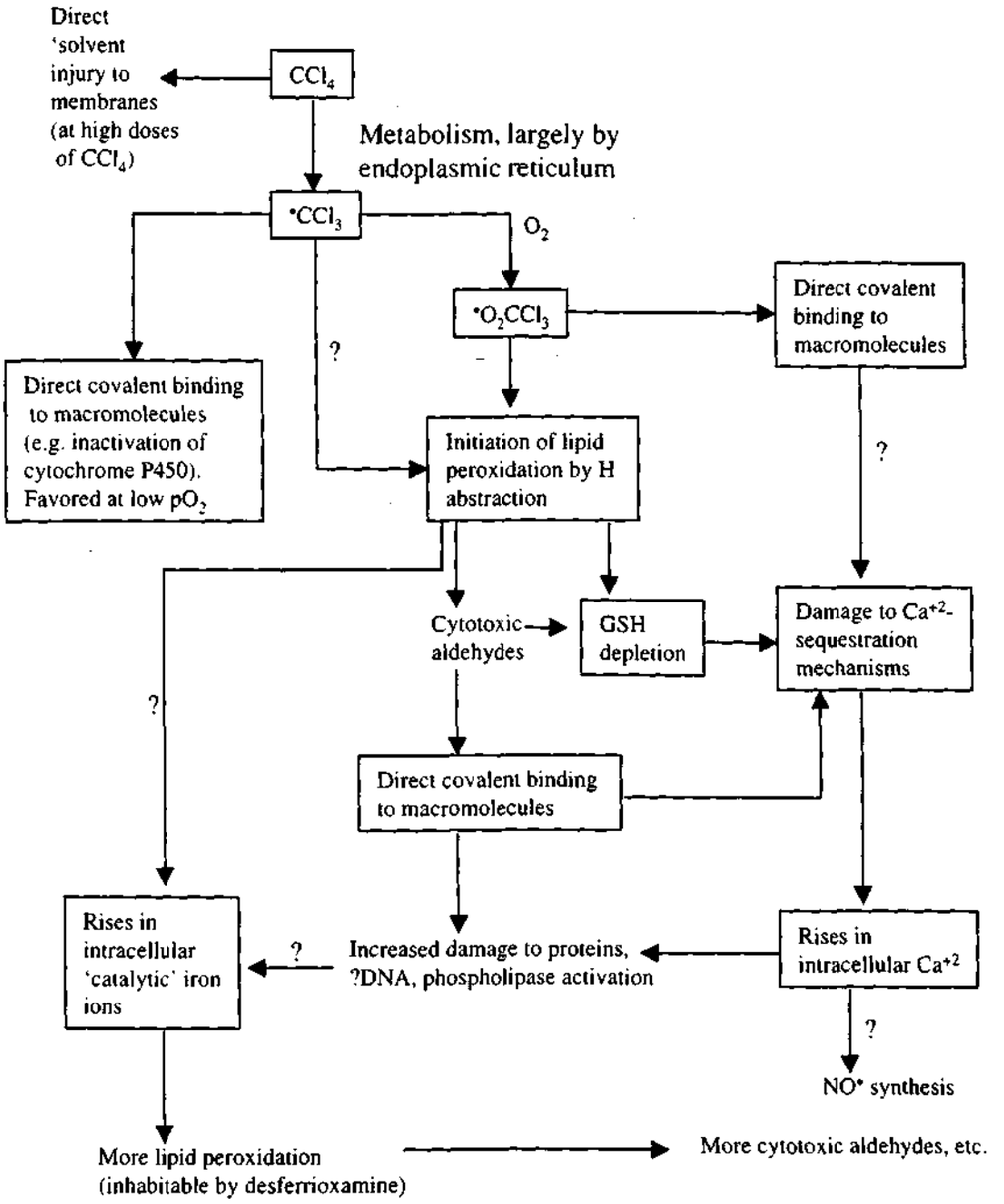

Fig. 13: Mechanisms of hepatotoxicity of CCl_4

CCl_4-treated rats show increased peroxidation. Levels of F_2-isoprostanes, products of arachidonic acid peroxidation, increase up to 50-fold in the plasma of rats given CCl_4.

Administration of a wide range of antioxidants, (including vitamin E, promethazine, propyl gallate and GSH) or inhibitors of P450, decreases CCl_4 toxicity in parallel with decreased lipid peroxidation in animals. Vitamin E-deficient animals are more susceptible to CCl_4. Neonatal animals have low P450 levels and are more resistant to CCl_4 toxicity.

As a result of various observations, it is believed that CCl_4 is metabolized by the P450 system to give $CCl_3^{\bullet}$, a carbon centered radical. In fact, several P450s are involved in the formation of carbon centered radical, including CYP2E1, the 'ethanol-inducible' cytochrome P450. The $CCl_3^{\bullet}$ radical might combine directly with biological molecules, causing covalent modification as well as abstracting hydrogen from membrane lipids, setting off the chain reaction of lipid peroxidation. Products of peroxidation are known to inhibit protein synthesis and the activity of certain enzymes. In fact, liver microsomal fractions from CCl_4-treated rats contain more protein-bound 'cytotoxic aldehydes' than untreated rats. Promethazine, an inhibitor of lipid peroxidation, decreases peroxidation in liver cells and prevents loss of glucose 6-phosphatase activity, but not the loss of P450. Probably cytochrome P450 is directly attacked by $CCl_3^{\bullet}$ or other radicals derived from it, whereas the inactivation of glucose 6-phosphatase is brought about by products of lipid peroxidation (**Fig. 12**).Conversion of CCl_4 into $CCl_3^{\bullet}$ appears to be brought about by P450 itself, although it is possible that the reductase might also interact with CCl_4. Destruction of P450 during the reaction makes CCl_4 toxicity, to some extent, a self-limiting event.

The free radical stress imposed on the liver by CCl_4 can lead to rises in intracellular Ca^{+2}, GSH depletion and iron release. Pre-treatment of mice with the iron chelator desferroxamine decreased the hepatotoxicity and decreased the exhalation of ethane caused by CCl_4. This suggests that the lipid peroxidation initiated by free-radical metabolites of CCl_4 is made worse by release of iron ions, a possible mechanism being illustrated in **Fig. 13**. Liver injury by almost any mechanism may provoke activation of resident macrophages (Kupffer cells) in this organ. Recruitment of neutrophils from the circulation can also occur; production of ROS by both types of phagocytes has also been suggested as a potential contributor to liver injury.

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CHAPTER 2: PART A

Purification and Characterization of C-Phycocyanin, a Biliprotein from *Spirulina platensis*

INTRODUCTION

There are more than 25.000 species of algae, living everywhere. They range in size from a single cell to the giant kelp, which is over 150 feet long. Most algae live off sunlight through photosynthesis, but some live off organic matter like bacteria.

Large algae, like seaweeds, are macroalgae. They have an important economic role with over 70 species being used for food, food additives, animal feed, fertilizers and biochemicals. Microalgae can only be seen under a microscope. Some serve a vital role for breaking down sewage, improving soil structure and fertility and generating methane and fuel for energy. Others are grown for animal and aquaculture feeds, human consumption, biochemicals and pharmaceuticals.

There are blue-green microalgae like *Spirulina* and *Aphanizomenon*, green algae like *Chlorella* and *Scenedesmus*, red algae like *Dunaliella*, and also brown, purple, pink, yellow and black microalgae. They are seen everywhere - in water and soil, on rocks and plants. Blue-green algae are the most primitive organisms, and contain no nucleus or chloroplast. Their cell walls evolved before cellulose, and are composed of soft mucopolysaccharides. Blue-green algae do not reproduce sexually but they simply divide. Some can fix atmospheric nitrogen into organic forms. This is very important because organic nitrogen is essential for building proteins and amino acid complexes in plants and animals. Although nitrogen gas comprises 78% of the atmosphere, it is not usable by most plants and animals. For more productive crops, nitrogen must be added to soil. Organic nitrogen can only come upon the addition of chemical fertilizers, from existing microbial mineralization of organic matter, by nitrogen-fixing bacteria in legume roots, or by nitrogen-fixing blue-green algae. Because of this ability to fix nitrogen, blue-green algae are often the first lifeforms to colonize a desolate land area - in deserts, in volcanic rocks, on coral reefs, and even in polar regions, working with lichen to fix nitrogen to the rocks to begin life in the tundra (1)

Nitrogen-fixing blue-green algae are being developed as natural biofertilizers, but they are not always safe to eat. Many kinds of *Microcystis*, *Anabaena* and *Aphanizomenon* are toxic just like some mushrooms and land plants. Harvesting wild blue green algae from lakes presents a risk of contamination by algal toxins.

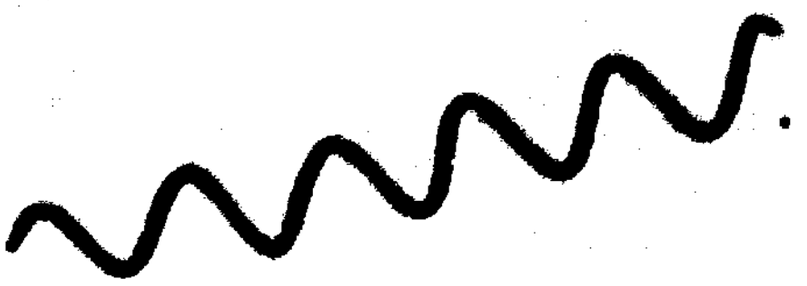


Fig. 1A: Spirulina maxima

Fig. 1B: Spirulina platensis

Spirulina, whose scientific name is *Arthrospira*, is an edible, non-nitrogen fixing blue-green algae. With a long history of safe human consumption and over 30 years of safety testing, it has all international food quality and safety standards. Specially designed farms where *Spirulina* for human consumption is cultivated under controlled conditions (out door mass cultivation) do not allow the growth of other contaminant blue-green algae (free of contaminants) as in lakes and waterways (2,3).

Spirulina maxima Geitler and *Spirulina platensis* Geitler (two traditional species) are planktonic cyanobacteria that form massive populations in tropical and subtropical water bodies characterized by high levels of carbonate and bicarbonate, and high pH (up to 11) (4-6). While *S. platensis* seems to be the widely distributed species, mainly found in Africa, Asia and South America, *S. maxima* appears to be confined to Central America.

S. platensis and *S. maxima* are cyanobacteria recognizable by the characteristic morphological features of the genus: the arrangement of the multicellular cylindrical trichomes in an open, left-handed helix along the entire length and is unbranched (**Fig. 1A and 1B**) (6).

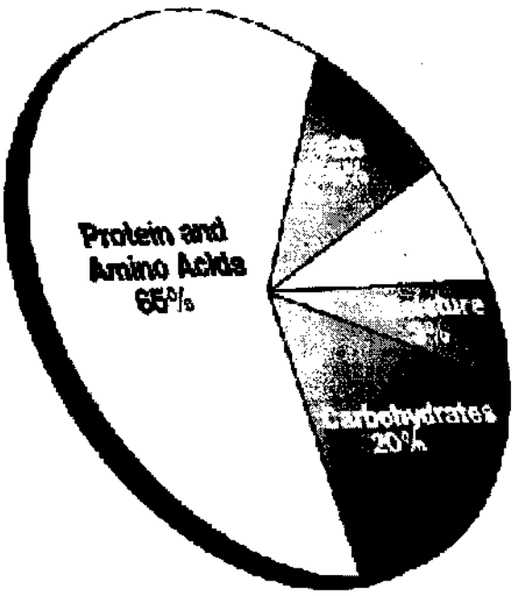
In one of the first books on microalgae, ***Spirulina*, the Whole Food Revolution**, Larry Switzer wrote (7):

> *"For the first time since the appearance of man, both wilderness and food productivity can be increased simultaneously with a new technology. This is a choice that man has never had before. The rediscovery of this ancient life as a human food has great implications for us all, now and in the 21st century. It is an example of the myriad of unexpected and astounding solutions to basic world problems that are now beginning to appear together on this planet."*(7)

Historians have discovered that algae were a major dietary component of ancient cultures and are still consumed by people today (8). *Spirulina* is one such major kind of blue green algae which has been commercialized for its use in health foods and for therapeutic purposes due to its valuable constituents particularly proteins and vitamins (2,9,10). *Spirulina* is used as a food supplement throughout the world and its nutritional and therapeutic values are very well documented (9,10). It is also used as animal feed, in

Table 1: Spirulina power typical analysis

Physical Properties		**General Analysis**	
Composition	100% Spirulina	Protein	55 -70 %
Appearance	fine powder	Carbohydrates	15 - 25 %
Color	dark blue-green	Fats (Lipids)	06 - 08 %
Odor and Taste	mild like seaweed	Minerals (Ash)	07 - 13 %
Bulk Density	0.35 to 0.60 kg/liter	Moisture	03 - 07 %
Particle Size	64 mesh through	Fiber	08 - 10 %



Natural Pigment Phytonutrients (per 10 grams / % total)

Pigments	**Color**	**per 10g**	*% Spirulina*
C-Phycocyanin	Blue	1400 mg	14.0%
Chlorophyll	Green	100 mg	1.0%
Carotenoids	Orange	47 mg	0.47%

Table 2: Biological effects of whole Spirulina sp.

Introduced as:	Test Species	Effects	Reference
Food	Human	Reversal of tobacco-induced oral cancer	32
Food	Mouse	Proportional reduction of IgE, increase of IgA	26a
Food	Mouse	Increased phagocytic activity Increased spleen cell proliferation Increased antibody production	26
Food	Chicken	Increased phagocytic activity Increased NK cell-mediated anti-tumor activity	24
Extract	In vitro, cat	Increased antibody production Increased phagocytic activity	25
IP injection	Rat	Inhibition of mast cells Decrease in local allergic reaction Decrease in serum histamine levels Reduced allergy-induced mortality	22 23
Food	Human	Reduced body weight	31
Food	Rat	Reduction of cholesterol	30a
Food	Rat	Increased activity of lipase	30
Food	Rat	Reduced glucose levels	31a
Food	Rat	Inhibition of maltase and sucrase	32a
Food	Mouse	Modulation of carcinogen metabolic enzymes	33a
Food	Mouse	Mouse Modulation of lead toxicity	34a
Food	Rat	Increased iron status during pregnancy and lactation	35a

Table 3: Bio-modulatory effects of purified compounds from Spirulina Sp.

Compound	Effects	Reference
C-Phycocyanin	Anti-inflammatory	60
	Anti-cancer	42, 43
Calcium Spirulan (Ca-Sp)	Selectively inhibits penetration of virus into host cell (Herpex Simplex, human cytomegalovirus, measles, mumps, Influenza A, HIV-1)	17
	Reduces lung metastasis of melanoma cells by inhibition of tumor cell invasion of basal membrane	18

fertilizers, for biochemicals and the development of pharmaceuticals (11). *Spirulina* is particularly rich in proteins and also contains vitamins, minerals, phytonutrients (phycocyanin, carotenoids, chlorophyll) and essential fatty acids (12), (**Table 1**) though its vitamin B_{12} content does not appear to be readily usable by people (12). Table 1, shows the typical analysis of *Spirulina* powder. Most health benefits to humans due to *Spirulina* and other blue-green algae supplementation come from anecdotes and not scientific research. Test tube and animal studies have demonstrated several properties of large amounts of *Spirulina* or *Spirulina* extracts (**Table 2**), including antioxidant (14), antimutagenic (15), antiviral (anti-herpes simplex virus activity) (16,17), anticancer (18-21), antiallergic (22,23), immune-enhancing (24-26,26a), liver-protecting (27,28), blood vessel-relaxing (29), and blood lipid-lowering (28,30) effects. It has been reported that *Spirulina* sp. reduce body weight in obese humans (31) as well as their total plasma cholesterol levels (**Table 2**)(30).

One controlled human study found that *Spirulina fusiformis* reversed precancerous lesions of the mouth (leukoplakia) in 45% of the group given 1 gram per day for one year, compared to only 7% of the group receiving placebo (32). Another small, controlled study found overweight individuals taking 8.4 grams per day of *Spirulina* lost an average of three pounds in four weeks compared to 1.5 pounds when taking placebo, though this difference was not significant and no effects on blood pressure or serum cholesterol were observed (31). A later controlled but unblinded trial found a small cholesterol-lowering effect when 4.2 grams of *Spirulina* per day were taken for eight weeks, but serum triglycerides, blood pressure, and body weight were unchanged (33).

As it is not an essential nutrient, blue-green algae is not associated with a deficiency state. However, individuals who do not consume several servings of vegetables per day could benefit from the carotenoids and other nutrients in blue-green algae. Since it is a complete food, it can be used in place of some of the proteins in a healthy diet.

Blue-green algae can be taken as a powder, flakes, capsules, or tablets. The typical manufacturer's recommended dose per day is 2,000–3,000 mg divided throughout the day. However, typical amounts shown to have beneficial properties in animal studies would be equivalent to 34 grams per day or more for a 150-pound human. So far no side effects have

been reported with blue-green algae. Toxicological studies of several *Spirulina* species have not revealed any toxic effect. Reproductive toxicity and mutagenicity tests have been performed in several rodent species with *spirulina*, and it was concluded that the algal diet used in these experiments did not cause any toxic effect (34-39). Animal studies have found *Spirulina* to be safe during pregnancy (37,39,40).

Spirulina maxima has been shown to decrease vascular tone of aortic rings in rats fed on a normal purified diet (29) as well as to prevent the development of fatty liver induced by a fructose-rich purified diet (28), or by carbon tetrachloride treatment (27).

All the observed biological and pharmacological properties of *Spirulina* suggest that this algae contains a factor or factors, which are responsible for the observed effects. Recently it has been shown that, two novel compounds isolated from *Spirulina*, namely calcium spirulan (17,18,41) and C-phycocyanin (42,43), possess anti-viral and anti-cancer properties, respectively. However, phycocyanin gained more attention because of its anti-cancer activity and is expected that it might enhance the body's immunity (44) to fight against diseases (**Table 3**).

The growing awareness regarding the importance of natural colors, especially food and cosmetic colorants, have placed great demand on biological sources of natural colors. Cyanobacteria and algae possess a wide range of colored components including carotenoids, chlorophyll and phycobiliproteins (45). The principal phycobiliproteins are phycocyanin, allophycocyanin and phycoerythrin, which are made up of dissimilar α and β polypeptide subunits (45). Phycobiliproteins are assembled into particles, named phycobilisomes, which are attached in regular arrays to the external surface of the thylakoid membranes and act as major light harvesting pigments in cyanobacteria and red algae. Phycobilisomes consist of allophycocyanin cores surrounded by phycocyanin on the periphery. Phycocyanin is the major constituent while allophycocyanin functions as the bridging pigment between phycobilisomes and the photosynthetic lamella (46). Phycocyanin is used as a colorant in food (chewing gums, dairy products, ice sherbaths, jellies etc) and cosmetics such as lipstick and eye liners in Japan, Thailand and China (47,48). It was also shown to have therapeutic value (immunomodulatory activity and anti

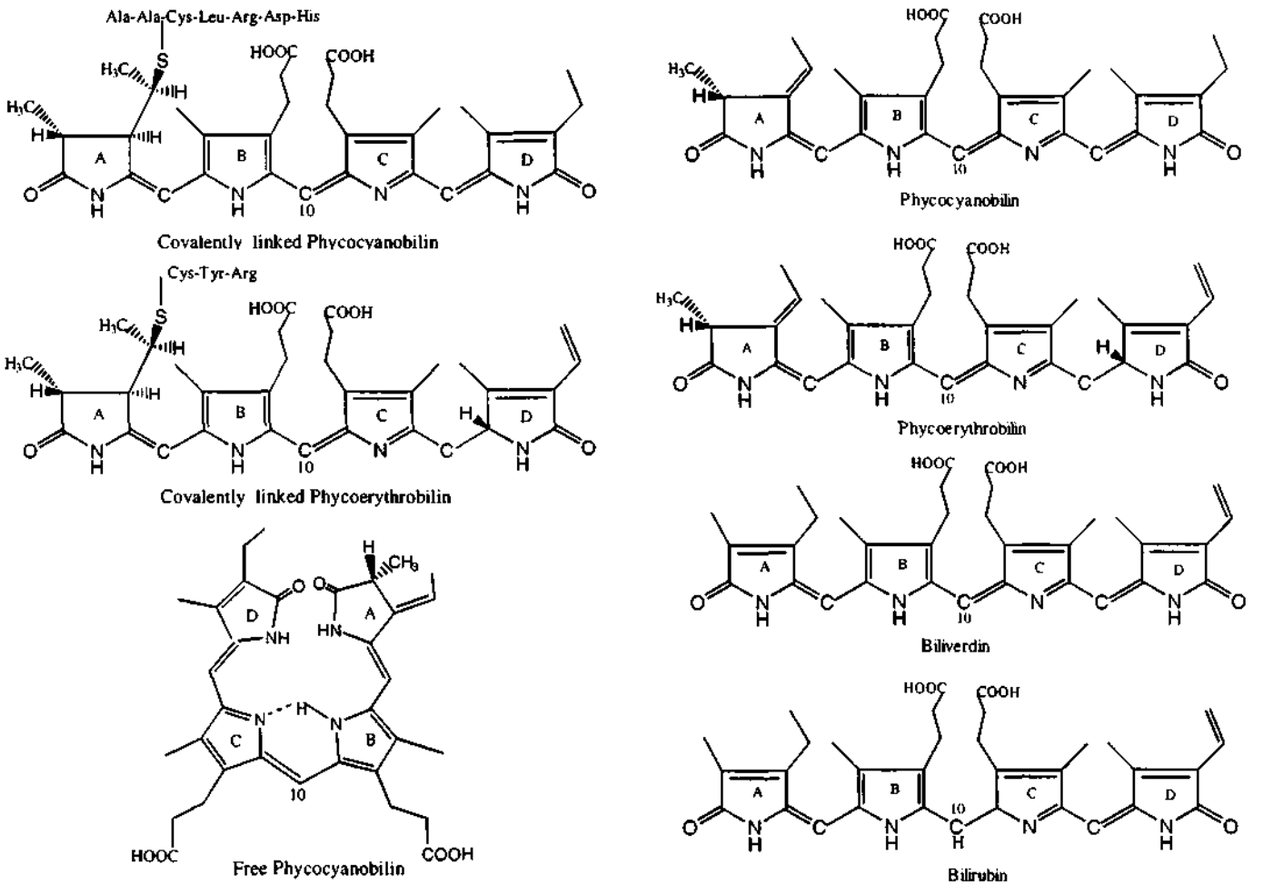


Fig. 2: Structures of selected free and covalently linked bilins

Phycobiliprotein	Mol. Wt. (g/mole)*	Absorption Maxima (nm)	Emission Maxima (nm)	Extinction Coefficient ($M^{-1}cm^{-1}$)
Allophycocyanin	104,000	652, 625	657	163,000
C-Phycocyanin	**264,000**	**620**	**635.5**	**279,000**
R-Phycocyanin	103,000	619, 566	635	139,000
B-Phycoerythrin	240,000	545, 563.5	572	2,410,000
R-Phycoerythrin	250,000	565, 498	573	1,960,000

Table 4: Spectral properties and physical characteristics of standard phycobiliproteins
**These are not the molecular weight of the monomers*

cancer activity) (42,43). Owing to its fluorescence properties, it has gained importance in the development of phycofluor probes for immunodiagnostics (49).

C-Phycocyanin is a photosynthetic accessory pigment found in *Spirulina* and other cyanobacteria, red algae and cryptomonads, and it is a part of the supramolecular complex, phycobilisomes, that are located on the surface of the photosynthetic membranes (50-52). Phycobilisomes are composed of rods and a core, which are highly organized by various phycobiliproteins and linker polypeptides. Allophycocyanin (APC), C-phycocyanin (CPC), phycoerythrin (PE) and phycoerythrocyanin (PEC) are the principal classes of phycobiliproteins in phycobilisome assembly. The brilliant blue colors of the phycobiliproteins originate from covalently attached, linear tetrapyrrole prosthetic groups known as phycobilins. Different phycobiliproteins contain different kinds and different numbers of chromophores, which are open-chain tetrapyrroles linked to the polypeptide chain at conserved positions either by one cysteinyl thioether bond through the vinyl constituent of the pyrrole ring A, or on both A and D, of the tetrapyrrole (52a). The chromophores are classified by structure as phycoerythrobilin (PEB), phycocyanobilin (PCB), phycoviolobilin (PVB) and phycourobilin (PUB) (50-52) (**Fig. 2**). The spectroscopic properties of individual phycobiliproteins depend in large measure on the chemical nature of the bilin they carry. The phycobiliproteins can be divided into three major classes according to their spectral features: phycoerythrins (PE; λ_{max} = 565 nm), phycocyanins (PC; λ_{max} = 620 nm) and allophycocyanins (AP; λ_{max} = 650 nm) (50-52) (**Table 4**). The antennae rods of phycobilisomes are composed in the order, APC at the core, CPC in the middle and PE/PEC at the tip. The energy transduction proceeds in the direction from tip to the core through PE/PEC → CPC → APC → Reaction center, with an overall efficiency greater than 95% (52,53).

All phycobiliproteins have a common subunit organization, which consists of α and β subunits, that form a heterodimer αβ (54). The heterodimer, called a 'monomer' in the phycobiliprotein assembly pathway, can aggregate together to form disc-shaped $(\alpha\beta)_3$ trimers. The $(\alpha\beta)_6$ hexamer is formed by tight association of two $(\alpha\beta)_3$ trimers (55). Hexameric phycobiliproteins together with the linker proteins, self-assemble *in vivo* into

macromolecular light-harvesting complexes (56). The crystal structure of several phycobiliproteins were solved earlier, including allophycocyanin from *S. platensis*. All the structures are very similar. However, the crystal structure of C-phycocyanin from *S. platensis* has never been solved earlier, even though it was crystallized (57). Recently we have crystallized and solved the crystal structure of phycocyanin from *S. platensis*.

The cyanobacterium *Spirulina platensis* contains only two phycobiliproteins, APC and CPC (58). CPC is the major light-harvesting pigment protein (~14% of dry weight of *Spirulina*) present in the antenna rods of *S. platensis*, whereas APC is a minor component present only at the core. As mentioned earlier, phycocyanin was extensively used as a food colorant and in cosmetics because of its blue color and it shows a strong fluorescence in the visible region. It is also non-carcinogenic. However, most of its pharmacological properties are not known except a few. Macros *et al.* have shown its photodynamic properties and its use in cancer treatment (42,43). They have shown that, phycocyanin specifically binds to cancer cells, and thus can be used for anatomical imaging of tumours *in vivo* (59). Recently its anti-inflammatory properties has been published (60).

The blue pigmentation of phycocyanin is due to the presence of linear tetrapyrrole chromophore, called phycocyanobilin (PCB), which is covalently attached to the apoprotein through thioether bond (51,61). Phycocyanin has three PCB chromophores attached to the $\alpha\beta$ monomer through thioether linkages at the $\alpha 84$, $\beta 84$ and $\beta 155$ positions. These chromophores are located within hydrophobic regions of the protein molecule (51).

Phycocyanobilin is structurally similar to biliverdin and bilirubin, degradative products of heme (51) (**Fig. 2**). Bilirubin and biliverdin are natural antioxidant and cytoprotective agents present in the mammalian body (62,63). Both bilirubin and biliverdin are hydrophobic in nature. Bilirubin is non-covalently bound to serum albumin *in vivo* and it also protects albumin (64). Few bilirubin molecules are covalently attached to amino acid residues of serum albumin and this bilirubin is called as delta bilirubin. It has been shown that, delta bilirubin is a potent antioxidant and cytoprotective agent (65). Animal experiments have proved that delta bilirubin (bilirubin-albumin complex) protects the liver from toxic injury (66) and enhances the growth of liver after partial hepatectomy (67), and it is identified as a liver growth factor (68). It has also been shown that albumin-bound

bilirubins (synthetic delta bilirubin) protect human ventricular myocytes against oxyradical damage (69). In phycocyanin, phycocyanobilin (PCB) is covalently attached to apoprotein and the PCB-apoprotein complex is similar to delta bilirubin. However, it is not known whether phycocyanin possesses hepatoprotective and cytoprotective properties and these aspects have not been looked into. Moreover, it is one of the major proteins (up to 14-17%) present in the *Spirulina* and it has been established earlier that *Spirulina* protects the fructose rich diet-induced fatty liver in mice (10). This suggests the presence of factor(s), in *Spirulina* responsible for the observed hepatoprotective effect and it is quite possible that phycocyanin may have this unique property. In present study, we have purified the phycocyanin to homogeneity from both fresh and spray dried *Spirulina platensis*. Molecular mass of phycocyanin monomer ($\alpha\beta$) was determined by using ESI-MS and MALDI-TOF spectrometry. The monomer molecular mass is compared with the computed molecular mass of this protein based on its DNA sequence, and the molecular mass corresponding to the chromophore which is tightly bound to the polypeptide. Its pH and temperature stability was studied. Phycocyanin was crystallized and its crystal structure were solved at 2.2Å resolution (work carried out in collaboration with the Department of Physics, IISc).

METHODS AND MATERIALS

Chemicals: DEAE-Sephadex A50 from Pharmacia (Uppsala, Sweden), Sephadex G-100, DEAE-cellulose (DE-52), Tris, β-Mercaptoethanol, Acrylamide, N, N-methylene bisacrylamide, TEMED, Ammonium persulfate, SDS, Coomassie Brilliant Blue R-250 were purchased from Sigma (St. Louis, MO, USA). All other materials were from standard suppliers and were of analytical grade. Initially, C-Phycocyanin (isolated from *Spirulina platensis*) was a generous gift from Cyanotec. Bio-products (P) Ltd. Bangalore, India.

Purification of C-Phycocyanin from *Spirulina platensis*: Freshly grown and spray dried *Spirulina platensis* was a generous gift from Ballarpur Industries Ltd. Bangalore, India. Phycocyanin was isolated and purified following the published procedure (58) with few modifications. Freshly grown cells were pelleted out by centrifugation at 3,000 rpm for 20 min at 4° C. The pellet was washed thoroughly with distilled water (twice), suspended in

Na-phosphate buffer (pH 7.0, 10 mM) and the suspension was frozen (at -20° C) and thawed. The mixture was subsequently centrifuged at 8,000 × *g* for 30 min at 4° C, and phycocyanin containing clear blue supernatant was collected. When dried *Spirulina* cells were used for the isolation of phycocyanin, the cells were soaked in Na-phosphate buffer (pH 7.0, 10 mM) before they were subjected to the above procedure. The clear blue supernatant obtained from both the procedures was further fractionated in order to get homogenous phycocyanin.

Separation and purification of phycocyanin:

The clear blue supernatant (8,000 × g supernatant) was subjected to ammonium sulfate fractionation and the protein precipitated between 30-50% saturation was dissolved in a small volume of 10 mM Na-phosphate buffer (pH 7.0) and dialyzed against the same buffer. The dialyzed sample containing C-phycocyanin was subjected to chromatography over DEAE-cellulose or DEAE-Sephadex A50. The column was equilibrated with 50 mM NaCl solution, washed extensively with equilibration buffer (10 mM Na-phosphate buffer, pH 7.0) and phycocyanin was eluted with 100 mM NaCl at a flow rate of 3 ml per min. The phycocyanin was collected as 5 ml fractions and those fractions having A620/A280 ratio more than 4 were pooled and concentrated by ultrafiltration. Concentrated phycocyanin was further desalted by passing through Sephadex G-100 column equilibrated with Na- phosphate buffer (pH 7.0) and the same buffer was used for eluting phycocyanin. Fractions (2 ml) containing phycocyanin were collected at a flow rate of 0.5 ml/min. The A620/A280 ratio was calculated for all the fractions and those fractions having A620/A280 ratio more than 4 were pooled and considered as pure phycocyanin (58,70).

The purity of phycocyanin prepared was examined by native PAGE (71) using 4% stacking and 10% resolving gel. Gels were stained using Coomassie Blue R-250.

SDS-PAGE: Electrophoresis was carried out according to Laemmli (72) using 4% stacking and 14% resolving gel, 1.5 mm thick, containing 0.1% (w/v) SDS. Samples were preincubated with 2% (w/v) SDS, 10% (v/v) glycerol, 4.5 % (v/v) β-mercaptoethanol, 0.025% (w/v) bromophenol blue and 60 mM Tris (pH 6.8) at 95° C for about 4 min.

Electrophoresis was carried out at room temperature and the gels were stained using Coomassie Blue R-250.

The fluorescence and UV-Visible spectra were recorded in Shimadzu RF 540 and Shimadzu UV 2100 thermostated spectrophotometer, respectively. Phycocyanin dissolved in phosphate buffer (0.1 M, pH 7.0) was dialyzed extensively against water and subjected to electrospray ionization mass spectrometry (ESI-MS) in acidic condition to determine the molecular mass. HP-1100MSD spectrometer was used for this purpose. The molecular mass of pure phycocyanin was also determined by MALDI-TOF (Matrix Assisted Laser Desorption / Ionization-Time Of Flight). Mass spectra were recorded on a Kratos Analytical (U.K) Kompact Seq model, which uses a 337 nm nitrogen laser for desorption and a 1.7 meter linear flight path (one micro liter of the protein sample was applied to a 20 slot MALDI plate, genetisic acid in 50% acetone/water was used as the matrix).

pH and temperature stability of C.phycocyanin:

Effect of pH and temperature on the stability (620/280 ratio) of C.phycocyanin was studied. Lyophilized phycocyanin was dissolved in distilled water just before use in order to prepare 1 mM phycocyanin stock solution. Phycocyanin (10 μM) was incubated at different pH (0.1 M, pH 3-10, acetate buffer, pH 3.0-5.6; phosphate buffer, pH 6.0-8.9; Tris buffer, pH 9.5, and 10.0) for 10 min, and the UV-Vis spectra were recorded between 250-700 nm at 25° C in a Shimadzu UV 2100 thermostated spectrophotometer. The temperature stability was studied by incubating phycocyanin (10 μM) at different temperatures (between 25° C-60° C) in phosphate buffer (0.1 M, pH 7.0) and the UV-Visible spectra were recorded in Shimadzu UV 2100 thermostated spectrophotometer at 5 min interval for 30 min. Zero time was defined as the time when the phycocyanin solution in cuvette reached the target temperature.

RESULTS

Purification of phycocyanin from *Spirulina platensis*:

Large-scale purification of phycocyanin from *Spirulina platensis* was achieved by a combination of salt precipitation and ion-exchange chromatography. Details of the

Fractionation step	A620/A280 ratio
Crude C-phycocyanin	0.932
30-50% Ammonium sulfate	1.861
DEAE-Cellulose	4.235

Table 5: Purification steps of C-phycocyanin from Spirulina platensis

Fig. 3: Pure C-phycocyanin: solid and liquid

Fig. 4: SDS-PAGE of the phycocyanin.
A) crude cell free extract,
B) 30-50% ammonium sulfate precipitate, and
C) Pure phycocyanin from DEAE-Cellulose column.

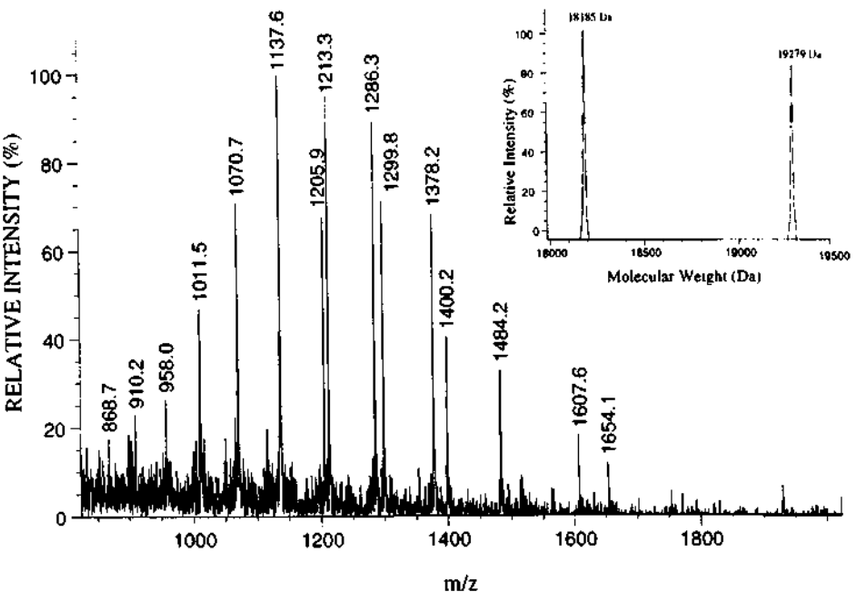


FIG. 5A. Electrospray ionization-mass spectrum of C-phycocyanin monomer. The molecular mass of α and β subunit derived from the deconvoluted spectrum is shown in the inset.

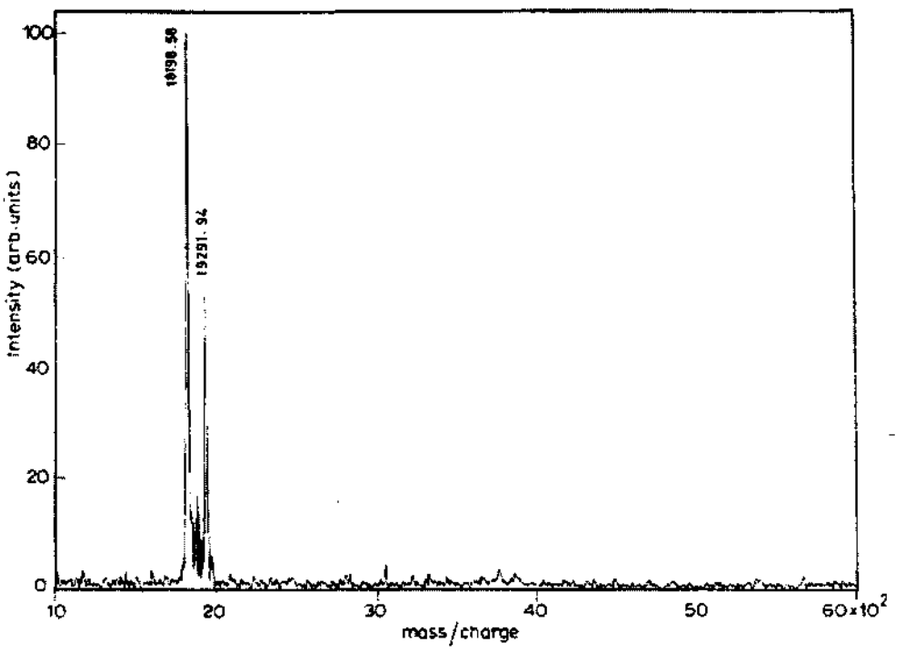


FIG. 5B: Molecular mass of C-phycocyanin monomer by MALDI-TOF

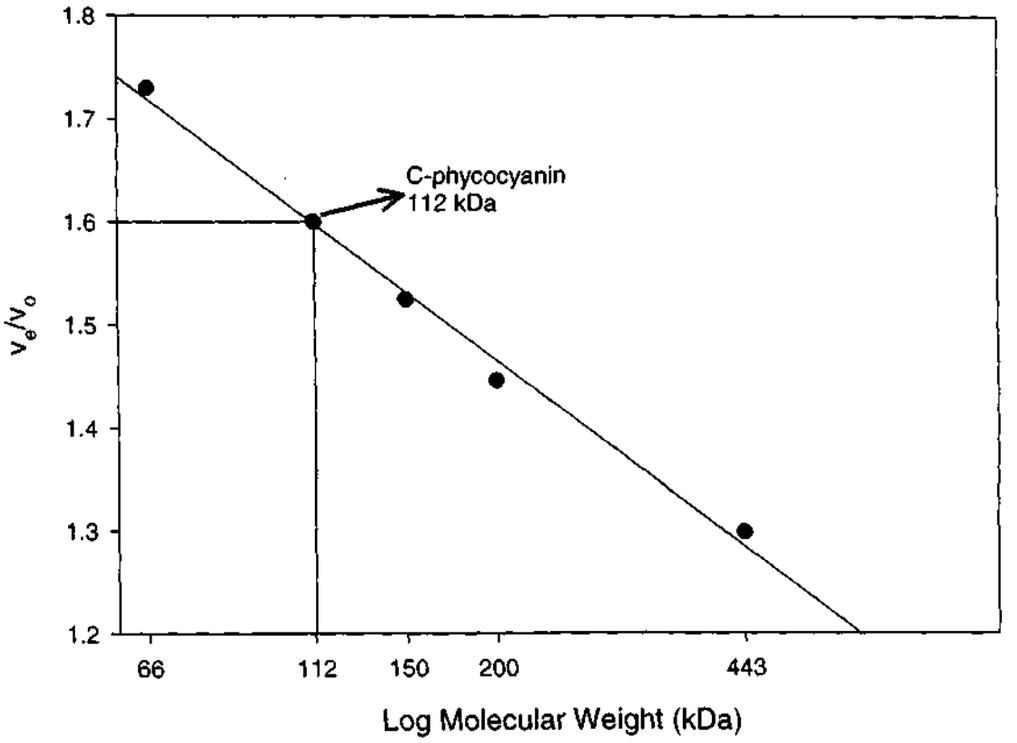


Fig. 6: Molecular weight determination by FPLC analysis for native C-phycocyanin from Spirulina platensis

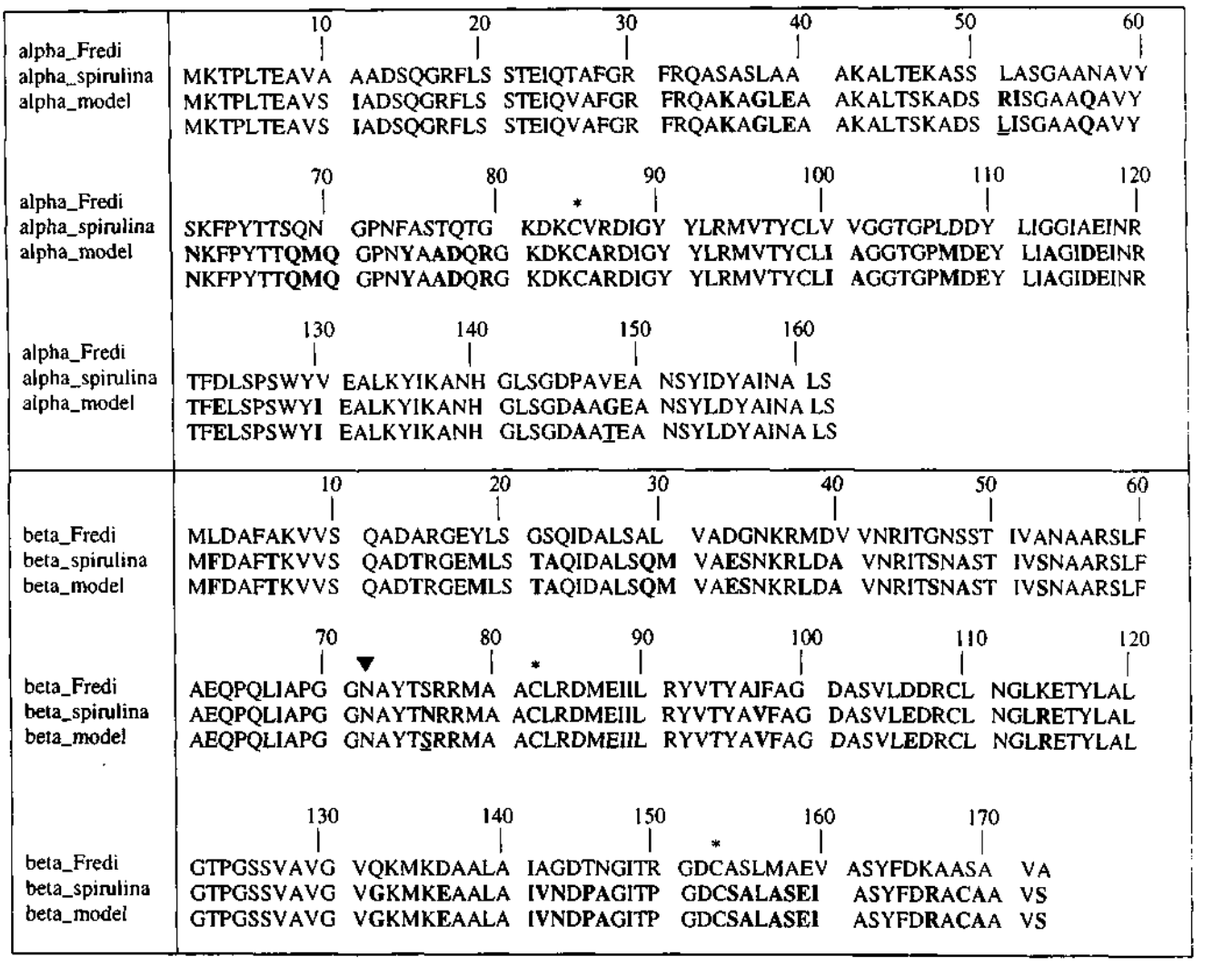


Fig. 7: Alignment of the C-phycocyanin amino-acid sequences for Fremyella diplosiphon (F. plosiphodin), Spirulina platensis (from SWISS-PROT database) and our model. The non-identical residues between the sequence from F. diplosiphon and that from the database are shown in bold. The differing residues between the model sequence and that from the database are underlined. The cysteinyl attachments for the chromophores are marked with an asterisk. ▼indicates the methylation site.

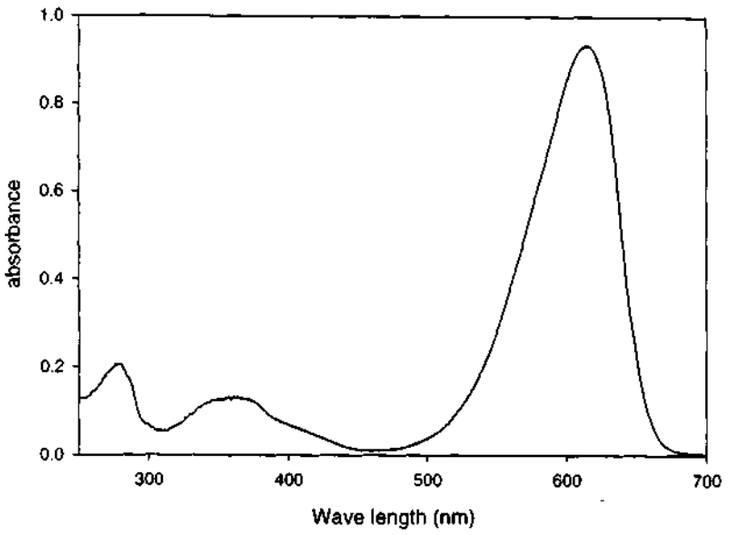


Fig. 8A: Absorption spectra of C-phycocyanin from spirulina platensis in phosphate buffer (0.1 M, pH 7.0 at 25 ° C

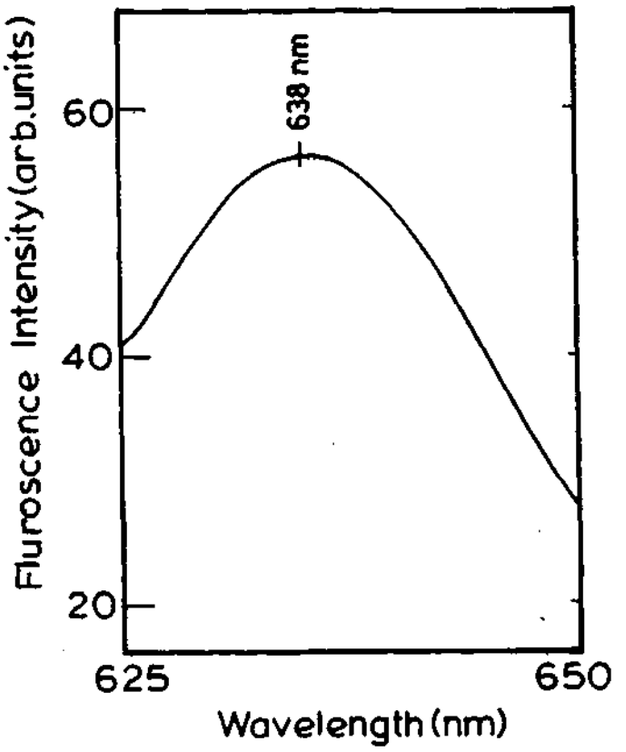


Fig. 8B: Fluorescence spectra of C-phycocyanin from spirulina platensis in phosphate buffer (0.1 M, pH 7.0 at 25 ° C. (Excitation wave length at 615nm)

purification steps are given in **Table 5**. Earlier investigators (58) have determined the purity of phycocyanin by A620/A280 ratio and the phycocyanin sample having this ratio above 4.0 was considered pure. Same procedure was followed to establish the purity of phycocyanin. In the ion-exchange column chromatography only fractions having A620/A280 ratio above 4.0 were pooled. From one kg of dry *Spirulina* cells, 6-7 grams pure phycocyanin was isolated. The yield was sacrificed for the sake of purity.

The polyacrylamide gel electrophoresis (PAGE) of the purified phycocyanin carried out under non-denaturing conditions revealed a single band and the SDS-PAGE performed under denaturing conditions showed the presence of two bands corresponding to α and β subunits (**Fig. 4**). This indicates that the linker polypeptides were effectively removed during the purification procedure. The absorption and fluorescence spectra of purified phycocyanin matched well with those reported earlier for C-phycocyanin (51). The molecular mass of phycocyanin monomer determined by ESI-MS mass spectrometry was found to be 37,468.5 mass units, which is 20.5 mass units less than the calculated mass of 37,489.0 for native phycocyanin monomer (**Fig. 5A**). However, when the molecular mass of phycocyanin monomer was determined by using MALDI-TOF, it was shown to be 37,490.5 mass units, which is only 1.5 mass units more than the calculated mass for phycocyanin monomer (**Fig. 5B**). Amino acid sequence of α and β chains were retrieved from SWISS-PROT database (73) with primary accession Nos. P72509 and Nos. P72508, respectively (**Fig. 6**). The molecular mass of α and β subunits were 18,186.56 and 19,281.94 mass units, respectively by ESI-MS (**Fig. 5**) and 18,198.58 and 19,291.94 mass units, respectively by MALDI-TOF (**Fig. 5B**). The calculated molecular mass for α and β subunits from amino acid sequence is 18,186 and 19,303 mass units, respectively (**Table 7**).

The spectroscopic properties of phycobiliproteins are critically dependent on the assembly state of the proteins; their molar extinction coefficients and fluorescence quantum yields decrease sharply upon dissociation to αβ monomers. In order to find out the aggregation state of αβ monomers of phycocyanin in solution, FPLC analysis was carried out. It was found that phycocyanin appears as a trimer $[(\alpha\beta)_3]$ in solution. The

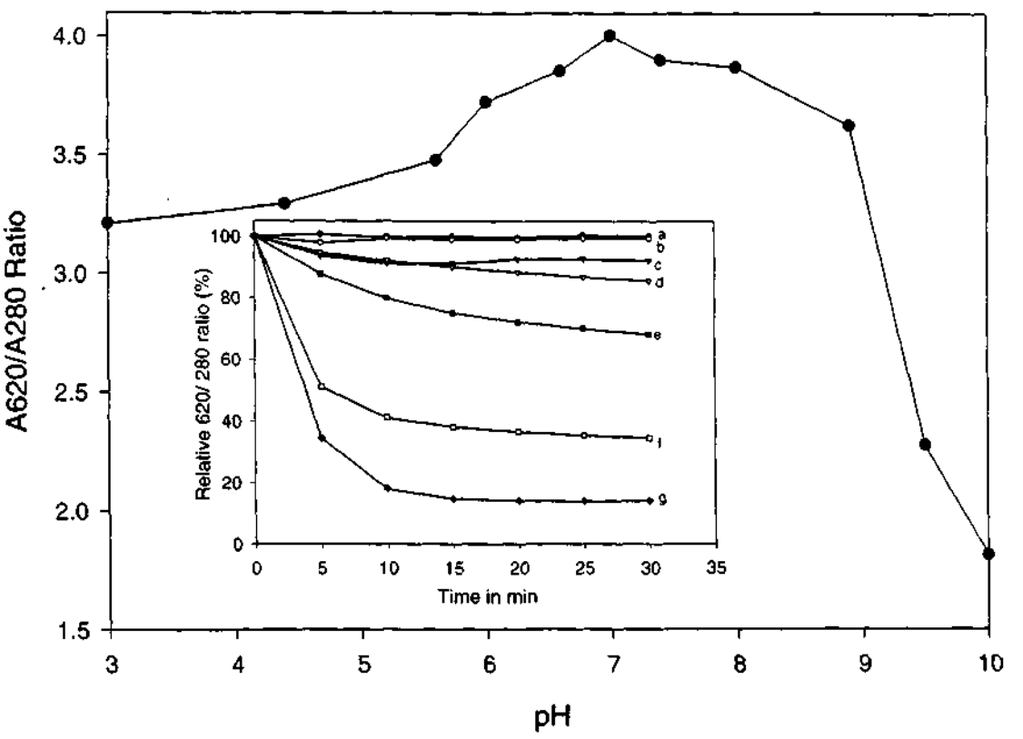


Fig. 9: Effect of pH on the stability (620/280 ratio) of phycocyanin. Inset: Time-dependent decrease in the relative 620/280 ratio of phycocyanin in different temperature. (a, b, c, d, e, f, and g corresponds to 25, 37, 42, 45, 50, 55, and 60 ^{0}C, respectively)

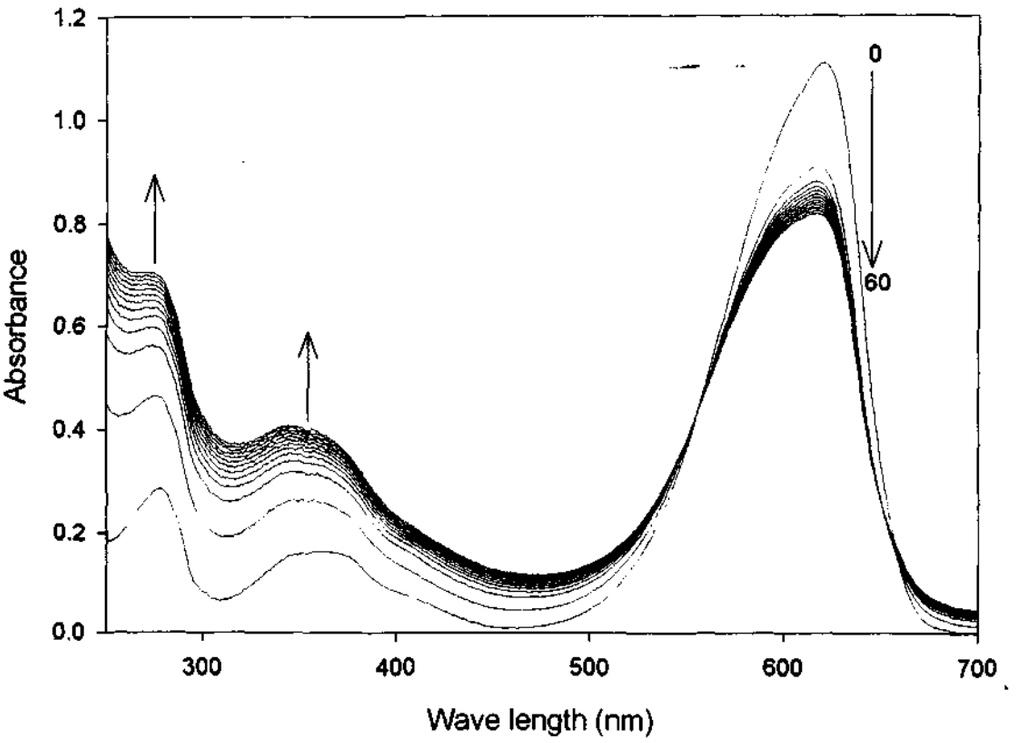


Fig. 10: Effects of temperature on the visible and near-UV absorption spectra of C-phycocyanin from spirulina platensis. Spectra was recorded for every 5 min up to 60 min.

Table 6. Summary of crystallographic analysis

Measurement	Value
Data collection:	
Resolution limit (Å)	2.2
Total reflections	1177344
Unique reflections	223480
Completeness %	99.3 (97.8)
R_{merge} %	8.9 (44.5)
Refinement Statistics:	
Resolution (Å)	25-2.2
Number of non-H protein atoms	31,572
Number of water molecules	2,290
R_{free} %	23.9 (30.3)
RMSD bond lengths (Å)	0.006
RMSD Bond angles	1.135
Average B-factor ($Å^2$)	27.3

Values in parenthesis correspond to the last Resolution shell from 2.28 to 2.20Å.

molecular mass of phycocyanin trimer was calculated, which was found to be 112 kDa by FPLC analysis using apo-ferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa) and BSA (66 kDa) as standard markers (**Fig. 7**). Superose-12 analytical column was used for this purpose.

Effect of pH and temperature on the spectral properties of phycocyanin:

The absorption spectra of purified phycocyanin in phosphate buffer (0.1 M, pH 7.0) showed peaks at 342 and 618.5 nm (**Fig.8A**) and this spectra is very similar to the reported spectra of phycocyanin isolated from *Spirulina platensis* (58). The fluorescence spectrum of purified phycocyanin (**Fig. 8B**) is also similar to the spectrum taken earlier for phycocyanin from *Spirulina platensis* (58).

Effect of pH (pH 3-10, 0.1 M) on C-phycocyanin was studied by incubating it at different pH for 10 min at room temperature and determining the A620/A280 ratio. It was noticed (**Fig. 9**) that the pH of the incubation medium markedly affected the stability of phycocyanin and it is more stable at neutral pH (pH 7.0). The A620/A280 ratio was significantly affected at pH values below and above 7.0, being 80% and 45% of the optimum ratio at pH 3.0 and 10, respectively (**Fig. 9**).

The temperature stability of phycocyanin was studied by incubating phycocyanin (10 μM) at different temperatures (between 25° C - 60° C) in phosphate buffer (0.1 M, pH 7.0) and the UV-Visible spectra were recorded in Shimadzu UV 2100 thermostated spectrophotometer at every 5 min interval till the duration of the incubation period (30 min). It was noticed that phycocyanin remained stable at 25° C with no changes in the A620/A280 ratio, whereas, the ratio was only 14% of the optimum at the end of 30 min at 60° C (**Fig. 9 inset**). It was observed that, at higher temperature phycocyanin undergoes rapid denaturation and absorption at 280nm was increased (**Fig. 10**).

Crystal structure of phycocyanin (collaborative work):

The crystal structure of C-phycocyanin from *Spirulina platensis* has been solved by using molecular replacement technique for the first time. The structure has been refined to a crystallographic R-factor of 19.2% (R_{free} = 23.9%) using the X-ray diffraction data extending upto 2.2 Å resolution. The quality of the final model is summarized in **Table 6**.

The asymmetric unit of the crystal cell consists of two $(\alpha\beta)_6$-hexamers (each hexamer being the functional unit in the native antenna rod of cyanobacteria). The molecular structure resembles that of other reported C-phycocyanins. However, the unique form of aggregation of two $(\alpha\beta)_6$-hexamers in the crystal asymmetric unit, suggests additional pathways of energy transfer in lateral direction between the adjacent hexamers involving β155 phycocyanobilin chromophores *in vivo*.

The amino-acid sequences of the α chain (SWISS-PROT database accession No. P72509) and the β chain (SWISS-PROT database accession No. P72508) of C-phycocyanin from *S. platensis* were aligned with those of C-phycocyanin from *Fremyella diplosiphon* (*F. diplosiphon*) (**Fig. 6**). The identities were 80.9 and 79.7%, respectively. Some changes in the amino acid sequence were noticed. The changed amino acid residues are shown in **Fig.7** based on the present crystal structure.

DISCUSSION

Phycocyanin is one of the major water soluble biliprotein present in *Spirulina platensis*. This water soluble protein pigment is gaining a lot of importance these days because of its various biological and pharmacological properties. However, the biochemical basis for the observed pharmacological properties has not been clearly understood. To undertake a critical analysis of some of the biochemical properties exhibited by *Spirulina platensis* it was necessary to have pure C-phycocyanin in sufficient quantities. So it was desirable to develop a simple method for the isolation and purification of C-phycocyanin from the cells of *Spirulina platensis* with a high purity. Earlier several investigators have used different methods for the isolation and purification of phycocyanin from various species of *Spirulina* (58). However, in these attempts, only small quantities of phycocyanin was isolated and the purity was relatively low. In the present study, we have purified phycocyanin in sufficiently large quantities from both fresh and spray dried cells of *Spirulina platensis*, to homogeneity. It was noticed that one Kg of dry cells of *Spirulina* yields 200-250 grams of protein containing phycocyanin. This crude phycocyanin was subjected to further purification as described under methods. Since our

objective was to get homogenous phycocyanin preparation, we sacrificed the yields for the sake of purity and hence only peak fractions containing phycocyanin was processed.

Phycocyanin from *Spirulina platensis* was isolated and purified to homogeneity and the procedure involved precipitation with ammonium sulfate (30-50% saturation), and ion exchange column chromatography on a DEAE-cellulose column. Compared to earlier reports (58), the present purification procedure is simpler. We have also noticed that phycocyanin from *Spirulina platensis* can be purified at room temperature

As reported earlier, the A620/A280 ratio of phycocyanin can be used to check the purity of phycocyanin preparation and if the A620/A280 ratio is >4, it can be considered to be pure (70). We have adopted the same method to check the purity of phycocyanin preparation. The UV-Visible spectrum exactly matches the reported spectra of phycocyanin from *Spirulina platensis* (58). The native and SDS PAGE clearly indicate that, the phycocyanin prepared is pure and free from linker peptide. It contains two subunits, α and β. In fact, biliproteins in general contain two subunits. The isoelectric point (pI) for C-phycocyanin from *Spirulina sp.* was reported to be between 4.3-4.6 (74).

It has been reported that the molecular mass of phycocyanin monomer (αβ) from *Spirulina platensis* is 44 kDa and for the α and β subunits 20.5 and 23.5 kDa, respectively (58). Recently Bermejo *et al.* reported the molecular mass of α and β subunits to be 21.5 and 19.0 kDa, respectively based on SDS-PAGE (75). The exact molecular mass of phycocyanin from *Spirulina platensis* has never been reported earlier. In fact, it is difficult to determine the exact molecular mass by SDS-PAGE. However, based on the amino acid sequence (from DNA sequence) it was reported to be 18,186 and 19,303 mass units for α and β subunits, respectively (73). For the first time we have determined the exact molecular mass of phycocyanin monomer from *Spirulina platensis* by ESI-MS and MALDI-TOF. Based on ESI-MS, the molecular mass of α and β subunits are 18,186.56 and 19,281.94 mass units, respectively. As determined by MALDI-TOF, the molecular mass of α and β subunits are 18,198.58 and 19,291.94 mass units, respectively. However, some changes in the amino acid sequence in both the subunits have been noticed based on the electron density in crystal structure data (our model). On the basis of crystal structure

Table 7: Comparison of the molecular mass of C-phycocyanin from spirulina platensis.

Subunit	Molecular mass from DNA sequence	Molecular mass from our model	Molecular mass by ESI-MS	Molecular mass by MALD-TOF
PHY _alpha	18,186	18,187	18,186.56	18,198.58
PHY_ beta	19,303	19,277	19,281.94	19,291.94
PHY_αβ monomer	37,489	37,464	37,468.85	37,490.52

of phycocyanin, the molecular mass for α and β subunits are 18,187 and 19,277 mass units, respectively. Based on the crystal structure the newly calculated mass for the αβ monomer is 37,464 mass units and that determined by ESI-MS is 37,468.85 mass units; i.e. 4.85 mass units more than the calculated mass (**Table 7**). Our results are in good agreement with the computed molecular mass of phycocyanin based on its crystal structure and DNA sequence. The computed molecular mass includes the mass corresponding to the chromophore, PCB.

Biliproteins from cyanobacteria are obtained as dissociation products of the phycobilisomes. In the phycobilisomes, C-phycocyanin is generally found as stacks of two or more disks, and each disk is an $\alpha_6\beta_6$ assembly of the α and β polypeptides, and the disks are connected by linker polypeptides. When the phycobilisomes from algae are extracted into dilute phosphate buffer, the rods detach from the allophycocyanin core and dissociate into $\alpha_6\beta_6$, $\alpha_3\beta_3$, αβ and $(\alpha_6\beta_6)_2$ units depending on the source of the organism (52). At pH 6.5-8.0, monomer and trimer of phycocyanin exist in equilibrium, but the protein is primarily in a monomeric form at concentration less than 8 μg/ml (76). The absorption and fluorescence spectra of C-PC in trimeric and monomeric forms show that the spectra are slightly affected by changes in the protein due to aggregation (76a). The colorless linker protein also modifies the absorption spectra. The linker-free and linker-biliprotein complexes had different optical spectra. We have carried out FPLC analysis in order to find out the aggregation of phycocyanin in buffered solution (0.1 M, pH 7.0). It was found that, phycocyanin appears as a trimer $(\alpha\beta)_3$ in solution.

Earlier it has been shown that, phycocyanin (from other species) undergo reversible association-dissociation of subunits with changes in pH (77). It has also been reported that, at a pH range of 5.0 to 7.0, phycocyanin exists in an equilibrium of hexamers $(\alpha\beta)_6$, trimers $(\alpha\beta)_3$ and monomers (αβ) (77). Hottori and others (77) have reported changes in phycocyanin absorption spectra with pH. The authors have concluded that at pH near the pI, the reduction of electrostatic repulsive forces between molecules lead to an association of the monomers, which results in alteration of the optical properties. Earlier it was concluded that, changes in the absorption spectra of phycocyanin is related to changes in

the aggregation pattern of this protein. At low pH, the protein gets denatured, and shows much lower visible absorption. This phenomenon was explained by the fact that, changes in the optical properties could be due to alterations in the conformation of the chromophore resulting from denaturation of the polypeptide. Similar kind of changes were noticed at higher pH also. As the pH increases from the pI, the protein becomes more soluble because of the increase in electrostatic repulsion that reduces aggregation and subsequent precipitation of the molecules (78). The chromophore phycocyanobilin (PCB) has a cyclic-helical conformation in solution, while it is extended in the native protein (79). This suggests that the native structure of the apoprotein forces the chromophore to adopt the liner configuration by hydrogen bonding to nearby side chains; the protein acts as a scaffold to hold the chromophore in the desired position. If the protein is unfolded, the scaffolding of nearby side chains is removed from the chromophore, which then folds into a cyclic lockwasher conformation. The absorption spectrum of the chromophore in the cyclic conformation is very different from that of the linear conformation. Moreover, cyclization of tetrapyrroles results in lower visible and higher near-UV absorbance (80). Therefore, the chromophore acts to report the integrity of the protein structure. When in its native form, the protein has a beautiful dark blue color. In the unfolded state, its blue color fades away. Therefore, it can be concluded that the spectral properties of phycocyanin are influenced by the structure of the protein molecule.

C-phycocyanin from *Spirulina platensis* is highly stable when it is stored as a 60% ammonium sulfate precipitate at 4° C. Phycocyanin from *Spirulina platensis* is more stable at room temperature in buffered solution. At higher temperature (60° C) phycocyanin rapidly undergoes irreversible denaturation resulting in complete loss of visible absorption but the near-UV band intensifies. The increase in the near UV absorption coupled with a loss of visible absorption demonstrates that the PCB has become cyclic. The bilins are linear in the ordered protein but become cyclic when the protein gets strongly disordered. Thermal unfolding of the protein will alter the polypeptide conformation to expose the chromophore that is contained in the hydrophobic regions of the protein (81). Unfolding of the protein may cause separation of chromophores that, under native condition, stay in close proximity and interact to produce the specific optical properties of the native protein

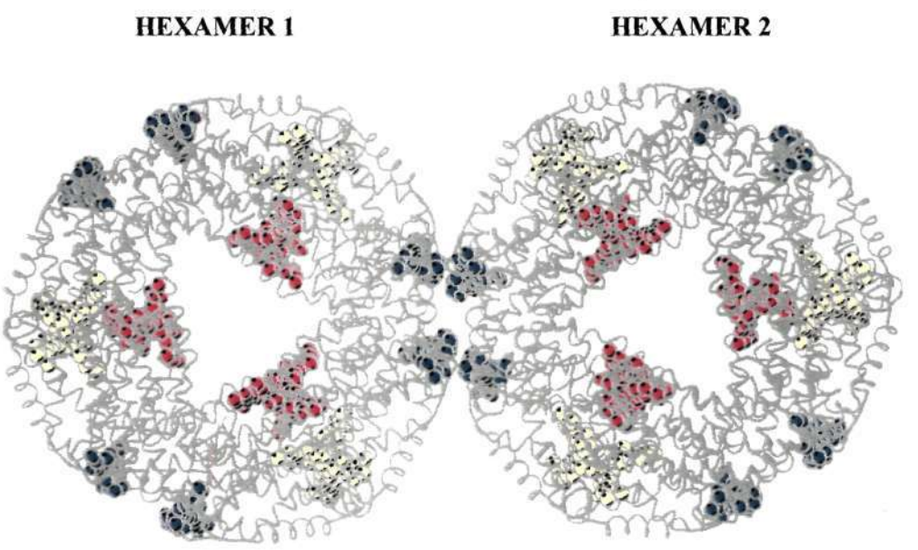


Figure 11. Coil representation of the two hexamers in the crystal asymmetric unit, a view through the approximate central axis of hexamers. Chromophres are shown in CPK representation. The figure illustrates the arrangement of chromophores at various locations within the hexamers.

(82). In addition, denaturation of the protein molecule may result in cyclization of chromophore with the subsequent loss of absorbance and fluorescence (79). These changes in spectral properties are due to changes in the protein conformation that result from chemical (pH) and physical (heat) denaturation.

Three-dimensional structure of any molecule gives a lot of information about its function. Crystal structure of phycocyanin from several other species has been solved earlier by many investigators. However, crystal structure of phycocyanin from *Spirulina platensis* has never been solved earlier, although it was crystallized before (57). In the recently solved structure, phycocyanin appears as "double hexameric" aggregation, where β155 PCBs are brought to a close proximity of each other within the region between the adjacent hexamers in the crystal asymmetric unit (**Fig. 11**). This suggests a possible additional role for β155 PCBs in terms of energy transfer in lateral direction between adjacent disks of CPCs. This could possibly be important considering the *in vivo* environs of CPCs.

The amino acid sequence of any protein gives an idea about its three-dimensional structure. It is also important to know the homology between closely related proteins, because it gives an idea about the probable three-dimensional structure. The primary sequence of C-phycocyanin from *S. platensis* was deduced from the gene sequence and deposited in the SWISSPROT database (accession Nos. P72508 and P72509) (73). Alignment against C-phycocyanin from *F. diplosiphon* (**Fig. 6**) showed that there are 31 differing residues in the α chain (19.1%) and 35 differing residues in the β chain (20.3%). The inspection of the electron density of the model indicated a few modifications in the sequence. The final model of sequences is shown in **Fig. 6**. Residue α51 was changed from Arg to Leu. This is consistent with the multiple alignment of known sequences of C-phycocyanin, where Leu at α51 is highly conserved. Inspection of extra density at α148 position prompted us to modify the existing residue from Gly to Thr. Another modification was done in β-chain corresponding to Asn to Ser at position β76.

The unusual methylation of Asn72 in the β chain has been proposed to be conserved in phycobiliproteins and may be involved in energy transfer (83). In C-

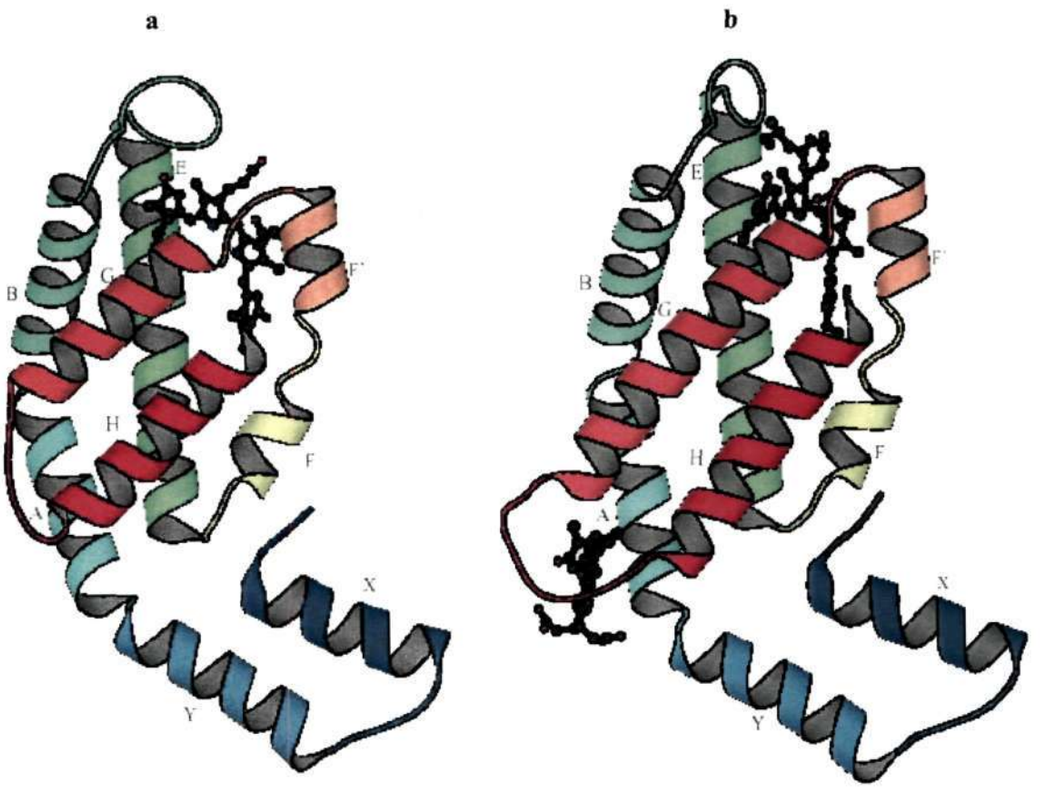


Figure 12. Ribbon representation of (a) CPC_SP α-subunit (b) CPC_SP β-subunit. Chromophores are shown in ball and stick representation.

phycocyanin from *S. platensis*, the side chain of Asn72 in the β chain was also found to be methylated. This side chain is involved in providing crucial contacts through polar interactions to the chromophore β84, thereby isolating it from interactions with the solvent.

In most of the known biliproteins, each polypeptide chain has eight α-helixes. In C-phycocyanin from *S. platensis*, both α- and β-subunits have similar three-dimensional structure, predominantly being α-helical (**Fig. 12**) and is similar to the known structures of C-phycocyanin, which is expected from the high level of sequence identity between them. The overall fold of each chain is a well-defined, helical globin-like domain with seven helices (A, B, E, F, F', G and H, **Fig. 12**) folded similar to that of myoglobin. The PCB chromophore attachment sites at α84 and β84 positions correspond to the heme-group binding site of myoglobin (83). The globin domain is complemented by an additional helical hairpin domain (X and Y) at the N-terminus of each chain, which is responsible for the stability of αβ-monomers (84). These features seen in the present structure are in agreement with the earlier reports of distant evolutionary relationship between globins and CPCs (83,84). Further, the α- and β- polypeptide chains of CPCs were predicted to be related to each other based on hydrophobicities of amino acid sequences (85). The evolutionary relationship between them is evident from the remarkably similar tertiary fold of the two chains in CPC_SP (**Fig. 12**) and in other phycobiliproteins. The structural superposition of CPC_SP α- and β-chains shows two regions of residue insertions with respect to each other. The first one corresponds to a two-residue insertion in α-chain at position 73-74 extending the loop region between B and E helices (**Fig. 12a**). The second insertion corresponds to a ten-residue stretch in β-chain. This occurs between the residues 146-155 in the loop region between the helices G and H, corresponding to a functional accommodation of an additional chromophore at β155 (**Fig. 12b**).

Each of the αβ-monomers has high affinity to assemble together to form a $(\alpha\beta)_3$-trimer. The $(\alpha\beta)_3$-trimers associate in a back to back fashion to form a $(\alpha\beta)_6$-hexamer, the functional unit of a phycobilisome assembly. However, in the present structure there are two such hexamers (**Fig. 11**) appearing in the single asymmetric unit. The overall hollow-

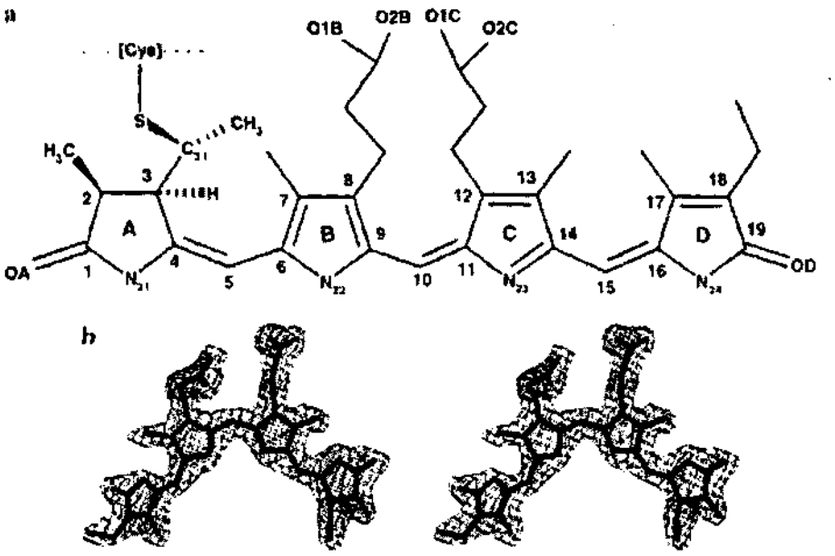


Figure 13. (a) Chemical structure of PCB chromophore with the atom labels (b) Stereo view of ($2F_o$-F_c) electron density map displayed at 1.0σ level for a β84-PCB chromophore

cylindrical structure of CPC_SP $(\alpha\beta)_6$-hexamer closely resembles that of other phycobiliproteins.

Chromophore structure and environment: There are a total of 36 PCB chromophores in the asymmetric unit, three chromophores for each αβ-monomer at α84, β84 and β155 positions. All the PCBs are covalently attached to the respective cysteinyl residues in protein by thioether linkages. The average B-factor for the PCBs is 22.6Å^2 except for PCB attached to β84 of T chain for which it is 44.5 Å^2. The conformation of PCB is similar to that seen in the model structure of CPC_FD (86). The stereoisomers for PCB at the C_{31} atom of ring A (**Fig. 13**) are (R) for α84, β84 and (S) for β155. All chromophores display Z configuration on the D ring as found in CPC_FD (86). The isolated chromophores are cyclic and have low visible absorption. In phycobiliproteins, the attachment to the protein by thioether bonds and the interactions with the surroundings cause the chromophores to be maintained in an extended state, which maximizes absorption in the visible region of the spectrum. The polar and ionic interactions between protein and chromophore are highly conserved and similar kind of environs for chromophore was seen in the present structure. These networks of interactions are responsible for maintaining the proper orientation of the chromophores. This in turn stabilizes the light absorption properties and energy transfer rates of each of the chromophores.

As an outcome of the "double hexameric" aggregation in the present structure, β155 PCBs are brought to a close proximity of each other within the region between the adjacent hexamers in the crystal asymmetric unit (**Fig. 11**). This suggests an additional possible role for β155 PCBs in terms of energy transfer in lateral direction between adjacent disks of C-phycocyanins, and this could be similar to the *in vivo* environs of phycocyanin.

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CHAPTER 2: PART B

Hepatoprotective Effect of C-Phycocyanin: Protection for Carbon Tetrachloride and R-(+)-Pulegone-Mediated Hepatotoxicty in Rats

INTRODUCTION

Spirulina platensis, a unicellular filamentous blue-green algae, is gaining more attention these days because of its nutritional and various medicinal properties (1,2). *Spirulina maxima* has a preventive effect on the fatty liver induced by a fructose-rich diet (2) or carbon tetrachloride in rat (3) suggesting that this alga contains a factor or factors which affect the fructose-induced alterations of triglyceride metabolism. It has also been shown that C- phycocyanin, one of the major biliproteins of *Spirulina platensis*, has anti-inflammatory properties (4). Phycocyanin is structurally similar to delta bilirubin, a liver growth factor (5), and it is also a hepatoprotective agent. It protects the liver against toxic injury (6). These studies have prompted us to evaluate the potential of C-phycocyanin in the prevention of chemical-induced liver injury.

Chemical-induced liver injury has been recognized as a toxicological problem for over 100 years. The vulnerability of the liver to chemical injury is as much a function of its anatomical proximity to the blood supply and digestive tract as to its ability to biotransform and concentrate xenobiotics. The liver response to chemical injury can be extremely varied, reflecting the chemical's properties, the exposure regimen and animal species examined. Carbon tetrachloride (CCl_4) is one of the compounds that is most studied with respect to the manifestation of toxic injury in liver (7,8). It is a well-known model compound for production of chemical hepatic injury, requires biotransformation by liver microsomal cytochrome P450 system to produce the hepatotoxic metabolite trichloromethyl free radical. Trichloromethyl can react with sulfhydryl groups such as glutathione and protein thiols. The covalent binding of trichloromethyl to cellular protein is considered the initial step in a chain of events eventually leading to membrane lipid peroxidation and finally to produce cell necrosis (9,10,11). Although several isoforms of P450 may metabolize carbon tetrachloride, attention has largely been focused on the P450 2E1 isoform, which is ethanol inducible (12,13). Cytochrome P450 2E1 is also involved in the metabolism of several other organic compounds. The reactive intermediates formed during the metabolism of therapeutic agents, toxicants, and carcinogens by this enzyme are frequently capable of covalently binding to tissue macromolecules, which may cause tissue damage (14,15). Like

carbon tetrachloride, R-(+)-pulegone, a monoterpene ketone is also a potent hepatotoxin (16,17) and liver microsomal cytochrome P-450 system is involved in its bioactivation to reactive metabolites responsible for the toxicity (18,19).

In the present study, we have tested the effect of C-phycocyanin from *Spirulina platensis* on carbon tetrachloride and R-(+)-pulegone -induced hepatotoxicity in rats.

MATERIALS AND METHODS

Chemicals

R-(+)-pulegone was obtained from Aldrich Chemical Co. (USA). It was purified by column chromatography as reported earlier. Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, $NADP^+$, Tris-HCl, α-ketoglutarate, and L-alanine were supplied by Sigma Chemicals (St. Louis, MO). All other materials were procured from standard suppliers and were of analytical grade.

C-phycocyanin from *Spirulina platensis*

C-Phycocyanin was isolated from *Spirulina platensis* and the crude phycocyanin obtained was further purified to homogeneity following the reported procedure (20) with few modifications as described in chapter 2: part A. The fractions showing an absorbance ratio of 618 nm/280 nm greater than 4 were pooled. The purity of phycocyanin prepared was examined by native PAGE and SDS-PAGE (details are given in chapter 2, part A).

Animals and treatment

Male albino rats (2-3 months old) weighing 160-180 g were used throughout the course of this investigation. Rats were housed in groups and were fed *ad libitum*. Six groups (A, B, C, D, E and F) of rats, each group with 6 animals, were used in the following way. Unless otherwise mentioned, all treatments were carried out intraperitoneally (i.p.). Group A and B (control rats) received coconut oil (0.3 ml); group C and D received R-(+)-pulegone at a dosage of 250 mg/kg as a suspension in 0.3 ml of coconut oil; group E and F received carbon tetrachloride at a dosage of 0.6 ml/kg as a suspension in coconut oil (0.3 ml). Rats from groups B, D and F were pretreated with phycocyanin (200 mg/kg) dissolved in water (0.5 ml) one hour prior to the administration of coconut oil (control rats), R-(+)-

pulegone and carbon tetrachloride, respectively. 24h after the administration of R-(+)-pulegone and carbon tetrachloride, the animals were sacrificed by cervical dislocation and blood was collected by cardiac puncture for SGPT (ALT) level determinations. The above experiment was repeated by pretreating the animals with phycocyanin three hours prior to the administration of R-(+)- pulegone and carbon tetrachloride. Urine samples were collected in bottles at 0-4° C from rats belonging to the groups C and D.

In time dependent studies, C-phycocyanin (200 mg/kg) dissolved in water, was administered i.p. 1h, 3h, 6h, 12h, and 24h prior to the administration of CCl_4 (0.6 ml/kg). 24h after the administration of CCl_4, the animals were sacrificed by cervical dislocation and blood was collected by cardiac puncture for SGPT (ALT) level determinations.

In dose dependent studies, different concentrations of C-phycocyanin (10-200 mg/kg) dissolved in water, was administered i.p. three hour prior to the administration of CCl_4 (0.6 ml/kg). 24h after the administration of CCl_4, the animals were sacrificed by cervical dislocation and blood was collected by cardiac puncture for SGPT (ALT) level determinations.

Preparation of microsomes

Microsomes were prepared from liver by a differential centrifugation method (21). The microsomal pellet was washed with Tris-HCl buffer (0.01 M, pH 7.4) containing 0.15 M KCl and 1 mM EDTA and centrifuged at 105,000 × g for 60 min. The microsomes were suspended in Tris-HCl buffer (0.05M, pH 7.8) containing 0.25M sucrose and 20% glycerol. Microsomal suspensions were stored at –70° C. Protein concentration was determined according to the method of Lowry *et al.*, using BSA as standard (22)

Enzyme assays

All UV-Visible spectrophotometric measurements were carried out using Hitachi Model 557,double beam double wavelength spectrophotometer. Cytochrome P-450 was estimated according to the method of Omura and Sato (23). Microsomal protein (1 mg) in Tris-HCl buffer (0.01 M, pH 7.4) containing EDTA (1 mM) and KCl (0.15 M) was reduced with sodium dithionite and the baseline correction was made. To the sample cuvette, carbon monoxide was bubbled for 30 sec and the spectrum was recorded. An

extinction coefficient of 91 cm^{-1} mM^{-1} for the difference between 450 nm and 490 nm was used to determine the amount of cytochrome P450.

Serum glutamate pyruvate transaminase (SGPT or ALT) was assayed according to the method of Reitman and Frankel (24). Blood was drawn from the heart by cardiac puncture and allowed to clot. Serum was separated by centrifugation. α-Ketoglutaric acid (29.2 mg) and L-alanine (1.78 g) were dissolved in a small amount of 1N NaOH, the pH was adjusted to 7.4 and the volume was made up to 100 ml in a volumetric flask with 0.1 M phosphate buffer, pH 7.4. From this buffered substrate, 1.1 ml was pipetted into a test tube and preincubated at 37° C for 5 min. Blank experiments consisted of 1.1 ml of buffer alone (without substrate). Serum (0.1 ml) was added to the tubes and then further incubated for 30 min at 37° C (Suitable dilutions of serum were made when activities were high). At the end of the incubation, 0.5 ml of color reagent (19.8 mg of 2,4-dinitrophenylhydrazine in 100 ml of 1 N HCl) was added. After 20 min, 5 ml of 0.4 N NaOH was added. The absorbance of the solution at 505 nm was measured 5 min after the addition of the NaOH. A standard curve was obtained using sodium pyruvate and the units of the enzyme were read from the standard curve after subtracting the blank.

Glucose 6-phosphatase activity was estimated according to the method of Traiger and Plaa (25). Glucose 6-phosphate (189 mg) was dissolved in 25 ml of 0.1 M Tris-maleate buffer, pH 6.2. To 0.4 ml of the buffer, 0.5 ml of glucose 6-phosphate was added and the tubes were preincubated at 37° C for 2 min. The reaction was started by the addition of microsomal protein (100-200 μg) in 0.2 ml of buffer and further incubated for 20 min. The reaction was stopped by the addition of 5 ml of 10% TCA. In blank experiments, TCA was added immediately after the addition of the enzyme. The protein precipitate was removed by centrifugation and the supernatant was assayed for the inorganic phosphate according to the method of Fiske and Subbarow (26).

Aminopyrine-N-demethylase was assayed according to the method of Werrihgloer (27). The assay was carried out in 0.01 M Tris-HCl buffer, pH 7.5, containing 1 mM EDTA. Incubations were carried out in a total volume of 1.5 ml buffer containing 1.5 mg microsomal protein and 0.25 mM 4-dimethyl amino antipyrene (substrate). The reaction

Enzyme activity	Control †	Phycocyanin	R(+)Pulegone	CCl_4	Phycocyanin + R(+)Pulegone	Phycocyanin + CCl_4
Cytochrome P450 (nmol/mg) %change	0.71 ±0.02 -	0.61 ±0.01 -15.0	0.27 ±0.01 - 61	0.26 ±0.01 - 64	0.58 ±0.01 -17	0.51 ±0.01 - 28.0
SGPT (units/ml) Fold change	33.25 ±1.1 -	28.0 ±2.0 - 0.15	504.0 ±36.0 +15.0	485.3 ±18.7 +14.6	31.27 ±1.0 - 0.06	30.34 ±1.8 - 0.09
G-6-Phosphatase(nmol/min/mg) %change	225.1 ±7.6 -	228.5 ±7.5 +1.5	152.2 ±7.9 -32.3	126.6 ±4.6 - 43.7	193.3 ±7.3 -14.0	147.5 ±1.2 - 34.5
Aminopyrine-N-demethylase(nmol/min/mg) %change	7.33 ±0.33 -	6.0 ±0.33 -18.0	3.24 ±0.45 -55.7	3.58 ±0.24 -51.0	6.16 ±0.32 -16.0	5.83 ±0.2 -20.3

Table 2: Effect of phycocyanin pretreatment [+] on CCl_4 (0.6 ml /kg) and R-(+)-pulegone (250 mg/kg)-induced hepatotoxicity in rats.

[+] Rats were pretreated with phycocyanin (200 mg/kg) 3h prior to the administration of CCl_4 and R-(+)-pulegone.

† Animals treated with vehicle alone.

Values represent mean ± S. D. of 3 independent experiments, each consisting of tissues pooled from 6 rats.

mixture was preincubated for 2 min at 37° C and the reaction was initiated by the addition of NADPH generating system (1 mM NADPH, 10 mM glucose 6-phosphtae and 1.5 IU glucose 6-phoshphate dehydrogenase) and further incubated for 30 min. The reaction was stopped by adding 1.5 ml of 10% TCA. Tubes were centrifuged at 3,000 g for 5 min and 2 ml of each supernatant fraction was transferred to a fresh tube. To each tube, 1 ml of Nash reagent (2 M ammonium acetate, 0.05 M acetic acid and 0.02 M acetylacetone) was added and heated for 8 min at 60° C and absorbance at 405 nm was measured. Formaldehyde concentration was estimated from the standard curve. Blank values were obtained by omitting microsomal proteins from the incubation medium.

Extraction of urinary metabolites

Urine samples collected from animals belonging to groups C and D were separately extracted with diethylether as reported earlier (28). Briefly, the urine samples were acidified to pH 4-5 with 1N HCl and extracted 3 times with diethylether. The ether extracts were concentrated and subjected to GC analyses.

Gas chromatography

Analyses were carried out on a Shimadzu GC model 14 A instrument equipped with a hydrogen flame ionization detector. The instrument was fitted with a Shimadzu HR-1 wide bore capillary column (15m × 0.5mm dia). N_2 at a flow rate of 30 ml/min was used as the carrier gas. The temperature of the column was maintained at 80° C for 10min, and then it was raised to 120° C at the rate of 5° C/min.

All statistical analyses were performed using student's t-test and levels of significance determined at $p<0.05$.

RESULTS

Effect of C-phycocyanin (200 mg/kg) pretreatment on CCl_4 and R-(+)-pulegone-induced hepatotoxicity in rats: The results are summarized in **Table 1 and 2**. Intra peritoneal administration of a single dose of R-(+)-pulegone (250 mg/kg) and CCl_4 (0.6 ml/Kg) to rats caused marked decrease in microsomal cytochrome P-450 (50-64%), aminopyrine-N-demethylase (50-57%) and glucose 6-phosphatase activities (31.5-49%) whereas a

Enzyme activity	Control †	Phycocyanin	R(+)Pulegone	CCl_4	Phycocyanin + R-(+)-Pulegone	Phycocyanin + CCl_4
Cytochrome P450 (nmol/mg) %change	0.78 ± 0.01 -	0.72 ± 0.03 -7.7	0.38 ± 0.01 -50.3	0.34 ± 0.05 -55.8	0.63 ± 0.04 -18.7	0.52 ± 0.02 -33.0
SGPT (units/ml) Fold Change	32.2 ± 3.6 -	32.2 ± 3.1 0.0	490.0 ± 39.2 +15.2	469.0 ± 21 +14.5	36.2 ± 3 +0.12	34.6 ± 1.4 +0.07
G-6-Phosphatase(nmol/min/mg) %change	191.5 ±4 -	180.0 ± 2 -6.0	131.2 ± 2.3 -31.5	97.5 ± 3.7 - 49.0	172.5 ± 3.75 -10.0	142.5 ± 7.5 -25.5
Aminopyrine-N-Demethylase (nmol/min/mg) %change	7.0 ± 0.3 -	6.34 ± 0.45 -9.4	3.0 ± 0.33 -57.0	3.5 ± 0.16 -50.0	5.67 ± 0.19 -19.0	5.33 ± 0.33 -23.8

Table 1: Effect of phycocyanin pretreatment [+]on CCl_4 (0.6 ml /kg) and R-(+)-pulegone (250 mg/kg)-induced hepatotoxicity in rats.

[+] Rats were pretreated with phycocyanin (200 mg/kg) 1h prior to the administration of CCl_4 and R-(+)-pulegone.

† Animals treated with vehicle alone.

Values represent mean ± S. D. of 3 independent experiments, each consisting of tissues pooled from 6 rats.

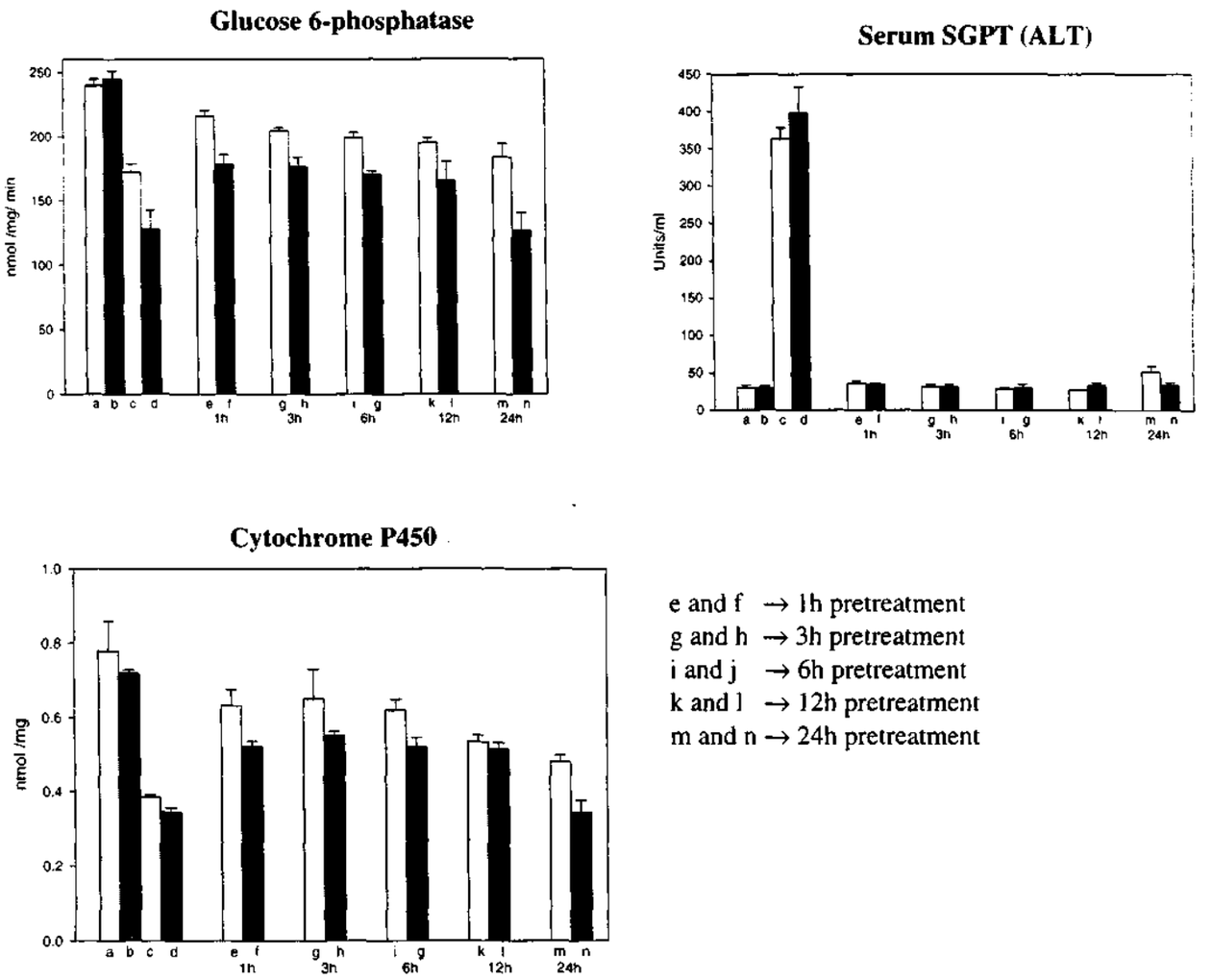

Fig. 1: Effect of phycocyanin pretreatment on CCl_4 (0.6 ml /kg) and R-(+)-pulegone (250 mg/kg)-induced hepatotoxicity in rats.

Rats were pretreated with phycocyanin (200 mg/kg) 1h, 3h, 6h, 12h, 24h prior to the administration of CCl_4 and R-(+)-pulegone.

a) control (vehicle alone)
b) Phycocyanin (200 mg/kg) + vehicle alone
c) R-(+)-pulegone (250 mg/kg) alone
d) CCl_4 (0.6 ml /kg) alone
e, g, i, k and m) Phycocyanin (200 mg/kg) + R-(+)-pulegone (250 mg/kg)
f, h, j, l and n) Phycocyanin (200 mg/kg) + CCl_4 (0.6 ml /kg)

significant increase in SGPT (ALT) level (14.5-15.2 fold) was observed. Phycocyanin (200 mg/kg) when administered alone did not alter any liver function. The levels of all the activities tested were similar to those of control values (**Table 1 and 2**). However, when phycocyanin was administered one or three hours prior to R-(+)-pulegone or CCl_4 administration, the response to these hepatotoxins were significantly reduced (**Table 1 and 2**).

SGPT (ALT) activities were approximately 15 fold greater in rats receiving R-(+)-pulegone or CCl_4 (**Table 1 and 2**). However, SGPT (ALT) levels were almost equal to control values in rats treated with phycocyanin, one or three hours prior to the administration of R-(+)-pulegone and CCl_4 (**Table 1 and 2**). When the rats were pretreated with phycocyanin one hour prior to the administration of R-(+)-pulegone, the reduction in cytochrome P450, glucose 6-phosphatase and aminopyrine-N-demethylase activities were only 18, 10 and 19%, respectively as compared to 50, 32 and 57% in animals treated with only R-(+)-pulegone (**Table 1**). Likewise, when CCl_4 was administered to phycocyanin pretreated rats, there was a 33, 26 and 27% decrease in cytochrome P450, glucose 6-phosphatase and aminopyrine-N-demethylase activities as against 56, 49 and 50% in rats treated with only CCl_4 (**Table 1**). The response to R-(+)-pulegone and CCl_4 was also significantly reduced when phycocyanin pretreatment was made three hours prior to the administration of R-(+)-pulegone and CCl_4 (**Table 2**).

Time course of C-phycocyanin (200 mg/kg) pretreatment on CCl_4 and R-(+)-pulegone-induced hepatotoxicity in rats: Phycocyanin was administered 1h, 3h, 6h, 12h, and 24h prior to the administration of CCl_4 or R-(+)-pulegone to rats. The results are summarized in **Fig. 1**. Time course studies clearly indicated that there was no significant change in the levels of cytochrome P450 and glucose 6-phosphatase as compared to control values (**Fig. 1**). SGPT (ALT) levels were almost equal to control values in rats treated with phycocyanin 1h, 3h, 6h, 12h and 24 hours prior to the administration of CCl_4 or R-(+)-pulegone (**Fig. 1**).

Effect of increasing doses of C-phycocyanin (10-200 mg/kg) pretreatment (3h) on CCl_4-induced hepatotoxicity in rats: Different concentrations of phycocyanin (10, 25, 50, 100,

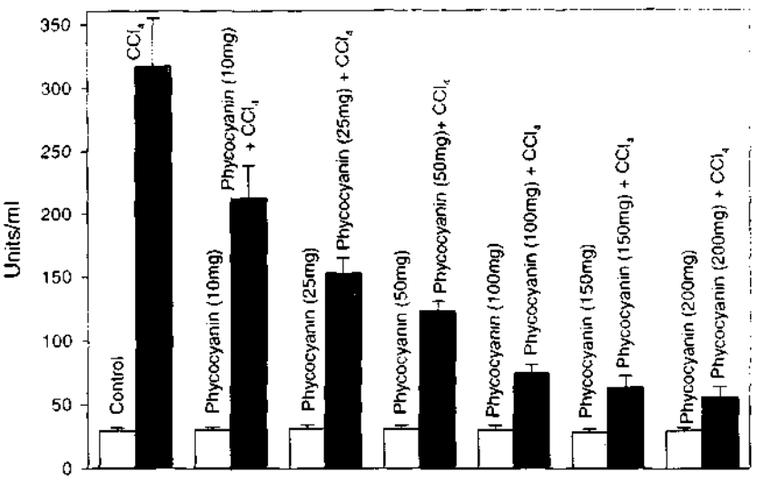

Fig. 2: Effect of different concentration of phycocyanin pretreatment on CCl_4 (0.6 ml /kg)-induced hepatotoxicity in rats.

Serum SGPT level 24 after the administration of CCl_4.

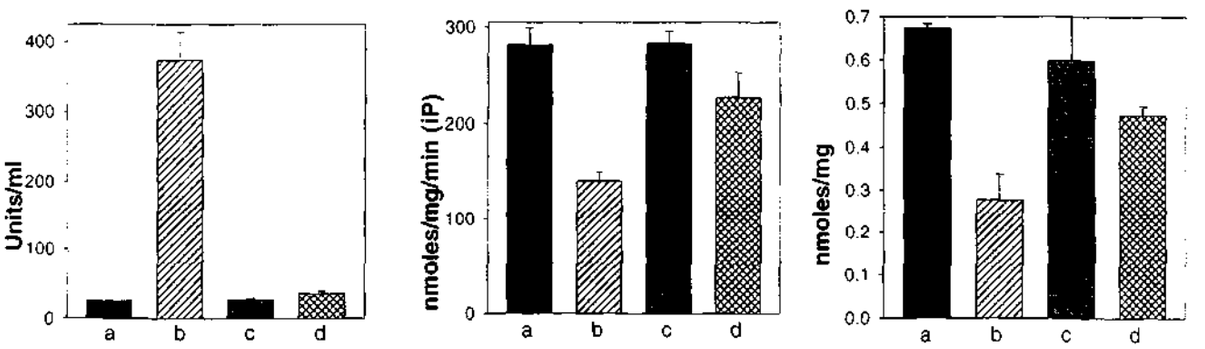

Fig. 3: Effect of phycocyanin (100 mg/kg) pretreatment on CCl_4 (0.6 ml /kg)-induced hepatotoxicity in rats.

Serum SGPT, liver cytochrome P450 and glucose 6-phosphatase activity 24h after the administration of CCl_4.

a) Control

b) CCl_4 (0.6ml/kg) alone

c) Phycocyanin alone (100 mg/kg)

d) Phycocyanin (100 mg/kg) + CCl_4 (0.6ml/kg)

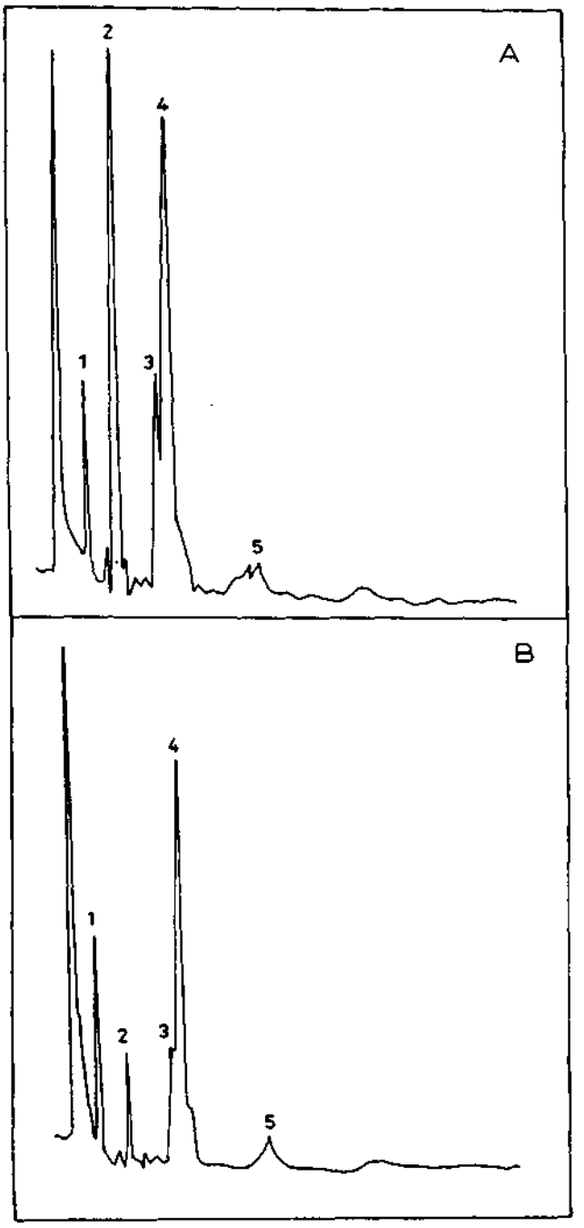

Fig. 4: GC separation of major urinary metabolites from (A) R-(+)-pulegone treated rats, (B) R-(+)-pulegone C. phycocyanin pretreated rats. (1) p-Cresol, (2) R-(+)-menthofuran, (3) R-(+)-pulegone, (4) piperitone, and (5) piperitenone. Experimental conditions are as reported under "Methods"

150, 200 mg/kg) was administered 3 hour prior to the administration of CCl_4. 24 Hour after the administration of CCl_4, the level of SGPT (ALT) was measured in serum. The results are summarized in **Fig. 2**. Phycocyanin protected the liver against CCl_4-induced hepatotoxicity in a dose dependent manner (**Fig. 2**). This is evidenced by the level of SGPT (ALT) in serum (**Fig. 2**). When 100 mg/kg of phycocyanin was used for pretreatment 3 hour prior to the administration of CCl_4, the SGPT (ALT) level in the serum is almost equal to control value. Similar results were also obtained when pretreatment of rats with phycocyanin (150 mg or 200 mg/kg) prior to the administration of CCl_4 (**Fig. 3**). When CCl_4 was administered to phycocyanin (100 mg/kg) pretreated rats, there was a 30% and 19% decrease in cytochrome P450 and glucose 6-phosphatase activities as against 63% and 50% in rats treated with only CCl_4 (**Fig. 3**).

Effect of phycocyanin on the mode of metabolism of R-(+)-pulegone in rats: The urine samples collected from rats treated with R-(+)-pulegone and rats treated with the combination of phycocyanin (200 mg/kg) and R-(+)-pulegone were analyzed by GC and the levels of various major metabolites present in the urine extracts from these two groups were compared (**Fig. 4**). The typical gas chromatogram shows (**Fig 4A and B**) that the level of menthofuran was significantly higher (nearly 70% more) in the urine of rats treated with R-(+)-pulegone alone compared to the urine of rats treated with the combination of phycocyanin and R-(+)-pulegone . However, there was only marginal changes in the levels of other major metabolites (**Fig 4**).

DISCUSSIONS

The biochemical mechanisms involved in the development of R-(+)-pulegone or CCl_4-induced hepatotoxicity has long been investigated. It is now generally believed that the formation of reactive metabolites and radicals from metabolism of these chemicals is a crucial factor in the pathogenesis of chemical-induced hepatotoxicity (29). The chemical-induced hepatotoxicity can be prevented or reduced by pretreatment with inhibitors of metabolism (30) or antioxidants and radical scavengers (31).

Effect of C-phycocyanin pretreatment on CCl_4 and R-(+)-pulegone-induced hepatotoxicity in rats is shown in **Table 1 and 2**. Consistent with earlier investigations (4-8), it was noticed that i.p. administration of a single dose of R-(+)-pulegone (250 mg/Kg) and CCl_4 (0.6 ml/Kg) to rats caused marked decrease in microsomal cytochrome P450, aminopyrine-N-demethylase and glucose 6-phosphatase activities and a significant increase in SGPT (ALT) level. Phycocyanin (200 mg/kg) when administered alone did not alter liver function. The levels of all the activities tested were similar to those of control values (**Table 1 and 2**). However, upon administration of phycocyanin one or three hours prior to R-(+)-pulegone or CCl_4 challenge, the response to these hepatotoxins were significantly reduced (**Table 1 and 2**). In fact SGPT (ALT) activities were approximately 15 fold greater in rats receiving R-(+)-pulegone or CCl_4 alone compared to those rats treated with the combination of phycocyanin and R-(+)-pulegone or phycocyanin and CCl_4 (**Table 1 and 2**). When the rats were pretreated with phycocyanin one hour before the administration of R-(+)-pulegone , the decrease in cytochrome P450, glucose 6-phosphatase and aminopyrine-N-demethylase activities were only 18, 10 and 19%, respectively as compared to 50, 32 and 57% in animals treated with only R-(+)-pulegone (**Table 1**). Likewise, when CCl_4 was administered to phycocyanin pretreated rats, there was a 33, 26 and 27% decrease in cytochrome P450, glucose 6-phosphatase and aminopyrine-N-demethylase activities as against 56, 49 and 50% in rats treated with only CCl_4 (**Table 1**). The response to R-(+)-pulegone and CCl_4 was also significantly reduced when phycocyanin pretreatment was carried out three hours prior to the administration of R-(+)-pulegone and CCl_4 (**Table 2**). Phycocyanin effectively prevented the R-(+)-pulegone and CCl_4-induced hepatotoxicity even when it was given 24 hours prior to the administration of R-(+)-pulegone and CCl_4 (**Fig. 1**). However, phycocyanin is more effective when it was given 3h prior to the administration of hepatotoxins. These studies have indicated that, pretreatment of rats with phycocyanin prior to the administration of R-(+)-pulegone and CCl_4 resulted in a significant protection of liver functions and prevention of toxicity. In fact the levels of SGPT (ALT) were almost equal to control values in rats treated with phycocyanin, one or three or 24 hours prior to the administration of R-(+)-

pulegone and CCl_4 (**Table 1 and 2 and Fig. 1**). Our results suggest that phycocyanin pretreatment has a greater effect on R-(+)-pulegone than CCl_4 induced hepatotoxicity.

Both CCl_4 and R-(+)-pulegone are metabolized by liver cytochrome P450 system to reactive metabolites and radicals. These reactive metabolites and radicals induce the lipid peroxidation in cell membranes and are responsible for cell necrosis and cell death. During this process the cytosolic enzyme leaks out into the blood and the serum SGPT (ALT) level increases. The SGPT (ALT) level in serum indicates the extent of liver damage. During acute liver injury, glucose 6-phosphatase activity in endoplasmic reticulum will decrease. The reactive metabolites damage and destabilize the membranes and inactivates the glucose 6-phosphatase, which is a membrane bound enzyme. More over, these reactive metabolites form adducts with cellular proteins. During all these processes the glucose 6-phosphatase gets inactivated. The serum SGPT (ALT) level tremendously decreased and the inactivation of glucose 6-phosphatase was prevented in phycocyanin pretreated rats than in the rats treated with only CCl_4 or R-(+)-pulegone. It suggests that phycocyanin may act as an antioxidant and inhibits the membrane lipid peroxidation in hepatocytes during chemical-induced liver injury. Earlier it has been reported that, *Spirulina maxima* prevents the fructose-rich diet or CCl_4-induced fatty liver in mice (2,3). It is quite possible that phycocyanin, one of the major constituents of *spirulina,* may be responsible for the observed hepatoprotective property of *spirulina.*

It is also important to know the effective dose of phycocyanin that is required to prevent the hepatotoxicity. Phycocyanin protects the CCl_4-induced liver injury in a dose dependent manner and at 100 mg phycocyanin/kg body weight, produced optimal effects (**Fig.2 and 3**). When phycocyanin (100 mg/kg) was given 3h prior to the administration of CCl_4, the level of SGPT (ALT) in serum was almost equal to normal in phycocyanin pretreated rats as compared to those treated with CCl_4 alone (**Fig. 3**). The inactivation of cytochrome P450 and glucose 6-phosphatase activities was reduced tremendously in phycocyanin pretreated rats (**Fig. 3**).

Many hepatotoxicants including carbon tetrachloride, R-(+)-pulegone, nitrosamines, and polycyclic aromatic hydrocarbons require metabolic activation,

especially by liver microsomal cytochrome P450 (P450) enzymes, to form reactive, toxic metabolites, that in turn produce liver injury in experimental animals and humans (32). It is an accepted fact that hepatotoxicity of carbon tetrachloride results from reductive dehalogenation catalyzed by reduced P450 2E1 to form a highly reactive trichloromethyl free radical, which interacts readily with molecular oxygen to form the trichloromethyl peroxy radical. Both radicals are capable of binding to proteins and lipids, or abstracting a hydrogen atom from an unsaturated lipid, thereby initiating lipid peroxidation and liver damage (11). In a similar way, R-(+)-pulegone is also metabolized by liver microsomal cytochrome P450 system and generates reactive metabolites. These reactive metabolites are capable of binding to proteins and lipids, or abstracting a hydrogen atom from an unsaturated lipid, thereby initiating lipid peroxidation and liver damage (15).

In order to find out the effect of phycocyanin on the mode of metabolism of R-(+)-pulegone, experiments were carried out *in vivo* where the urine samples collected from rats treated with R-(+)-pulegone and rats treated with the combination of phycocyanin and R-(+)-pulegone were analyzed by GC and the levels of various major metabolites present in the urine extracts from these two groups were compared (conditions for GC analyses were as mentioned under "methods"). The typical gas chromatogram shows (**Fig 4A and B**) that the level of menthofuran was significantly higher (nearly 70% more) in the urine of rats treated with R-(+)-pulegone alone than in the urine of rats treated with the combination of phycocyanin and R-(+)-pulegone . However, there was only marginal changes in the levels of other major metabolites (**Fig. 4**). This is a significant observation since menthofuran is considered as the proximate toxin of R-(+)-pulegone and is responsible for at least half of the hepatocellular necrosis caused by R-(+)-pulegone (33). It is known that microsomal cytochrome P-450 system carries out the regiospecific oxidation of R-(+)-pulegone to its allylic alcohol (9-hydroxypulegone)which, upon cyclization followed by dehydration yields menthofuran (18,19). The cytochrome P-450 system further converts menthofuran to its epoxide which could easily give rise to an α, β -unsaturated- γ -ketoaldehyde, a highly reactive metabolite known to covalently interact with tissue macromolecules generating toxicity (34,35). So it is quite possible that phycocyanin could interact preferentially with

individual species of cytochrome P450 and thus could affect the formation of 9-hydroxypulegone, which is the precursor of menthofuran. It is also possible that the cytochrome P450 mediated reaction involved in the conversion of menthofuran to its epoxide may be inhibited so that the reactive metabolite viz.α, β-unsaturated-γ-ketoaldehyde may not be formed in sufficient quantities to elicit toxicity. So it appears that prior administration of phycocyanin protects against CCl_4 and R-(+)-pulegone mediated toxicity by lowering the biotransformation of these hepatotoxins into toxic intermediates. This assumption is supported by the fact that higher levels of menthofuran was shown to be present in the urine of rats treated with R-(+)-pulegone alone than in the urine of rats treated with the combination of phycocyanin and R-(+)-pulegone (**Fig. 4**). It is also possible that the haloalkane free radicals produced from CCl_4 and reactive metabolites formed from R-(+)-pulegone by the liver microsomal cytochrome P-450 systems are being scavenged by phycocyanin. Recent reports suggest that phycocyanin has the ability to scavenge alkoxy and hydroxyl radicals (4). Natural compounds that inhibit or reduce the chemical activating enzymes or antioxidants could be considered as good candidates to protect against chemical-induced toxicity. More experiments have to be carried out to establish the mechanisms involved in the hepatoprotection by phycocyanin.

CONCLUSIONS

The results presented here demonstrate that C-phycocyanin, one of the major biliproteins of *Spirulina platensis*, can significantly reduce R-(+)-pulegone and CCl_4 induced liver injury in rats. The responses to both of these hepatotoxins are significantly reduced in the presence of phycocyanin possibly due to lower levels of reactive metabolites formed. Phycocyanin may inhibit some of the cytochrome P450 mediated reactions involved in the formation of reactive metabolites may also contribute to its hepatoprotective action. It is also possible that phycocyanin may act as an efficient radical scavenger and antioxidant *in vivo*.

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CHAPTER 2: PART C

C-Phycocyanin: A Potent Peroxyl Radical Scavenger *in Vivo* and *in Vitro*

INTRODUCTION

The nutritional and therapeutic values of *Spirulina*, a blue green algae, are very well documented (1-3). Recent studies indicated that *Spirulina maxima* prevent the fructose rich diet and CCl_4-induced fatty liver in rat (3,4). C-phycocyanin, a water soluble protein pigment, is one of the major constituents of *Spirulina platensis*. Its various medicinal as well as pharmacological properties have been reported earlier (5-8). We have demonstrated that C-phycocyanin significantly reduces carbon tetrachloride (CCl_4) and R-(+)-Pulegone-induced hepatotoxicity in rats (8). The hepatoprotective effect of phycocyanin could be due to the inhibition of some of the cytochrome P450 mediated reactions involved in the formation of reactive metabolites or its ability to act as an efficient radical scavenger or both (8). In fact it has been shown that the hepatotoxic effects of CCl_4 are mainly due to its metabolic activation in the liver endoplasmic reticulum to reactive metabolites such as haloalkane free radicals with toxic consequences (9). These free radicals interact with membrane lipids and initiate the chain reaction of lipid peroxidation leading to tissue damage (10). Membrane lipid peroxidation is in fact responsible for the leakage of cytosolic enzymes to serum during chemical-induced toxicity. Pretreatment with inhibitors of CCl_4 metabolism (11) or antioxidants (12) protects the CCl_4-induced liver injury. In our earlier studies we have demonstrated that phycocyanin completely inhibits the leakage of glutamate pyruvate transaminase (SGPT or ALT) to serum after a massive dose of CCl_4 (8). So, it is reasonable to assume that phycocyanin efficiently scavenges free radicals, inhibits the membrane lipid peroxidation and protects the liver against chemical-induced damage. Recently the anti-inflammatory properties of phycocyanin have been reported and it was suggested that the antioxidant property might be involved in the anti-inflammatory property of phycocyanin (5,6).

Serious attention is now paid to the cytotoxicity of active oxygen/free radicals as the cause of various pathological conditions. Lipid peroxides, produced from unsaturated fatty acids via radicals, cause cytotoxicity and promote the formation of additional free radicals in a chain reaction-type manner. It is believed that, if the radicals generated are not efficiently scavenged or their formation is not prevented, various diseases such as arteriosclerosis, liver disease, diabetes, inflammation, renal failure or accelerated aging

may result (13,14). The role of dietary antioxidants and their potential benefits in health and disease have attracted great attention (15). *Spirulina*, a unicellular filamentous blue-green alga, is one such example. It is a natural food having various beneficial properties. Recently it has been shown that *Spirulina* contains antioxidant activities and this activity has been shown to be present only in a few species of this algae (16,17).

In the present study, we evaluated the relationship between pharmacological and antioxidant effect and radical scavenging property of phycocyanin *in vivo* and *in vitro*. We have also demonstrated that phycocyanin with a reduced chromophore inhibits radical induced lipid peroxidation. We present evidence to support the involvement of chromophore in the radical scavenging property of phycocyanin.

MATERIALS AND METHODS

Chemicals

2-Deoxyribose, thiobarbituric acid (TBA), egg phosphotidyl choline (PC), butylated hydroxytoluene (BHT), β-nicotinamide adenine dinucleotide (reduced form, NADH), xanthine, xanthine oxidase, phenazine methosulfate (PMS) were purchased from Sigma (St. Louis, MO). Sodium borohydride ($NaBH_4$) and 2,2'-Azo-bis(2-amindinopropane) hydrochloride (AAPH) was obtained from Aldrich Chemical Co. (St. Louis, MO). All other chemicals from standard suppliers and were of analytical grade.

Crocin was isolated from saffron by water/methanol extraction, which was initially repeatedly extracted with diethyl ether to eliminate possible interfering substances as reported earlier (18,19). The extract containing crocin was diluted with 10 mM phosphate buffer (pH 7.4), and estimated using extinction coefficient 89,000 M^{-1} cm^{-1} reported for crocin in aqueous solution (20).

C-phycocyanin from *Spirulina platensis*

C-phycocyanin was isolated and purified to homogeneity from *Spirulina platensis* as described in the previous chapter (Chapter 2, part A). Highly pure phycocyanin was used throughout the study (A618 nm/A280 nm, >4). The pure phycocyanin was dialyzed extensively against water and then subjected to electrospray ionization mass spectrometry to determine the molecular mass. HP-1100 MSD mass spectrometer was used for this

purpose. Molecular mass of phycocyanin was found to be 37,468.5 mass units and this value was used to calculate the concentration of phycocyanin throughout the study.

Reduction of covalently linked chromophore (PCB)

The chromophore (PCB) in phycocyanin was chemically reduced using $NaBH_4$ and the reaction was monitored spectrophotometrically. Reaction was carried out in deionized water. Procedure followed was similar to that used for the reduction of biliverdin-protein complex using $NaBH_4$ (21). Pure C-phycocyanin and $NaBH_4$ (1:0.5 w/w) were dissolved in water at 4° C. After the reaction, it was dialyzed against water and freeze dried.

CCl_4-induced lipid peroxidation in rat liver *in vivo*

The liver injury in rat was induced as reported earlier (8,22). Male albino rats weighing 160-180 g were used. All the treatments were carried out intraperitoneally (i.p.). Carbon tetrachloride (CCl_4) at a dosage of 0.6 ml/kg was administered as a suspension in coconut oil (0.3 ml). To find out the effect of phycocyanin on CCl_4-induced lipid peroxidation, rats were pretreated with phycocyanin (10-200 mg/kg body wt.) dissolved in water (0.5 ml) three hours prior to the administration of CCl_4 (0.6 ml/kg). Animals were sacrificed by cervical dislocation 24h after CCl_4 administration. Two sets of control experiments were carried out; (i) rats receiving only the vehicle (ii) rats receiving vehicle with phycocyanin. Both the control and experimental rats were housed separately in cages with free access to food and water.

The liver was removed after perfusion *in situ* with ice-cold KCl (1.15%) and 10% liver homogenate was prepared in ice-cold KCl (1.15%) solution. The extent of hepatic lipid peroxidation was assayed by measuring malondialdehyde (MDA) in liver homogenate using thiobarbituric acid (TBA) as described earlier (23). Briefly, 3 ml of 1% phosphoric acid and 1 ml of 0.6% TBA aqueous solution were added to 0.5 ml of liver homogenate. The reaction mixture was boiled for 45 min, cooled, 4 ml of n-butanol was added and mixed vigorously and centrifuged. The absorbance of n-butanol phase was measured at 532 nm. 1,1,3,3-Tetraethoxypropane was used as standard MDA. The results are expressed as nmols of MDA/gram wet weight of liver.

Peroxyl radical or iron-ascorbate-induced lipid peroxidation in rat liver microsomes:

Microsomal fraction was prepared from the liver of male albino rats (160-180 g body wt.) as previously described (24). Microsomes were prepared from liver by a differential centrifugation method. The liver homogenate was prepared and centrifuged at 10000 g for 20 min. The supernatant obtained was centrifuged at 105000 g for 60 min. The microsomal pellet was washed (twice) and suspended in 1.15% KCl solution and stored at -20° C. The protein concentration was determined by the method of Lowry, *et al*, (25).

Freshly prepared microsomes (1.5 mg) were pre-incubated in potassium phosphate buffer (10 mM, pH 7.4), with or with out phycocyanin (1-250 μM) in a total volume of 1.0 ml for 10 min at 37° C. The lipid peroxidation was initiated either by the addition of AAPH (20 mM final concentration) or iron (10 μM)-ascorbate (100 μM) mixture and incubated for a further period of 1 hour at 37° C. The extent of lipid peroxidation was assayed by measuring thiobarbituric acid (TBA)-reactive substances (TBARS) in microsomal membranes as reported earlier (26) with minor modification. Briefly, 3 ml of stopper solution [TCA (15%, w/v)-TBA (0.375%, w/v)-HCl (0.125 M)-BHT (0.6 mM)] was added, mixed and centrifuged at 10,000 g for 15 min at 4° C. Phycocyanin reacts with TBA at higher temperature yielding a pink chromogen with an absorption peak at 528 nm. In order to prevent this reaction, phycocyanin was removed by centrifugation after addition of stopper solution. The supernatant was transferred, boiled for 30 min, cooled and used to quantify TBARS spectrophotometrically at 532 nm. The results are expressed as % inhibition, which represents the degree of protection by phycocyanin against the AAPH or iron-ascorbate-induced membrane lipid peroxidation.

Peroxyl radical or iron-ascorbate-induced lipid peroxidation in phosphatidylcholine (PC) liposomes:

Multilamellar phosphatidylchoine (PC) liposomes were prepared immediately before the experiments by dissolving PC in chloroform and removing the solvent under vacuo. The dried lipid film was hydrated with 5 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, by vigorous shaking for 2 min followed by ultrasonic irradiation with a

BRASON Ultrasonicator B-12 (55 kHz) for 30 sec at ambient temperature. Liposomes (4 mg/ ml) in 5 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl were preincubated with or without phycocyanin (0-75 μM). Lipid peroxidation was initiated by the addition of AAPH (20 mM final concentration) or iron-ascorbate mixture (10 μM and 100 μM respectively) and incubated for a further period of 1 h at 37° C. The extent of lipid peroxidation was assayed by measuring thiobarbituric acid (TBA)-reactive substances (TBARS) in PC liposomes as described for microsomal lipid peroxidation. The results are expressed as % inhibition, which represents the degree of protection by phycocyanin against the AAPH or iron-ascorbate-induced membrane lipid peroxidation.

Time dependent effect of native and reduced phycocyanin on peroxyl radical-induced lipid peroxidation in rat liver microsomes:

Freshly prepared microsomes (1.5 mg) were pre-incubated in potassium phosphate buffer (10 mM, pH 7.4), with or without native/reduced phycocyanin (10 and 20 μM) in a total volume of 1.0 ml for 10 min at 37° C. Then the lipid peroxidation was initiated by the addition of AAPH (20 mM, final concentration) and the mixture was further incubated at 37° C and aliquots were taken at different time intervals (0, 15, 30, 45, 60, 75, 90, 120, 150 min). The extent of lipid peroxidation was assayed by measuring thiobarbituric acid (TBA)-reactive substances (TBARS) in microsomal membranes as mentioned above. The results are expressed as % inhibition.

Interaction of phycocyanin with peroxyl and hydroxyl radicals:

In these studies, AAPH and Fenton reagent were used for the generation of peroxyl and hydroxyl radicals, respectively. Phycocyanin (10 μM) dissolved in 1.0 ml of phosphate buffer (10 mM, pH 7.4) was taken in a 1 ml quartz cuvette thermostated at 37° C. The reaction was initiated by adding freshly prepared AAPH solution (0.5 M in 20 μl) or a mixture of ferrous iron solution (20 μM), EDTA (100 μM), H_2O_2 (1.42 mM) and ascorbate (100 μM). While generating the hydroxyl radicals, ferrous iron solution and EDTA were added to phycocyanin and the reaction was initiated by the addition of a mixture of H_2O_2 and ascorbate. The final reaction mixture contained phycocyanin (10 μM), phosphate buffer (10 mM, pH 7.4) and AAPH (10 mM)/or Fenton reagent in a total volume of 1.0 ml.

The changes in the UV-Visible spectra of phycocyanin were recorded between 300-700 nm for every 5 min in a Shimadzu UV2100 thermostated spectrophotometer.

Interaction of peroxyl radical with chemically reduced phycocyanin was carried out in the phosphate buffer (10 mM, pH 7.4). The changes in the absorption of native and reduced phycocyanin at 618 nm were recorded continuously up to 40 min.

Crocin bleaching assay for peroxyl radical scavenging activity of C-phycocyanin

The reactivity of phycocyanin with peroxyl radicals was measured by competition kinetics of crocin bleaching in the presence of peroxyl radicals generated by the thermal decomposition of an azo compound (18). The test was carried out at 40° C in phosphate buffer (10 mM, pH 7.4) containing crocin (10 μM) and increasing concentrations (0-50 μM) of phycocyanin in a total volume of 1 ml. Uric acid (0-10 μM) was used as a known peroxyl radical scavenger. The peroxyl radicals were generated by adding AAPH (10 mM) and the rate of crocin bleaching was recorded at 440 nm in a thermostated spectrophotometer. The bleaching rate was linear 1.5 min after the addition of AAPH and the rate from 2 to 5 min was used for calculations. Bleaching rates were plotted as Vb/Va versus [A]/[C], according to the equation Vb/Va = 1 + Ka/Kc. [A]/[C], where Vb is the basal bleaching rate of crocin in the absence of phycocyanin, Va is bleaching rate of crocin in the presence of phycocyanin, [C] and [A] are the concentrations of crocin and phycocyanin, respectively. Ka and Kc are the rate constants for the reaction of the peroxyl radical with phycocyanin and crocin, respectively. This plot gives a straight line, intersecting the ordinate, with a slope of Ka/Kc.

Measuring hydroxyl radical scavenging:

Hydroxyl radical scavenging activity of phycocyanin was estimated by inhibition of deoxyribose degradation as described earlier (27). Hydroxyl radicals were generated by a mixture of ascorbic acid, H_2O_2 and Fe^{2+}-EDTA (27). Each assay contained 2-deoxyribose (2.8 mM), ferrous iron solution (20 μM), EDTA (100 μM) and different concentrations of C-phycocyanin (0-250 μM) in a total volume of 1.2 ml of phosphate buffer (10 mM, pH 7.4). The ferrous iron solution and EDTA were premixed before they were added to the assay mixture. The reaction was initiated by the addition of a mixture of H_2O_2 (1.42 mM)

and ascorbate (100 μM), and incubated at 37° C for 30 min. Then 1 ml of thiobarbituric acid (1%, w/v) in NaOH (50 mM) and 1 ml of TCA (2.8%, w/v) were added, centrifuged at 5,000 rpm for 10 min to remove fine suspended particles, boiled for 20 min, cooled, and the absorbance was measured at 532 nm. The results were expressed as % inhibition by phycocyanin. Reciprocal absorption values obtained for different concentrations were plotted against the concentrations of phycocyanin and from the graph the rate constant for the reaction of phycocyanin with hydroxyl radical was determined assuming that deoxyribose reacts with hydroxyl radical with a rate constant of 3.1×10^9 M^{-1} S^{-1} (27). In a similar way the rate constants for established scavengers of hydroxyl radical such as mannitol and dimethylsulfoxide (DMSO) were determined.

Superoxide anion scavenging activity:

Superoxide anion radical scavenging activity of C-phycocyanin was determined by monitoring the competition kinetics of cytochrome c reduction by superoxide anion generated by xanthine- xanthine oxidase system (enzymatically) (28) or NADH-PMS system (non-enzymatically) (29) in phosphate buffer (50 mM, pH 7.8). Various concentrations of phycocyanin (0-200 μM), 40 μM cytochrome c, 0.1 mM EDTA and xanthine oxidase (the enzyme activity was adjusted by dilution to produce a linear flux of superoxide anions that gave a spectrophotometrically initial rate of 0.2 OD/min at 550 nm by the reduction of cytochrome c) in phosphate buffer (50 mM, pH 7.8) were preincubated for 1 min at room temperature in a 1 ml cuvette. The reaction was started by the addition of 100 μM of xanthine and change in the absorption at 550 nm was recorded for 2 min. Superoxide anion radical was also generated using PMS-NADH system where PMS (10 μM) was added instead of xanthine oxidase and the reaction was started by the addition of NADH (78 μM). The results were expressed as % inhibition of cytochrome c reduction.

Fluorescence quenching experiments:

Fluorescence quenching experiment was carried out in sodium acetate buffer (50 mM, pH 5.2) in order to check the iron chelating ability of C-phycocyanin. C-phycocyanin (0.65 μM) was taken in sodium acetate buffer (50 mM, pH 5.2) and the fluorescence quenching studies were carried out as described earlier (30). Different concentrations of

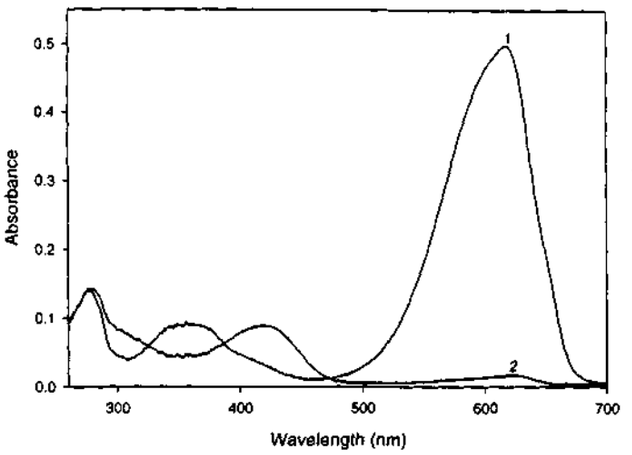

Fig. 1: UV-Visible spectrum of (1) native phycocyanin and (2) $NaBH_4$ reduced C-phycocyanin

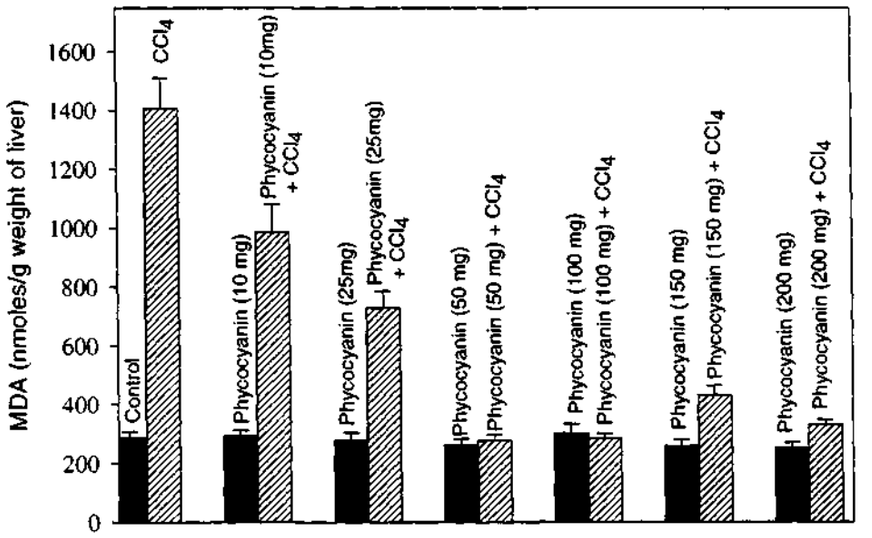

Fig. 2: Effect of c.phycocyanin on CCl_4-induced hepatic lipid peroxidation in vivo in rats. The extent of tissue lipid peroxidation was measured as MDA. Results were expressed as nmols of MDA / gram wet weight of liver. Different doses of phycocyanin/kg, administered 3 h prior to CCl_4 (0.6 ml / kg) administration (i.p.). Values represent mean ± S.D. of 3 independent experiments, each consisting of tissues pooled from 4-6 rats.

ferric iron (0-200 μM) was added after which the change in the fluorescence of phycocyanin was noted using Hitachi 650-10S Fluorescence Spectrophotometer.

The fluorescence intensities were analyzed according to the Stern-Volmer equation: $F_0/F = 1 + KM$, by plotting F_0/F versus M, where F_0 and F are the fluorescence intensities in the absence and in the presence of quencher M (iron ion), respectively and K is the Stern-Volmer quenching constant.

RESULTS

Native phycocyanin was chemically reduced by using $NaBH_4$. Chemical reduction of covalently linked chromophore phycocyanobilin (PCB) was monitored by changes in the UV-visible spectrum of phycocyanin (**Fig. 1**). Addition of $NaBH_4$ rapidly reduces the C-10 methine bridge of the PCB as detected by a color change (blue-green to yellow) and also by UV-visible spectroscopy (**Fig. 1**). Reduction resulted in the color change (deep blue to greenish yellow) and disappearance of absorption at 360 and 618 nm and appearance of strong absorption near 418 nm in the UV-visible spectrum (**Fig. 1**). The spectral characteristics resemble to that of phycocyanorubin, an analogue of bilirubin (31).

Effect of C-Phycocyanin pretreatment on CCl_4-induced liver lipid peroxidation in rats *in vivo*, measured as MDA (nmols/gm wet weight of liver) is shown in **Fig. 2**. Exposure of animals to CCl_4 results in cell damage and lipid peroxidation is generally invoked as an explanatory event that leads to injury (32). Single i.p. administration of CCl_4 (0.6 ml / kg) to rats caused marked increase in liver MDA level (**Fig. 2**). The liver MDA level was nearly 5 fold greater in CCl_4 treated rats than in control rats or rats treated with the combination of phycocyanin and CCl_4 (**Fig. 2**). Different doses of phycocyanin when administered alone did not change the level of MDA and it was found to be similar to that of the control value. However, administration of phycocyanin (10-200 mg / kg body wt.) three hours prior to CCl_4 treatment resulted in significantly lower production of MDA than that found in rats receiving only CCl_4. Maximum inhibition of liver lipid peroxidation was noticed when phycocyanin (100 mg/kg body weight) was administered 3h prior to the CCl_4 administration (**Fig. 2**). Phycocyanin inhibits the CCl_4-induced hepatic lipid peroxidation in a dose dependent manner (**Fig. 2**).

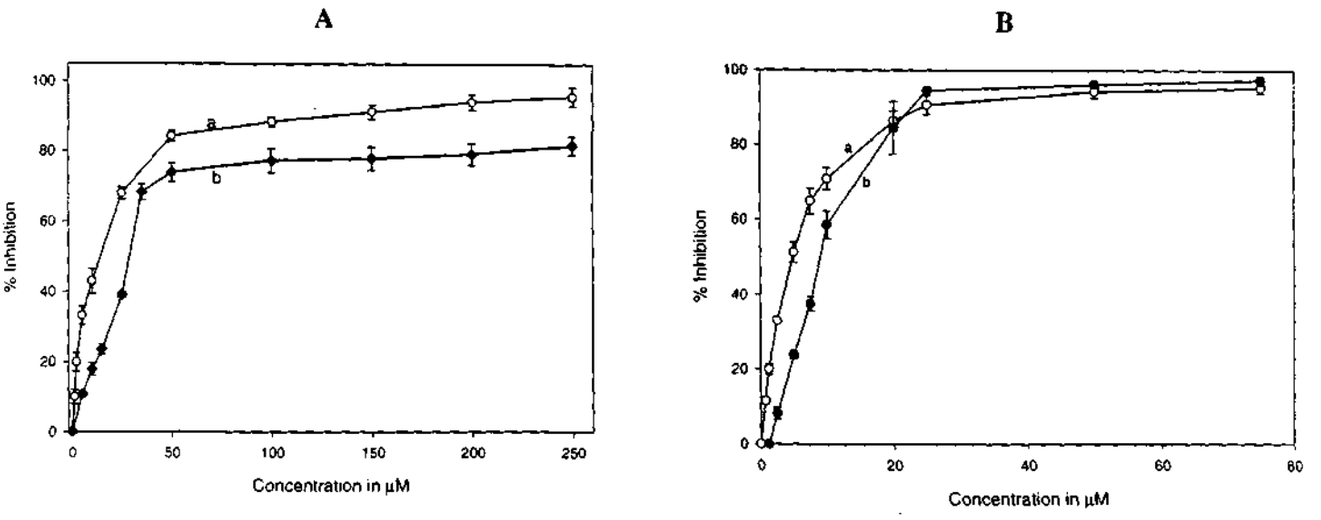

Fig. 3: The inhibitory effect of native phycocyanin and on a) peroxyl radical and b) iron-ascorbate-induced lipid peroxidation (TBARS formation) in rat liver microsomes (A) and in PC liposomes (B). The results are expressed as % inhibition of lipid peroxidation. The experimental details are as described in the Materials and Methods section.

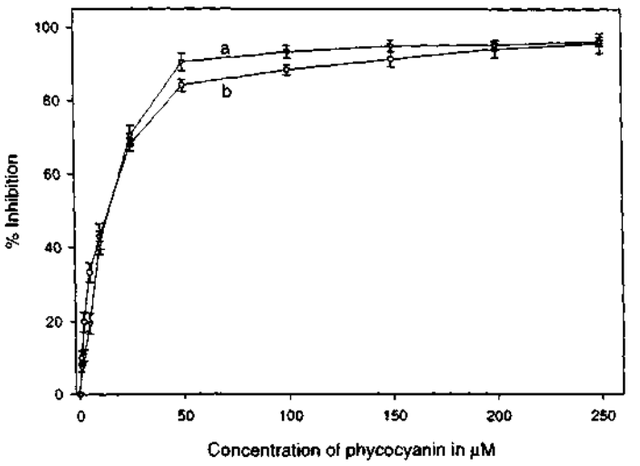

Fig. 4: The inhibitory effect of native phycocyanin (a) and $NaBH_4$ reduced phycocyanin (b) on peroxyl radical-induced lipid peroxidation (TBARS formation) in rat liver microsomes. The results are expressed as % inhibition of lipid peroxidation. The experimental details are as described in the Materials and Methods section.

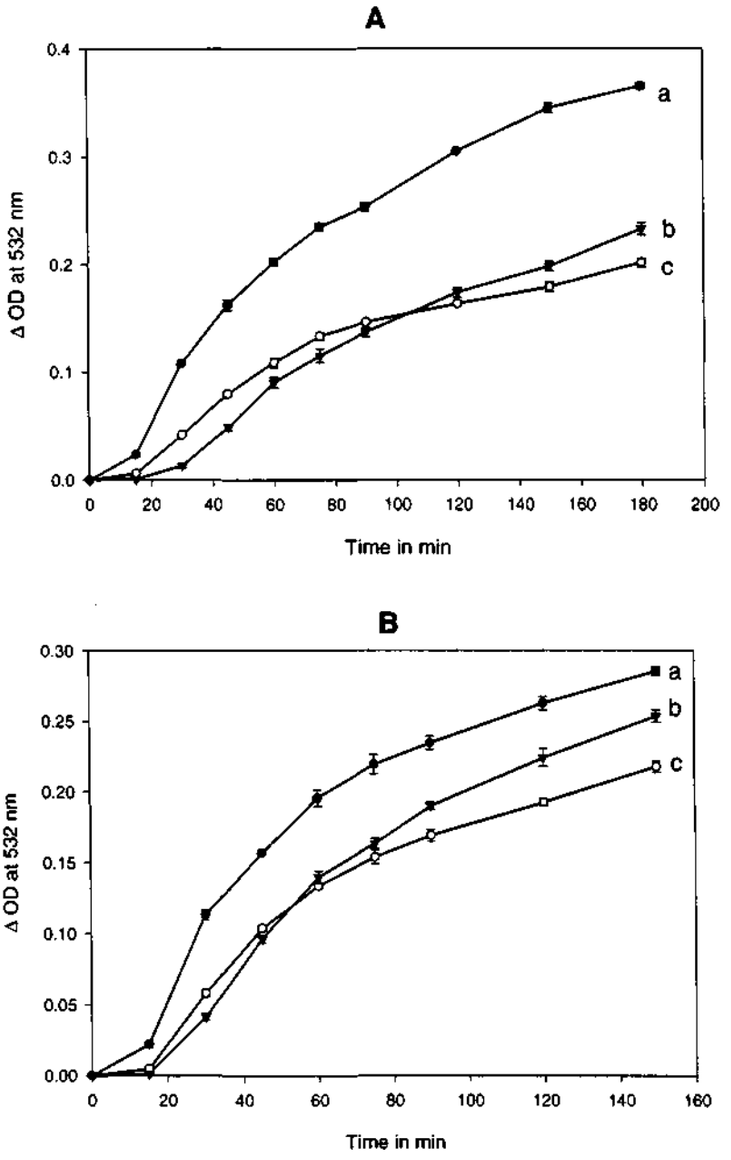

Fig. 5: Time dependent inhibitory effect of native phycocyanin (b) and $NaBH_4$ reduced phycocyanin (c) on peroxyl radical-induced lipid peroxidation (TBARS formation) in rat liver microsomes, (a) control.

(A) 20 μM native and reduced phycocyanin

(B) 10 μM native and reduced phycocyanin

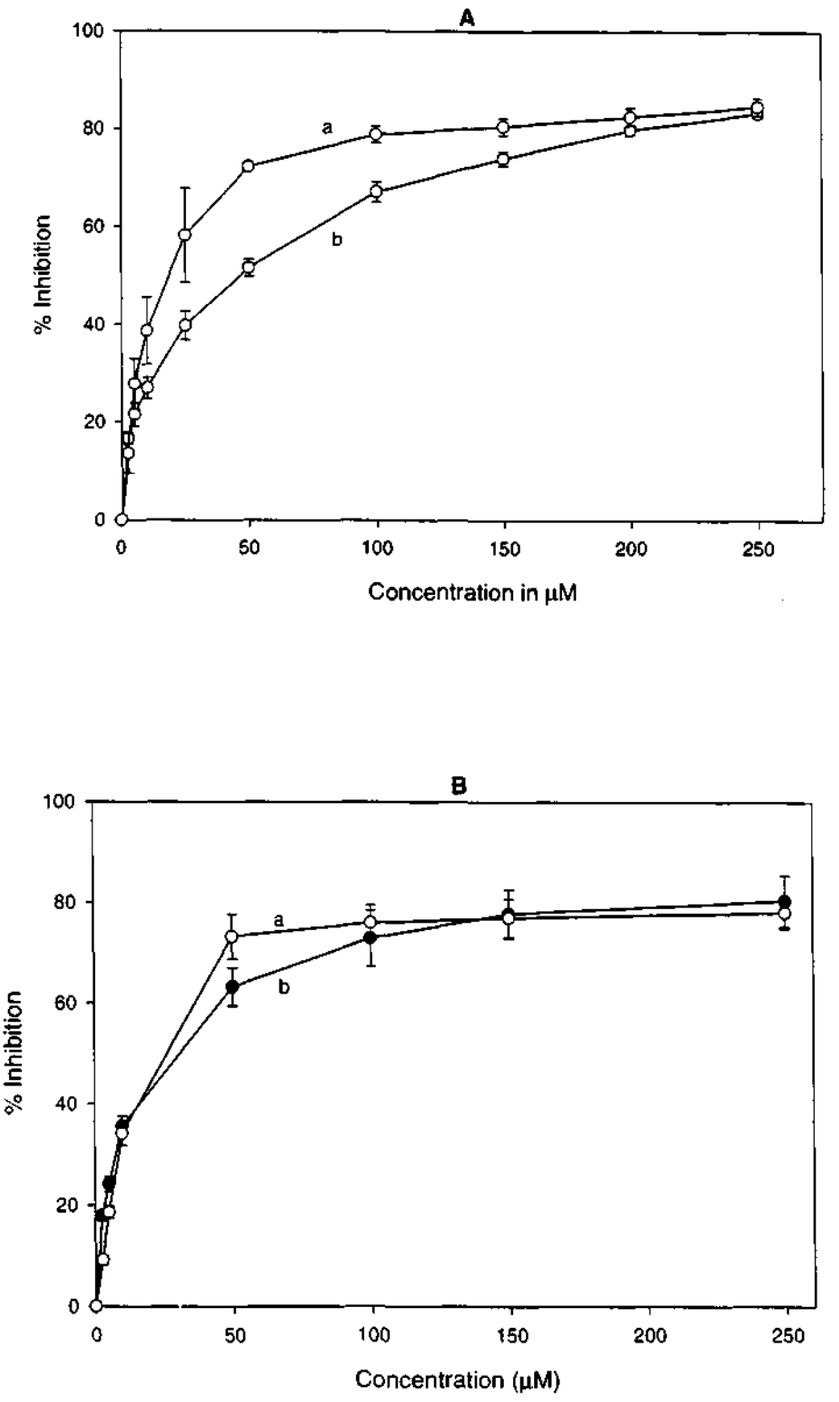

Fig. 6: ***Deoxyribose assay for hydroxyl radical scavenging by native and reduced phycocyanin (a) without EDTA, and b) with EDTA.***
A) Native phycocyanin
B) Reduced phycocyanin

The effect of phycocyanin on iron-ascorbate or peroxyl radical-induced lipid peroxidation in rat liver microsomes (**Fig. 3A**) and PC liposomes were studied *in vitro* (**Fig. 3B**). Extent of lipid peroxidation was measured by the formation of TBARS. Phycocyanin inhibits the iron-ascorbate as well as azo-initiated lipid peroxidation in a concentration dependent fashion, with an IC_{50} value of 38.8 μM and 11.35 μM, respectively in rat liver microsomes and 9.3 μM and 4.7 μM, respectively in PC liposomes (**Fig. 3A and B**). Phycocyanin effectively inhibits the peroxyl radical-induced lipid peroxidation than iron-ascorbate-induced lipid peroxidation in both microsomal systems (**Fig. 3A**). Phycocyanin at 200 μM concentration inhibits nearly 95 % of peroxyl radical-induced and 79% of iron-ascorbate-induced lipid peroxidation in rat liver microsomes (**Fig. 3A**).

Both native phycocyanin and chemically reduced phycocyanin inhibits peroxyl radical-induced lipid peroxidation in rat liver microsomes in a dose dependent fashion with an IC_{50} value of 11.35 and 12.67 μM, respectively (**Fig. 4**). Time dependent effects of native phycocyanin and reduced phycocyanin were studied at two different concentrations '10 and 20 μM' (**Fig. 5A and B**). As shown in **Fig. 5A and B**, both native and reduced phycocyanin inhibit peroxyl radical induced lipid peroxidation in rat liver microsomes in a time dependent manner at both 10 μM and 20 μM concentrations. Reduced phycocyanin is more effective inhibitor than native phycocyanin in initial period of peroxyl radical-induced lipid peroxidation in rat liver microsomes **Fig. 5A and B.**

Hydroxyl radical scavenging ability of native and reduced phycocyanin was studied by using deoxyribose degradation assay. Both native and reduced phycocyanin inhibits hydroxyl radical-induced deoxyribose degradation in a concentration dependent fashion with an IC_{50} value of 30.5 ± 5.5 and 27.4 ± 1.6 μM, respectively (**Fig. 6A and B**). The second order rate constant for the interaction of native and reduced phycocyanin with hydroxyl radical is 1.5-2.1 × 10^{11} M^{-1} S^{-1} and 1.05-1.62 × 10^{11} M^{-1} S^{-1}, respectively. Deoxyribose assay was carried out in the absence of EDTA and it was found that both native and reduced phycocyanin were more potent inhibitors of deoxyribose degradation with an IC_{50} value of 16.5 ± 2.8 and 25.0 ± 1.2 μM, respectively (**Fig. 6A and B**). In the

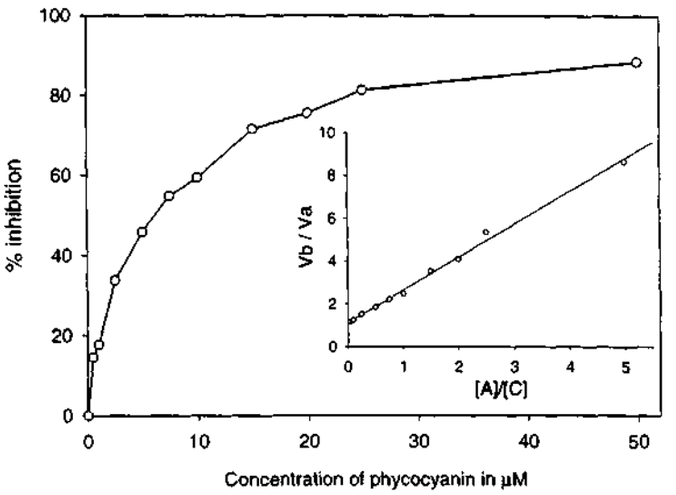

Fig. 7: Dose-inhibition curve of phycocyanin. The inhibitory effect of phycocyanin on peroxyl radical-induced bleaching of crocin in phosphate buffer (10 mM, pH 7.4) at 40^0 C. Inset: competition kinetic plot of phycocyanin towards crocin in the AAPH-induced radical reaction. Slope of the straight line indicates the relative capacity of phycocyanin to interact with peroxyl radical according to Eq. presented in the methods section.

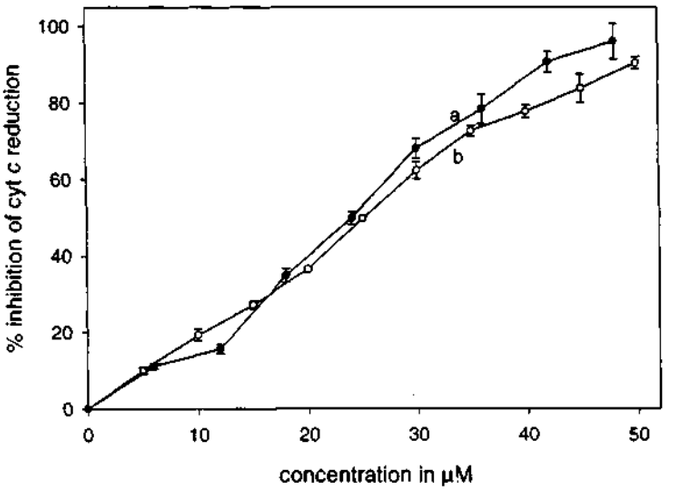

Fig. 8: Superoxide anion radical scavenging activity of phycocyanin. Concentration dependent scavenging of superoxide anion radicals by monitoring the competition of phycocyanin with cytochrome c for superoxide radicals generated by (a) PMS-NADH system (non-enzymatic) and (b) xanthine-xanthine oxidase system.

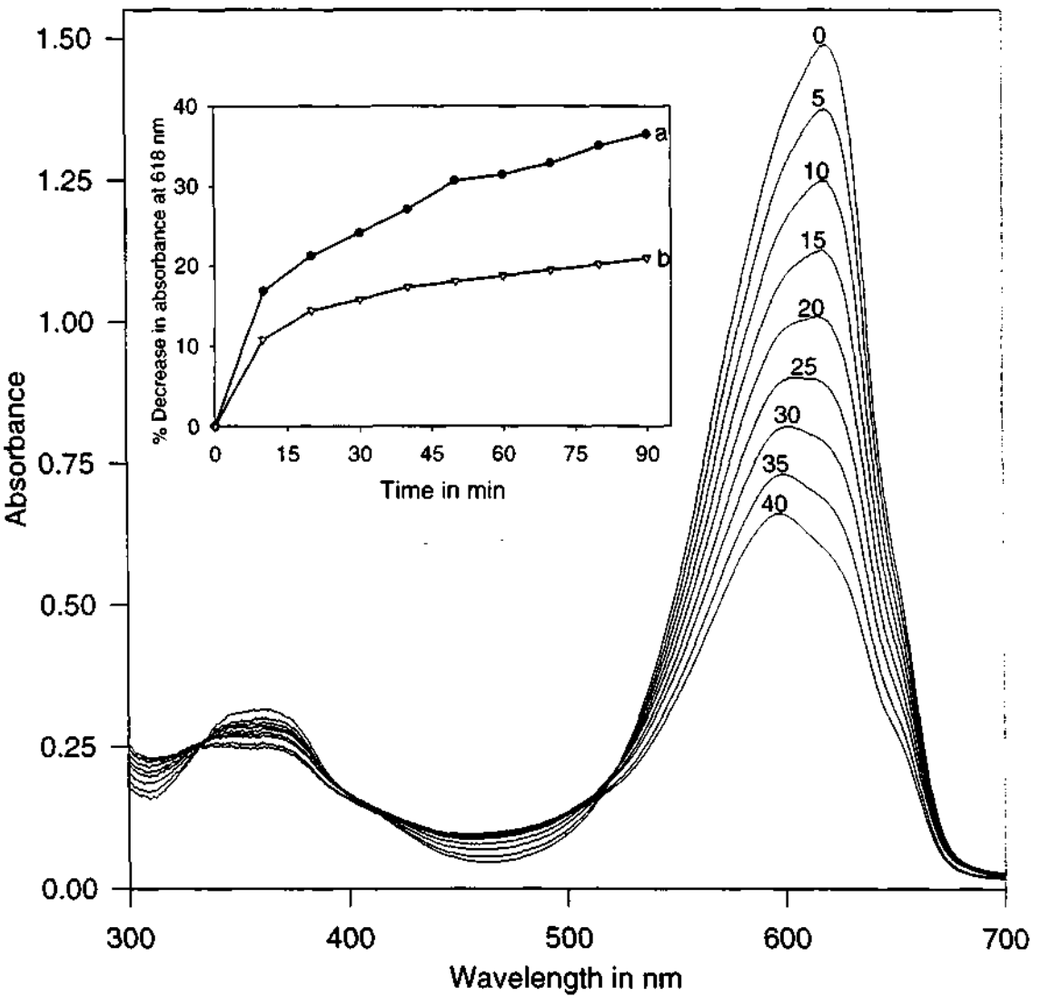

Fig. 9: Time-dependent spectral changes associated with the AAPH-induced oxidation of phycocyanin (10 μM) in phosphate buffer (10 mM, pH 7.4) at 37^0 C. The reaction was started by the addition of AAPH (10 mM) to the reaction mixture. Number indicate the time in minutes elapsed after the addition of AAPH. Inset: Time-dependent decrease in the absorbance at 618 nm associated with the hydroxyl radical induced oxidation of phycocyanin (10 μM) in phosphate buffer (10 mM, pH 7.4) at 37^0 C. a) In the presence of EDTA, and b) in the absence of EDTA. The experimental details are as described in the Materials and Methods section.

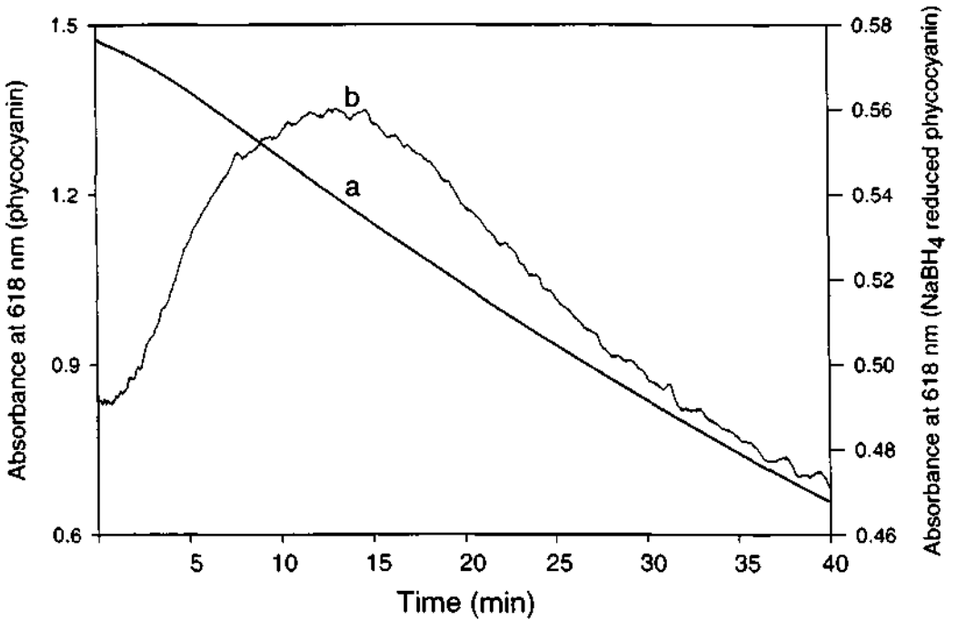

Fig. 10: Time-dependent changes in the absorbance at 618 nm associated with the peroxyl radical-induced oxidation of, a) native phycocyanin (10 μM) and b) $NaBH_4$ reduced phycocyanin in phosphate buffer (10 mM, pH 7.4) at 37^0 C.

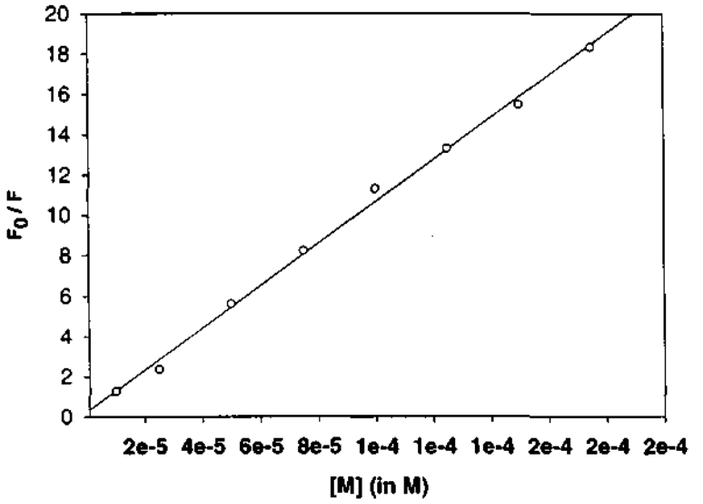

Fig. 11: Stern-Volmer plot for fluorescence quenching of phycocyanin by iron ion.

absence of EDTA, such a deoxyribose assay helps to assess the ability of a compound to interfere with the site-specific generation of hydroxyl radicals (33,34). Our results suggest that both native and reduced phycocyanin chelates free iron ion and hence protect the target molecule, deoxyribose.

Peroxyl radical scavenging ability of phycocyanin was analyzed by the competition kinetics of crocin bleaching (**Fig. 7**). In these experiments, peroxyl radicals were generated by the thermal decomposition of the azo compound AAPH. Phycocyanin is a potent peroxyl radical scavenger with an IC_{50} value of 5.0 μM. Under these experimental conditions, uric acid, a known peroxyl radical scavenger had an IC_{50} value of 1.9 μM. The rate constant ratios (K_{rel}) obtained for phycocyanin and uric acid were of 1.54 and 3.5, respectively (**Fig. 7**).

Superoxide anion radical scavenging ability of phycocyanin was studied by the competition kinetics of cytochrome c reduction (**Fig. 8**). Superoxide anion radical was generated by using xanthine-xanthine oxidase (enzymatic) or PMS-NADH (non-enzymatic) system. Phycocyanin appeared to be an efficient scavenger of superoxide radical generated both enzymatically and non-enzymatically, and it scavenges in a dose dependent manner with an IC_{50} of 20.84 and 20.0 μM, respectively (**Fig. 8**).

The involvement of the covalently linked chromophore in phycocyanin, phycocyanobilin (PCB), in the radical scavenging activity was established by studying the reactivity of phycocyanin with peroxyl and hydroxyl radicals. When phycocyanin was incubated with AAPH (10 mM) at 37° C, there was a significant decrease in the absorption at 618 nm (60% decrease) and a shift in the absorption maxima at 618 nm (21.5 nm shift) towards lower wavelength was noticed (**Fig. 9**). The decrease in the absorption is accompanied by disappearance of color. Similar spectral changes have also been noticed when phycocyanin was incubated with hydroxyl radical generating system (Fenton reagent) containing EDTA (**Fig. 9**). Under these conditions, there was a significant decrease in the absorption at 618 nm (36.5 % decrease). However, the decrease was less significant (20 %) when incubation was carried out in the absence of EDTA (**Fig. 9**).

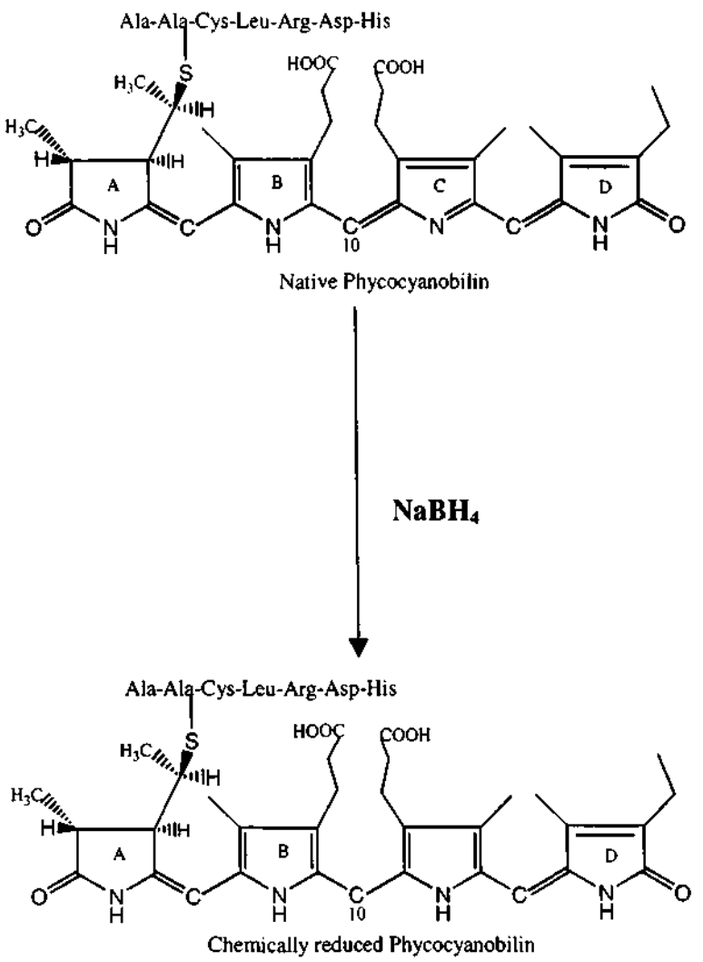

Fig. 11: $NaBH_4$ reduction of native phycocyanin, where C-10 methine bridge of phycocyanobilin was reduced to phycocyanorubin

When chemically reduced phycocyanin was used instead of native phycocyanin, the absorption at 618 nm rapidly increased initially and then the absorption gradually decreased (**Fig. 10**). However, the absorption of native phycocyanin at 618 nm decreased continuously (**Fig. 10**).

Iron chelating ability of phycocyanin was established by using fluorescence quenching experiments. The fluorescence of phycocyanin was quenched after each addition of iron ion. The fluorescence of phycocyanin decreases with increasing concentration of ferric iron (**Fig. 11**). Phycocyanin interacts with iron ion with an association constant of $1.11 \pm 0.06 \times 10^5 M^{-1}$ (Stern-Volmer quenching constant) (**Fig. 11**).

DISCUSSION:

Exposure of animals to CCl_4 results in cell damage and lipid peroxidation is generally invoked as an explanatory event that leads to injury (32). In the present study, the effect of C-Phycocyanin pretreatment on CCl_4-induced hepatic lipid peroxidation was investigated and the results are presented in **Fig. 2**. Malondialdehyde (MDA) was used as an index of hepatic lipid peroxidation. Consistent with the earlier report (36), a single i.p. administration of CCl_4 (0.6 ml/kg) to rats caused marked increase in liver MDA level (**Fig. 2**). Phycocyanin when administered alone did not change the level of MDA and it was found to be similar to that of control value. However, administration of phycocyanin (10-200 mg/kg body wt.) three hours prior to CCl_4 treatment resulted in significantly lower production of MDA than that found in rats receiving only CCl_4. The liver MDA level was nearly 5 fold greater in CCl_4 treated rats than control rats or rats treated with the combination of phycocyanin and CCl_4 (**Fig. 2**). It is known that, in CCl_4 intoxication, free radicals arising from its biotransformation induce lipid peroxidation. The biotransformation is catalyzed by liver microsomal cytochrome P450 system (32-37, 38). The trichlormethyl radical ($CCl_3^{\bullet}$) initially formed is relatively unreactive and this carbon centered radical readily reacts with O_2 to form a peroxyl radical ($CCl_3O_2^{\bullet}$) which is a good initiator of lipid peroxidation (10,39). The free radical, in the presence of oxygen, leads to autooxidation of the fatty acids present in the cytoplasmic membrane phospholipids (32,36,37) and causes functional and morphological changes in the cell membrane.

Furthermore, influx of extracellular Ca^{+2}-ions into cell is claimed to be an important step leading to cell death. Therefore, studies on the prevention of liver damage caused by CCl_4, may give an indication of the liver-protective action of drugs in general. Earlier we have noticed that, phycocyanin when administered alone to rats did not alter the liver function and the level of cytochrome P450 was similar to that of control value (8). This suggests that, the protection by phycocyanin against CCl_4-induced lipid peroxidation may not be related to a less formation of reactive metabolites of CCl_4, but due to the unique ability of phycocyanin to scavenge reactive radicals. Although scavenging of reactive species by phycocyanin may be mostly responsible for the observed protection from CCl_4-induced liver damage. One cannot rule out the possibility that, in the presence of phycocyanin, liver produces lower levels of reactive radicals. Further studies are required to ascertain these aspects.

It has been hypothesized that, one of the principal causes of CCl_4-induced liver injury is lipid peroxidation by free radicals derived from CCl_4. It has been suggested that, any compound possessing antioxidant activity or having the ability to inhibit the formation of free radicals from CCl_4 could easily provide protection against CCl_4-induced liver damage (40,41).

In liver microsomes as well as in PC liposomes, lipid peroxidation takes place non-enzymatically in vitro. It is known that Fe^{2+} and ascorbic acid or azo compounds stimulate lipid peroxidation in rat liver microsomes and PC liposomes. The effect of phycocyanin on iron-ascorbate and peroxyl radical-induced lipid peroxidation in rat liver microsomes and PC liposomes were studied *in vitro* (**Fig. 3**). Iron-ascorbate generates hydroxyl radical and azo compounds generate peroxyl radical. The extent of membrane lipid peroxidation was measured by the formation of TBARS. In the present study it was noticed that, phycocyanin inhibits the iron-ascorbate and azo-initiated lipid peroxidation in a concentration dependent fashion with an IC_{50} value of 38.8 and 11.35 μM, respectively in rat liver microsomes and 9.3 μM and 4.7 μM, respectively in PC liposomes (**Fig.3**). So it appears that phycocyanin scavenges peroxyl radicals more efficiently than hydroxyl radicals *in vitro*. Phycocyanin at 200 μM concentration almost completely inhibits (95%) peroxyl radical-induced lipid peroxidation in rat liver microsomes (**Fig. 3**). However, only

79% inhibition was noticed in case of iron-ascorbate-induced lipid peroxidation in rat liver microsomes (**Fig. 3**). Phycocyanin at 75 μM concentration almost completely inhibits (~95%) both peroxyl and hydroxyl radical-induced lipid peroxidation in PC liposomes (**Fig. 3**). These results suggest that phycocyanin acts as a good radical scavenging antioxidant. It scavenges the radicals generated *in situ* and inhibits the initiation of membrane lipid peroxidation.

Phycocyanin monomer contains two protein sub-units (α and β) with three bilin chromophores, phycocyanobilin, covalently attached to the cysteine residues of the apoprotein by thioether bonds to cysteine residues. Phycocyanobilin is an analogue of biliverdin and it has been reported earlier that, addition of $NaBH_4$ rapidly reduces the biliverdin-albumin complex to bilirubin-albumin complex as detected by a color change (blue-green to yellow) and by UV-visible spectroscopy (21). It has also been shown that, during this facile transformation, the C-10 methine bridge in the chromophore is reduced (21). Following this method we have reduced the chromophore in the native phycocyanin using solid $NaBH_4$. Reduction resulted in the color change (deep blue to greenish yellow) and disappearance of absorption at 360 and 618 nm and appearance of strong absorption near 418 nm in the UV-visible spectrum. Although reduced phycocyanin has not been characterized, the spectral characteristics resemble to that of phycocyanorubin, an analogue of bilirubin (31). This suggests that $NaBH_4$ possibly could have reduced the C-10 methine bridge in the chromophore (**Fig 12**). It is interesting to note that the reduced (chemically modified) phycocyanin also efficiently inhibited peroxyl radical-induced lipid peroxidation in rat liver microsomes and the inhibition was dose dependent with an IC_{50} value of 12.7 μM (**Fig. 4 and 5**). In fact, both native and reduced phycocyanin inhibited lipid peroxidation almost to the same extent (**Fig. 4**). Both native and reduced phycocyanin inhibit the peroxyl radical-induced lipid peroxidation in rat liver microsomes in a time dependent fashion (**Fig. 5**). Reduced phycocyanin is a more effective inhibitor of lipid peroxidation than native phycocyanin during early stages of the reaction. This could be due to the reduced chromophore, phycocyanorubin, reacting with the peroxyl radical and getting oxidized to phycocyanobilin which further reacts with another molecule of peroxyl radical. This explanation is supported by the fact that, when reduced phycocyanin was used

as an inhibitor of lipid peroxidation, it changes its color from greenish yellow to deep blue indicating that the chromophore gets re-oxidized to phycocyanobilin. This was further supported by our observation that when reduced phycocyanin was incubated with AAPH (10 mM) at 37° C, there was a rapid decrease in the absorption at 418 nm with the concomitant appearance of peaks at 618 and 360 nm in the UV-visible spectrum indicating the oxidation of phycocyanorubin to phycocyanobilin by peroxyl radical. In fact **Fig. 10** clearly shows the transient formation of native phycocyanin during the interaction of peroxyl radical with reduced phycocyanin. Similar observations have been made earlier during the peroxyl radical mediated transformation of bilirubin to biliverdin (42). The aforementioned results clearly indicate that the chromophore (bilin) and not the apoprotein is directly involved in the antioxidant and radical scavenging properties of phycocyanin.

The involvement of chromophore, phycocyanobilin, in the radical scavenging activity was established by studying the reactivity of phycocyanin with peroxyl radicals. When phycocyanin was incubated with AAPH (10 mM) at 37° C, there was a significant decrease in the absorption at 618 nm (60% decrease) and a shift in the absorption maxima at 618 nm (21.5 nm shift) towards lower wavelength was noticed (**Fig. 1**). The decrease in the absorption is accompanied by disappearance of color. Similar spectral changes have also been noticed when phycocyanin was incubated with hydroxyl radical generating system (Fenton reagent) containing EDTA (**Fig. 9**). Under these conditions, there was a significant decrease in the absorption at 618 nm (36.5% decrease). However the decrease was less significant (20%) when incubation was carried out in the absence of EDTA (**Fig. 9**). This is possibly due to the fact that, in the absence of EDTA, phycocyanin removes the free iron ion from the medium, which affects the hydroxyl radical formation, and in turn its interaction with phycocyanin. To confirm the iron binding property of phycocyanin, fluorescence quenching experiment was carried out in 50 mM sodium acetate buffer, pH 5.2 and it was found that phycocyanin interacts with iron ion with an association constant of $1.11 \pm 0.06 \times 10^5 M^{-1}$ (**Fig. 11**). In fact, iron binding properties of C-phycocyanin from *Phormidium luridum* has been reported earlier (43).

Phycocyanobilin, the chromophore of phycocyanin, is structurally similar to biliverdin and bilirubin, the end products of heme catabolism in mammals. It was

demonstrated earlier that bilirubin, at micromolar concentrations *in vitro* efficiently scavenges peroxyl radicals generated chemically in either homogeneous solution or multilamellar liposomes (44). In fact, both bilirubin and its metabolic precursor biliverdin inhibited the peroxyl radical-induced oxidation of linoleic acid in homogenous solution (42,44,45). It was suggested that, peroxyl radical-induced oxidation of lipids result in the formation of chain carrying fatty acid peroxyl radicals which interact with bilirubin and inhibit the lipid peroxidation. It has been proposed that bilirubin scavenges the chain-carrying peroxyl radicals by donating a hydrogen atom attached to the C-10 bridge of the tetrapyrrole molecule to form a carbon-centered radical which may then react with another peroxyl radical to give rise to a non-radical product. So bilirubin acts as a physiological, chain-breaking antioxidant and this activity is probably due to the formation of resonance-stabilized carbon-centered radical (42,44,45). It is interesting to note that both phycocyanobilin and bilirubin contain an extended system of conjugated double bonds and a reactive hydrogen atom suggesting that both may be following the same mechanism for their antioxidant activity. It was noticed earlier that biliverdin has a higher radical scavenging ability due to the difference in the rate constant for the addition of fatty acid peroxyl radical to these pigments.

Formation of peroxyl radicals is a necessary proximate step in the liberation of TBARS during lipid peroxidation. The interaction of peroxyl radical with phycocyanin and its ability to scavenge peroxyl radical was further analyzed by the competition kinetics of crocin bleaching (**Fig. 7**). In these experiments peroxyl radicals were generated by the thermal decomposition of the azo compound AAPH. Since transition metals were not used for peroxyl radical generation, the effect was not due to the transition metal ion chelation by phycocyanin. These studies demonstrated that phycocyanin is a potent peroxyl radical scavenger with an IC_{50} value of 5.0 μM. Under these experimental conditions, uric acid, a known peroxyl radical scavenger had an IC_{50} value of 1.9 μM. The rate constant ratios (K_{rel}) obtained for phycocyanin and uric acid were of 1.54 and 3.5, respectively (**Fig. 7**). The high rate constant for the interaction of phycocyanin with peroxyl radical suggests that the hepatoprotective effect of phycocyanin is due to its ability to scavenge reactive radicals.

It is well known that hydroxyl radical is a highly potent oxidant that reacts with almost all biological macromolecules found in living cells (46,47). Iron salts damage deoxyribose with the release of thiobarbituric acid-reactive substances (TBARS) (48), and this damage is due to the site-specific iron-dependent generation of hydroxyl radicals (34). EDTA-iron complex allows sufficient generation and release of hydroxyl radicals into the medium, rather than site-specific generation of hydroxyl radical from localized iron-binding site of the substrates. Earlier it was shown that phycocyanin is a hydroxyl radical scavenger by deoxyribose degradation assay (5). Consistent with the earlier report (5), we have noticed that phycocyanin interacts with hydroxyl radical with a reaction rate constant (Ks) of 1.5-2.1 $\times 10^{11}$ M^{-1} S^{-1} and inhibits the deoxyribose degradation in a concentration dependent fashion with an IC_{50} value of 30.5 ± 5.5 μM (**Fig. 6A**). Reduced phycocyanin also readily interacts with hydroxyl radical with a reaction rate constant (Ks) of 1.05-1.62 $\times 10^{11}$ M^{-1} $S^{-1}$and inhibits the deoxyribose degradation with an IC_{50} value of 27.4 ± 1.6 μM (**Fig. 6B**). When the deoxyribose assay was carried out in the absence of EDTA, both native and reduced phycocyanin were found to be a more potent inhibitors of deoxyribose degradation with an IC_{50} value of 16.5 ± 2.8 and 25.0 ± 1.2 μM (**Fig. 6**). This suggests that phycocyanin chelates iron ion and hence protect the target molecule, deoxyribose. Our results clearly suggest that phycocyanin not only has the ability to scavenge hydroxyl radicals but also chelates iron.

There are many sources of superoxide anion radical generation during pathological events (49). Superoxide is known to cause severe damage to various macromolecules either by directly interacting with them (50) or other reactive oxygen species (ROS) such as hydrogen peroxide (via dismutation reaction) or hydroxyl radicals (via metal-catalyzed Haber-Weiss reaction) derived from superoxide can bring about the deleterious effects (51,52). Therefore, removing superoxide anion radical is probably one of the most effective defences of a living body against diseases. Phycocyanin scavenges the superoxide anion radical generated by xanthine/xanthine-oxidase (enzymatic) and PMS-NADH (non-enzymatic) systems in a concentration dependent manner and inhibits the reduction of cytochrome c (**Fig. 8**). Xanthine/xanthine-oxidase and PMS-NADH systems generates

more superoxide anion radicals *in vitro* than during pathological conditions *in vivo*. Low IC_{50} value suggests that phycocyanin may act as a good cytoprotective agent against oxyradical-induced tissue damage during infection and inflammation.

Active oxygen species and free radicals are involved in a variety of pathological events, cancer, aging, chronic inflammation, heart disease, etc (1,2). To prevent the formation of reactive oxygen species as well as to repair oxidative damage caused by these ROS, all living systems possess enzymatic and non-enzymatic antioxidant defenses. Enzymes such as superoxide dismutase, catalase, glutathione peroxidase etc (14), dietary components such as vitamin E, and C and β-carotene (14), uric acid, the end product of purine metabolism (53), taurine, the end product of oxidative metabolism of cysteine (54), bilirubin, the degradative product of hemoprotein (42,44) are some of the participants in the process of antioxidant defense. Any compound, natural or synthetic, with antioxidant properties that might contribute towards the partial or total alleviation of damage caused by ROS, may have a significant role in maintaining health when continuously taken as components of dietary foods, spices and drugs. Therefore, removing (scavenging) reactive oxygen species is probably one of the most effective defences of a living body against many types of diseases.

In conclusion, the antioxidant property of phycocyanin was established on the basis of experiments carried out both in *vivo* and *in vitro*. The radical assisted bleaching of chromophore (bilin group) in phycocyanin clearly indicates its involvement in the scavenging of reactive oxygen radicals. The study provides an explanation for the hepatoprotective and anti-inflammatory properties of phycocyanin.

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CHAPTER 2: PART D

Phycocyanobilin from *Spirulina Platensis*, a Potent Antioxidant, Radical Scavenger and iron chelator

INTRODUCTION

Spirulina platensis, a blue green algae, is used as a food supplement and its nutritional as well as therapeutic values are very well documented (1-4). One of the major constituents of this algae is C-phycocyanin, a water soluble biliprotein. C-Phycocyanin is used as a coloring agent in food and cosmetic preparations (5,6). It has significant antioxidant and radical scavenging (7,8), hepatoprotective (9), neuroprotective (10) and anti-inflammatory properties (7, 11). Studies have also established its ability to effectively inhibit lipid peroxidation both *in vivo* and *in vitro* (8).

The chromophore of phycocyanin is phycocyanobilin (PCB), a linear tetrapyrrole, which is covalently attached to the apoprotein by thioether bonds to cysteine residues. PCB is structurally similar to the bile pigment bilirubin (BR), a heme degradative product. In fact, PCB is an analogue of biliverdin (BV), a metabolic precursor for both BR and PCB (12). It has been suggested that, the bile pigments BR and BV may protect vitamin A and linoleic acid from oxidative destruction in the intestinal tract (13). In support of this, it was demonstrated later that albumin-bound bilirubin exhibits significant antioxidant activity and has the ability to protect linoleic acid from peroxyl radical-induced oxidation *in vitro* (14). These findings support the view that, naturally occurring bile pigments serve as a physiological antioxidant. Studies carried out in our laboratory (8) as well as elsewhere (15,16) clearly suggest that, the chromophore phycocyanobilin (PCB) is responsible for the antioxidant and radical scavenging properties of phycocyanin (8,15,16) It has been observed that, when the native phycocyanin is exposed to free radicals, bleaching of the chromophore absorption and fluorescence occurs (8,15,16). However, these findings have not been substantiated by carrying out experiments using phycocyanobilin. Although the antioxidant and radical scavenging properties of biliverdin (BV) and bilirubin (BR) have been studied earlier, both *in vivo* and *in vitro* (14,17,18), similar studies have never been carried out with phycocyanobilin. Attempts have not been made to evaluate the functional role of phycocyanobilin, the chromophore of phycocyanin.

In the present study we compare the antioxidant and radical scavenging properties of PCB with those obtained for phycocyanin. In addition, the iron chelating property of PCB has also been investigated.

METHODS AND MATERIALS

Chemicals

2-Deoxyribose, Thiobarbituric acid (TBA), Butylated Hydroxytoluene (BHT), Adenosine 5'-diphoshate (ADP), β-Nicotinamide adenine dinucleotide phosphate (reduced form, NADPH), 1,1-Diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma (St. Louis, MO., USA). 2,2'-Azo-bis(2-amindinopropane) hydrochloride (ABAP) was obtained from Aldrich Chemical Co. (St. Louis, MO., USA). Crocin was isolated from saffron by water/methanol extraction as reported earlier (19). The extract containing crocin was diluted with 10 mM phosphate buffer (pH 7.4), and concentration was estimated using the extinction coefficient of 89,000 M^{-1} cm^{-1} reported for crocin in aqueous solution (20). All other materials were procured from standard suppliers and were of analytical grade.

Preparation of C-phycocyanin and Phycocyanobilin

Freshly grown *Spirulina platensis* was a generous gift from Ballarpur Industries (P) Ltd., Bangalore, India. C-phycocyanin was isolated from freshly grown cells of *Spirulina platensis* as described in chapter 2: part A (21). The purity and molecular mass of phycocyanin were determined as previously described (8). The phycocyanobilin (PCB) was cleaved from the pure freeze-dried phycocyanin by alcoholysis as described earlier (22). The PCB obtained was re-dissolved in chloroform containing 5 % methanol, washed several times with water to remove impurities and evaporated under vacuum to dryness. The purity of phycocyanobilin was examined by HPLC analysis, using ODS reverse phase column with acetone:water (2.5 : 1.5, v/v) containing 2 mM H_3PO_4 and 0.5 mM NaH_2PO_4 as a solvent system (1.5 ml/min) and the column elute was monitored at 365 nm. The UV-Visible spectra of PCB was recorded between 280-800 nm in methanol/2% HCl and the concentration was estimated at 374 nm using the extinction coefficient of 47,900 $M^{-1}cm^{-1}$ (23). The mass of the PCB was determined using MALDI-TOF mass spectrometry.

Stock solution of PCB was prepared in ethanol. Working solution was prepared by taking known amount of PCB from the stock solution, and dried under vacuum. The PCB solution (0.25 mM) was prepared by dissolving dry PCB in 10-25 µl of NaOH (25 mM) and further diluted with assay buffer. The pH was adjusted as required.

Phospholipid Liposomes and Rat Liver Microsomes Preparation

Multilamellar phosphatidylcholine (PC) liposomes were prepared immediately before the experiments by dissolving PC in chloroform and removing the solvent under vacuo. The dried lipid film was hydrated with 5 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, by vigorous shaking for 2 min, followed by ultrasonic irradiation with a BRASON Ultrasonicator B-12 (55 kHz) for 30 sec at ambient temperature.

Rat liver microsomes were prepared from the liver of male albino rats (160-180 g body wt.) as previously reported (24). The microsomal pellet was washed and suspended in 1.15 % KCl solution. The protein concentration was determined by the method of Lowry, *et al.* (25).

Lipid Peroxidation Induction and Assay

Preincubation: Liposomes (4 mg/ ml) in Tris-HCl buffer (5 mM, pH 7.4) containing NaCl (150 mM) were preincubated with or without PCB (0-10 μM) or phycocyanin (0-75 μM) in a total volume of 1.0 ml for 10 min at 37° C. Similarly, freshly prepared microsomes (1.0 mg/ml) in phosphate buffer (10 mM, pH 7.4) were preincubated with or without PCB (0-150 μM) or phycocyanin (0- 250 μM) in a total volume of 1.0 ml for 10 min at 37° C. In case of enzymatic lipid peroxidation, rat liver microsomes (1 mg/ml) in phosphate buffer (0.1 M, pH 7.4) were preincubated with PCB (0-150 μM) and CCl_4 (5 mM in 2.5 μl ethanol) in a total volume of 1.0 ml for 10 min at 37° C.

Induction: Liposomal lipid peroxidation was initiated by the addition of AAPH (20 mM, final concentration). Microsomal non-enzymatic lipid peroxidation was initiated by $FeSO_4$ (10 μM) and ascorbate (100 μM), whereas enzymatic lipid peroxidation was initiated by adding NADPH generating system (1 mM $NADP^+$, 10 mM glucose 6-phosphate, and 1 unit glucose 6-phosphate dehydrogenase). After the initiation of lipid peroxidation, the reaction mixture was incubated for a further period of 1 h at 37° C. The extent of lipid peroxidation in liposomes as well as microsomes was estimated by measuring thiobarbituric acid (TBA)-reactive substances (TBARS) as reported earlier (26) with minor modifications. Briefly, 3 ml of stopper solution [TCA (15%, w/v)-TBA (0.375%, w/v)-HCl (0.125 M)-BHT (0.6 mM)] was added, mixed and centrifuged at 5,000 g for 10 min. The

supernatant was transferred to a fresh tube, boiled for 30 min, cooled and used to quantify TBARS photometrically at 532 nm. Appropriate blanks (without liposomes or microsomes) were also run along with the experiment to find out the effect of PCB during TBA reaction. It was noticed that, PCB reacts with TBA to generate a pink color having absorption at 528 nm. The results are expressed as % inhibition, which represents the degree of protection, by the phycocyanobilin/phycocyanin against the AAPH or iron-ascorbate or CCl_4-induced membrane lipid peroxidation.

Interaction of Phycocyanobilin with Peroxyl and Hydroxyl Radicals

Interaction of peroxyl and hydroxyl radicals with phycocyanobilin and its bleaching were studied as described earlier (8). The changes in the UV-Visible spectra of PCB were recorded between 260-800 nm at different time intervals (0, 5, 10, 20, 30, 40, 50 and 60 min) in a Shimadzu UV2100 thermostated spectrophotometer.

Iron Chelation Study

Spectra of the complexes of PCB with Fe^{+2} or Fe^{+3} were recorded in a Shimadzu UV2100 thermostated spectrophotometer. Iron chelation was studied by titrating different concentrations of either $FeCl_3$ or $FeSO_4$ with phycocyanobilin (10 μM) in phosphate buffer (10 mM, pH 7.4) or in acetate buffer (10 mM, pH 5.0) at 37° C. After each addition of either $FeCl_3$ or $FeSO_4$, the mixture was incubated for 5 min and then spectra were recorded against the corresponding iron blank.

Ferrozine Assay

Iron oxidation-reduction experiments were carried out as reported earlier with minor modifications (27). Assay was carried out in phosphate buffer (10 mM, pH 7.4). PCB (50 μM) was incubated with free Fe^{+2} or Fe^{+3} (50 μM) or ADP (250 μM) complex of Fe^{+2} or Fe^{+3} (50 μM) for 1 h at 25° C. The ferrozine assay for Fe^{+2} (28) was performed by addition of excess ferrozine (14 mM) to the reaction mixture at the end of 60 min. The stable complexes formed between ferrozine and Fe^{+2} were quantitated by measuring absorbance at 562 nm continuously after the addition of ferrozine up to 30 min.

Measuring Hydroxyl Radical Scavenging

Hydroxyl radicals were generated by a mixture of ascorbic acid, H_2O_2 and Fe^{2+}-EDTA, and estimated using the 2-deoxyribose method (29). Each assay contained 2-deoxyribose (2.8 mM), ferrous iron solution (20 μM), EDTA (100 μM) and different concentrations of PCB (1-100 μM) in a total volume of 1.2 ml of phosphate buffer (10 mM, pH 7.4). The ferrous iron solution and EDTA were premixed before they were added to the assay mixture. The reaction was initiated by the addition of a mixture of H_2O_2 (1.42 mM) and ascorbate (100 μM), and incubated at 37° C for 30 min. Then, 1 ml of thiobarbituric acid (1%, w/v) in NaOH (50 mM) and 1 ml of TCA (2.8%, w/v) were added, centrifuged at 5,000 rpm for 10 min to remove suspended fine particles, boiled for 20 min, cooled, and the absorbance was measured at 532 nm. The results are expressed in % inhibition by PCB. Reciprocal absorption values obtained for different concentrations were plotted against the concentrations of PCB and from the graph, the rate constant for the reaction of PCB with hydroxyl radical was determined, assuming that deoxyribose reacts with hydroxyl radical with a rate constant of 3.1×10^9 M^{-1} S^{-1} (29). In a similar way, the rate constants for established scavengers of hydroxyl radical such as mannitol and dimethylsulfoxide (DMSO) were also determined.

Crocin Bleaching Assay for Peroxyl Radical Scavenging Activity of Phycocyanobilin

The reactivity of the PCB with peroxyl radicals was measured by competition kinetics of crocin bleaching in the presence of peroxyl radicals generated by thermal decomposition of an azo compound (19). The test was carried out at 40°C in phosphate buffer (10 mM, pH 7.4) containing crocin (10 μM) and increasing concentrations (0-10 μM) of PCB in a total volume of 1 ml. Uric acid (0-10 μM) was used as a known peroxyl radical scavenger. The peroxyl radicals were generated by adding AAPH (10 mM) and the rate of crocin bleaching was recorded at 440 nm in a thermostated spectrophotometer. The bleaching rate was recorded 2 min after the addition of AAPH and the rate from 2 to 5 min was used for calculations. Bleaching rates were plotted as Vb/Va versus [A]/[C], according to the equation $Vb/Va = 1 + Ka/Kc.\ [A]/[C]$, where Vb is the basal bleaching rate of crocin in the absence of PCB, Va is bleaching rate of crocin in the presence of PCB, [C] and [A]

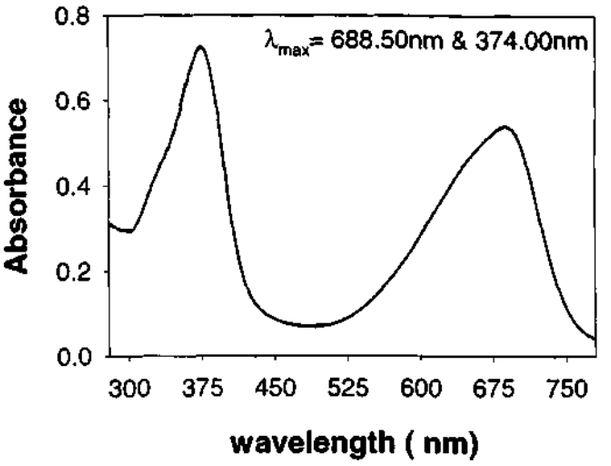


Fig. 1A: Absorption spectrum of pure phycocyanobilin in methanol/2% HCl

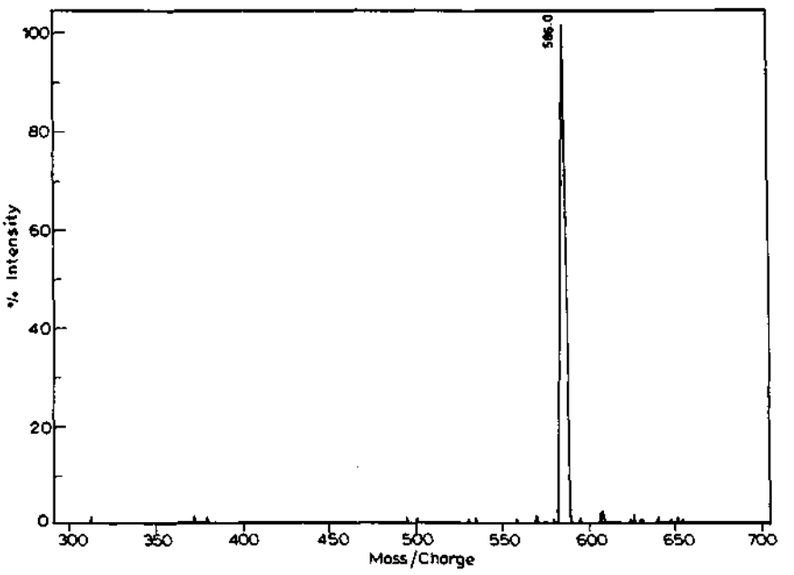
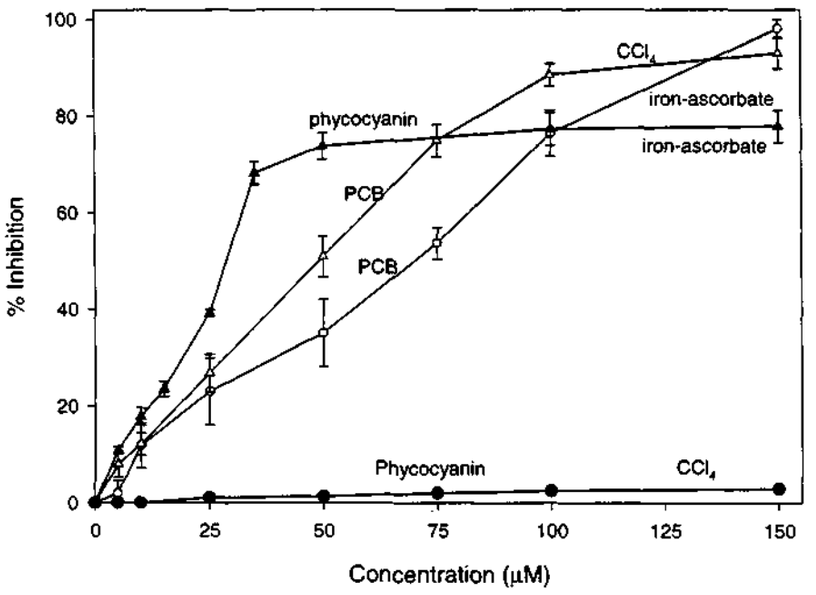


Fig. 1B: MALDI-TOF mass spectrum of pure phycocyanobilin



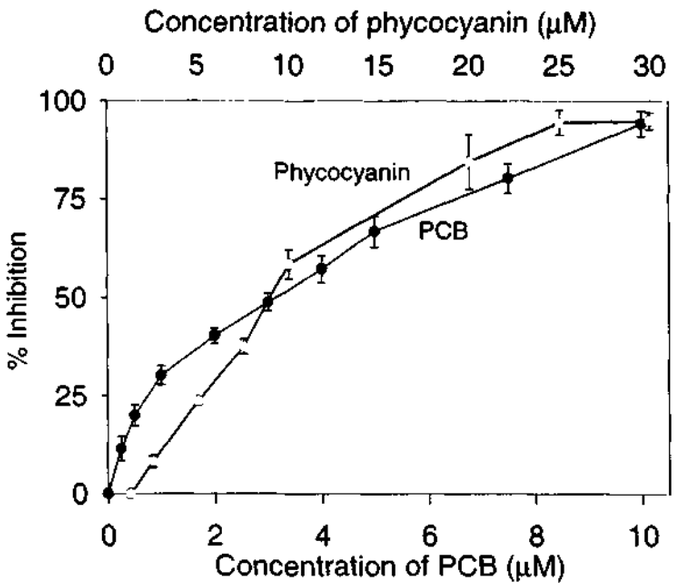


Fig. 2: Effect of PCB and phycocyanin on lipid peroxidation

(A) Inhibition of non-enzymatic (iron-ascorbate) and enzymatic (CCl_4)-induced lipid peroxidationin in rat liver microsomes.

(B) Inhibition of peroxyl radical (AAPH) induced lipid peroxidation in PC liposomes by phycocyanobilin and phycocyanin

are the concentrations of crocin and PCB, respectively. Ka and Kc are the rate constants for the reaction of the peroxyl radical with PCB and crocin, respectively. This plot gives a straight line, intersecting the ordinate, with a slope of Ka/Kc.

DPPH Radical Scavenging

Scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH), a stable nitrogen-centered free radical was measured as described earlier (30). Briefly, The reaction was started by mixing different concentrations of PCB (5-100 μM) to 100 μM DPPH in 1 ml cuvette and the time course of the optical density change was determined at 517 nm for 30 min (all dissolved in ethanol). DPPH radical scavenging activity was expressed in terms of $IC_{0.20}$ for 5 min, final concentration of scavengers required to decrease the 0.2 OD of DPPH at 517 nm. Uric acid, BHT, and glutathione were used as known DPPH radical scavenger in this assay.

RESULTS

Isolation and characterization of phycocyanobilin (PCB)

The blue color of C-phycocyanin is due to the chromophore, phycocyanobilin (PCB), which is an analogue of biliverdin. The PCB was isolated from pure phycocyanin by alcoholysis using Soxhlet extraction method as described earlier (22). Purity of PCB isolated was ascertained by HPLC analysis using ODS reverse phase column with acetone:water (2.5 : 1.5, v/v) containing H_3PO_4 (2 mM) and NaH_2PO_4 (0.5 mM) as a solvent system. The HPLC analysis showed a single peak for PCB with a retention time of 3.5 min. The absorption spectrum of pure PCB is shown in **Fig. 1A**. The spectrum shows absorption maxima at 374 nm and 688.5 nm and the spectral properties match well with the reported spectra for PCB (23). Its molecular mass was determined by MALDI-TOF (matrix assisted laser desorption/ionization-time of flight) and found to be 586 mass units (**Fig. 1B**), which corresponds to the reported molecular mass for PCB (22).

Non-enzymatic lipid peroxidation

(a) rat liver microsomes:

Lipid peroxidation was initiated in rat liver microsomes by the addition of iron and ascorbate. **Figure 2A** shows the antioxidative activities of PCB and phycocyanin at

Table 1: Inhibitory effect of PCB and phycocyanobilin on enzymatic and non-enzymatic lipid peroxidation.

Scavenger	IC_{50} (μM)
Iron-ascorbate-induced lipid peroxidation in rat liver microsomes	
Phycocyanobilin (PCB)	45.8 ± 4.7
Phycocyanin	38.8 ± 5.3
AAPH-induced lipid peroxidation in PC liposomes	
Phycocyanobilin (PCB)	2.2 ± 0.1
Phycocyanin	4.7 ± 0.5
CCl_4-induced lipid peroxidation in rat liver microsomes	
Phycocyanobilin	35.9 ± 4.0
Phycocyanin	< 500.0*

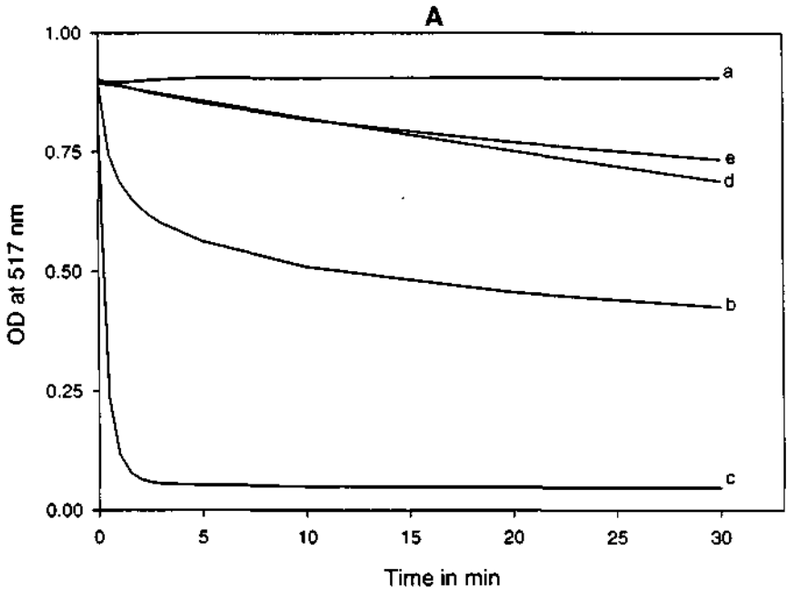
****at lower concentrations (up to 300 μM), phycocyanin did not inhibit CCl4-induced lipid peroxidation in rat liver microsomes***

different concentrations. Both PCB and phycocyanin inhibited peroxidation of membrane lipids in a concentration dependent manner with an IC_{50} value of 45.8 ± 4.7 μM and 38.8 ± 5.3 μM, respectively (**Table 1**). At 150 μM concentration, PCB inhibited the iron-ascorbate-induced lipid peroxidation up to 93% whereas at the same concentration, phycocyanin inhibited up to 73% (**Fig. 2A**). However, at lower concentrations, phycocyanin is a more efficient inhibitor than PCB (**Fig. 2A, Table 1**).

Iron-ascorbate-induced lipid peroxidation in rat liver microsomes is initiated by hydroxyl radicals generated by Fenton reaction (31). However, in the presence of iron chelators, generation of hydroxyl radicals are inhibited due to the removal of the iron from the reaction medium. To find out the ability of PCB and phycocyanin to scavenge radicals in an iron free reaction medium, water soluble AAPH, which generates peroxyl radical was used to induce lipid peroxidation in PC liposomes. The inhibition of AAPH-induced lipid peroxidation was studied using both PCB and phycocyanin and the results are compared (**Table 1, Fig. 2B**). At 10 μM concentration, both PCB and phycocyanin inhibited AAPH-induced lipid peroxidation in PC liposomes to the extent of 94% and 60%, respectively (**Fig. 2B**). In fact, PCB at 10 μM concentration almost completely inhibited lipid peroxidation and the inhibition was concentration dependent with an IC_{50} value of 2.2 ± 0.1 μM, which is significantly lower than the value obtained for phycocyanin (IC_{50} is 4.5 ± 0.7 μM, **Table 1**). PCB is a better inhibitor of lipid peroxidation than phycocyanin even at very low concentrations and this could be possibly due to its lipophilic nature.

Effect of PCB and Phycocyanin on CCl_4-induced enzymatic lipid peroxidation in rat liver microsomes

In the previous section, the ability of phycocyanin to effectively inhibit the CCl_4-induced lipid peroxidation *in vivo* is presented. [Earlier we have reported that, phycocyanin effectively inhibits the CCl_4- induced lipid peroxidation *in vivo* (8)]. Studies pertaining to the effect(s) of PCB and phycocyanin on CCl_4-dependent lipid peroxidation in rat liver microsomes is presented in this section. It was noticed that PCB effectively inhibited CCl_4-induced lipid peroxidation in a concentration dependent manner (**Fig. 2A**) with an IC_{50} value of 35.9 ± 4.0 μM (**Table 1**). A similar IC_{50} value was obtained for PCB for the



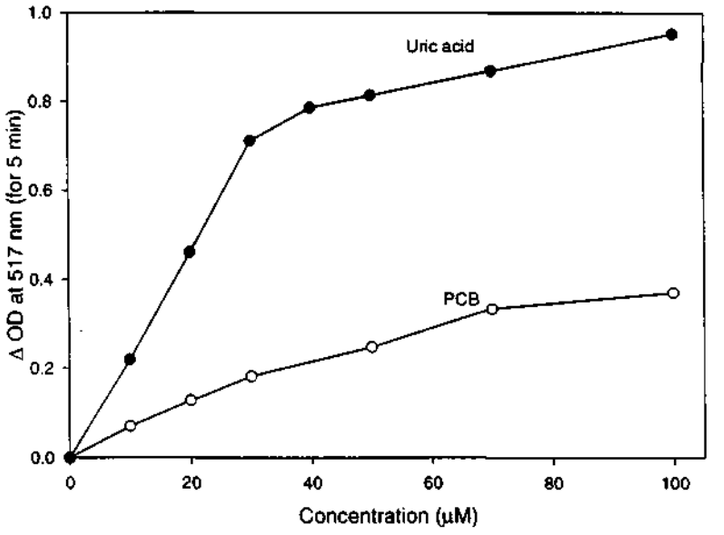


Fig. 3: DPPH radical scavenging ability of PCB

A) DPPH radical scavenging as a function of time. a) no addition; b) 100 μM phycocyanobilin; c) 100 μM Uric acid; d) 100 μM BHT; and e) 100 μM GSH

B) Dose dependent interaction of PCB and uric acid with DDPH stable free radical

inhibition of lipid peroxidation induced by iron/ascorbate system in rat liver microsomes. It is interesting to note that, phycocyanin failed to inhibit CCl_4-induced lipid peroxidation even at 200 μM concentration (**Fig. 2A**). However, at 400 μM concentration, a moderate inhibition (~45 %) was observed (data not shown).

Since cytochrome P450 mediated reactions are involved in the formation of reactive metabolites responsible for CCl_4-induced hepatotoxicity, it was of interest to find out whether PCB and phycocyanin have any effect on this enzyme system. It was noticed that, PCB at 200 μM concentration inhibited 45% of the cytochrome P450 activity, measured as aminopyrine N-demethylase activity. In fact, it is a more potent inhibitor of cytochrome P450 reductase and at 50 μM concentration, PCB inhibited nearly 50% of the cytochrome P450 reductase (measured as NADPH cytochrome c-reductase) activity. However, phycocyanin failed to inhibit aminopyrine N-demethylase at all concentrations tested, but at higher concentrations (50 μM) phycocyanin marginally (~25%) inhibited NADPH cytochrome c-reductase activity.

Interaction of PCB with stable free radical, DPPH

Stable free radical 2,2-diphenyl-1-picryl-hydrazyl (DPPH) is a useful reagent to study the radical scavenging ability of a compound. In this reaction, DPPH abstracts a hydrogen atom from the compound and this reaction involves a color change from violet to yellow that can easily be monitored at 517 nm (**Fig. 3 A**). The ability of PCB to scavenge DPPH stable radical was assayed spectrophotometrically and compared with that of uric acid, BHT and glutathione, the known DPPH radical scavengers. The results are summarized in **Fig. 3A and 3B**. It was noticed that, PCB interacts with DPPH radical in a time dependent manner and the rate at which it interacted with DPPH was significantly slower than that observed for uric acid, but the rate was significantly higher than that observed for BHT and glutathione (**Fig. 3A**). At 100 μM concentration, PCB removes nearly 40% of DPPH in 5 min, whereas during the same time interval (5 min) uric acid at 100 μM concentration almost completely scavenges DPPH (**Fig. 3A**). The other known scavengers (BHT and glutathione) tested were not efficient as PCB and they scavenged nearly 5% in 5 min (**Fig. 3A**). Both PCB and uric acid interact with DPPH in a

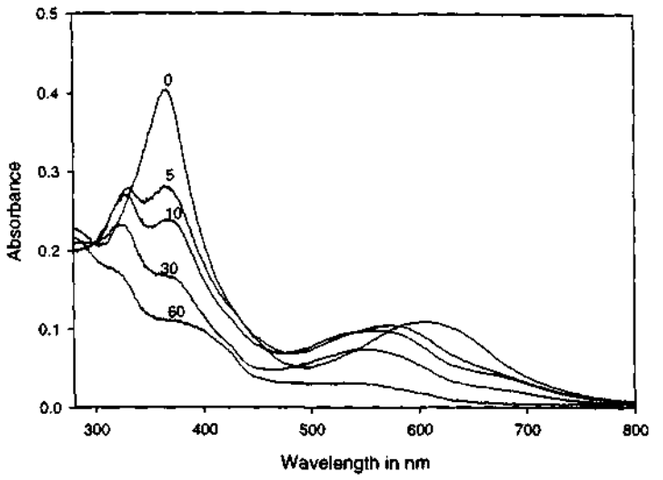


Fig. 4: Time-dependent spectral changes associated with the AAPH-induced oxidation of PCB (10 μM) in phosphate buffer (10 mM, pH 7.4) at 37° C. Number indicate the time in minutes elapsed after the addition of AAPH.

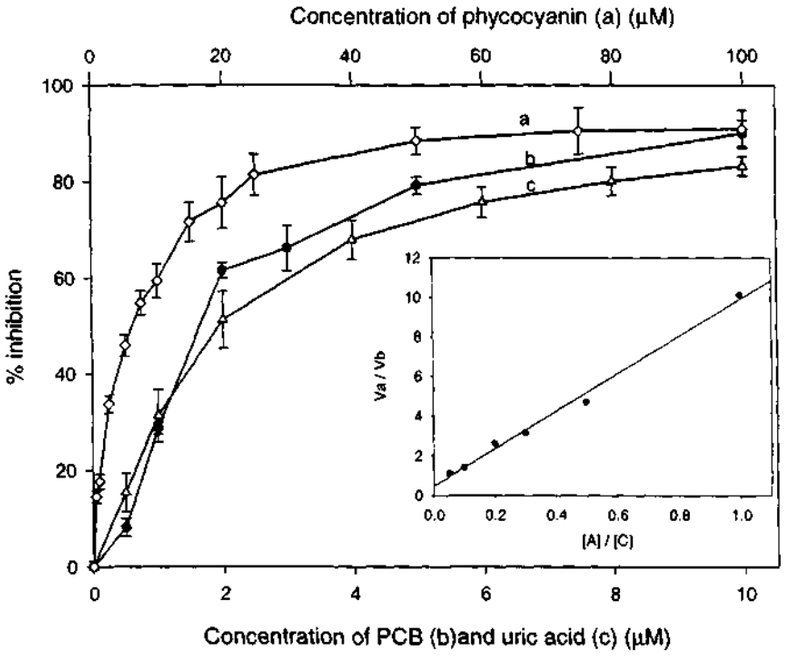


Fig. 5: Dose-inhibition curve. The inhibitory effect of a) Phycocyanin, b) PCB and c) Uric Acid on peroxyl radical-induced bleaching of crocin in phosphate buffer (10 mM, pH 7.4) at 40° C.
Inset: The slope of the straight line indicates the relative capacity of phycocyanobilin to interact with peroxyl radical according to the equation presented in the text.

Table 2: Antioxidant effect of phycocyanobilin (PCB) and uric acid on the DPPH test

Scavenger	Antioxidant effect $IC_{0.20}$ (μM)
Phycocyanobilin (PCB)	35.2 ± 3.8
Uric acid	9.2 ± 1.1

The stable free radical scavenging action was evaluated as the concentration of the compound that decrease to 0.20 the absorbance of the stable free radical DPPH, as described under methods and materials section.

Table 3: Inhibitory effects of PCB, phycocyanin and uric acid on peroxyl radical-induced bleaching of crocin

Scavenger	IC_{50} (μM)	Ka / Kc (Rate constant ratio)
Phycocyanobilin (PCB)	1.92 ± 0.06	9.5
Phycocyanin	5.3 ± 0.3	1.54
Uric acid	2.4 ± 0.5	3.5

Table 4: Inhibitory effects of PCB, phycocyanin, reduced phycocyanin, mannitol, DMSO and uric acid on hydroxyl radical induced deoxyribose degradation in the presence or absence of EDTA in assay mixture

Scavenger	IC_{50} (μM) In presence of EDTA	IC_{50} (μM) In the absence of EDTA
Phycocyanobilin (PCB)	67.2 ± 14.8	52.13 ± 8.2
Phycocyanin	30.5 ± 5.5	16.5 ± 2.8
Chemically reduced phycocyanin	27.4 ± 1.6	25.0 ± 1.2
Mannitol	3433 ± 152	n.d
DMSO	584.4 ± 50.2	n.d
Uric Acid	419.1 ± 80.9	n.d

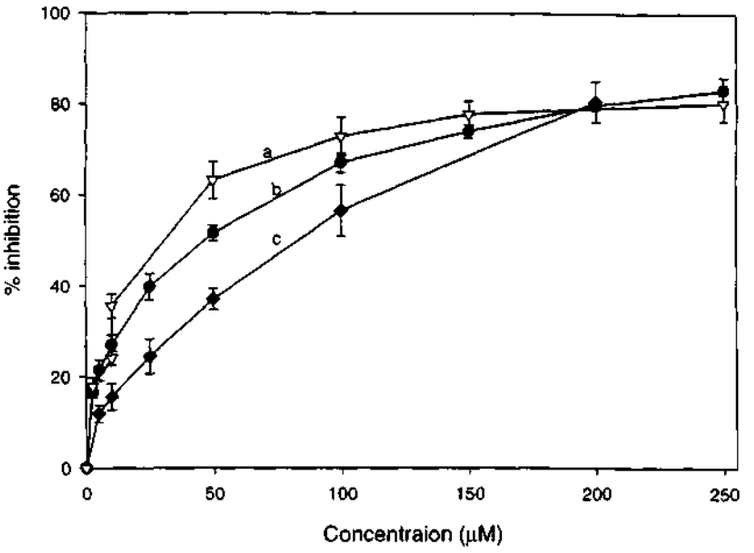


Fig. 6: Effect of (a) chemically reduced phycocyanin, (b) native phycocyanin, and (c) PCB on hydroxyl radical-induced deoxyribose degradation

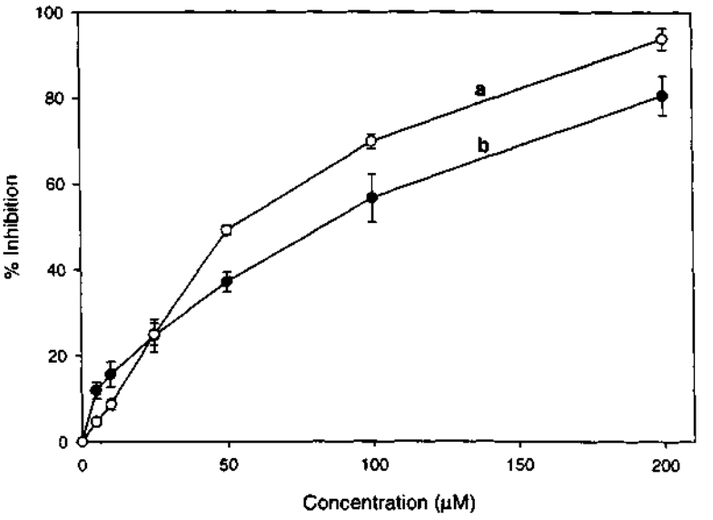


Fig. 7: Effect of PCB on hydroxyl radical-induced deoxyribose degradation. a) without EDTA and b) with EDTA

concentration dependent manner (**Fig. 3B**) with an $IC_{0.20}$ value of 35.2 μM and 9.2 μM, respectively (**Table 2**). The interaction of phycocyanin with DPPH was not tested since it precipitates in ethanol, the medium used for the assay.

Interaction with peroxyl radical and scavenging activity of PCB

It was observed earlier that, during peroxyl radical-induced lipid peroxidation, the color of PCB disappears. Results presented in the earlier sections clearly indicate that, the chromophore in phycocyanin directly interacts with peroxyl and hydroxyl radicals (8,15). The interaction of peroxyl radical with PCB (**Fig. 4**) and its ability to scavenge peroxyl radical were analyzed by the competition kinetics of crocin bleaching (**Fig. 5**). It was noticed that, incubation of PCB (10 μM) with peroxyl radical generator (AAPH) at 37° C for 1 h results in a significant decrease and shift in the absorption maxima towards lower wavelength at 610 nm (50 nm shift) and 370 nm (10 nm shift) (**Fig. 4**). The peroxyl radical scavenging ability of PCB was determined by crocin bleaching assay (**Fig. 5**). The results clearly indicate that, PCB is a potent peroxyl radical scavenger with an IC_{50} valve of 1.92 ± 0.06 μM. Under these assay conditions, uric acid, a known peroxyl radical scavenger has an IC_{50} value of 2.1 ± 0.3 μM, which is almost similar to the value obtained for PCB and 2.6 times lesser than the value obtained for the parent compound phycocyanin (5.3 ± 0.3 μM) (**Table 3**). The rate constant ratios obtained for the reactions of peroxyl radical with PCB, uric acid and phycocyanin are 9.5, 3.5 and 1.54, respectively (**Table 3**).

Hydroxyl radical scavenging activity of PCB by deoxyribose assay

The deoxyribose assay was used to test the hydroxyl radical scavenging and iron chelating properties of PCB. In the absence of EDTA, the deoxyribose degradation is a method to assess the ability of a compound to interfere with site-specific generation of hydroxyl radicals (32,33). In order to assess the hydroxyl radical scavenging and site specific inhibition of deoxyribose degradation by PCB, the deoxyribose degradation assay was carried out in the presence and absence of EDTA with appropriate controls (34). The scavenging activity of PCB was compared with that of phycocyanin and chemically reduced phycocyanin. The results are summarized in **Fig 6 and Table 4**. At 200 μM concentration, PCB inhibited nearly 80% of deoxyribose degradation by hydroxyl radical

Table 5: Comparison of the Second Order Rate Constants of PCB, phycocyanin, reduced phycocyanin, Dimethylsulfoxide, Mannitol and uric acid

Second Order Reaction rate constants

Scavengers	K_s
Phycocyanobilin (PCB)	$2.15\text{-}3.04 \times 10^{11}$
Phycocyanin	$1.5\text{-}2.1 \times 10^{11}$
Chemically reduced phycocyanin	$1.05\text{-}1.62 \times 10^{11}$
Mannitol	$1.9\text{-}2.5 \times 10^{9}$
DMSO	$1.3\text{-}1.6 \times 10^{10}$
Uric Acid	$1.4\text{-}1.6 \times 10^{10}$

The second order rate constant is calculated from the slope of the line from the plot of concentration of test compounds against 1/ absorbance (k = slope $\times k_{DR} \times [DR] \times A^0$), where A^0 is the absorbance, measured in the absence of hydroxyl radical scavenger, $k_{DR} = 3.1 \times 10^9\ M^{-1}\ S^{-1}$, derived from pulse radiolysis studies (29) and [DR] = 2.8 mM.

generated by iron-EDTA mixture (**Fig. 6 and 7**). PCB inhibited the degradation of deoxyribose in a dose dependent manner (**Fig. 6 and 7**) with an IC_{50} value of 67.2 ± 14.8 μM (**Table 4**). We have demonstrated earlier (chapter 2: part C) that, phycocyanin protects deoxyribose degradation more efficiently when the deoxyribose assay was carried out in the absence of EDTA (8) and it was also shown by fluorescence quenching experiments that phycocyanin acts as a chelator of iron (8). Similar observations were also made with PCB. When the deoxyribose assay was carried out in the absence of EDTA (iron alone was added in the hydroxyl radical generating system), PCB at 200 μM concentration was found to be a more potent inhibitor (~90%) of deoxyribose degradation (**Fig. 7**) with an IC_{50} value of 52.13 ± 8.2 μM (**Table 4**). This value is significantly lower than the IC_{50} value obtained for the inhibition of deoxyribose degradation when the assay was carried out in the presence of EDTA. These results suggest that, PCB can act as a potent chelator of iron and a site specific inhibitor.

The second order rate constant for the reaction of a scavenger molecule with hydroxyl radical was determined. From the graph with concentrations of the test compounds on x-axis and reciprocal absorption values obtained for different concentrations of test compounds used on y-axis, the second order rate constants (Ks) for the reaction of the test compounds with hydroxyl radical were calculated as reported earlier (29). In a similar way, the rate constants for established scavengers of hydroxyl radical such as mannitol, dimethylsulfoxide (DMSO), uric acid and parent molecule phycocyanin were also determined and the values obtained for them compared well with the reported values (**Table 5**). The second order rate constant (Ks) for PCB and other compounds with hydroxyl radicals, using deoxyribose assay, are presented in **Table 5**. The second order rate constant for PCB is in the range of 2.15-3.04 × 10^{11} M^{-1} S^{-1} whereas for phycocyanin, mannitol, dimethylsulfoxide and uric acid are in the range of 1.5 – 2.1 × 10^{11} $M^{-1}S^{-1}$, 1.9-2.5 ×10^{9} M^{-1} S^{-1}, 1.3-1.6 × 10^{10} M^{-1} S^{-1} and 1.4-1.6 × 10^{10} M^{-1} S^{-1}, respectively (**Table 5**). This result clearly suggests that, PCB is an efficient hydroxyl radical scavenger with effectiveness better than mannitol and uric acid, and is similar to the parent compound phycocyanin (**Table 5**).

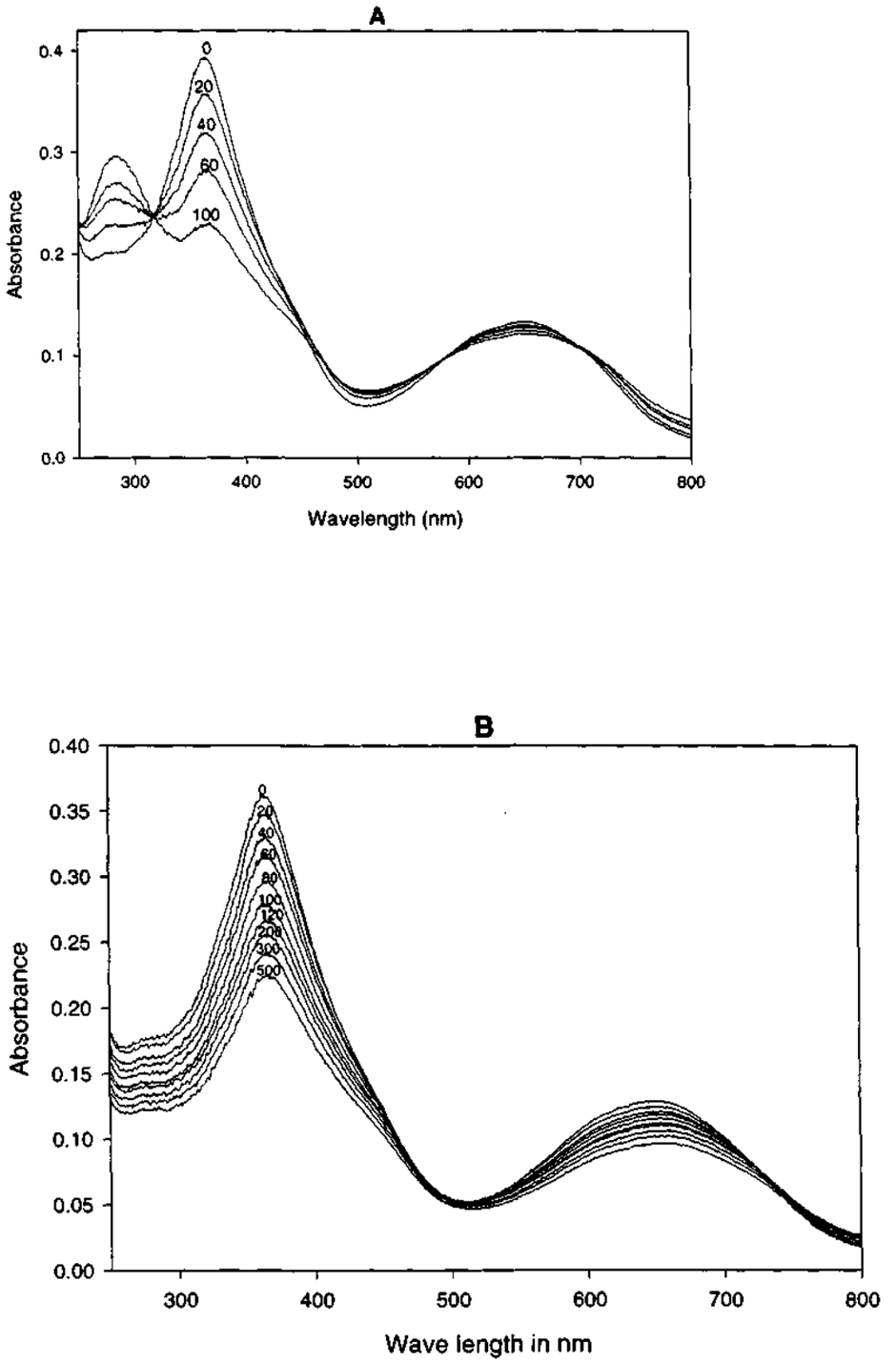


Fig. 8: Interaction of PCB with iron ions: Absorption spectra of PCB-iron complex
A) Interaction of PCB with Fe^{+3} in pH 5.0
B) Interaction of PCB with Fe^{+2} in pH 5.0
Numbers indicate the concentration of iron ion

At the end of deoxyribose assay, it was noticed that, the color of PCB decreased when iron-EDTA was used in hydroxyl radical generating system. Surprisingly, when EDTA was omitted in hydroxyl radical generating system, the color intensity of PCB did not change. The direct bleaching of PCB by hydroxyl radicals was carried out by using iron-EDTA mixture and iron alone in the hydroxyl radical generating system. At the end of one hour incubation with hydroxyl radical generated in the presence of iron-EDTA, there was about 80% and 70% decrease in the absorption at 370nm and 610 nm, respectively. Besides, there was a red shift at 610 nm (50nm shift) and 370 nm (20nm). When EDTA was omitted in hydroxyl radical generating system, there was no change or shift in the UV-Visible spectra of PCB (data not shown).

Iron chelation studies

Addition of different concentrations of Fe^{+2} (0-500 μM) or Fe^{+3} (0-100 μM) ions to PCB results in an iron-dependent UV-Visible spectral changes (**Fig. 8A and B**). When Fe^{+2} ions (0-500 μM) were titrated against PCB (10 μM taken in 10 mM acetate buffer, pH 5.0), there was a significant change in the UV-Visible spectrum of PCB with decrease in the absorption at 365 nm and 650 nm (**Fig. 8B**). In contrast to this, when Fe^{+3} (0-100 μM) was titrated against PCB (10 μM taken in 10 mM acetate buffer, pH 5.0), there was a significant decrease in the absorptions at 365nm and 650 nm with a concomitant appearance of a new peak at 285.5nm (**Fig. 8A**). Surprisingly, when the same experiment was repeated in phosphate buffer (10 mM, pH 7.4), there was no noticeable change in the UV-Visible spectrum of PCB in the presence of Fe^{+3} or Fe^{+2} ions (data not shown). These studies indicate that, PCB chelates iron (both Fe^{+3} and Fe^{+2}), and observable changes in the UV-Visible spectrum occur only in the acidic pH but not in the neutral pH. In fact, there was no change in the UV-Visible spectrum of PCB when it was treated with hydroxyl radical generating system at pH 7.4, in the absence of EDTA. This suggests that, PCB inhibited hydroxyl radical generation in the Fenton reaction possibly by chelating iron.

Characteristics of iron-PCB complexes

To find out whether PCB interacts with iron at neutral pH, studies have been carried out using ferrozine at pH 7.0. Ferrozine is a reagent that avidly binds to Fe^{+2},

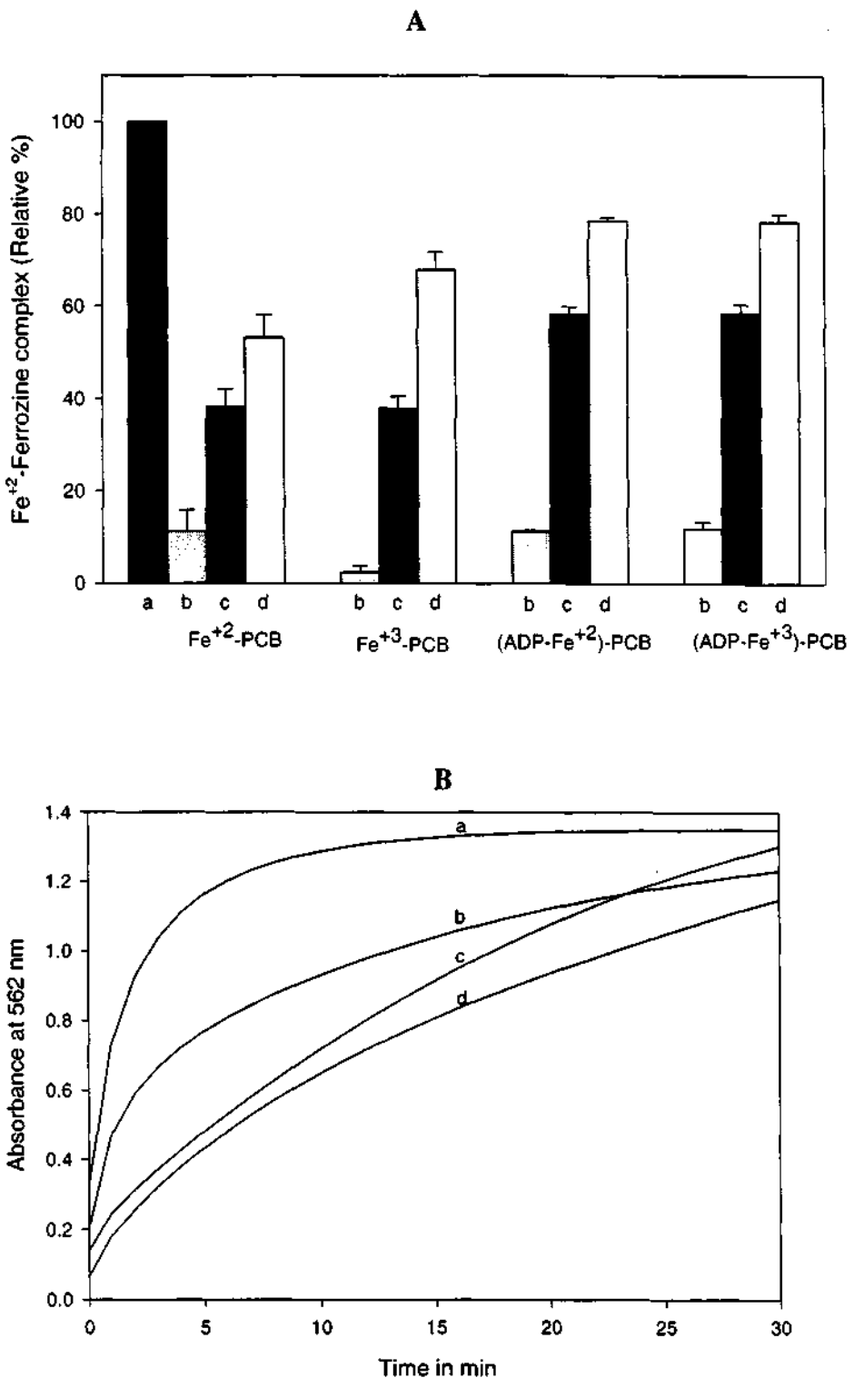


Fig. 9: PCB-iron complex

A) Effect of PCB on Fe^{+2}-Ferrozine complex formation (time dependent). a) Control (Fe^{+2} alone); b) 0 min; c) 10 min; d) 30 min

B) Effect of PCB and ascorbate on iron reduction. a) Fe^{+3}-ADP + Ascorbate; b) Fe^{+3}-ADP + PCB; c) Fe^{+3}+ascorbate and d) Fe^{+3} + PCB

forming a complex with a very high extinction coefficient at 562 nm (27,28). It was noticed that, nearly 55 % of iron was detectable as Ferrozine:Fe^{+2} complex., when PCB-Fe^{+2} complex was incubated with ferrozine for 30 min (these results are compared to ferrozine:Fe^{+2} complex without PCB). The same experiment was repeated with Fe^{+3} to find out the iron reduction capacity of PCB and it was observed that, nearly 70 % of iron appeared as ferrozine:Fe^{+2} complex at the end of 30 min, which clearly indicates the ability of PCB to reduce ferric iron to the ferrous state (**Fig. 9A**). However, the rate of reduction and formation of ferrozine:Fe^{+2} complex is much slower as compared to the reduction mediated by ascorbate, a known iron reductant (**Fig. 9B**). Our studies also indicate that, reduction of Fe^{+3} to Fe^{+2} by PCB and formation of ferrozine-Fe^{+2} chromophore is time dependent.

The ability of PCB to reduce iron in Fe^{+3}-ADP complex was also examined. The Fe^{+3}-ADP complex was preincubated with PCB (50 μM) or ascorbate (50 μM) for 1h and subsequently incubated with ferrozine for 30 min. As expected, ascorbate reduces the Fe^{+3} to Fe^{+2} much faster than PCB (**Fig. 9B**). In fact, both PCB and ascorbate reduce iron in Fe^{+3}-ADP complex much faster than free Fe^{+3} (**Fig. 9B**).

It was of interest to find out whether PCB has the ability to remove iron from Fe^{+2}-ADP complex. This was evaluated by preincubating Fe^{+2}-ADP complex with PCB for 1 hour followed by incubation with ferrozine for 30 min and the results were compared with the same experiment carried out in the absence of PCB (control). In the absence of PCB, at 0 min, all the iron from Fe^{+2}-ADP complex appears as ferrozine:Fe^{+2} complex. However, the formation of ferrozine:Fe^{+2} was much slower when PCB was added to Fe^{+2}-ADP mixture (**Fig. 9B**) under the same assay conditions. When Fe^{+3}-ADP was used instead of Fe^{+2}-ADP, the removal of Fe^{+3} from Fe^{+3}-ADP and its reduction to Fe^{+2} is similar to that observed with Fe^{+2}-ADP complex and PCB-Fe^{+3} (**Fig. 9B**).

DISCUSSION

Highly reactive oxygen-containing free radicals are formed as a consequence of a variety of biochemical reactions. These free radicals have the potential to damage cells and tissues. The membrane phospholipid bilayers and other tissue macromolecules (viz.

nucleic acids, proteins, sugars, etc) at cellular and subcellular levels, are undoubtedly major targets for the free radicals. In fact, the unsaturated bonds in membrane lipids are the targets for free radical reactions. Oxyradicals, including those associated with lipid peroxidation, cause cellular damage. The compound that inhibits membrane phospholipid peroxidation seems to exert a pharmacological effect in the prevention of radical-induced pathological events (35,36).

Most investigators believe that, lipid peroxidation is promoted by hydroxyl radical ($^{\bullet}OH$) and iron serves to catalyze the reactions responsible for the generation of $^{\bullet}OH$ (37,38). Contrary to this popular belief, it has been suggested that, lipid peroxidation is mediated by iron possibly in the form of an iron-oxygen complex (39,40). It is believed that, hydroxyl radical or an iron-oxygen complex abstracts hydrogen from polyunsaturated fatty acids and forms a lipid radical ($L^{\bullet}$), which will rapidly interact with molecular oxygen to form lipid peroxyl radical ($LOO^{\bullet}$) and eventually $LOO^{\bullet}$ gets converted to LOOH by abstracting hydrogen from a neighbouring allylic bond. During the propagation step, LOOH undergoes an iron-catalyzed breakdown to $LO^{\bullet}$, which in turn re-initiate lipid peroxidation via the hydrogen abstraction pathway. In fact, it is known that, both heme (cytochrome P450) and non-heme iron can catalyze the breakdown of LOOH (40,41). Termination of lipid peroxidation is brought about by non-enzymatic or enzymatic scavengers of reactive species that are formed during initiation and propagation. From the results of the rat liver microsomal studies, a mechanism of lipid peroxidation directly induced by ferrous ion has been proposed in which, the transition metal ion site-specifically binds in the vicinity of membrane phospholipids and leads to the peroxidative chain reaction (31,42,43). It is known that, liver microsomal NADPH-dependent lipid peroxidation is catalyzed by NADPH cytochrome P450 reductase and proceeds in the presence of iron ions (passing electrons directly or indirectly to some Fe^{+3} complexes), thus generating Fe^{+2} and eventually setting off the same reaction (44,45). It is very well documented that, transition metals are involved in both initiation and propagation of lipid peroxidation (46,47). Undoubtedly, the compounds interfering with the catalytic activity of iron could affect the peroxidative process (lipid peroxidation). On the other hand, lipid peroxidation taking place during carbon tetrachloride-induced hepatotoxicity does not

require iron ions (48). The involvement of lipid peroxidation in CCl_4-induced hepatotoxicity is widely accepted, although covalent binding of the reactive metabolites to cellular macromolecules may also contribute to the damage. In the present investigation we have demonstrated that PCB, the chromophore of phycocyanin, is a potent inhibitor of membrane lipid peroxidation in microsomal (enzymatic and non-enzymatic) as well as PC liposomal systems and these activities were compared with those obtained with the parent compound, phycocyanin.

PCB inhibited microsomal iron-ascorbate induced lipid peroxidation with an IC_{50} value of about 45.8 ± 4.7 μM (**Table 1**) and this value is not significantly different from the IC_{50} value (38.8 ± 5.3 μM) obtained for phycocyanin (**Table 1**). However, each phycocyanin molecule has three PCB molecules covalently attached to cysteine residues. So one molecule of phycocyanin is equivalent to three molecules of PCB. Our results show that, one mole of free PCB inhibits lipid peroxidation to the same extent as that observed with three molecules of bound PCB (as phycocyanin). Thus it is reasonable to state that, PCB is a more efficient inhibitor of lipid peroxidation in microsomal as well as PC liposomal systems. One possible explanation could be that, phycocyanin is hydrophilic, whereas PCB is hydrophobic in nature. Due to its lipophlic nature, PCB can easily reach the membrane sites where iron ion initiate and propagate the peroxidation. So, PCB can act as radical scavenger as well as chain breaking antioxidant. It can inhibit the generation of radicals by interfering with Fenton reaction due to its iron chelating property (**Fig. 8**). It is also possible that in the biliprotein phycocyanin, the three bound molecules of PCB are sterically inaccessible to membrane sites where peroxidation takes place. Earlier studies have demonstrated that, tetrapyrroles like bilirubin, biliverdin, porphyrins, tetraarylpyrrols, etc inhibit the iron-induced membrane lipid peroxidation (49-51). Preliminary studies on the inhibition of diene conjugation formation in linoleic acid dispersed micelles carried out as reported earlier (52) indicated that, PCB effectively inhibited diene conjugation (IC_{50} 0.90 ± 0.11 μM) when iron-EDTA was used as inducer of lipid peroxidation in linoleic acid micelles (data not shown). In this experiment, hydroxyl radicals ($^{\bullet}OH$) are involved in the initiation of lipid peroxidation and PCB inhibits effectively by scavenging hydroxyl radicals. Efficient inhibition of peroxidation in micelles by PCB may be due to its lipophlic

nature. This suggests that PCB can easily reach the metal ion binding sites in the membrane phospholipid during lipid peroxidation and inhibits oxidative process either by chelating iron or scavenging radicals or both. It is also quite possible that, PCB may act as a powerful chain breaking antioxidant.

Studies on CCl_4-induced hepatotoxicity provided clear evidence for the metabolism of CCl_4 to free radicals such as $CCl_3^{\bullet}$ and $O_2CCl_3^{\bullet}$, which can initiate lipid peroxidation (53,34). It has been demonstrated that, CCl_4 is metabolized by the liver microsomal cytochrome P450 system to give the trichloromethyl radical ($CCl_3^{\bullet}$), which abstracts hydrogen from membrane lipids and initiates the chain reaction of lipid peroxidation. In the earlier section (Chapter 2: part C) it is presented that, phycocyanin effectively inhibits CCl_4-mediated lipid peroxidation in rat liver *in vivo* and it also protects the liver enzymes (8,9). The chromophore, PCB, has been shown to effectively inhibit CCl_4-induced lipid peroxidation in rat liver microsomes *in vitro* with an IC_{50} value of 35.9 ± 4.0 μM (**Table 1**). This value is almost similar to the IC_{50} value of PCB for the inhibition of iron-ascorbate-induced lipid peroxidation (**Fig. 2**). Interestingly, the parent compound phycocyanin failed to inhibit the CCl_4-induced lipid peroxidation *in vitro* at all concentrations tested (0-250 μM, **Fig. 2**). However, at high concentrations (500 μM), phycocyanin moderately inhibited (20-40%) lipid peroxidation (results are not shown). It is quite possible that, under *in vivo* conditions, phycocyanin may get converted to PCB, which is responsible for the observed inhibition of lipid peroxidation by phycocyanin *in vivo*.

Involvement of cytochrome P450 in CCl_4 or NADPH-induced microsomal lipid peroxidation is known. It is also known that, inhibitors of cytochrome P450 prevent the CCl_4-mediated lipid peroxidation (54). Hence it was of interest to find out whether PCB and phycocyanin have any effect on cytochrome P450 system. It was noticed that, PCB moderately inhibited both cytochrome P450 and NADPH-cytochrome P450 reductase. In fact, reductase was inhibited comparatively to a greater extent than cytochrome P450. However, PCB did not elicit any binding spectra with cytochrome P450 although some change in cytochrome P450 spectra were noticed (data not shown). It is known that, some of the cytochrome P450 inhibitors do not elicit any binding spectra, but still inhibit the

reactions mediated by cytochrome P450 (55). It is also known that, microsomal cytochrome P450 has the ability to catalyze the decomposition of lipid peroxide (LOOH) to alkoxyl ($LO^{\bullet}$) or alkyl ($L^{\bullet}$) radicals (41,56), which in turn re-initiate lipid peroxidation via the hydrogen abstraction pathway. Even transition metal ions such as iron, copper, etc can catalyze the decomposition of lipid peroxide (LOOH) to alkoxyl ($LO^{\bullet}$) or alkyl ($L^{\bullet}$) radicals. It is well known that, some of the antioxidants inhibit the enzymatic lipid peroxidation by inhibiting either NADPH-cytochrome P450 reductase or cytochrome P450 activity (55). So it appears that, PCB inhibits lipid peroxidation by virtue of its ability to inhibit cytochrome P450 system and also to chelate transition metal ions (Fe^{+2} or Fe^{+3}) (**Fig. 8**). Besides this, PCB also has the ability to scavenge reactive oxygen species (**Table 3 and 4**) such as hydroxyl and peroxyl radicals. It is interesting to note that, phycocyanin does not inhibit cytochrome P450 *in vitro*.

In the previous section it is being mentioned that, phycocyanin efficiently inhibits the peroxyl radical-induced lipid peroxidation (8). The results presented in this section clearly indicate that, PCB has significant antiperoxidative activity against AAPH-induced lipid peroxidation in PC liposomes (**Fig. 2B**), with an IC_{50} value of 2.17 ± 0.1 μM, which is much lower (> 5 fold) than that observed for phycocyanin (11.61 ± 1.18 μM, **Table 1**). These studies indicate that, free PCB is a better radical scavenger and antiperoxidative compound than covalently bound PCB (phycocyanin), and this could be due to its lipophlic nature. PCB also has the ability to interact with stable DPPH radical in a time dependent manner. Although it is not as efficient as uric acid, but significantly better than BHT and glutathione in scavenging stable DPPH radical (**Fig. 3A**). The potency of PCB in scavenging stable free radical, DPPH, is nearly four times lower than that observed for uric acid, a known DPPH radical scavenger (**Table 2**). However, the antioxidant potency of any compound need not correlate to its DPPH radical scavenging property. In fact, earlier it has been shown that, the antiperoxidative activity of synthetic tetraarypyrroles does not correlate with DPPH radical scavenging activity (51).

Generation of peroxyl radical is a necessary proximate step leading to the formation of TBARS during lipid peroxidation (57). Both bilirubin (BR) and biliverdin (BV), the structural analogues of PCB, are known potent peroxyl radical scavengers (17,18,58). In

fact, albumin-bound bilirubin serves as a physiological antioxidant in plasma. It has been shown that, both BR and BV protect the rat hepatocytes and erythrocytes against peroxyl radical induced damage (58). Results presented in the earlier section have established the radical scavenging property of phycocyanin by studying its reactivity with the peroxyl and hydroxyl radicals, and also by competition kinetics of crocin bleaching (8). The peroxyl radical scavenging ability of PCB was also studied by crocin bleaching assay (**Fig. 5**). It was noticed that, PCB reacts with peroxyl radical more efficiently than phycocyanin and uric acid (**Fig. 5, Table 3**). PCB inhibited bleaching of crocin with an IC_{50} value of 1.92 ± 0.06 μM, which is significantly lower than that observed for phycocyanin (5.3 ± 0.3 μM **Table 3**). Since these assays were carried out in the absence of iron, the ability of PCB to scavenge peroxyl radicals is not due to its iron-chelating property. In fact, these results compare well with the inhibition of AAPH-induced lipid peroxidation in PC liposomes studied using both PCB and phycocyanin (**Table 1**). These studies also revealed that, PCB is a better inhibitor of lipid peroxidation than phycocyanin. This could be possibly due to its lipophilic nature. The protection of erythrocytes against AAPH-induced hemolysis by phycocyanin has been reported earlier (59).

We have demonstrated that, the covalently linked chromophore, PCB is involved in the antioxidant and radical scavenging activity of phycocyanin (8). This is supported by the fact that both peroxyl and hydroxyl radicals bleach PCB (**Fig. 4**). Interaction of radicals with PCB initially results in a shift in the absorption maxima towards lower wavelength and this spectrum is similar to the reported UV-Visible spectra of mesobiliviolin (60). Continued incubation of PCB with radicals is accompanied with a gradual decrease in the absorption of all the peaks indicating that, PCB is undergoing oxidative fragmentation. In fact, both bilirubin and biliverdin interact with different kinds of free radicals to undergo oxidative degradation towards one-ring system (61).

It is well known that, hydroxyl radical is a highly potent oxidant that reacts with almost all biomacromolecules found in the living cells (62-64). Hydroxyl radical is very short-lived and will react at the site where it is formed, in a site-specific fashion. It is known that, iron salts damage deoxyribose with the release of TBARS (65), and this damage can be related to the site-specific iron-dependent generation of hydroxyl radicals

(66). The chelating agent EDTA binds iron tightly and helps to release hydroxyl radicals into free solution, rather than being localized to iron-binding sites of the substrates (67). Hence, any hydroxyl radical generated by the iron-EDTA complex will have to migrate a short distance to reach the substrate (deoxyribose), and addition of a hydroxyl radical scavenger should inhibit the substrate (deoxyribose) degradation to an extent depending on its second order rate constant for the reaction with the hydroxyl radical. In the earlier section (8) it has been reported that, phycocyanin interacts with hydroxyl radical with a reaction rate constant (Ks) of 1.9×10^{11} M^{-1}. S^{-1} and inhibits the deoxyribose degradation with an IC_{50} value of 28 μM. However, in the absence of EDTA phycocyanin was found to be a more potent inhibitor of deoxyribose degradation since it readily interacts with hydroxyl radical scavenger and also chelates with iron ion and thus protect the target molecule, deoxyribose. Bleaching of phycocyanin in the presence of radicals suggest its interaction with them. Studies carried out with PCB have shown that, it is a potent scavenger of hydroxyl radicals *in vitro* and in fact, it is more potent than phycocyanin, uric acid, DMSO and mannitol (**Table 4**). PCB is a more potent inhibitor than phycocyanin of the site-specific degradation of deoxyribose in the absence of EDTA. The IC_{50} valve is much lower than that of the deoxyribose degradation induced by hydroxyl radicals generated using iron-EDTA mixture (**Table 4**) and in this respect, it behaves like phycocyanin. Both PCB and phycocyanin interact with hydroxyl radical and undergo degradation. Both of them are strong iron chelators. Iron-PCB complex can be gradually oxidized by peroxyl radical generated by AAPH indicating the ability of this complex to scavenge radicals. Our results suggest that, PCB possess radical scavenging and iron chelating properties.

Iron-mediated oxidative stress is thought to be involved in several pathologies, including hemochromatosis, β-thalassemia, atherosclerosis and ischemic heart disease and cancer (68-72). Recent studies have highlighted that, oxidative stress and iron may be important in the activation of HIV-1 (73). Iron is directly involved in cell damage by oxyradicals since it catalyzes the formation of highly reactive $^{\bullet}OH$ radicals through Haber-Weiss reactions (47,47,75) Therefore, substances that are able to trap `free iron' and make it unavailable for Haber-Weiss reactions act as antioxidants (76-78). Transition metals like

iron are known to convert poorly reactive species into more reactive ones. Autoxidation of thiols, diphenols, etc produces reactive radicals and these transformations require metal ions. Lipid peroxides decompose under physiological conditions in the presence of iron to generate highly cytotoxic aldehydes (79). Available evidences clearly suggest that, iron ions are the promoters of radical reactions. Due to all these reasons, organisms have evolved to keep transition metal ions sequestered in storage or transport proteins as much as possible. So, metal sequestration is an important part of extracellular antioxidant defense (62). We have demonstrated that, both phycocyanin and PCB have the ability to scavenge reactive radicals as well as chelate iron ions (8). The iron chelating property of PCB was studied spectroscopically and it was noticed that, a change in the UV-Visible spectra of PCB depends upon the pH as well as the oxidation state of iron (**Fig 8A & B**). These studies have also revealed the iron reducing property of PCB. It is well known that, some of the polyphenolic compounds enhance the oxidation of reduced iron or other transition metals required for the production of superoxide and hydroxyl radicals. However, some nonflavonoid polyphenolics can complex with iron, and these complexes cannot react with oxygen. There are compounds, which show potent iron reducing ability as well as inhibitory effect on lipid peroxidation (80). PCB also appears to possess several interesting properties. PCB can bind both reduced and oxidized form of iron and it is also an iron reducing agent. PCB also has the ability to remove and reduce iron from iron-ADP complex. However, the rate of reduction is much slower than ascorbate, a known iron reductant (**Fig. 9B**). Iron-PCB complex cannot participate in Fenton reaction, but it efficiently scavenges radicals. Based on these results one can state that PCB can act as a 'site specific scavenger'. Summarizing, our results clearly indicate that, PCB has the ability to chelate iron and block the generation of hydroxyl radical by Fenton reaction, efficiently scavenge reactive oxygen radicals and inhibits the membrane lipid peroxidation induced by iron-ascorbate system.

Conclusions:

Antioxidant activity of phycocyanin, isolated from *Spirulina platensis,* is largely due to radical scavenging and iron chelating properties of PCB. However, one cannot rule out the involvement of apoprotein in the antioxidant properties of phycocyanin. The

present work supports our earlier finding on the involvement of phycocyanobilin in radical scavenging and antioxidant properties of phycocyanin (8). Our studies show that, PCB displays the multiple property of antioxidant activities. It is [i] an inhibitor of iron-ascorbate, CCl_4 and peroxyl radical-induced lipid peroxidation; [ii] a scavenger of peroxyl radical in aqueous system; [iii] a scavenger of stable free radical, DPPH; [iv] a scavenger of hydroxyl radical; and [v] an iron chelator, iron reductant and an inhibitor of Fenton reaction. In addition, PCB is an inhibitor of rat liver microsomal cytochrome P450 and cytochrome P450 reductase activity. The hepatoprotective activity of phycocyanin is partly due to antioxidant and radical scavenging activities of PCB. Inhibition of rat liver microsomal cytochrome P450 and cytochrome P450 reductase activities also contributes to the observed hepatoprotective activity of phycocyanin. The anti-inflammatory activity of phycocyanin is partly due to the radical scavenging and iron-chelating properties of PCB. The inhibition of arachidonic acid metabolism by phycocyanin and PCB also contributes to the observed anti-inflammatory and hepatoprotective properties of phycocyanin. The studies carried out so far show that, PCB present in *Spirulina* (a natural food supplement) as C-phycocyanin may play an important role in human health and disease. However, more research is needed on the uptake, transportation and metabolism of phycocyanin and PCB in living cells.

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CHAPTER 2: PART E

Scavenging of Peroxynitrite by Phycocyanin and Phycocyanobilin from *Spirulina platensis:* Protection against Oxidative Damage to DNA

INTRODUCTION

Phycocyanin, a water soluble biliprotein, is one of the major constituents of *Spirulina platensis*, a blue-green algae. Phycocyanin has significant antioxidant, anti-inflammatory, hepatoprotective and radical scavenging properties (1-5). It has been suggested that the anti-inflammatory and hepatoprotective effect could be due to its ability to scavenge oxygen free radicals and inhibit enzymes involved in the formation of inflammatory prostaglandins (2,3,6). Recently we have reported its ability to selectively inhibit cyclooxygenase-2 (COX-2) (7). In fact, oxidative stress is considered as one of the pathogenic factors causing inflammation and COX-2 is implicated in oxidative stress-induced diseases (8). When polymorphonuclear leukocytes and macrophages are activated by contact with a foreign substance, they produce superoxide radical catalyzed by NADPH oxidase enzyme complex. Superoxide radical dismutates into hydrogen peroxide (H_2O_2), which is further converted to highly reactive hydroxyl radical. In addition, lot of hypohalous acid, a strong oxidant, is also generated and this reaction is catalyzed by lysosomal myeloperoxidase. Phycocyanin contains an open chain tetrapyrrole chromophore known as phycocyanobilin (PCB) which is covalently attached to the apoprotein. Available evidences suggest that PCB plays an important role in some of the biological properties exhibited by phycocyanin (3,4,9). PCB has a chemical structure similar to that of bilirubin, a bile pigment which is known to scavenge various reactive oxygen species *in vivo* (10,11). Bilirubin is also known to inhibit $ONOO^-$-mediated oxidations (12). However, it is not known whether PCB can be a useful antioxidant defense against $ONOO^-$-induced oxidations.

Peroxynitrite ($ONOO^-$) anion is an inorganic toxin of biological importance and this potent oxidant can be generated *in vivo* from nitric oxide and superoxide (13-15). Activated neutrophils and macrophages simultaneously generate both NO and $O_2^{\cdot-}$ during the inflammatory response, and these species rapidly react to form $ONOO^-$ at a rate of $2 \times 10^{10}\ M^{-1}\ s^{-1}$ *in vivo* (16.17). Recent reports provided the evidence for the *in vivo* formation of $ONOO^-$ in, for example, human atherosclerotic coronary vessels, during acute lung injury, toxic liver injury and chronic inflammation (18). At physiological pH peroxynitrite anion can get protanated to peroxynitrous acid (ONOOH), an unstable species, which

decomposes spontaneously to a mixture of products (13). It is a relatively stable species, which can oxidize various biomolecules such as lipids, proteins and thiols through a two-electron-transfer process (19-23). It causes extensive base modification as well as single strand breaks in both supercoiled plasmid DNA (23-26) and mammalian cellular DNA (27). Recent studies have implicated $ONOO^-$ in several inflammatory disorders and oxidative damage to DNA is one of the events taking place during chronic inflammation (28,29). $ONOO^-$-mediated reactions can lead to profound biological consequences such as apoptosis (30) and even mutation of the cell (31). In fact, oxidative damage to DNA has long been considered an important underlying event in chronic inflammation leading to carcinogenesis (32). Thus, antioxidant defenses of cells and extracellular fluids appear to be crucial in reducing tissue damage and cancer. A suitable $ONOO^-$ scavenger can possibly prevent or at least reduce some of the deleterious effects caused by this toxin during inflammation. Therefore, there is a great demand to identify compounds that show pharmacological activity against $ONOO^-$.

The present study demonstrates that phycocyanin and its chromophore, phycocyanobilin (PCB), a naturally occurring linear tetrapyrrole interact and efficiently scavenge peroxynitrite anion. Evidence is also presented to support that PCB significantly inhibit $ONOO^-$-mediated strand breaks in supercoiled pBR322 DNA.

MATERIALS AND METHODS

Chemicals

Plasmid DNA (pBR322) was obtained from Bangalore Genei (P) Ltd. (Bangalore, India). Agarose was purchased from GibcoBRL (Gaithesburgh, MD). Pyrogallol Red was obtained from SISCO Research Laboratories (P) Ltd. (Mumbai, India). Tris and Diethylenetriaminepentaacetic acid (DTPA) were purchased from Sigma (St. Louis, MO, USA). All other materials were procured from standard suppliers and were of analytical grade.

Phycocyanin was isolated from *Spirulina platensis* and purified as described in chapter 2; part A).

Phycocyanobilin (PCB) was prepared from pure phycocyanin and purity ascertained as described earlier (7,33). The UV-Visible spectra of PCB was recorded between 280-800 nm in methanol/2% HCl and the concentration was estimated at 374 nm using the extinction coefficient of 47,900 $M^{-1}cm^{-1}$ (34). PCB stock solution (20 mM) was prepared in ethanol and further diluted with assay buffer.

Synthesis of peroxynitrite ($ONOO^-$)

Peroxynitrite was synthesized by reacting nitrite with acidified hydrogen peroxide as described earlier (35). Acidified hydrogen peroxide (1M in 0.5 M HCl, 20 ml) and sodium nitrite (200 mM, 20 ml) solutions were drawn into two separate syringes, analogous to a stop flow set up. Simultaneous injection of the contents of both syringes into an ice-cooled beaker containing 1.5 M potassium hydroxide (40 ml) through a 'Y'-shaped junction, leading to rapid mixing to form peroxynitrous acid followed by stabilization of the resulting peroxynitrite anion and treated with manganese dioxide (3 mg/ml for 30 min at 4° C) to remove residual hydrogen peroxide. Manganese dioxide was removed by centrifugation (for 5 min at 4° C and 15,000 g) and filtration (0.45 μm; Millipore, Molsheim, France). Concentration of peroxynitrite was determined spectrophotometrically at 302 nm in 1 M NaOH (ε_{302} = 1670 $M^{-1}cm^{-1}$). The typical yield of freshly prepared peroxynitrite ranged from 50-90 mM. The solution of peroxynitrite was stable for 15 days (-80° C) with negligible changes in its concentration. The frozen fraction of peroxynitrite solution forms a yellow top layer, which was retained for further studies.

Peroxynitrite treatment and analysis of DNA strand breaks

Single and double strand breaks in supercoiled DNA were analyzed by agarose gel electrophoresis as described earlier (24). Plasmid pBR322 DNA (0.5 μg) was treated with $ONOO^-$ (50 μM) at room temperature in a buffered reaction mixture containing sodium phosphate (50 mM, pH 7.0), NaCl (10 mM), DTPA (diethylenetriaminepentaacetic acid, 0.1 mM) and different concentrations of PCB (0.5-25 μM) in ethanol (0.00125-0.125%) in a total volume of 0.25 ml (stock solution of PCB was prepared in ethanol). The control experiments were carried out by adding the same amount of ethanol (0.00125-0.125%)

without PCB. The reaction mixture was vigorously shaken during the addition of $ONOO^-$, since under these conditions $ONOO^-$ spontaneously decays with a half-life of less than 2s (25) and incubated at room temperature for 5 min. After the incubation period, DNA was precipitated with ethanol and left at -80°C for 3 h. Samples were centrifuged and the DNA pellet was washed with 70 % ethanol and resuspended in TE buffer (10 µl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0), mixed with 5 µl of electrophoresis loading buffer (0.05% bromophenol blue, 40% sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5), and loaded onto a 0.8% agarose gel prepared in Tris-acetate (40 mM, pH 8.3) containing EDTA (1 mM) and ethidium bromide (1.5 µg/ml). Electrophoresis was carried out at 60-70 mV for 1.30-2.0 h. The DNA bands in the gel were visualized under UV light and photographed. DNA quantification was carried out using UVi Tech gel documentation system with UVI BAND V.97 software.

Oxidation of PCB and phycocyanin by peroxynitrite ($ONOO^-$)

The oxidation was carried out at room temperature in a 1 ml cuvette and monitored spectrophotometrically. PCB or phycocyanin (10 µM) was taken in 1.0 ml of phosphate buffer (pH 7.0, 100 mM) to which different concentrations of $ONOO^-$ (0-125 µM in 0-10 µl of 0.5 M NaOH for PCB; 0-200 µM in 0-20 µl of 0.5 M NaOH for phycocyanin) were added directly through the sides of the cuvette, mixed immediately and after 5 min the UV-Visible spectrum was recorded using Shimadzu UV2100 spectrophotometer. The addition of $ONOO^-$ did not affect the pH of the reaction mixture.

Pyrogallol Red (PR) bleaching assay for peroxynitrite scavenging

Pyrogallol Red (PR) bleaching assay was carried out as reported earlier (36). Briefly, assay was carried out at 25° C in phosphate buffer (100 mM, pH 7.0), containing 50 µM Pyrogallol Red and increasing concentrations (0-50 µM) of PCB or phycocyanin (0-100 µM) in a total volume of 1 ml. Reaction was started by the addition of 25 µM of $ONOO^-$, mixed immediately, and the decrease in the absorbance at 542 nm was recorded after 5 min. Relative antioxidant activities (k_a/k_p) were calculated as reported earlier (36) where k_a and k_p are reaction rate constants of antioxidant and Pyrogallol Red with $ONOO^-$, respectively. The ratio k_a/k_p was calculated from the slope of the straight line plotting

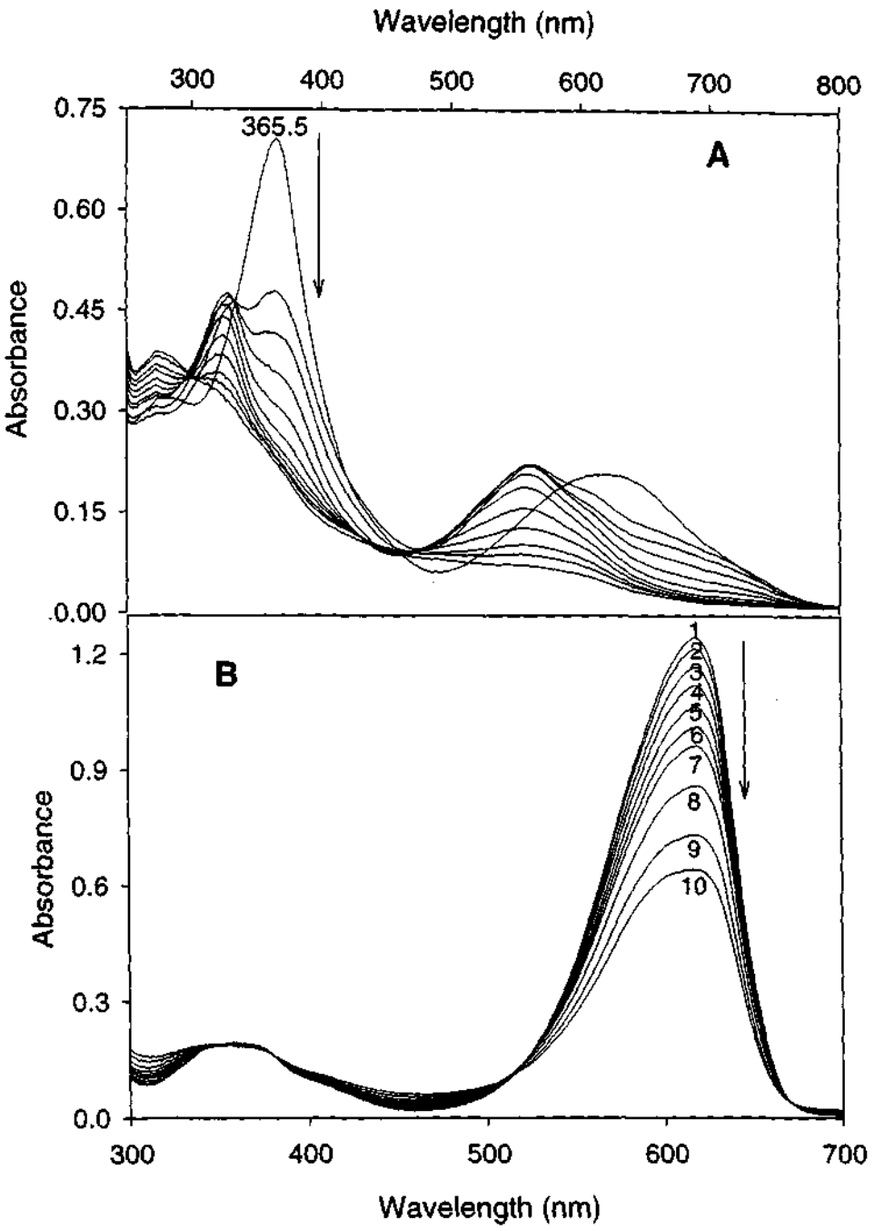

Fig. 1: Spectral changes of phycocyanobilin and phycocyanin caused by peroxynitrite. A: Phycocyanobilin (10 μM) was treated with different concentrations of ONOO⁻ (0-125 μM) in phosphate buffer (100 mM, pH 7.0). Absorbance spectra were recorded 5 min after addition of ONOO⁻. Each line in the descending order correspond to 0, 12.5, 25, 37.5, 50, 67.5, 75, 87.5, 100, 112.5, 125 μM of ONOO⁻. B: Absorbance spectra of phycocyanin (10 μM) were recorded 5 min after addition of ONOO⁻ in phosphate buffer (100 mM, pH 7.0). ONOO⁻ concentrations (μM) were as follows: 0 (1), 10 (2), 20 (3), 30 (4), 40 (5), 50 (6), 60 (7), 100 (8), 150 (9), and 200 (10).

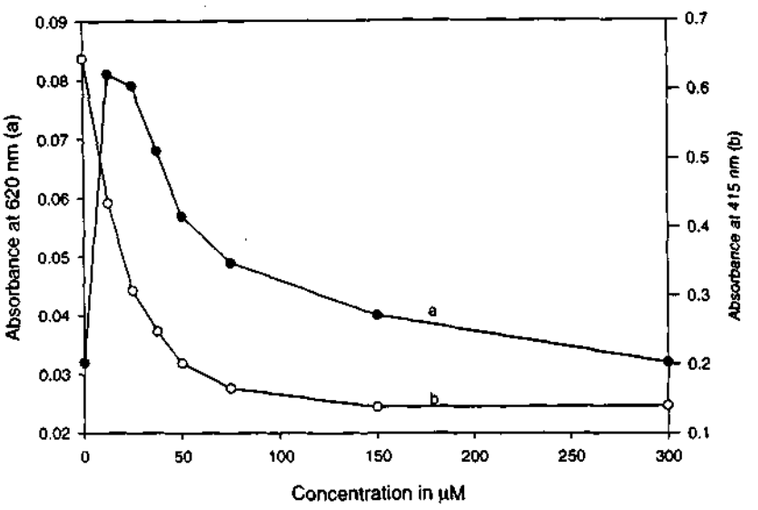

Fig. 3: Concentration dependent changes in the absorbance at 618 nm and 415 nm associated with the peroxynitrite-induced oxidation of NaBH4 reduced phycocyanin in phosphate buffer (100 mM, pH 7.0). Absorption was recorded 5 min after each addition of peroxynitrite.

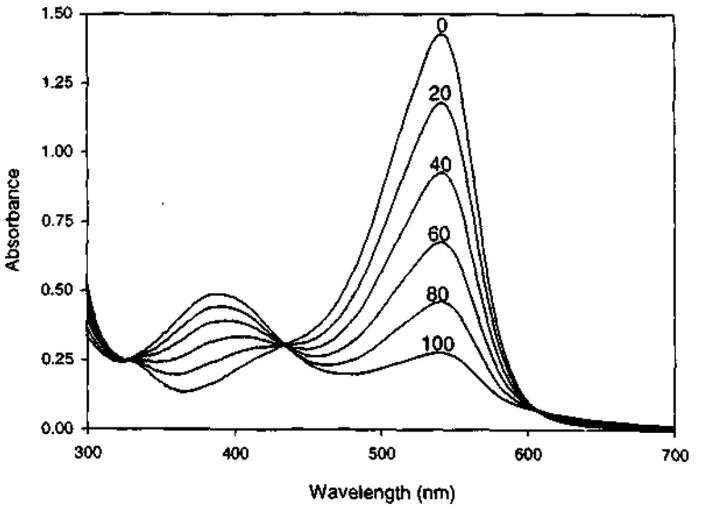

Fig. 4: Spectral changes of pyrogallol red (PR) caused by peroxynitrite. Pyrogallol red (50 µM) was treated with different concentrations of $ONOO^-$ (0-100 µM) in phosphate buffer (100 mM, pH 7.0). Absorbance spectra were recorded 5 min after addition of $ONOO^-$ (5 µ l each addition). Each line in the descending order corresponds to 0, 5, 10, 15, 20 µl of $ONOO^-$. Numbers indicate the concentration of $ONOO^-$ in µM

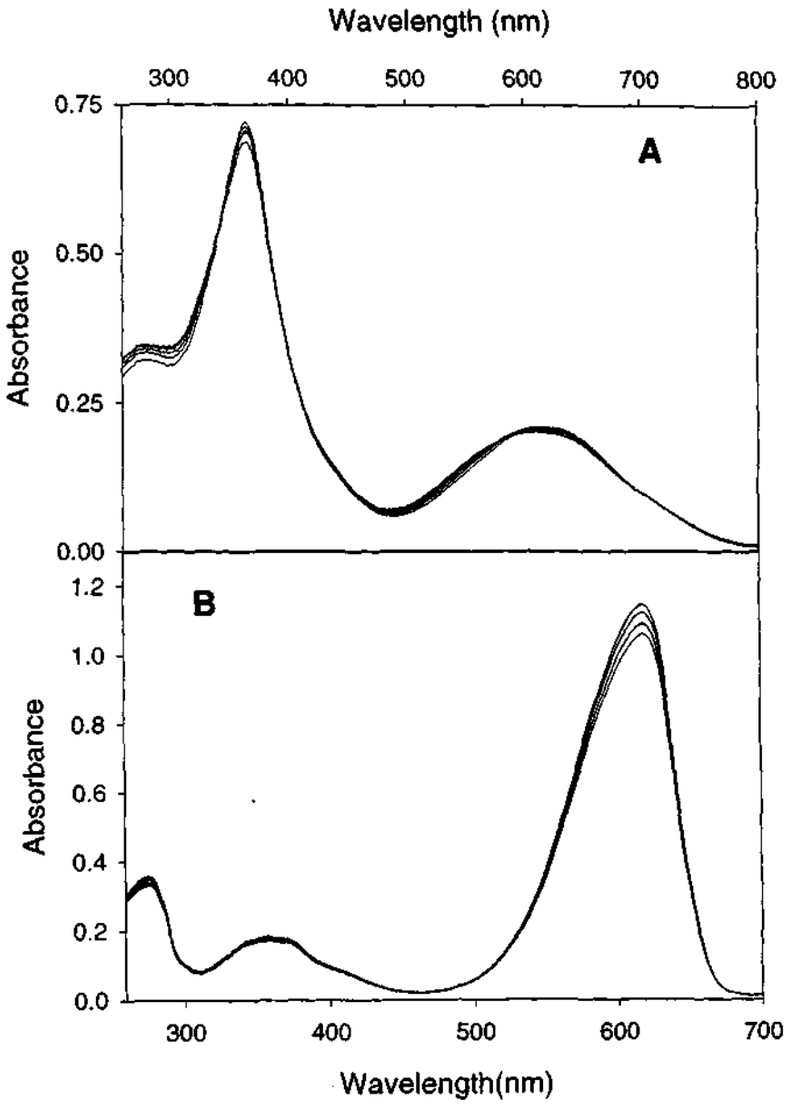

Fig. 2: Spectral changes of phycocyanobilin and phycocyanin caused by decomposed peroxynitrite. A: Phycocyanobilin (10 μM) was treated with different concentrations of $ONOO^-$ (0-20 μl) in phosphate buffer (100 mM, pH 7.0). Absorbance spectra were recorded 5 min after addition of decomposed $ONOO^-$ (5 ml each addition). Each line in the descending order corresponds to 0, 5, 10, 15, 20 μl of $ONOO^-$. B: Absorbance spectra of phycocyanin (10 μM) were recorded 5 min after addition of decomposed $ONOO^-$ in phosphate buffer (100 mM, pH 7.0). Each line in the descending order corresponds to 0, 5, 10, 15, 20 μl of $ONOO^-$.

$\Delta[PR]_0/\Delta[PR]_a$ against $[A]/[PR]$, where $\Delta[PR]_0$ and $\Delta[PR]_a$ are consumptions of PR in the absence and presence of antioxidant by the same amount of $ONOO^-$. $[A]_0$ and $[PR]_0$ are the initial concentrations of antioxidant and pyrogallol red, respectively.

RESULTS

Interaction of phycocyanin and phycocyanobilin (PCB) with peroxynitrite ($ONOO^-$)

Interaction of phycocyanin and PCB with $ONOO^-$ was studied spectroscopically at pH 7.0 in phosphate buffer (**Fig. 1A and B**). The spectra of phycocyanin is characterized by absorption maxima at 360 and 618 nm. The addition of increasing concentrations of $ONOO^-$ (0-200 μM) to phycocyanin (10 μM) significantly decreased the absorption at 618 nm with no change in the absorption at 360 nm (**Fig. 1B**). Nearly 50 % of absorption at 618 nm was lost in the presence of 200 μM $ONOO^-$, although there was no shift in the absorption maxima. The effect of $ONOO^-$ (0-125 μM) on the UV-Visible spectra of PCB (10 μM) is shown in **Fig. 1A**. The spectra of PCB is characterized by absorption maxima at 610 and 365.5 nm. At lower concentrations of $ONOO^-$, the absorbance peak at 610 nm and 365.5 nm were decreased with a shift towards lower wavelength and the two peaks appeared at 563 nm and 329.5 nm, respectively (**Fig. 1A**). At higher concentration of $ONOO^-$ (125 μM), there was no further shift in the absorption maxima. However, the absorption at 563 and 329.5 nm were almost abolished and PCB (10 μM) was completely bleached. There was no significant change in UV-Visible spectra of PCB or phycocyanin when decomposed $ONOO^-$ was used (**Fig. 2A and 2B**).

The change in the spectral properties of chemically reduced phycocyanin is given in **Fig. 3**. During peroxynitrite titration, when chemically reduced phycocyanin was used instead of native phycocyanin, at lower concentrations of peroxynitrite the absorption at 618 nm rapidly increased and at higher concentrations of peroxynitrite the absorption was gradually decreased (**Fig. 3**). However, the absorption at 415 nm continuously decreased during peroxynitrite addition (**Fig. 3**). The absorption maxima at 415 nm and 618 nm corresponds to reduced and native phycocyanin, respectively.

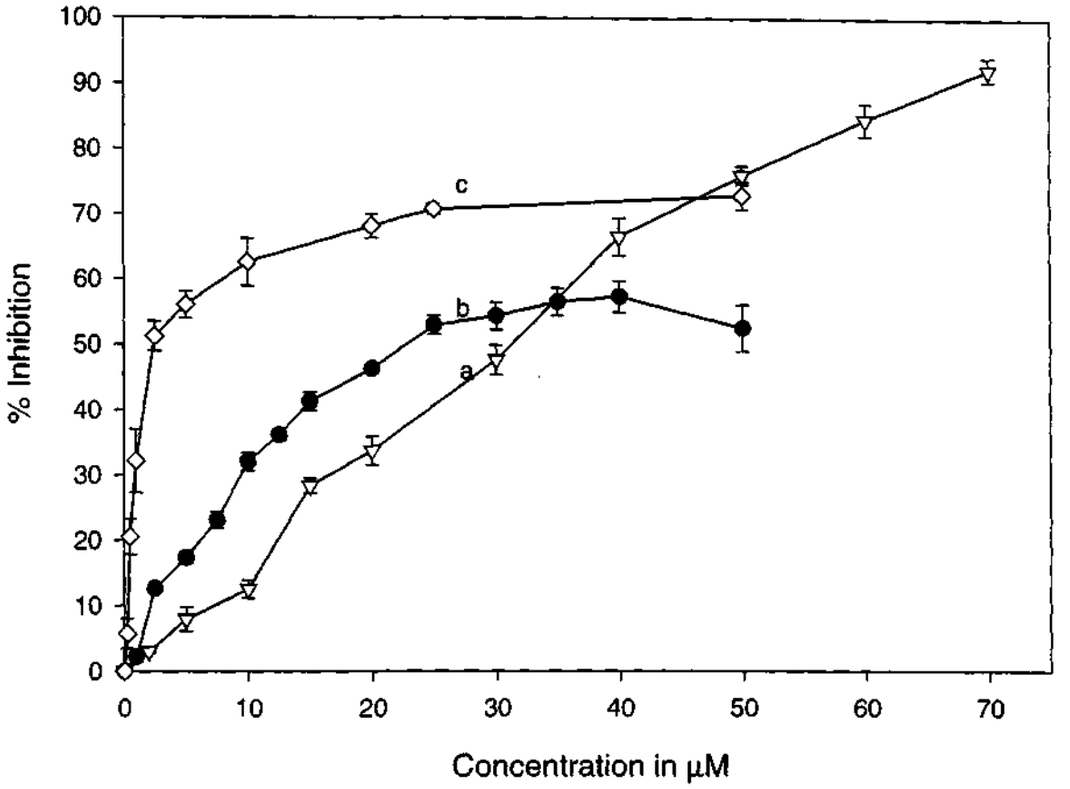

Fig. 5: Inhibitory effect of phycocyanobilin, phycocyanin and glutathione on peroxynitrite-mediated oxidation of pyrogallol red. $ONOO^-$ (25 µM), pyrogallol red (50 µM) and various concentrations of (a) phycocyanin (0-70 µM) or (b) PCB (0-50 µM) or (c) glutathione (0-50 µM) in potassium phosphate buffer (100 mM, pH 7.0 at 25 °C) were incubated for 5 min in 1 ml cuvette, and change in absorbance was recorded at 542 nm. Each value represents the mean ± S.D. of three independent experiments.

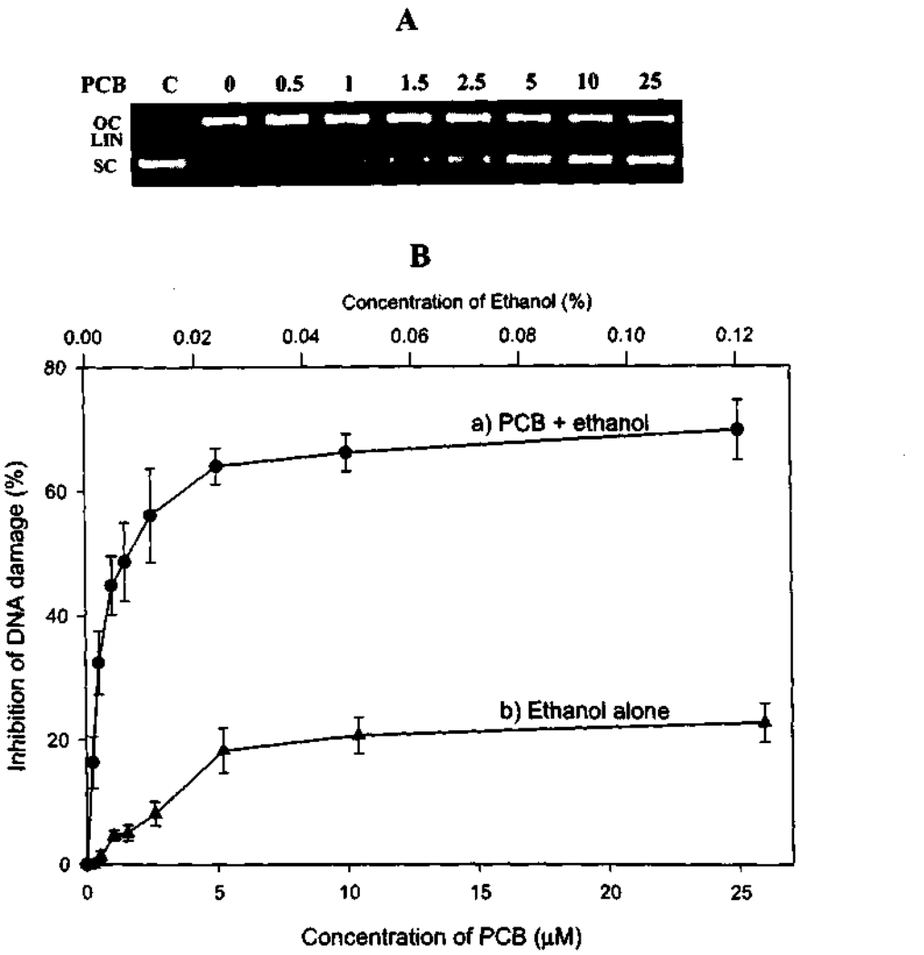

Fig. 6: Effect of phycocyanobilin on peroxynitrite-induced DNA damage.
6A: Inhibition of $ONOO^-$-induced strand breaks in DNA by phycocyanobilin (PCB) (lanes 3-9). Supercoiled (SC) pBR322 DNA (0.5 μg) was incubated in the presence of 50 μM $ONOO^-$ in 50 mM phosphate buffer, 10 mM NaCl, 0.1 mM DTPA, pH 7.0, and analyzed for single (OC, open circular) and double (LIN, linear) strand breaks by agarose gel electrophoresis. PCB was added to the reaction at the concentrations indicated above each lane (μM). Lane 1 represents untreated control. Lane 2 represents effect of $ONOO^-$ in the absence of PCB.

6B: Protection by different doses of PCB against the DNA damage from $ONOO^-$ (50 μM). Inhibition of DNA damage is represented by the percentage of SC DNA to the sum of OC and SC DNA. a) PCB in the presence of ethanol (ethanol concentration is similar to b); and b) ethanol alone.

Scavenger	IC_{50} (μM)	k_a/k_{PR}
Phycocyanobilin	30.5 ± 0.8	1.8
Phycocyanin	21.8 ± 2.6	3.9
Glutathione	4.8 ± 1.2	5.3

Table 1: Effect of phycocyanobilin (PCB), Phycocyanin, and glutathione on ONOO⁻-dependent oxidation of pyrogallol red (PR). The IC_{50} values and relative antioxidant activity ratios (k_a/k_{PR}) of phycocyanobilin (PCB), phycocyanin, and glutathione were calculated by using ONOO⁻-induced oxidation of pyrogallol red. The experimental details are as mentioned in Fig. 5. The IC_{50} values were calculated from curves fitted in Fig. 5. The relative antioxidant activity (k_a/k_{PR}) was calculated from data obtained by oxidation of PR in the presence and absence of scavengers (data not shown). Data are means ± S.D. of three independent experiments.

Scavenging of peroxynitrite by phycocyanin and PCB: Pyrogallol Red (PR) bleaching assay

Several methods can be used to test the peroxynitrite scavenging ability of an antioxidant, *viz*, nitration of tyrosine, fluorescence quenching of dyes like dehydrorodomine 123, etc. It has been reported recently that bleaching of dyes by $ONOO^-$ can be used efficiently for the evaluation of relative antioxidant activity (36). PR is one of the more efficient dyes, which can be used to evaluate the $ONOO^-$ scavenging activity of any compound in aqueous solution (36). PR readily interacts with $ONOO^-$ and undergoes rapid bleaching (**Fig. 4**) The ability of phycocyanin, PCB and glutathione (a known $ONOO^-$ scavenger) to scavenge $ONOO^-$ was quantified by using competitive kinetics of PR bleaching assay (36) and the results are summarized in **Fig. 5 and Table 1**. It was demonstrated that phycocyanin is an efficient scavenger of $ONOO^-$, and at 70 μM concentration, it inhibited PR bleaching to the extent of nearly 90 % (**Fig. 5**). However, both PCB and glutathione appeared to be more efficient scavenger of $ONOO^-$ at lower concentrations than phycocyanin (**Fig. 5**). Decomposed $ONOO^-$ did not bleach PR. It was also noticed that phycocyanin, PCB and glutathione inhibited bleaching of PR in a concentration dependent manner with an IC_{50} value of 21.8 ± 2.6 μM, 30.5 ± 0.8 μM and 4.8 ± 1.2 μM, respectively (**Table 1**). The relative antioxidant activity ratios (k_a/k_{PR}) for phycocyanin, PCB and glutathione calculated as reported earlier (36) are 3.9, 1.8, and 5.2, respectively (**Table 1**). Glutathione was used as a known $ONOO^-$ scavenger (36).

Inhibition of peroxynitrite-induced strand breaks in pBR322 plasmid DNA by PCB

Agarose gel electrophoresis was used to demonstrate the ability of PCB to protect DNA from $ONOO^-$-induced cleavage (**Fig. 6A**). It was noticed that exposure of DNA (0.5 μg) to $ONOO^-$ (50 μM) converts more than 95% of the native supercoiled (SC) form of DNA to an open circular (OC) form with single strand breaks as well as a lower yield of a linearized (LIN) form caused by double strand breaks (**lane 2, Fig. 6A**). In the presence of PCB (**Fig. 6A, lane 3-9**), the extent of $ONOO^-$-mediated DNA damage decreased in a dose-dependent manner (**Fig. 6B**) with an IC_{50} value of 2.9 ± 0.6 μM. At 10 μM concentration of PCB, nearly 50 % of the DNA was recovered in the supercoiled form after

exposure to $ONOO^-$ (50 μM). Since PCB was added as a solution in ethanol, the effect of ethanol on $ONOO^-$-induced DNA damage was also determined. It was noticed that ethanol marginally inhibited DNA damage caused by $ONOO^-$ (**Fig. 6B**). In fact, earlier it was determined that ethanol at a final concentration of 10 mM, was shown to poorly inhibits DNA damage caused by $ONOO^-$ (37). Incubation of DNA with PCB alone or decomposed $ONOO^-$ did not affect the supercoiled form of plasmid DNA (data not shown). However, we have not determined photodynamic or prooxidant activity of PCB. All our experiments were carried out in dark.

DISCUSSION

In the present study we have demonstrated for the first time that phycocyanin and phycocyanobilin (PCB), a naturally occurring linear tetrapyrrole, the chromophore of phycocyanin, efficiently scavenge peroxynitrite anion, a potent inorganic toxin (**Table 1, Fig. 5**). Although PCB appears to be more efficient $ONOO^-$ scavenger than phycocyanin at lower concentrations (**Fig. 5**), the relative antioxidant ratio as well as IC_{50} value (**Table 1**) clearly suggest that phycocyanin is more efficient than PCB. This could be due to the interaction of $ONOO^-$ with tyrosine and tryptophan residues of the apophycocyanin. In fact, nitration of aromatic amino acids by $ONOO^-$ has been reported earlier (35). Spectroscopic studies also indicate that both phycocyanin and PCB interact with $ONOO^-$ and undergo oxidation (**Fig. 1A and B**). Earlier studies have shown that the interaction of phycocyanin with peroxyl and hydroxyl radicals decreases the absorption at 618 nm with a shift in the absorption maxima towards lower wavelength (4). The decrease in the absorption is accompanied by disappearance of color (4). The radical assisted bleaching of chromophore in phycocyanin clearly indicates its involvement in the scavenging of reactive oxygen species. At lower concentrations of $ONOO^-$, the absorption maxima of PCB shifted towards lower wavelength (**Fig. 1A**) and the new absorption maxima at 563 and 329.5 nm matches well with the reported spectra of mesobiliviolin (38). At higher concentrations of $ONOO^-$, the peaks at 563 and 329.5 nm were almost abolished suggesting its oxidative fragmentation towards biliviolins (a three-ring system) which

further gets oxidized to a one-ring system (38). However, further studies are required to understand the mode of $ONOO^-$-mediated metabolism of phycocyanin and PCB.

Structurally PCB is very similar to bilirubin, a heme degradative product. Both are polar molecules and at physiological pH and ionic strength they are poorly water soluble. Bilirubin is considered to be a physiologically important antioxidant against reactive oxygen species (10,39). It inhibits oxidative modification of plasma proteins and aromatic amino acid residues (39). Scavenging of oxygen radicals by bilirubin has been shown to protect serum albumin as well as other biological targets (10,11). Interestingly, earlier studies have shown that phycocyanin is a potent peroxyl and hydroxyl radical scavenger and its chromophore PCB is directly involved in the antioxidant and radical scavenging properties of phycocyanin (4,9). The peroxyl radical has been shown to transform the chromophore of reduced phycocyanin (chemically modified) to phycocyanobilin (4). This observation appears to be similar to the peroxyl radical-mediated transformation of bilirubin to biliverdin (10). Addition of $ONOO^-$ to bilirubin results in a rapid destruction of pigment as indicated by the decrease in the absorption at 435 nm (12) and it has been suggested that mono- and bipyrrols are formed as degradation products (40). It is also suggested that $ONOO^-$ abstracts two hydrogen atoms from bilirubin and gets oxidized to biliverdin (12). However, biliverdin is not the major oxidative product during $ONOO^-$-mediated oxidation of bilirubin (12). When reduced phycocyanin was used instead of native phycocyanin, there was a rapid increase in the absorption at 618 nm at lower concentrations of $ONOO^-$ and at higher concentrations the absorption gradually decreases (**Fig. 3**). However, when native phycocyanin was used, the absorption at 618 nm linearly decreased during the addition of $ONOO^-$.This suggests that initially $ONOO^-$ abstracts proton from phycocyanorubin to yield phycocyanobilin which further gets oxidized to mono- and bipyrrols. The present study suggests that PCB undergoes oxidative degradation when treated with $ONOO^-$.

In view of the fact that $ONOO^-$ and products derived from it have the potential to inactivate important cellular targets (19-27), it is of great importance to look for compounds that can scavenge this potential physiological oxidant. Several natural plant constituents such as flavonoids (41) and polyphenols (42,43) protect against $ONOO^-$-

mediated oxidations. Polyphenols, the natural constituents of tea and chocolate, possess significant antioxidant activity and also protect the biological systems against $ONOO^-$-mediated oxidations (43,44). In fact, dietary polyphenols have been shown to inhibit DNA damage and strand breakage caused by $ONOO^-$ (42,43). Oxidative DNA damage induced by $ONOO^-$ is one of the major reasons for cancer during chronic inflammation (45). In the present study it was observed that PCB significantly inhibits the $ONOO^-$-mediated DNA damage (**Fig 6A and B**). The DNA nicking activity of peroxynitrite is most probably mediated by an activated form of peroxynitrous acid, or a peroxynitrite/CO_2 adduct, which abstracts hydrogen atoms from deoxyribose groups in DNA (23). Any peroxynitrite scavenger has the ability to inhibit the strand breakage in supercoiled plasmid DNA by scavenging either the intermediates or the peroxynitrite. Several anticarcinogenic agents are known to inhibit oxidative DNA damage and tumor promotion (46) and so it is quite possible that phycocyanin may act as an anticarcinogenic agent.

Earlier we have shown that C-phycocyanin effectively inhibits the chemical-induced liver injury in rats (3,4). It is well known that chemicals which are hepatotoxic get metabolized to reactive intermediates and free radicals (18). These reactive intermediates and free radicals are mainly responsible for the observed toxic effect. Recently it has been noticed that the level of nitric oxide and $ONOO^-$ increases during chemical induced liver injury (47). These reactive nitrogen species are responsible for inflammatory and generalized liver toxicity during liver injury (47). Our results clearly suggest that phycocyanin provides protection against chronic liver injury induced by chemicals and appears to inhibit the initiation of cancer during chronic liver injury.

Earlier studies have clearly demonstrated that phycocyanin has anti-inflammatory and hepatoprotective properties (1,3,6). In fact, in chronic inflammation one of the events taking place is the oxidative damage to DNA and reactive oxygen species are involved in this disorder. It has been established that phycocyanin not only scavenges peroxyl, hydroxyl (4) and superoxide radicals (1) but also acts as a potent antioxidant and inhibits the lipid peroxidation mediated by reactive oxygen species (1-6). In the present study we have shown that both phycocyanin and PCB efficiently scavenge $ONOO^-$ (**Table 1 and Fig. 5**) and PCB inhibits $ONOO^-$-mediated DNA damage (**Fig. 6A and B**). Moreover,

phycocyanin is a very good scavenger of superoxide radical, which is involved in the generation of $ONOO^-$. So it appears that the anti-inflammatory property of phycocyanin is attributable to its ability to scavenge endogenous $ONOO^-$ as well as other reactive oxygen species responsible for promoting oxidative stress, one of the pathological conditions for diseases related to inflammation.

It is well known that, during inflammatory response, macrophages and neutrophils release both nitric oxide (NO) and superoxide ($O_2^{\cdot}$-). These two reactive species rapidly react to yield $ONOO^-$ *in vivo* (16). Neutrophils also generate hypochlorous acid. The inorganic physiological toxin $ONOO^-$ has been implicated in several inflammatory disorders such as rheumatoid arthritis, myocardial dysfunction and atherosclerosis (18,45) and oxidative damage to DNA is one of the events taking place during chronic inflammation (45). The ill effect of $ONOO^-$ can be prevented either by inhibiting formation of or by scavenging the nitric oxide or superoxide or both (48). In fact inhibitors of xanthine oxidase or NADPH oxidase decrease $O_2^{\cdot}$- production. Even superoxide dismutase and its mimics (e.g. metalloporphyrins) can decrease $O_2^{\cdot}$- levels there by formation of $ONOO^-$ is decreased (49). However, it is practically impossible to completely inhibit the generation of NO or completely scavenge $O_2^{\cdot}$-. Since low level of NO is required for important physiological processes, the deleterious effects of $ONOO^-$ can be prevented or reduced by suitable antioxidants with the ability to scavenge $O_2^{\cdot}$-, $ONOO^-$ and various reactive oxygen species. In fact, oxidative stress is considered as one of the pathologic factors of inflammation and reactive oxygen species are also involved in this disorder. Our studies have clearly established that phycocyanin is a good scavenger of both superoxide and peroxynitrite. Earlier studies have demonstrated that phycocyanin interacts with cytotoxic as well as powerful oxidant, hypochlorous acid (5), generated during the immune response. The chromophore of phycocyanin, phycocyanobilin (PCB), also has the ability to scavenge both superoxide and peroxynitrite. Our studies have also demonstrated that phycocyanin possesses significant antioxidant and radical scavenging properties. It is quite possible that the anti-inflammatory property of phycocyanin could be not only due to its ability to scavenge oxygen free radicals such as peroxyl or hydroxyl but also its ability

to inhibit enzymes involved in the formation of inflammatory prostaglandins. Our results also suggest that the antioxidant activity of phycocyanin is attributable to its chromophore, phycocyanobilin (PCB). The results presented in this chapter as well as in other chapters clearly indicate that both phycocyanin and PCB are powerful radical scavengers and have the potential to inactivate oxidants *in vivo* during inflammation and oxidative stress. Thus, PCB and phycocyanin could be used as potential therapeutic agents in many diseases, to reduce oxidative damage caused by $ONOO^-$ and other radicals. A functional, broad spectrum antioxidant should be able to scavenge a variety of toxic free radical species and phycocyanin certainly belongs to this class of compounds. Above all, phycocyanin is a natural compound, least toxic and hence may find utility as a therapeutic agent. Although further study is required to fully understand the characteristics of PCB protection against $ONOO^-$-induced DNA damage, the present study provides the first evidence that PCB protects from $ONOO^-$-induced DNA damage and has the ability to scavenge $ONOO^-$. More research has to be carried out to find out the uptake and metabolism of PCB and phycocyanin in living cells.

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CHAPTER 2: PART F

Selective Inhibition of Cyclooxygenase-2 by C-Phycocyanin, a Biliprotein from *Spirulina platensis*

INTRODUCTION

Cyclooxygenase (COX, Prostaglandin H Synthase) is a bifunctional enzyme catalyzing the first two steps in the biosynthesis of prostaglandins (PGs) from the substrate arachidonic acid (1). This bifunctional enzyme having two activities, the cyclooxygenase activity in which arachidonic acid is converted to prostaglandin G_2 (PGG_2) and the peroxidase activity in which PGG_2 undergoes a bi-electron reduction to prostaglandin H_2 (PGH_2) (1). PGH_2 is then tissue specifically metabolized to a variety of biologically active molecules like prostaglandins, thromboxanes and prostacyclin, collectively termed as prostanoids. Studies carried out so far suggest that there is a link between cancer and prostaglandins and it has been observed that tumor tissues contain higher levels of PGs (2). It is now known that at least two forms of cyclooxygenase enzyme exist (3). One of these forms, cyclooxygenase-1 (COX-1) is considered a constitutive form and is responsible for maintaining normal physiological function and the PGs produced by this enzyme play a protective role. The other known form of the enzyme, cyclooxygenase-2 (COX-2) is an inducible form and its expression is affected by various stimuli such as mitogens, oncogenes, tumor promoters and growth factors (4). COX-2 is the principal isoform that participates in inflammation whose induction is responsible for the production of PGs at the site of inflammation (4). It was shown that COX-2 and not COX-1 activity increases in malignant tissues from colorectal cancer as well as human gastric and breast tumors (5).

In 1971, John R. Vane proposed that the mechanism of action of the aspirin-like drugs (non-steroidal anti-inflammatory drugs; NSAIDs) is through the inhibition of prostaglandin biosynthesis (6). It is well known that non-steroidal anti-inflammatory drugs (NSAIDs) are effective against inflammation and are shown to inhibit PG biosynthesis which are the inflammatory mediators (6). However, the deleterious side effects of NSAIDs are that, while they inhibit PG biosynthesis at inflammatory sites, they are also known to inhibit constitutive biosynthesis of PGs through the mediation of COX-1 (5,7). Recently the isoform of COX (COX-2) was discovered (3,8). COX-2 is inducible and is short lived (8,9). Inhibition of COX-1 by NSAIDs would result in ulcer and bleeding in gastrointestinal tract and also kidney failure. So the adverse effects of currently available NSAIDs have limited their clinical usefulness. COX-2 is involved in the mediation of

inflammation, arthritis and certain types of cancers, especially in colon carcinogenesis (10). Hence selective inhibition of COX-2 would prevent the formation of inflammatory PGs without affecting the COX-1 activity and in fact, there is a concerted effort by various investigators to develop such inhibitors for therapeutic uses.

One of the processes involved in carbon tetrachloride (CCl_4)-induced hepatotoxicity is the free radical-catalyzed lipid peroxidation or oxidative injury (11). It is known that lipid peroxidation mediated by free radicals activate cyclooxygenases resulting in the formation of PGs from arachidonic acid (12). Non-enzymatic free radical catalyzed and enzymatic cyclooxygenase mediated oxidation of arachidonic acid have been shown to be involved during CCl_4-induced hepatotoxicity (12). Earlier studies have indicated that oxidative injury is the primary and cyclooxygenase mediated inflammatory response is the secondary effect of CCl_4-induced hepatotoxicity (12). Recent studies have also demonstrated that one of the end products of lipid peroxidation *viz.* 4-hydroxy-2-nonenal, a breakdown product of hydroperoxy, fatty acid, is a specific inducer of *COX-2* expression (13). It is well known that, CCl_4 induces the lipid peroxidation. This suggests that during CCl_4-induced hepatotoxicity, the level of COX-2 gets elevated and COX-2 may be involved in the generalized liver injury.

C-Phycocyanin is one of the major biliproteins of *Spirulina platensis*, a blue-green algae. This water soluble protein pigment has significant antioxidant and radical scavenging properties (14-16). Earlier we have demonstrated that phycocyanin significantly reduces R-(+)-pulegone (a potent hepatotoxin) and CCl_4-induced hepatotoxicity in rats (17). It effectively inhibited CCl_4-induced lipid peroxidation in rat liver *in vivo* (16). Recently it was demonstrated that, oral administration of phycocyanin exerted anti-inflammatory effects on arthritis induced by zymosan in mice (18). It was suggested that the anti-inflammatory activity of phycocyanin could be due to its ability to inhibit arachidonic acid metabolism and to scavenge oxygen free radicals (18,19). In fact, oxygen free radicals are believed to be involved in rheumatoid arthritis (20) and inhibitors of arachidonic acid metabolism are commonly used in the treatment of arthritis (21). However, the mechanism of action of phycocyanin is not clearly understood. The present study reports for the first time that phycocyanin is a selective COX-2 inhibitor with very

low COX-2/COX-1 ratio and its potency is comparable to those of rofecoxib and celecoxib, the known selective COX-2 inhibitors.

MATERIALS AND METHODS

Chemicals

Arachidonic acid, N, N, N', N'-tetramethyl-p-phenylenediamine (TMPD), Tris, Diethyldithiocarbomate (DDC) and Lipopolysaccharide (LPS) were purchased from Sigma Chemicals Co. (St. Louis, MO). Indomethacin was supplied by Cayman Chemicals Co. (Ann Arbor, MI). PGE_2 EIA kit was from Assay Designs, Inc (Ann Arbor, MI). Celecoxib and rofecoxib were generous gifts from Unichem Laboratories, Mumbai, India. Recombinant human *COX-2* was a generous gift from Shozo Yamamoto, School of Medicine, The University of Tokushima, Japan. All other materials were procured from standard suppliers and were of analytical grade.

C-Phycocyanin was isolated from *Spirulina platensis* and purified as reported earlier (22) with few modifications (Chapter 2: Part A). The fractions showing absorbance ratio of 618 nm/280 nm greater than 4 were pooled and used in the present study. The purity and molecular mass of the purified phycocyanin were determined as reported earlier (16). The chromophore (phycocyanobilin), covalently attached to phycocyanin, was reduced using solid $NaBH_4$ (16,23). The phycocyanobilin (PCB) was cleaved from pure freeze-dried phycocyanin by alcoholysis (24). The PCB obtained was re-dissolved in chloroform containing 5% methanol, washed several times with water to remove impurities and evaporated under vacuum to dryness. The purity of phycocyanobilin was examined by HPLC analysis, using ODS reverse phase column with acetone : water (2.5 : 1.5 v/v) containing 2 mM H_3PO_4 and 0.5 mM NaH_2PO_4 as solvent system (1.5 ml/min) and the column eluate was monitored at 365 nm. The UV-Visible spectra of PCB was recorded between 280-800 nm in methanol / 2% HCl and the concentration was estimated at 374 nm using the extinction coefficient of 47,900 $M^{-1}cm^{-1}$ (25).

Preparation of COX-1 and COX-2

Microsomal fraction of ram seminal vesicles containing cyclooxygenase-1 (COX-1) activity was prepared and the enzyme was purified as described earlier (26,27). Briefly, the ram seminal vesicles were minced into small pieces and homogenized in the Tris-HCl

buffer (100 mM, pH 8.0) containing EDTA (5 mM), and Diethyldithiocarbomate (DDC, 5 mM). The homogenate was subjected to differential centrifugation to obtain microsomal pellet. The microsomal pellet (105,000 × g pellet) was suspended in Tris-HCl buffer (25 mM, pH 8.0) containing EDTA (1 mM) and Triton X-100 (1 %) and then centrifuged at 105,000 × g for 1 h at 4° C. The supernatant was subjected to DEAE-cellulose column chromatography and the fractions containing COX-1 activity were pooled and used as the source of enzyme.

Recombinant human cyclooxygenase-2 (COX-2) was expressed in *Spodoptera frugiperda* (Sf9) cells infected with bacculovirus containing human *COX-2*. After 72 hrs of infection, the cells were collected by centrifugation at 5000 rpm for 5 min. The pellet was suspended in Tris-HCl buffer (pH 7.2, 50 mM) containing EDTA (5 mM), sucrose (300 mM), DDC (5 mM), pepstatin (1 µg/ml) and phenol (1 mM) and sonicated for 3 min. The cell lysate was subjected to centrifugation (100,000 × g for 1 h) at 4° C and the microsomal pellet obtained was suspended in Tris-HCl buffer (pH 7.2, 25 mM) containing glycerol (0.5%), Tween-20 (0.8%) and phenol (1 mM). Protein concentration was determined by the method of Bradford (28).

Spectrophotometric assay for COX-1 and COX-2

Enzymatic activity of COX-1 and COX-2 were measured as described earlier with little modification (29) using a chromogenic assay based on the oxidation of N, N, N', N'-tetramethyl-p-phenylenediamine (TMPD) during the reduction of PGG_2 to PGH_2. The assay mixture contained Tris-HCl buffer (pH 8.0, 100 mM), hematin (15 µM), EDTA (3 µM), enzyme (COX-1 OR COX-2, 100 µg) and test compound, phycocyanin (for COX-1, 1-100 µM in 12 µl of buffer, for COX-2, 0.03-30 µM in 12 µl of buffer). The mixture was preincubated at 25° C for 15 min. and then the reaction was initiated by the addition of arachidonic acid (100 µM in 5 µl of ethanol) and TMPD (120 µM in 3 in µl of ethanol) in a total volume of 1.0 ml. The enzyme activity was measured by estimating the initial velocity of TMPD oxidation for the first 25 sec of the reaction following the increase in absorbance at 603 nm. A low rate of non-enzymatic oxidation observed in the absence of COX-1 and COX-2 was subtracted from the experimental value while calculating the

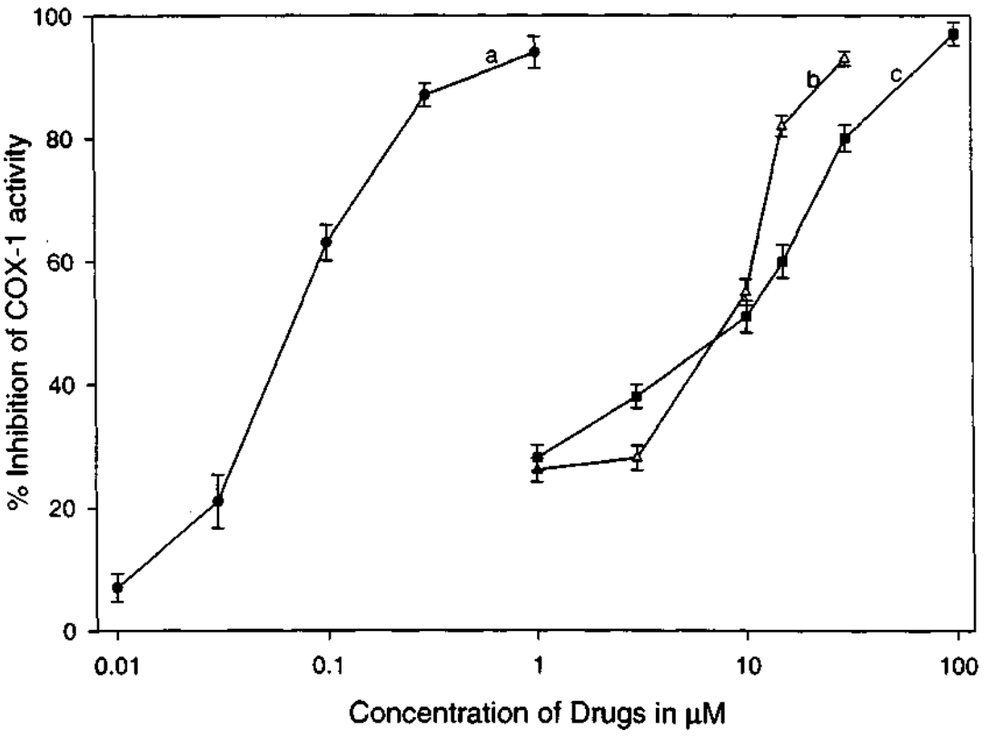

Fig. 1: The inhibitory effect of (a) indomethacin (0.01-1.0 µM), (b) phycocyanin (1.0-30.0 µM), and (c) celecoxib (1.0-100 µM) on ram seminal vesicle COX-1 activity. The results are expressed as % inhibition of COX-1 activity by inhibitors. The experimental details are as described under Materials and Methods.

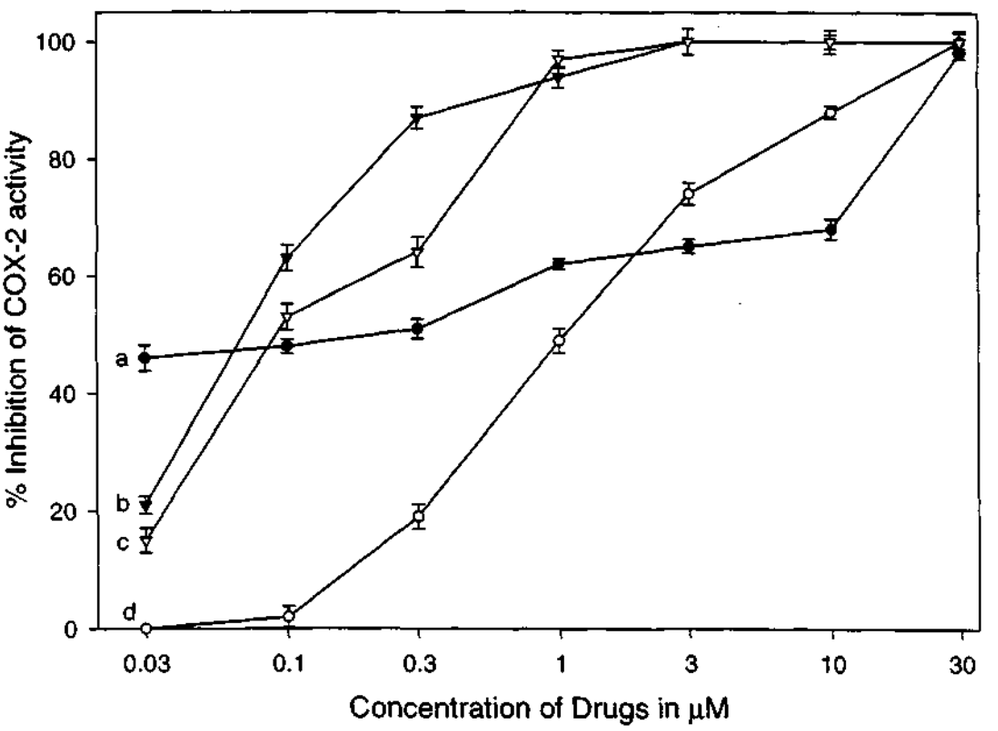

Fig. 2: The inhibitory effect of (a) phycocyanin, b) rofecoxib, c) celecoxib, and d) indomethacin (0.03-30.0 µM) on human recombinant COX-2 activity. The results are expressed as % inhibition of COX-2 activity by inhibitors. The experimental details are as described under Materials and Methods.

Drugs	IC_{50} (μM)		COX-2/COX-1
	COX-1	COX-2	
Phycocyanin	4.5	0.18	0.04
Reduced Phycocyanin	5.6	9.7	1.73
Phycocyanobilin	9.9	39.0	3.93
Celecoxib	16.3	0.26	0.015
Rofecoxib	> 300*	0.4	< 0.0013
Indomethacin	0.22	1.74	7.9

TABLE 1: The IC_{50} Values of phycocyanin, reduced phycocyanin, phycocyanobilin, celecoxib, rofecoxib, and indomethacin in ram seminal vesicle COX-1 and human recombinant COX-2 activity. *Maximum dissolved concentration.

Fig. 3: Preincubation time dependent inhibition of ram seminal vesicle COX-1activity and human recombinant COX-2 activity by 1 μM C-phycocyanin

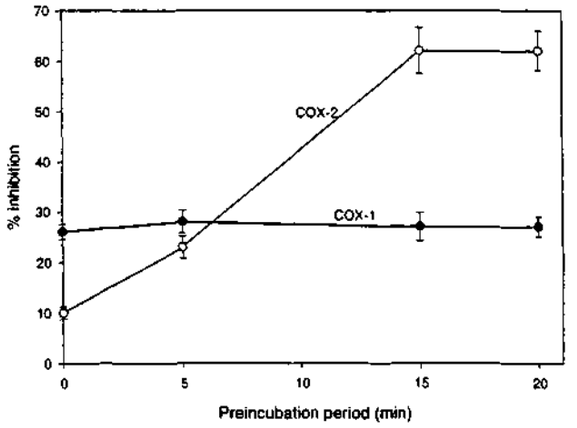

Fig. 3: Preincubation time dependent inhibition of ram seminal vesicle COX-1activity and human recombinant COX-2 activity by 1 μM C-phycocyanin

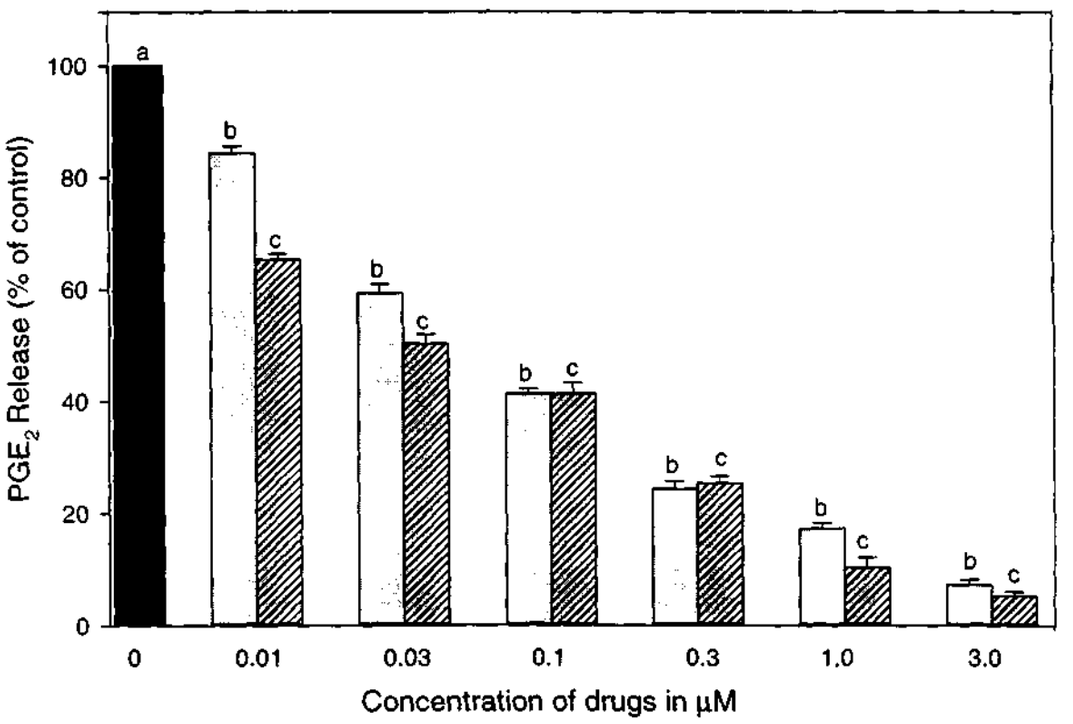

Fig. 4: Effect of celecoxib and phycocyanin on LPS-stimulated PGE_2 synthesis in human whole blood assay. PGE_2 levels determined without drug taken as the maximal PGE_2 synthesis (i.e., 100%) was used as control. a) Control, b) celecoxib, and c) phycocyanin. The results are expressed as % of PGE_2 release to plasma (% of control). The experimental details are as described under Materials and Methods.

percent inhibition. The effect of different concentrations of indomethacin, celecoxib and rofecoxib (known inhibitors of COX-1 and COX-2) were examined under the same experimental conditions.

Human Whole Blood Assay for COX-2

The assay was carried out as previously described (30). Briefly, freshly heparinized human whole blood was incubated with lipopolysaccharide (LPS) from *E. coli* (100 μg/ml) and indicated concentrations of test compound [0.01-3.0 μM, phycocyanin in buffer (5 μl); celecoxib in DMSO (5 μl)] for 24 h at 37° C. The level of PGE_2 formed was measured using EIA kit as per the protocol given by the company (Assay Designs, Inc., USA)

RESULTS

Inhibition of COX-1 and COX-2 by phycocyanin was analyzed in both cell-free and whole blood assay systems. The partially purified enzyme from ram seminal vesicles served as the source of COX-1, while the human recombinant enzyme formed the source of COX-2. The inhibition of COX-1 by phycocyanin, celecoxib and indomethacin is shown in **Fig. 1**. It was demonstrated that phycocyanin and celecoxib at a concentration of 100 μM inhibited COX-1 activity almost completely (~95%), where as much lower concentration (~1 μM) of indomethacin was sufficient to exhibit the same effect (**Fig. 1**). Indomethacin is the most potent inhibitor of COX-1 with IC_{50} value of 0.216 μM followed by phycocyanin (IC_{50}, 4.47 μM) and celecoxib (IC_{50}, 16.3 μM). The inhibition of COX-1 by phycocyanin was dose dependent, but independent of the period of preincubation with the enzyme. Phycocyanin at a concentration of 1 μM inhibited COX-1 activity by 26% with or without preincubation of the enzyme with the inhibitor.

The present studies revealed that phycocyanin is a potent inhibitor of human recombinant COX-2 with an IC_{50} value of 180 nM (**Table 1**) which is much lower than the values obtained for celecoxib (255 nM) and rofecoxib (401 nM), the known selective inhibitors of COX-2. The extent of inhibition of COX-2 by phycocyanin was dependent on the preincubation period of the enzyme with inhibitor before the initiation of the reaction with arachidonic acid (**Fig. 3**). Inhibition of COX-2 by phycocyanin dependents on the

preincubation period of enzyme with phycocyanin. When 1 mM of phycocyanin was preincubated with COX-2 for 0, 5, 15, and 20 min, the inhibition was 10%, 23%, 62%, and 62%, respectively (**Fig. 3**). However, COX-1 inhibition by phycocyanin is not depending on perincubation period (**Fig. 3**). It was noticed that at 0.03 μM concentration of the inhibitors, highest percentage of inhibition of COX-2 was recorded for phycocyanin (48%) as compared to rofecoxib (21%) and celecoxib (15%) (**Fig. 2**). Based on the IC_{50} values for COX-1 and COX-2, the relative ratios of IC_{50} COX-2/IC_{50} COX-1 were calculated and the data is presented in Table1. The COX-2/COX-1 ratio obtained for phycocyanin (0.04) is comparable to the values obtained for well-known selective COX-2 inhibitors such as celecoxib (0.015) and rofecoxib (0.0013) (**Table 1**). However, these values are significantly different from that obtained for indomethacin (7.9), the selective COX-1 inhibitor.

COX-2 activity was also determined in the human whole blood stimulated by LPS and measuring the release of prostaglandin E_2 (PGE_2). Phycocyanin at a concentration of 1 μM inhibited the release of PGE_2 to a significant extent (nearly 80% inhibition) with an IC_{50} value of 80 nM. The results (**Fig. 4**) also indicate that phycocyanin inhibits PGE_2 synthesis in a dose-dependent manner. The IC_{50} value determined for celecoxib in the whole blood assay was found to be 28 nM.

To understand the role of chromophore in the inhibition of COX-1 and COX-2 by phycocyanin, we studied these activities in the presence of reduced phycocyanin as well as free phycocyanobilin (PCB), the chromophore of phycocyanin. It was observed that both these compounds significantly lost the selectivity towards COX-2 inhibition (**Table 1**). However, there was not much change in the inhibition of COX-1 by these two compounds and as a result, the relative ratios of IC_{50} COX-2/IC_{50} COX-1 for reduced phycocyanin and phycocyanobilin increased by several folds as compared to native phycocyanin.

DISCUSSION:

Cyclooxygenase, a key enzyme involved in the biosynthesis of prostaglandin (1) plays an important role in inflammation and a variety of other disorders (2,5,12). With the discovery of inducible form of cyclooxygenase, COX-2 (3), it has been postulated that PGs

that contribute to inflammatory process are derived exclusively from COX-2, while many of the "house keeping" effects of COX appear to be mediated by COX-1. Selective inhibitors of COX-2 would exhibit anti-inflammatory and analgesic effects without the deleterious side effects of NSAIDs. Hence lot of interest is being shown to develop drugs that could selectively inhibit COX-2 without affecting COX-1 activity. These efforts have led to the introduction of selective COX-2 inhibitors such as celecoxib by Searle and rofecoxib by Merck, as new class of NSAIDs into the market.

In the present study it has been shown that phycocyanin is a selective inhibitor of COX-2. It has more potent (IC_{50} 180 nM) inhibitory effect on COX-2 than celecoxib (IC_{50} 255 nM) and rofecoxib (IC_{50} 401 nM). The human whole blood COX-2 assay provides an additional and a more relevant measure of COX-2 inhibition selectivity under a pathophysiological environment rich in plasma protein and cells (30,31). In the present study it was observed that, phycocyanin very efficiently inhibited COX-2 activity with an IC_{50} value of 80 nM in the human whole blood assay, wherein, the freshly heparinized human whole blood was incubated with LPS and the PGE_2 formed is measured. In fact, the IC_{50} value obtained in the whole blood assay (80 nM) is much lower than the value obtained with the partially purified enzyme (180 nM). Inhibition of COX-2 activity is a favorable condition for treating inflammation, arthritis and preventing cancer (32). Earlier studies have demonstrated the anti-inflammatory property of phycocyanin (18) and this property can be explained, in part, by the specific inhibition of COX-2. Recently, Romay *et al.*, suggested that phycocyanin inhibits the arachidonic acid metabolism in mouse ear inflammation test (33,34).

The commercially available NSAIDs belong to a diverse array of chemical structural classes such as salicylates, acetic and propionic acid derivatives, fenamates, pyrazoles and oxicams. COX inhibitors have been categorized into time dependent and time-independent inhibitors, according to their kinetic behavior towards the enzymes (35). Some of these drugs (ibuprofen, mefenamate) are competitive inhibitors of both COX-1 and COX-2, whereas flurbiprofen and indomethacin inhibit the two isoforms in a slow time-dependent process. Another mode of inhibition is exhibited by diarylheterocycles such as Celecoxib and Dup-697. These are weak, competitive inhibitors of COX-1 but they

inhibit COX-2 selectively in a slow, time-dependent manner. Phycocyanin definitely belongs to this class. The mechanism of inhibition of COX activity by phycocyanin appears to be similar to those reported for COX-2 selective inhibitors, occurring via a time dependent mechanism leading to a possible formation of a tightly bound inhibitor complex (29,36). It is well known that, the selective inhibitors of COX bind to the enzyme active site and subsequently cause slow irreversible event that leads to significant inactivation of the enzyme (29,36). Phycocyanin inhibits COX-2 in a time dependent mechanism but not COX-1. These results suggest binding of phycocyanin to COX-2 which evokes a conformational change resulting in a tighter or possibly an irreversible binding of the phycocyanin to the enzyme, COX-2. In fact, Kulmacz and Lands (37) have shown that indomethacin, a time-dependent inactivator of COX-1, induces a conformational change in the enzyme that leads to essentially irreversible binding without any chemical modification of the enzyme or inhibitor. It is well known that a selective COX inhibitor inhibits enzyme activity by a time-dependent, irreversible mechanism, whereas, non-selective inhibitors carry out the inhibition by a rapid, competitive, and reversible mechanism (38,39).

Additionally, phycocyanin has been shown earlier to possess radical scavenging and antioxidant properties (14-16) which could also contribute substantially towards its anti-inflammatory and anti-arthritic effect. It is increasingly recognized that reactive oxygen species are involved in rheumatoid arthritis (20,40). Recently we have reported that phycocyanin and its chromophore PCB are efficient scavengers of peroxynitrite anion, a potent oxidizing agent (41). An attractive feature of the involvement of peroxynitrite as an activator of COX-2 in inflammatory cells is the fact that both the inducible form of nitric oxide synthase and COX-2 are immediate-early genes that are induced by many of the same agonists and with very similar time courses (42,43). It is quite possible that scavenging of peroxynitrite anion and inhibition of COX-2 by phycocyanin may act synergistically during inflammation in inflammatory cells.

Chronic use of nonsteroidal anti-inflammatory drugs (NSAIDs), in arthritis or other inflammatory diseases, develop ulcers or other serious gastrointestinal complications. These toxicities result from the inhibition of prostaglandin synthesis by COX-1, the predominant cyclooxygenase isozyme in the stomach lining (epithelium). When compared

to the toxicities associated with the currently available anti-inflammatory drugs (NSAIDs) and their activity as COX-2 inhibitors, phycocyanin will likely provide safer therapeutic alternatives since it is as efficacious as currently used drugs (NSAIDs), if not more. In addition, this water-soluble biliprotein is from a natural source and is least toxic. Recently it has been shown that resveratrol, a phytoalexin found in grapes and other foods inhibit COX-2 activity and suppress the activation of *COX-2* gene expression (44). It is also known that compounds such as radicicol, genistein, curcumin and retinoids inhibit *COX-2* gene expression (45).

Earlier we have demonstrated that phycocyanin significantly reduces R-(+)-pulegone and CCl_4-induced hepatotoxicity in rats (17). One of the processes involved in CCl_4-induced hepatotoxicity is the free radical-catalyzed lipid peroxidation (11), which is accompanied by activation of cyclooxygenase and increased synthesis of PGs (12). Recently it has been shown that one of the major end products of lipid peroxidation, 4-hydroxy-2-nonenal, is a highly specific inducer of *COX-2* gene expression (13). It is a well known fact that free radicals and reactive oxygen species are readily produced during various pathological conditions including chemical induced liver injury (46). These free radicals and reactive oxygen species oxidize membrane lipids and the end products of lipid peroxidation, mainly aldehydes, are involved in most of the pathophysiological effects associated with oxidative stress in cells and tissues (46). The hepatoprotective effect of phycocyanin could be due to its ability to efficiently scavenge free radicals (16) and inhibit lipid peroxidation as well as COX-2 activity.

Selective inhibition of COX-2 isozyme, while preserving the functional activity of COX-1 necessary for normal production of gastroprotective and other prostaglandins, would be ideal. Until recently, none of the NSAIDs were COX-2 specific, although some more recent drugs can be classified as equipotent inhibitors. At doses of these drugs that inhibit COX-2, the majority of NSAIDs also inhibit COX-1, in most cases quite effectively (47). To provide a quantitative figure which indicates the relative potency of NSAIDs against COX-1 and COX-2, many authors have expressed the ratio of the concentration required to produce 50% inhibition of COX activity (IC_{50}) such that a low COX-2 IC_{50}/COX-1 IC_{50} ratio indicates a more potent inhibition of COX-2 than of COX-

1, and a high COX-2 IC_{50}/COX-1 IC_{50} ratio indicates a more potent inhibition of COX-1. IC_{50} COX-2/IC_{50} COX-1 ratios provide a useful comparison of relative values for a series of NSAIDs tested in the same system. However, this ratio for a particular NSAID will vary according to whether it is measured using intact cells, cell homogenates, purified enzymes or recombinant proteins expressed in bacterial, insect or animal cells. It will also vary when measured in different types of cells derived from various species (48,49). Studies indicate that a high degree of *in vitro* biochemical selectivity for COX-2 will be required in order to achieve effective functional selectivity *in vivo*. The ratio demonstrates the relative selectivity of NSAIDs towards the two COX isoforms and low ratios indicate a preferential inhibition of COX-2. In the present study, COX-2/COX-1 ratio of the IC_{50} values calculated for phycocyanin *in vitro* with the partially purified enzymes is very low and comparable to those calculated for celecoxib and rofecoxib.

It is known that the active site of COX-2 is larger than that of COX-1 so that it can accommodate bigger structures (50). Phycocyanin is significantly much bigger in size (~37.5 kDa) than NSAIDs. Its three dimensional structure would probably facilitate its proper binding with the active site of COX-2. In fact, reduced phycocyanin, where the C-10 methine bridge in the chromophore is reduced (25), is a significantly less active COX-2 inhibitor that has also lost COX-2 selectivity (**Table 1**). It is quite possible that the reduced phycocyanin with an altered native conformation may not favor proper binding to the active site. This appears to be true with phycocyanobilin, the chromophore of phycocyanin, which also has significantly lost COX-2 selectivity (**Table 1**) suggesting that the apoprotein plays an important role in the inhibition of COX-2. It is also quite possible that the chromophore is an active inhibitor, but its conformation is important for selective inhibition. The apoprotein may keep the chromophore in the proper conformation, which is required for the selective inhibition.

Preliminary studies indicate that phycocyanin induced apoptosis in RAW 264.7 macrophages in which COX-2 activity was stimulated by LPS. Some of the selective COX-2 inhibitors exhibit this property (51). However, more experiments have to be carried out to substantiate these findings. Hence these aspects are not discussed in this chapter. It

has also been observed that phycocyanin failed to inhibit human 5-lipoxygenase and rabbit reticulocyte 15-lipoxygenase (unpublished data).

In summary, we have demonstrated that phycocyanin is a selective inhibitor of COX-2 activity with potency comparable to celecoxib and rofecoxib, the known selective COX-2 inhibitors. Reduced phycocyanin and phycocyanobilin are poor inhibitors of COX-2 activity whose COX-2 specificity is also abolished.

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CHAPTER 3: PART A

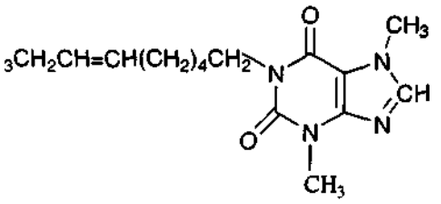
Preparation of Novel Analogues of 1,3,7-Trimethyluric Acid by using Mixed Culture consortium

Isolation, Purification and Partial Characterization of Caffeine Oxidase

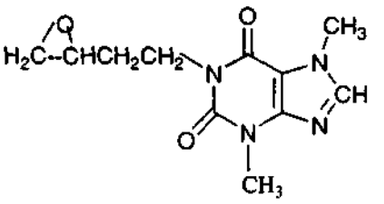
INTRODUCTION

Oxygen is a highly reactive atom that is capable of becoming part of the potentially damaging molecules commonly called "free radicals". Free radicals are capable of attacking the healthy cells of the body, causing them to lose their structure and function. Cell damage caused by free radicals appears to be a major contributor to aging and degenerative diseases such as cancer, cardiovascular disease, cataracts, immune system decline and brain dysfunction. Overall, free radicals have been implicated in the pathogenesis of at least 100 diseases. These radicals interact with membrane lipids and initiate the chain reaction of lipid peroxidation leading to tissue damage (1,2). Fortunately, free radical formation is controlled naturally by various beneficial compounds known as antioxidants. It is interesting to note that, in the biological system, there is an in-built defense mechanism against free radical-induced deleterious effects through scavenging effects of various endogenous antioxidants (1,2).

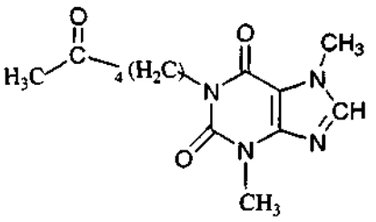
One of the effective endogenous antioxidants, which participate in the prevention of oxidative damage in humans is uric acid (3). In most of the lower animals, from fish to rhesus macaques, uric acid (UA), a product of purine metabolism, is rapidly oxidized to allantoin by the enzyme urate oxidase and found only at a low concentration in the serum (4). However, in humans and higher primates, functional urate oxidase is not present and serum level of UA is ~10-fold higher (5), and its level in human plasma is about 2.0-7.5 mg/100ml (0.12-0.45 mM) (6). The observation that UA has antioxidant properties, which are not shared by either its precursor xanthine or product allantoin (7) has led to the speculation that, uric acid may have an important role in protecting hominoids from oxidative damage mediated by free radicals. It has been shown that, uric acid and related analogues at concentrations similar to their physiological levels, suppress oxidative degradation of low density lipoprotein (LDL) components (8), inhibit oxygen free radical-induced DNA damage (9), function as efficient antioxidant and free radical scavengers (3,10,11) and protect erythrocyte membranes from lipid peroxidation (12,13). It is well known that, UA is a very important antioxidant in respiratory tract lining fluid (14). Recently it has been demonstrated that, UA is a very effective peroxynitrite scavenger (7,15) and appeared as a neuroprotective agent (16). The antioxidant activity of uric acid



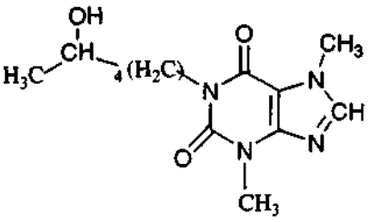
1-(6-NONENYL)-3,7-DIMETHYLXANTHINE



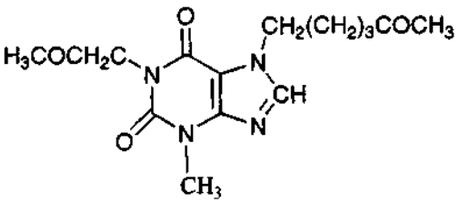
1-(4-OXIRANYLBUTYL)-3,7-DIMETHYLXANTHINE



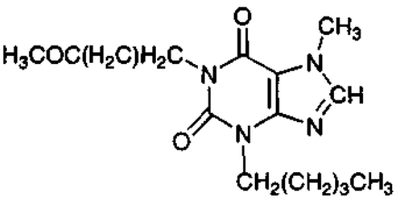
PENTOXIFYLLINE



LISOFYLLINE



1-(2-OXOPROPYL)-7-(5-OXOHEXYL)-3-METHYLXANTHINE



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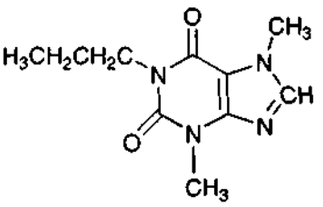
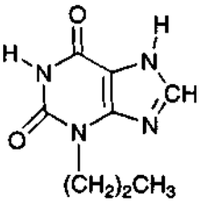


1-PROPYL-3,7-DIMETHYLXANTHINE



ENPROFYLLINE

Fig. 1: Various xanthine analogues used as drugs

has been attributed to its ready formation of urate anion radical through one electron oxidation, and 8-oxo group seems to be necessary for the stabilization of the urate anion radical (10,17). In fact, it has been suggested that, 8-oxo group is an important functional moiety for urates' antioxidant property (10,17). Uric acid is highly insoluble in lipophlic environment, such as biological membranes (18). It is reported that, the lipophilicity of uric acid increases by alkylating at 1, 3, 7 or 9^{th} position of UA structure (18). Studies have indicated that, methylation at certain nitrogens in uric acid can enhance, as well as retard, the antioxidant property of uric acid (19). Alkyl substituted uric acid analogues are better antioxidants in hydrophobic environment like membrane lipid/water interphase (18). Recent reports indicate that, purine molecule such as xanthine, caffeine, theophylline and theobromine without the 8-oxo group lack antioxidant activity and hence these molecules do not possess any significant protective effects towards LDL constituents (8).

Some of the mono-, di-, and trimethyluric acids and their corresponding xanthines have been examined earlier for their preventive effects on lipid peroxidation (13). One of the compounds which has high potency in the prevention of hydrogen peroxide-induced lipid peroxidation in human erythrocyte membranes is 1,3,7-trimethyluric acid (13). This observation has prompted us to prepare analogues of 1,3,7-trimethyluric acid in order to evaluate their bio-modulatory property. We chose to prepare analogues of 1,3,7-trimethyluric acid with N-1 methyl replaced by various groups mainly because, N-1 position is not in proximity to the 8-oxo group, an important functional moiety of urates (10,17). Hence, any bulky substitution at N-1 position would not affect the formation of urate radical. It is known that, some of the long chain-substituted uric acids exhibit better inhibiting activity against oxygen radical-induced lipid peroxidation compared to known antioxidants such as α-tocopherol (18).

Most of the alkyl xanthines are used as drugs, which are having a wide range of activity (**Fig. 1**). However, the corresponding 8-oxo compounds have never been prepared and hence their biological activities have not been ascertained. 8-Oxopurine is available naturally and only a few 8-oxo compounds are evaluated for their biological activity. It is interesting to note that, 8-oxomethylxanthines and their derivatives are used as one of the

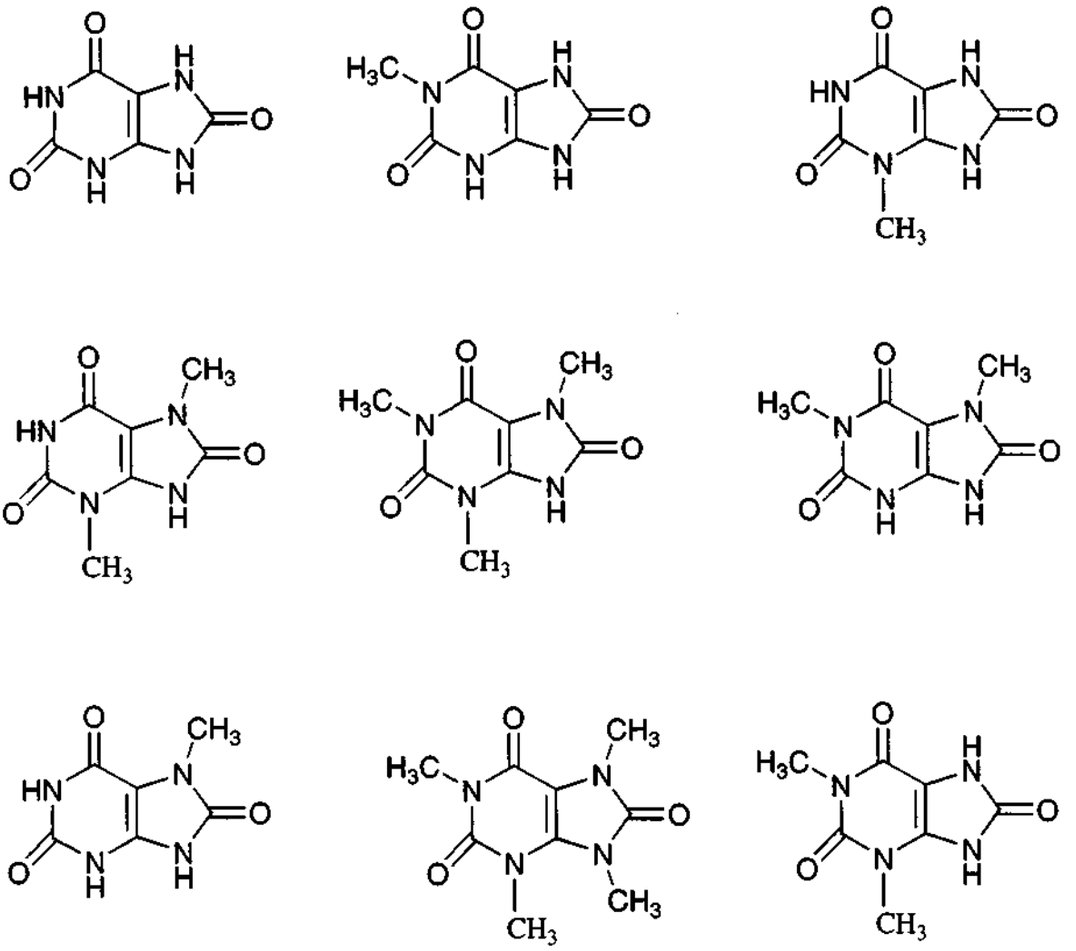


Fig. 2: Naturally occurring uric acids

components in obesity-treating pharmaceuticals, cosmetics and antidandruff preparations (20-22). Mono-, di-, tri-, and tetramethyl uric acids occur naturally (**Fig. 2**) and these compounds are formed during the metabolism of the corresponding xanthines in the mammalian system.

In the present study, we wanted to prepare different new analogues of 1,3,7-trimethyluric acid with N-1 methyl group replaced by alkyl, hydroxyethyl, benzyl, 2-oxopropyl, allyl, propargyl and butenyl groups and evaluate their biological potentials like protective effect against lipid peroxidation and ability to scavenge oxygen free radicals.

Synthesis of analogues of 1,3,7-trimethyluric acid involves a lengthy procedure with stringent experimental conditions. There are many reports on the synthesis of 8-oxopurines and one of the limitations of these methods is that, most of the purines are insoluble in organic solvents, and hence they should be protected as acetyl or trityl groups to increase the solubility (23-26). In the process the number of steps involved in the synthesis increases. The methods developed for the chemical synthesis of these compounds are not satisfactory since they involve the usage of expensive reagents, yields are varying and suitable for compounds soluble in organic solvents (27,28), but not for water-soluble ones. Under these circumstances, it is reasonable to suggest that, microbial transformations may be the best suited for these highly water soluble compounds. Xanthine oxidase is the enzyme, which is responsible for carrying out the C-8 oxidation of xanthine and converts it to uric acid. Although xanthine oxidase has fairly broad substrate specificity, it does not accept dimethyl and trimethyl xanthine as substrates. It accepts only xanthine and hypoxanthine as substrates and converts to uric acid. However, it has been shown that, xanthine oxidase accepts 1-methylxanthine as substrate and converts it to 1-methyluric acid (29).

Sometimes, microbes bring about highly selective transformations of prototype compounds leading to the products that are rare or only available in very low yield by conventional chemical approaches. Interesting new analogues of 1,3,7-trimethyluric acid may be prepared without resorting to new and lengthy total synthetic chemical methods.

In recent years, the most striking and significant development in the field of synthetic organic chemistry is the application of biological systems to chemical reactions.

Biological systems display a far greater specificity than the conventional organic reagents. Exploration of microbes in carrying out transformations of natural products has been an important and fascinating method in the synthetic organic chemistry. Microbes offer the opportunity to effect selective transformation, a feature of great utility in organic synthesis that would otherwise be difficult to carryout by conventional methods. Microbial reactions are versatile, covering many reactions such as (i) oxidation, (ii) reduction, (iii) hydrolysis, (iv) dehydration and condensation, (v) degradation, (vi) formation of C-C or hetero atom bonds, (vii) isomerization and rearrangements, etc (30-35). Some advantages in selecting microbial reactions as a rational supplement to chemical synthesis are the following:

1. ***Microbial reactions can be used to attack positions in the molecules which are not normally affected by chemical methods because of lack of sufficient activation or, require a number of intermediate synthetic stages to impart chemical accessibility. Oxygen function or other substituents can be introduced stereospecifically or regiospecifically or altered with a possible formation of optically active centers.***
2. ***Several reactions can be combined in one fermentation step and actually programmed to occur in a specific sequence if a suitable microorganism with a number of appropriate enzyme systems can be used.***
3. ***The conditions under which microbial reactions take place are mild. Hence, compounds that are sensitive to heat, acid, and base become amenable to such transformations.***
4. ***Microbial synthesis also has advantages in the preparation of optically active compounds since chemical synthesis leads to racemic mixtures that must subsequently be resolved.***
5. ***It is often cheaper to use a microorganism for the preparation of an organic compound than to synthesize it chemically. Chemistry is yet to catch up with biological processes for the economic production of many substances that are useful to mankind. Above all, the processes mediated by microbes are ecofriendly and so these processes produce minimum environmental pollution unlike processes mediated by chemical reagents.***

Hence, it is not surprising to note that, about fifty antibiotics and several of the medicinally important steroid hormones are now produced on a large scale by microbial processes.

There is probably no naturally occurring organic compound, which cannot be used as a substrate for respiratory metabolism by some microorganism. Biotransformations of organic compounds are carried out either by constitutive or substrate-induced enzymes of the secondary metabolism by taking advantage in some cases of an unpredictable lack of substrate specificity of essential enzymes in microorganisms. The observed transformations of organic compounds are consistent with respect to the following generalizations:

1. ***Enzymes catalyzing the reactions have a broad substrate specificity.***
2. ***Relatively few reaction types are involved in the early stages of the inducible pathway of metabolism.***
3. ***The pathways of degradation of structurally related compounds tend to converge on common intermediates early in their sequence.***

Considerable progress has been made in the microbial transformations of various classes of organic compounds such as steroids, carbohydrates, lipids, lignins, terpenoids, alkaloids and other aromatic compounds. Bacteria mostly degrade the organic compounds to the level of CO_2 and water to derive energy whereas, fungi normally transform the substances mostly into their oxygenated products.

The metabolism of caffeine (1,3,7-trimethylxanthine) and related methylated xanthines has been investigated both in microbes (36-39) and mammals (40-44). The work carried out so far on the biodegradation of caffeine and related compounds in the living systems clearly indicate that, there exists a significant difference between the mode of metabolism of these compounds taking place in the microbial and mammalian systems. Although caffeine is ubiquitous and extensively used throughout the world, microbial transformation of caffeine did not receive much attention because of its anti-microbial nature. Caffeine is generally toxic to bacteria, although the concentration required is often relatively high. In microbial system, degradation of caffeine is initiated by partial or complete N-demethylation followed by oxidation at C-8 position to produce the

corresponding uric acids. In fact, caffeine is N-demethylated in two parallel ways via theobromine (3,7-dimethylxanthine) and paraxathine (1,7-diemthylxanthine), the former appears to be the major pathway for degradation (38,39). Uric acid is degraded to allantoin and then to allantoic acid. Further degradation of allantoic acid results in the formation of urea and glyoxalic acids as the end products. Mammals metabolize caffeine via two common metabolic reactions, the N-demethylation and direct oxidation of caffeine, yielding 1,3,7-trimethyluric acid, which further gets oxidized to 3,6,8-trimethylallantoin by cleavage of 6-membered ring or to substituted diaminouracil derivative by cleavage of 5-membered ring.

Microbial biotransformations are best suited for these highly water soluble compounds. Xanthine oxidase is the enzyme, which is responsible for carrying out the C-8 oxidation of xanthine and converts it to uric acid. Although xanthine oxidase has fairly broad substrate specificity, it does not accept dimethyl and trimethyl xanthine as substrates. It accepts only xanthine and hypoxanthine as substrates and converts to uric acid. However, it has been shown that xanthine oxidase accepts 1-methylxanthine as substrate and converts it to 1-methyluric acid.

Earlier in our laboratory we have isolated a microbial system, which degrades caffeine and related compounds. This microbial system is a mixed culture bacterial consortium consisting of strains belonging to the genera *Klebsiella* and *Rhodococcus*, capable of utilizing caffeine as the sole source of carbon and nitrogen (45). In fact, there has not been any study carried out so far on the degradation of caffeine by a mixed culture. Although caffeine is the sole source of carbon and nitrogen, 0.1% glucose is required in the media in order to enhance the growth rate. This mixed culture completely metabolizes caffeine in 36h (45). The C-8 oxidation is the initial step in the metabolism of caffeine (45) and this mixed culture does not carry out N-demethylation reaction (45). It also does not use theophylline, theobromine and paraxanthine as carbon source. Fermentation of caffeine by this mixed culture does not accumulate 1,3,7-trimethyluric acid. If the fermentation is carried out in the presence of N-methylmaleimide, 1,3,7-trimethyluric acid accumulates in the medium.

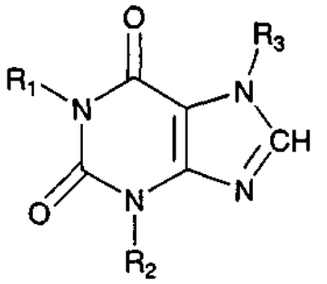
The mixed culture degrades caffeine following a pathway hitherto not reported in the microbial system (45). Although the mixed culture does not accept theophylline and theobromine as substrates, the caffeine grown cells quantitatively convert these dimethylxanthines into their corresponding dimethyluric acids very efficiently (46). Recently our laboratory has reported that, caffeine grown cells of mixed culture consortium uses 1-, 3-, and 7- substituted xanthines as substrates and quantitatively convert them to their corresponding uric acids (46). However, none of the xanthine oxidases or xanthine dehydrogenases isolated so far, either from the microbial or from the mammalian sources, has been shown to accept caffeine or dimethylxanthines as substrates.

The major pathway for the metabolism of caffeine (1,3,7-trimethylxanthine), in both microbial (36,37) and mammalian (47,48) systems, is initiated by N-demethylation, either partial or complete, followed by oxidation at C-8 position to yield the corresponding uric acids. Direct oxidation of caffeine at C-8 position is also known to take place in the mammalian system resulting in the formation of 1,3,7-trimethyluric acid and the hepatic cytochrome P-450 system catalyzes this reaction in the presence of reduced pyridine nucleotide (48-50).

The mixed culture consisting of strains belonging to the genera *Klebsiella* and *Rhodococcus* does not initiate degradation of caffeine by N-demethylation, instead carries out direct oxidation at C-8 position to 1,3,7-trimethyluric acid (45). In fact, the mixed culture very efficiently converts 1-, 3-, and 7- substituted xanthines to their respective 8-oxo compounds (46). It is known that, xanthine oxidase catalyzes the C-8 oxidation of xanthine to uric acid (51,52). However, none of the xanthine oxidases or xanthine dehydrogenases (53) isolated so far, either from the microbial or from the mammalian sources, have been shown to accept caffeine or dimethylxanthines as substrates. This has prompted us to characterize the enzyme system from the mixed culture, which efficiently carries out C-8 oxidation of caffeine.

The present chapter deals with the preparation of several new analogues of 1,3,7-trimethyluric acid using a mixed culture consortium consisting of strains belonging to the genera *Klebsiella* and *Rhodococcus*. The uric acids obtained were characterized by various spectral analyses. The new uric acid analogues prepared have been evaluated for their bio-

Table 1: Chemical Structures of N-1 substituted theobromines (I-XI), 3-Propyl-1,3-dimethylxanthine (XII) and 3-Propylxanthine (XIII)



Compounds	R_1	R_2	R_3
1-Ethyl-3,7-dimethylxanthine (I)	CH_3CH_2-	-CH_3	-CH_3
1-Propyl-3,7-dimethylxanthine (II)	$CH_3CH_2CH_2$-	-CH_3	-CH_3
1-Butyl-3,7-dimethylxanthine (III)	$CH_3CH_2CH_2CH_2$-	-CH_3	-CH_3
1-Pentyl-3,7-dimethylxanthine (IV)	$CH_3CH_2CH_2CH_2CH_2$-	-CH_3	-CH_3
1-(β-hydroxyethyl)-3,7-dimethylxanthine (V)	-CH_2 CH_2OH	-CH_3	-CH_3
1-Benzyl-3,7-dimethylxanthine (VI)	Ph-CH_2-	-CH_3	-CH_3
1-(5-Oxohexyl)-3,7-dimethylxanthine (VII	$CH_3CO(CH_2)_3CH_2$	-CH_3	-CH_3
1-(2'-Oxopropyl)-3,7-dimethylxanthine (VIII)	CH_3CO CH_2-	-CH_3	-CH_3
1-Allyl-3,7-dimethylxanthine (IX)	CH_2=CH-CH_2-	-CH_3	-CH_3
1-Propargyl-3,7-dimethylxanthine (X)	CH≡C-CH_2-	-CH_3	-CH_3
1-Butenyl-3,7-dimethylxanthine (XI)	CH_2=CH- CH_2CH_2-	-CH_3	-CH_3
3-Propyl-1,7-dimethylxanthine (XII)	CH_3	-$CH_2CH_2CH_3$	-CH_3
3-Propylxanthine (XIII)	-H	-$CH_2CH_2CH_3$	-H

modulatory properties. The present study also deals with the isolation, purification to homogeneity and partial characterization of a novel caffeine (1,3,7-trimethylxanthine) oxidase from a mixed bacterial culture grown on caffeine. The results presented here clearly indicate that, caffeine oxidase possesses properties distinctly different from what has been reported for xanthine oxidase isolated from various sources (54). The purified caffeine oxidase also uses 1-, 3-, and 7- substituted xanthines as substrates and converts them to their corresponding C-8 oxidized compounds.

MATERIALS AND METHODS

Chemicals

Tris, β-mercaptoethanol, Nitroblue tetrazolium (NBT), Phenazine ethosulfate (PES), Dichlorophenol indophenol (DCPIP), Cytochrome c, Acrylamide, N, N-methylene bisacrylamide, TEMED, Ammonium persulfate, SDS, BSA, Coomassie Brilliant Blue R-250, p-Chloromercuric benzoate, N-methylmaleimide, Iodoacetamide, Phenyl sepharose, DEAE-cellulose (DE-52), Caffeine, Theophylline, Theobromine and the corresponding uric acids were purchased from Sigma. Enprofylline (3-propylxanthines, **XIII, Table 1**) was a generous gift from Dr. Hans Jurgen Federsel, Astra Production Chemicals AB, Sweden. All other materials were from standard suppliers and were of analytical grade.

Synthesis of *N*-1 substituted theobromines (1–XI)

Substituted theobromines **I–XI (Table 1)** were synthesized as reported earlier (46,55). A mixture of theobromine (1.0 g, 5.55 mmol), sodium hydroxide (8 cm^3, 3.2 mmol), alkyl/allyl/propynyl/but-3-enyl/hydroxyethyl/benzyl bromides (11 mmol) and propan-2-ol (12 cm^3) was taken in a sealed tube and heated at 120° C for 24 h. The reaction mixture was allowed to cool to room temperature and extracted with chloroform (50 cm^3 × 4). The organic layer, evaporated and purified by column chromatography over silica gel using $CHCl_3$–CH_3OH (97: 3, v/v), yielded pure **I-XI** and the yields varied from 45–83%.

Synthesis of 3-propyl-1,7-dimethylxanthine (XII)

The compound **XII** was prepared following the published procedure (56). To a stirred suspension of 3-propylxanthine (0.97 g, 5 mmol) and anhydrous K_2CO_3 in 8 cm^3 of DMF was added dropwise 15 mmol of methyl iodide. The reaction mixture was heated at

35° C for 4 h and the volatile material removed under vacuum. The product was isolated by adding water and extracted with ethyl acetate. The crude product was purified by column chromatography over silica gel using $CHCl_3$–CH_3OH (97:3, v/v) and yielded pure 3-propyl-1,7-dimethylxanthine **(XII)**.

Spectroscopic methods

Infrared spectra (IR) were recorded on a Perkin-Elmer model 599 IR spectrophotometer. Proton Magnetic Resonance (PMR) spectra were recorded with either JEOL FX-90Q or 300 MHz using tetramethyl silane as an internal standard. Mass spectra were recorded on a JEOL-JMX-DX 303 instrument with JMA-DA 5000 data system. UV-visible absorption was monitored on Hitachi 557, double wavelength, double beam spectrophotometer.

Analytical methods

Thin layer chromatographic (TLC) analysis were performed on silica gel GF_{254} plates (0.5 mm) developed with $CHCl_3$:CH_3OH (85:15, system I) as the solvent system. Compounds were visualized by exposing the plates to UV-light or Iodine vapour. HPLC analysis were carried out on Shimadzu CR7A instrument using ODS reverse phase column with H_2O:CH_3OH:CH_3CN (70:10:20, v/v/v) as the solvent system (1 ml/min) and eluants were monitored with a UV-detector at 254 nm.

Maintenance and propagation of the mixed culture (organism and growth conditions)

The mixed culture consisting of *Klebsiella* and *Rhodococcus sp.* was maintained on nutrient agar slants, of the following composition: Peptone (1.0%), Sodium Chloride (0.5%), Yeast extract (0.5%), Beef extract (0.5%), Agar (2.0%), in distilled water.

The first four constituents were mixed with water and the pH was adjusted to 7.0 with 2N NaOH solution. The agar (2%) was added and the mixture was steamed for 1h to melt the agar after which, 0.3% caffeine was added to the mixture. For the preparation of the slants, 10 ml aliquots were distributed into test tubes and autoclaved at 15 psi for 20 min. The tubes were left slanting while hot and allowed to solidify. The slants were inoculated either from broth or stock cultures and incubated at 28-30° C for 48 hours and stored at 4° C.

For maintenance of the mixed culture in liquid medium with caffeine as a source of carbon, a chemically defined mineral salts medium having the following composition described by Seubert (57) was used.

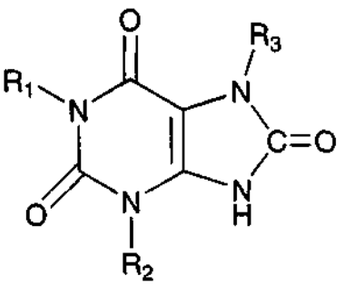
Dipotassium hydrogen phosphate	63.0 g
Potassium dihydrogen phosphate	18.2 g
Ammonium nitrate	10.0 g
Magnesium sulfate	1.0 g
Calcium chloride, dihydrate	1.0 g
Manganese sulfate	0.006 g
Sodium molybdate	0.006 g
Ferrous sulfate, heptahydrate	0.1 g

The phosphates were first dissolved together and the solutions of the other salts were added to the phosphate solution. The volume was made up to 10 liters and the pH was adjusted to 7.2. Aliquots of 100 ml medium were distributed into 500 ml Erlenmeyer flasks and autoclaved at 15 psi for 20 min. Whenever a starter culture was required, an aliquot of 5 ml from the maintenance culture (A_{660nm}= 1.1) was transferred to 100 ml sterile mineral salts medium containing 0.04% of caffeine and 100 mg of glucose and incubated aerobically on a rotary shaker (220 rpm) at 28-30° C for 36 hrs.

General procedure for biotransformation

The mixed culture utilizes caffeine as the sole source of carbon (45). Caffeine-induced cells are required to transform various substituted xanthines to their corresponding uric acids. Hence fermentation was carried out in the presence of small quantities (0.04%) of caffeine to induce the cells. The caffeine added gets completely metabolized within 24 hours. A batch of 10 flasks containing 100 ml of sterile mineral salts medium (57) (pH 7.2) containing 0.1% glucose, 0.04% of caffeine (inducer) and 0.05% of substrates (**I–XIII, Table 1**) was inoculated from a 36 h old culture (5 ml, A_{660} = 1.1) and incubated on a rotary shaker at 29–30° C for 48 h. The transformation of substrates was checked periodically by taking aliquots at different time intervals. At the end of the incubation period, the contents of all flasks were pooled, acidified to pH 5–6 and centrifuged. An aliquot from the supernatant was subjected to HPLC analysis, which indicated the absence

Table 2: Chemical Structures of 1,3,7-Trimethyluric Acid Analogues (XIV-XXV)



Compounds	R_1	R_2	R_3
1-Ethyl-3,7-dimethyluric acid (XIV)	CH_3CH_2-	-CH_3	-CH_3
1-Propyl-3,7-dimethyluric acid (XV)	$CH_3CH_2CH_2$-	-CH_3	-CH_3
1-Butyl-3,7-dimethyluric acid (XVI)	$CH_3CH_2CH_2CH_2$-	-CH_3	-CH_3
1-Pentyl-3,7-dimethyluric acid (XVII)	$CH_3CH_2CH_2CH_2CH_2$-	-CH_3	-CH_3
1-(β-hydroxyethyl)-3,7-dimethyluric acid (XVIII)	-CH_2 CH_2OH	-CH_3	-CH_3
1-Benzyl-3,7-dimethyluric acid (XIX)	Ph-CH_2-	-CH_3	-CH_3
1-(5-Oxohexyl)-3,7-dimethylxanthine (XX)	$CH_3CO(CH_2)_3CH_2$	-CH_3	-CH_3
1-(2′-Oxopropyl)-3,7-dimethyluric acid (XXI)	CH_3CO CH_2-	-CH_3	-CH_3
1-Allyl-3,7-dimethyluric acid (XXII)	CH_2=CH-CH_2-	-CH_3	-CH_3
1-Propargyl-3,7-dimethyluric acid (XXIII)	CH≡C-CH_2-	-CH_3	-CH_3
1-Butenyl-3,7-dimethyluric acid (XXIV)	CH_2=CH- CH_2CH_2-	-CH_3	-CH_3
3-Propyl-1,7-dimethylxanthine (XXV)	CH_3	-$CH_2CH_2CH_3$	-CH_3
3-Propyluric acid (XXVI)	-CH_3	-$CH_2CH_2CH_3$	-H

of substrate and its complete conversion to the corresponding C-8 oxidized product (**XIV-XXVI, Table 2**). For isolating the biotransformed product, the supernatant was extracted with $CHCl_3$–CH_3OH (2: 1, v/v), the organic phase dried over Na_2SO_4 and solvent removed under reduced pressure. The residue was passed through a small pad of silica gel. eluted with $CHCl_3$–CH_3OH (95: 5, v/v) to obtain pure 8-oxo compounds (uric acids, **XIV-XXVI, Table 2**) which were completely characterized by various spectral analyses (IR, NMR, MS). Although the HPLC analyses indicated the complete conversion (100%) of the substrates (**I-XIII, Table 1**) to the corresponding C-8 oxidized products, the isolated yields of the uric acids (**XIV-XXVI**) varied from 95–97% (**Table 2**).

Growth of mixed culture for purification of caffeine oxidase

For large scale preparation, 80 Erlenmeyer flasks (500 ml) containing 100 ml sterile mineral salts medium (pH 7.2) to which 0.04% caffeine, 0.1% glucose and 5% of 36h old inoculum (A_{660nm}= 1.1) were added and incubated as described above. After 36h of growth, cells were harvested by centrifugation in a Sorvall RC-5B centrifuge (3000 g, 20 min), washed thoroughly with ice-cold phosphate buffer (0.05 M, pH 7.8) and processed further for enzyme purification.

Enzyme purification

All operations were carried out between 0-4° C unless otherwise specified. Washed cells (9.0 g wet weight) were suspended (5 ml/g wet weight) in phosphate buffer (0.05 M, pH 7.8) containing 10% glycerol (v/v, buffer A). The cell suspension was sonicated using a Branson B-30 sonifier for 5 min with intermittent cooling for every 30 sec at maximum output and the sonicate was centrifuged (10,000 g, 30 min). The supernatant, designated as the crude cell-free extract (270 mg), was applied on a DEAE-Cellulose column (2 × 30 cm) previously equilibrated with buffer A. After washing the column with buffer A (300 ml), the enzyme was eluted with a linear concentration gradient of NaCl in buffer A from 0 to 0.6 M. The fractions containing significant caffeine oxidase activity were pooled (32 mg) and loaded on to a Phenyl sepharose column (1.5 × 5 cm) equilibrated with buffer A. After washing the column with phosphate buffer (0.02 M, pH 7.8) containing 10% glycerol until the eluate showed less than 0.005 absorbance at 280 nm, the enzyme was specifically eluted with phosphate buffer (0.005 M, pH 7.8) containing 10% glycerol. The fractions

rich in caffeine oxidase activity were pooled and concentrated by ultra filtration using Amicon PM 10 membrane filter.

Protein estimation

The protein concentration in the enzyme preparations was determined by the Folin-Ciocalteau method (Lowry et al., 1951) using BSA as standard (58).

Enzyme assays

PES-DCPIC coupled assay: This assay is based on the fact that, the reducing equivalents from the substrate are passed on to the enzyme thereby reducing PES. The reduced PES in turn transfers the reducing equivalents to DCPIP thereby reducing it. The reduction of DCPIP was followed spectrophotometrically at 600 nm, which is the measure of enzyme activity. This well-known assay system is used for estimating dehydrogenases like succinic acid dehydrogenase. Caffeine oxidase (1,3,7-trimethyl xanthine oxidase) activity was monitored at room temperature (~26° C) using phenazine ethosulphate (PES) to couple electron transfer from the reduced enzyme to dichlorophenol indophenol (DCPIP). The assay mixture contained caffeine (500 µM), PES (40 µM), DCPIP (40 µM) and appropriate amount of enzyme (2-50 µg depending upon the purity of enzyme) in a total volume of 1.0 ml of phosphate buffer (0.05 M, pH 7.8). The reaction was initiated by the addition of caffeine and the reduction of DCPIP was measured at 600 nm. The specific activity was calculated using an extinction coefficient of 21×10^3 M^{-1} cm^{-1} (59).

PES-Cytochrome c coupled assay: Caffeine oxidase activity was also measured spectrophotometrically, by an assay using PES to couple electron transfer from the reduced enzyme to cytochrome c. The rates of reduction were monitored at 550 nm at room temperature. Reaction mixture contained protein (50 µg), caffeine (500 µM), PES (40 µM), and cytochrome c (40 µM) in a total volume of 1.0 ml of phosphate buffer (0.05 M, pH 7.8). The reaction was initiated by the addition of caffeine and the reduction of cytochrome c was measured at 550 nm. The specific activity was calculated using an extinction coefficient value of 21×10^3 M^{-1} cm^{-1} (59).

NAD^+ and $NADP^+$ as electron acceptors: Spectrophotometric assay was carried out to determine whether the enzyme is an oxidase or dehydrogenase using NAD^+ and $NADP^+$. It

is known in the literature that, both xanthine oxidase and xanthine dehydrogenase convert xanthine to uric acid; when xanthine oxidase requires artificial electron acceptors, xanthine dehydrogenase requires pyridine nucleotides. Reaction mixture contained protein (50 μg), caffeine (500 μM), NAD^+ or $NADP^+$ (40 μM), in a total volume of 1.0 ml of phosphate buffer (0.05 M, pH 7.8). The reaction was initiated by the addition of caffeine and reduction of NAD^+ or $NADP^+$ was monitored at 340 nm. The enzyme did not accept either NAD^+ or $NADP^+$ as electron acceptors confirming that, the enzyme is an oxidase but not dehydrogenase.

Transformation *in vitro*

Cell-free extract (0.6 mg) was incubated with caffeine/theobromine/theophylline (7.0 mM) in the presence of PES (100 mM) in a total volume of 1.0 ml of phosphate buffer (0.05 M, pH 7.8) at 30° C for 15 min. At the end of the incubation period, the contents were acidified to pH 4-5, extracted with $CHCl_3$:CH_3OH (2:1) and the organic layer was subjected to HPLC analysis. The enzymatically formed product was also isolated as reported earlier and characterized (37). In a similar way, incubations were also carried out using purified enzyme and the product formed was identified by HPLC and mass spectral analysis.

Similarly, the other substituted theobromines were used as substrate and the product formation was identified by HPLC and mass spectral analysis.

Polyacrylamide gel electrophoresis (PAGE)

Native-PAGE: Native polyacrylamide gel electrophoresis (PAGE) was carried out under non-denaturing conditions according to the method of Davis (60) on a 7.5% polyacrylamide gel.

SDS-PAGE: SDS-PAGE was done according to the method of Laemmli (61) using 10% polyacrylamide gels. The protein samples were mixed with sample buffer (2% SDS, 5% β-mercaptoethanol, 30% glycerol, 1.2% glycine and 0.002% bromophenol blue in 0.5 M Tris-HCl buffer, pH 6.8), boiled for 4 min and allowed to cool to room temperature before loading onto the gel. Electrophoresis was initially carried out at 75 volts till the sample entered the resolving gel and separation was effected at 130 volts. The molecular mass of

Table 3: Substrate specificity of caffeine oxidase

Substrates	Relative[1] activity(%)
Caffeine (1)	100.0
Theobromine (2)	29.9
Theophylline (3)	1.22
Ethyltheobromine ((4)	62.7
Propyltheobromine (5)	30.7
Butyltheobromine (6)	24.6
Pentyltheobromine (7)	18.4
β-Hydroxylethyltheobromine (8)	12.28
Benzyltheobromine (9)	3.7
Pentoxifylline (10)	25.8
Allyltheobromine (11)	48.0
Propargyltheobromine (12)	48.0
Butenyltheobromine (13)	31.95
3-propylxanthine(Enprofylline) (14)	3.7
3-propyl-1,7-Dimethylxanthine (15)	11.06
Xanthine (16)	2.45
Hypoxanthine (17)	2.37
Adenine (18)	0.0

[1]***Assays were carried out using 1.75 μg of the purified enzyme and 300 μM of the substrate listed. Other conditions were as described in the text. 100% activity represents 2.2 μmol of DCPIP reduced per min per mg of protein.***

the enzyme was deduced from SDS-PAGE profiles with reference to the standard protein molecular weight markers, which were phosphorylase b (97.3 kDA), Bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14.3 kDa).

The gels after electrophoresis were stained with 0.25% Coomassie Brilliant Blue R-250 in methanol : acetic acid : water (5:1:5, v/v/v) for 3-4h. Destaining was done using a mixture of 50% methanol and 10% acetic acid.

Enzyme activity staining: Enzyme activity was detected *in situ,* after Native-PAGE using nitroblue tetrazolium-phenazine methosulfate at 4° C, as reported earlier (62). Gel was incubated for 15 min in the dark in the assay mixture containing caffeine (10 µmol), NBT (4 µmol) and PMS (4 µmol) in a total volume of 100 ml Tris-HCl buffer (0.03 M, pH 9.0). The gel incubated in the above mixture minus substrate was used as a control. Following the color development, transferring the gel to the destaining solution stopped the reaction.

Substrate specificity

Spectrophotometric assay was carried out to determine the substrate specificity of caffeine oxidase using PES-DCPIP coupled assay. The assay mixture contained substrates 1-15 (300 µM, **Table 3**), PES (40 µM), DCPIP (40 µM) and enzyme (50 µg) in a total volume of 1.0 ml of phosphate buffer (0.05 M, pH 7.8). The reaction was initiated by the addition of substrates 1-15 (0.5 µmole, **Table 3**) and the reduction of DCPIP was measured at 600 nm at room temperature. Caffeine is the preferred substrate for the caffeine oxidase, and activities of substrates 1-15 (Table 3) are given relative to caffeine. In order to confirm the product formation, the assay mixture was extracted with $CHCl_3$:CH_3OH (2:1, 5 ml), the organic layer was concentrated and subjected to TLC and HPLC analysis.

RESULTS

Transformation of N-1 substituted theobromine to corresponding uric acids

A mixed culture grown on caffeine transformed a range of 1-, 3-, and 7- substituted xanthines **I-XIII (Table 1)** quantitatively to their corresponding C-8 oxidized compounds. It was observed that, caffeine grown cells convert quantitatively analogues of theobromine

with N-1-H replaced by various groups such as alkyl (**I–IV**), hydroxyethyl (**V**), benzyl (**VI**), 5-oxohexyl (**VII**), 2-oxopropyl (**VIII**), allyl (**IX**), propargyl (**X**) and but-3-enyl (**XI**) to their corresponding C-8 oxidized compounds (**XIV-XVI, Table 2**). The compounds **XIV-XVI** (**Table 2**) were fully characterized by various spectral analyses (IR, NMR, Mass) and as far as we know, these uric acids **XIV-XVI** were hitherto unknown. In all these cases, the HPLC analysis of the medium after 48 hrs, clearly indicated quantitative conversion of 4-17 to their respective 8-oxo derivatives (**XIV-XVI**). However, the isolated yield in all these cases ranged from 95-97%. The spectral characteristics of 8-oxo compounds **XIV-XVI** are given below.

Spectral analysis of new analogues of uric acids

1-Ethyl-3,7-dimethyluric acid (XIV)
Mp: 295° C ($CHCl_3$–MeOH).
IR (Nujol) ν_{max} **cm^{-1}**: 1640, 1680, 1695 (–NC=O–).
^{1}H NMR (300 MHz, $CDCl_3$): 1.2 (3H, t, *J* 7.2, CH_3CH_2–), 3.5 (3H, s, $-NCH_3$), 3.58 (3H, s, $-NCH_3$), 4.0 (2H, q, *J* 7.2, CH_2CH_3).
^{13}C NMR (75 MHz, $CDCl_3$): 14.2, 30.0, 30.8, 41.2, 99.0, 136.7, 150.9, 153.7, 154.0.
LRMS (*m/z*): 224 (M^+, 65%), 153 (40%), 82 (100%), 67 (55%).
HRMS: found M^+, 224.0900, $C_9H_{12}N_4O_3$ requires 224.0909.

1-Propyl-3,7-dimethyluric acid (XV)
Mp: Mp 280° C ($CHCl_3$–MeOH).
IR (Nujol) ν_{max} **cm^{-1}**: 1640, 1680, 1695 (–NC=O–).
^{1}H NMR (300 MHz, $CDCl_3$): 0.97 (3H, t, *J* 7.2, $CH_3CH_2CH_2$–), 1.67 (2H, m, $CH_3CH_2CH_2$–), 3.5 (3H, s, $-NCH_3$), 3.56 (3H, s, $-NCH_3$), 3.9 (2H, t, *J* 7.2, $-NCH_2$–).
^{13}C NMR (75 MHz, $CDCl_3$): 12.4, 22.3, 29.0, 31.2, 41.4, 98.9, 136.7, 150.0, 153.0, 154.5
LRMS (*m/z*): 238 (M^+, 100%), 219 (M^+ -H_2O, 50%), 196 (M^+ -C3H7, 70%), 153 (60%), 82 (75%).
HRMS: found M1, 238.1061, $C_{10}H_{14}N_4O_3$ requires 238.1065.

1-Butyl-3,7-dimethyluric acid (XVI)
Mp: 268–270° C ($CHCl_3$–MeOH).
IR (Nujol) ν_{max} **cm^{-1}**: 1640, 1680, 1700 (–NC=O–).
^{1}H NMR (300 MHz, $CDCl_3$): 0.95 (3H, t, *J* 7.2, $CH_3(CH_2)_3$–), 1.4–1.6 (4H, m, CH_2CH_2–CH_2–N–), 3.5 (3H, s, $-NCH_3$), 3.57 (3H, s, $-NCH_3$), 3.97 (2H, t, *J* 7.2, $-NCH_2$-).
^{13}C NMR (22.5 MHz, $CDCl_3$): 13.8, 20.2, 28.8, 30.0, 30.8, 41.4, 99.0, 136.0, 150.0, 153.0, 153.7.
LRMS (*m/z*): 252 (M1, 100%), 225 (M^+ -H_2O, 55%), 196 (M^+ -C_4H_9, 95%), 153 (90%), 82 (80%).
HRMS: found M^+, 252.1218, $C_{11}H_{16}N_4O_3$ requires 252.1222.

1-Pentyl-3,7-dimethyluric acid (XVII)
Mp: 265° C ($CHCl_3$–MeOH).
IR (Nujol) ν_{max} **cm^{-1}**: 1640, 1680, 1695 (–NC=O–).
^{1}H NMR (200 MHz, $CDCl_3$): 0.97 (3H, t, *J* 7.2, CH_3–$(CH_2)_4$–), 1.35 (4H, m, $CH_3CH_2CH_2$), 1.6 (2H, m, $–CH_2CH_2CH_2–N–$), 3.5 (3H, s, $–NCH_3$), 3.56 (3H, s, $–NCH_3$), 3.97 (2H, t, *J* 7.2, $–NCH_2–$).
^{13}C NMR (75 MHz, $CDCl_3$): 13.9, 22.3, 27.6, 29.0, 29.5, 33.4, 41.0, 107.0, 141.0, 148, 151, 155.
LRMS (*m/z*): 250 (M^+, 70%), 180 (M^+ -C_5H_{11}, 100%).
HRMS: found M^+, 250.1435, $C_{12}H_{18}N_4O_2$ requires 250.1429.

1-(2-Hydroxyethyl)-3,7-dimethyluric acid (XVIII)
Mp: 255° C ($CHCl_3$–MeOH).
IR (Nujol) ν_{max} **cm^{-1}**: 3300 ($–CH_2OH$), 1640, 1680, 1690 (–NC=O–);
^{1}H NMR (90 MHz, DMSO-d_3): 3.58 (6H, s, 2 ×-NCH_3), 3.8 (2H, t, *J* 7.2, $–CH_2–OH$), 4.15 (2H, t, *J* 7.2, $–CH_2N–$).
^{13}C NMR (22.5 MHz, DMSO-d_3): 29.0, 30.8, 45.6, 59.0, 97.0, 136.1, 150.2, 153.3, 153.9.
LRMS (*m/z*): 240 (M^+, 70%), 196 (M^+ -CH_2-CH_2OH, 60%), 153 (65%), 82 (100%).
HRMS: found M^+, 240.0852, $C_9H_{12}N_4O_4$ requires 240.0858.
C,H&N analysis: Found: C, 44.89; H, 4.92; N, 23.12; $C_9H_{12}N_4O_4$ requires C, 45.0, H, 5.0, N, 23.37%).

1-Benzyl-3,7-dimethyluric acid (XIX)
Mp: 315° C ($CHCl_3$–MeOH).
IR (Nujol) ν_{max} **cm^{-1}**: 1640, 1680, 1695 (–NC=O–).
^{1}H NMR (200 MHz, $CDCl_3$): 3.4 (3H, s, $–NCH_3$), 3.5 (3H, s, $–NCH_3$), 5.0 (2H, s, $–NCH_2–$), 7.2–7.4 (5H, m, aromatic)
^{13}C NMR (22.5 MHz, DMSO-d_3): 28.0, 30.8, 43.0, 97.0, 126.9, 127.3, 127.6, 136.9, 137.5, 149.8, 151.6, 152.5.
LRMS (*m/z*): 286 (M^+, 100%), 195 (30%), 91 (C_6H_5, 75%).
HRMS: found M^+, 286.2896. $C_{14}H_{14}N_4O_3$ requires 286.2899
C,H&N analysis: Found: C, 58.64; H, 4.81; N, 19.48. $C_{14}H_{14}N_4O_3$ requires C, 58.74; H, 4.89; N, 19.58%.

1-(5-Oxohexyl)-3,7-dimethyluric acid (XX)
Mp: 223–225 ° C ($CHCl_3$–MeOH).
IR (Nujol) ν_{max} **cm^{-1}**: 1700 (–C=O–), 1640, 1680 (–NC=O–).
^{1}H NMR (300 MHz, $CDCl_3$): 1.65 (4H, m, $–CH_2CH_2$), 2.1 (3H, s, CH_3–CO–), 2.5 (2H, t, *J* 7.2, $–CO–CH_2–$), 3.5 (3H, s, $–NCH_3$), 3.57 (3H, s, $–NCH_3$), 3.9 (2H, t, *J* 7.2, $–NCH_2–$).
^{13}C NMR (22.5 MHz, $CDCl_3$): 21.0, 27.4, 28.9, 29.7, 30.8, 41.6, 43.0, 99.2, 136.0, 150.2, 153.3, 153.5, 208.0.
LRMS (*m/z*): 294 (M^+, 100%), 196 (M^+ -$C_6H_{11}O$, 95%), 153 (70%), 82 (55%).
HRMS: found M^+, 294.1295. $C_{13}H_{18}N_4O_4$ requires 294.1325

C,H&N analysis: Found: C, 52.88; H, 6.04; N, 18.95. $C_{13}H_{18}N_4O_4$ requires C, 53.06; H, 6.12; N, 19.04%.

1-(2-Oxopropyl)-3,7-dimethyluric acid (XXI)
Mp: 240° C ($CHCl_3$–MeOH).
IR (Nujol) ν_{max} **cm^{-1}**: 1700 (–C=O–), 1640, 1680 (–NC=O–).
^{1}H NMR (90 MHz, $CDCl_3$): 2.1 (3H, s, CH_3CO–), 3.5 (3H, s, –NCH_3), 3.57 (3H, s, –NCH_3), 4.7 (2H, s, –NCH_2–).
^{13}C NMR (75 MHz, $CDCld_3$): 28.0, 30.6, 33.8, 47.8, 98.2, 136.1, 150.0, 153.3, 153.5, 204.0.
LRMS (*m/z*): 252 (M^+, 100%), 209 (M^+ -CH_3CO, 75%), 196 (M^+ -C_3H_5O, 85%).
HRMS: found M^+, 252.0852, $C_{10}H_{12}N_4O_4$ requires 252.0858.

1-Allyl-3,7-dimethyluric acid (XXII)
Mp: 305–306 ° C ($CHCl_3$–MeOH).
IR (Nujol) ν_{max} **cm^{-1}**: 1640, 1690 (–NC=O–).
^{1}H NMR (200 MHz, $CDCl_3$): 3.5 (3H, s, –NCH_3), 3.56 (3H, s, –NCH_3), 4.59 (2H, td, *J* 4.5, 13, –N–CH_2–CH=CH_2), 5.2 (1H, d, *J* 10, *cis* CH=CH_2), 5.3 (1H, d, *J* 17, *trans* CH=CH_2), 5.97 (1H, t of dd, *J* 17, 10.3, 4.5, C*H*=CH_2).
^{13}C NMR (22.5 MHz, DMSO-d_3): 28.2, 30.9, 42.5, 97.8, 116.5, 133.0, 136.8, 149.5, 151.8, 152.3.
LRMS (*m/z*): 236 (M^+, 100%), 219 (M^+ -H_2O, 15%), 153 (40%), 82 (45%).
HRMS: found M^+, 236.0900. $C_{10}H_{12}N_4O3$ requires 236.0909.

1-Prop-2-ynyl-3,7-dimethyluric acid (XXIII) (1-Propargyl-3,7-dimethyluric acid)
Mp: 276 ° C ($CHCl_3$–MeOH).
IR (Nujol) ν_{max} **cm^{-1}**: 1640, 1680 (–NC=O–).
^{1}H NMR (300 MHz, $CDCl_3$): 2.1 (1H, t, *J* 2.4, HC=C–CH_2), 3.5 (3H, s, –NCH_3), 3.59 (3H, s, –NCH_3), 4.6 (2H, d, *J* 2.7, –NCH_2–).
^{13}C NMR (22.5 MHz, $CDCl_3$): 29.7, 30.3, 34.0, 71.4, 79.1, 97.0, 136.0, 150.7, 153.0, 153.8.
LRMS (*m/z*): 234 (M^+, 100%), 153 (25%), 84 (45%), 82 (85%), 67 (57%).
HRMS: Found: found M^+, 234.0749, $C_{10}H_{10}N_4O_3$ requires 234.0752.

1-But-3-enyl-3,7-dimethyluric acid (XXIV) (1-Butenyl-3,7-dimethyluric acid)
Mp: 262 ° C ($CHCl_3$–MeOH).
IR (Nujol) ν_{max} **cm^{-1}**: 1640, 1690 (–NC=O–).
^{1}H NMR (90 MHz, $CDCl_3$): 2.4 (2H, q, CH_2=CH–CH_2–CH_2), 3.5 (3H, s, –NCH_3), 3.58 (3H, s, –NCH_3), 4.0 (2H, t, *J* 7.2, –N–CH_2–CH_2), 5.0 (1H, d, *J* 10, *cis* CH=CH_2), 5.2 (1H, d, *J* 17, *trans* CH=CH_2), 5.8 (1H, t of dd, *J* 17, 10.3, 4.5, C*H*=CH_2).
^{13}C NMR (22.5 MHz, $CDCl_3$): 28.0, 30.7, 31.8, 40.3, 97.8, 116.6, 135.1, 136.4, 149.5, 151.5, 152.5.
LRMS (*m/z*): 250 (M^+, 40%), 196 (M^+ -C_4H_7, 100%), 153 (45%), 82 (50%).
HRMS: found M^+, 250.1061. $C_{11}H_{14}N_4O_3$ requires 250.1065.

Table 4: Purification steps of caffeine oxidase from mixed culture

Fractionation step	Total protein (in mg)	Specific activity[1] (µmols/min/mg)	Fold purification	% yield
Crude enzyme	270.4	0.18	--	100
DEAE- cellulose	32.1	0.66	4.0	43.7
Phenyl sepharose	0 .70	7.9	44.0	11.4

[1]***Micromoles of DCPIP reduced per minute per mg of protein: Assay was carried out as described under materials and methods using caffeine (500 µM) as substrate.***

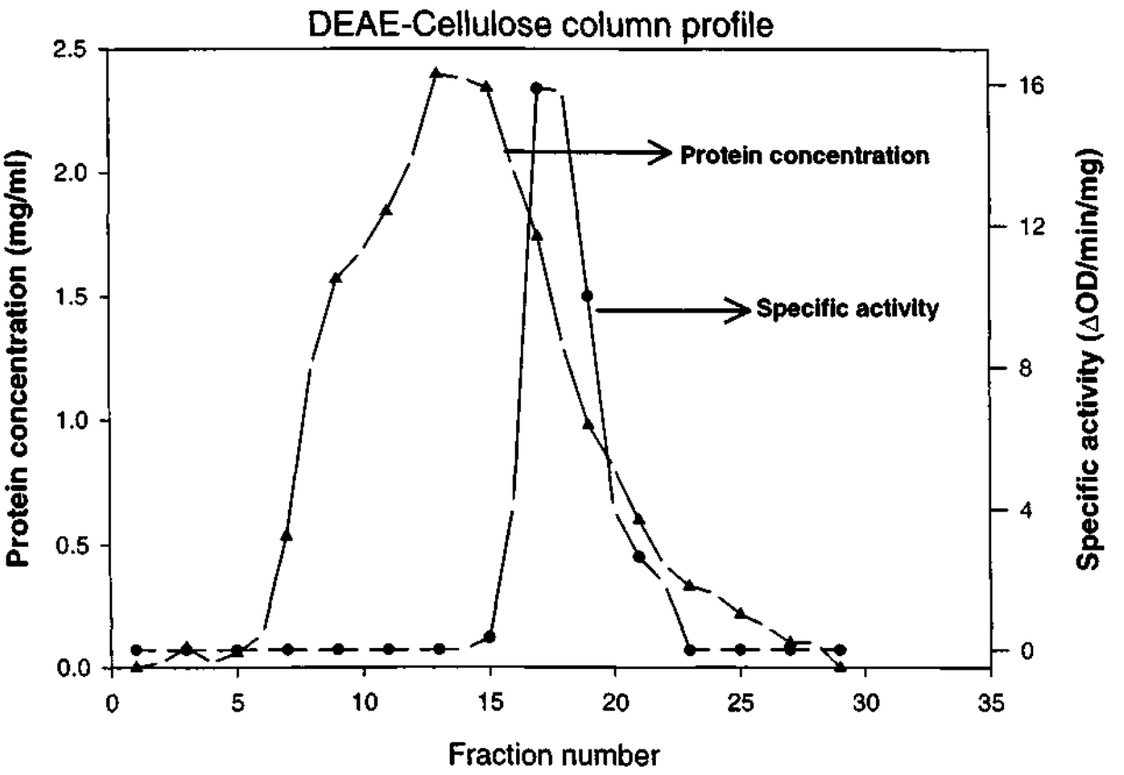


Fig. 3: DEAE-Cellulose column profile

C,H&N analysis: Found: C, 52.73; H, 5.58; N, 22.18. $C_{11}H_{14}N_4O_3$ requires C, 52.8; H, 5.6; N, 22.4%.

3-Propyl-1,7-dimethyluric acid (XXV)
Mp: 282° C ($CHCl_3$–MeOH).
IR (Nujol) ν_{max} **cm^{-1}**: 1640, 1680 (–NC=O–).
^{1}H NMR (90 MHz, $CDCl_3$): 1 (3H, t, *J* 7.2, CH_3CH_2–), 1.6 (2H, m, $CH_3CH_2CH_2$–), 3.4 (3H, s, $-NCH_3$), 3.5 (3H, s, $-NCH_3$), 3.9 (2H, t, *J* 7.2, $-NCH_2$–).
^{13}C NMR (75 MHz, $CDCl_3$): 10.8, 21.6, 28, 28.9, 46.0, 99.2, 135.7, 150.0, 153.0, 153.9.
LRMS (*m/z*): 238 (M^+, 100%), 196 (M^+ -C_3H_7, 70%), 152 (M^+ -$C_3H_4NO_2$, 50%), 139 (65%).
HRMS: found M1, 238.1059, $C_{10}H_{14}N_4O_3$ requires 238.1065.

3-Propyluric acid (XXVI)
Mp: 279° C ($CHCl_3$–MeOH).
IR (Nujol) ν_{max} **cm^{-1}**: 1640, 1680 (–NC=O–).
^{1}H NMR (90 MHz, $CDCl_3$): 0.95 (3H, t, *J* 6.6, CH_3CH_2–), 1.64 (2H, m, $CH_3CH_2CH_2$–), 3.9 (2H, t, *J* 7.5, $-NCH_2$–), 11.0 (1H, s, –NH).
^{13}C NMR (22.5 MHz, $CDCl_3$): 10.9, 21.5, 44.5, 98.9, 137.7, 151, 153.4, 153.9.
LRMS (*m/z*): 210 (M^+, 100%), 168 (M^+ -C_3H_7, 95%), 137 (M^+ -$C_2H_2NO_2$, 75%).
HRMS: found M1, 210.0748, $C_8H_{10}N_4O_3$ requires 210.0752.

Growth of mixed culture

The ability of mixed culture consortium to grow on caffeine, a purine alkaloid, was investigated. Maximum growth was noticed at 18h and complete mineralization of caffeine was found to take place at the end of 36hours. Hence the cells were harvested after 36h. Cell-free extracts of caffeine grown mixed culture possessed significant caffeine oxidase activity. The activity was located in the soluble fraction of the cell lysate.

Enzyme purification

A simple two step procedure involving ion exchange and hydrophobic column chromatographies were employed to purify caffeine oxidase to homogeneity. The results are summarized in **Table 4.** The adapted protocol resulted in nearly 44-fold increase in the specific activity with 11.3% yield. The values presented here are the average of several independent preparations, and variations between different batches are minimum.

The crude cell-free extract was directly loaded on DEAE-cellulose column previously equilibrated with phosphate buffer (0.05 M, pH 7.8). The protein was eluted using a linear gradient of 0 to 0.6 M NaCl in the phosphate buffer. Though we did not get

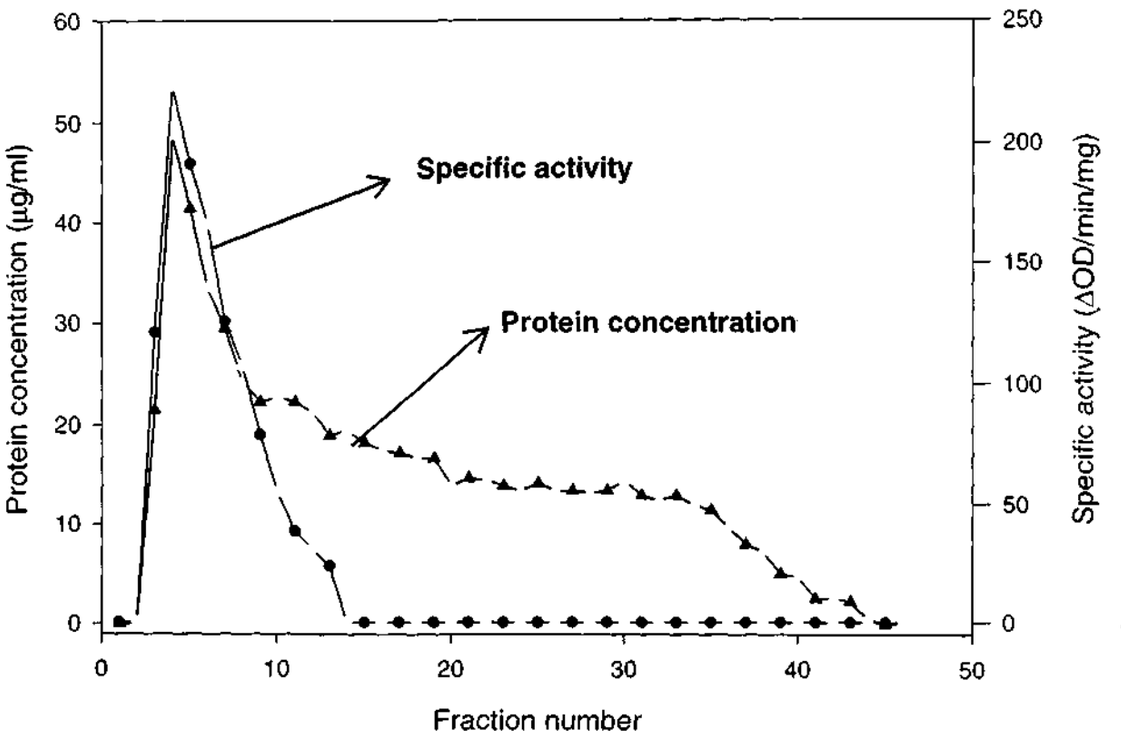


Fig. 4: Phenyl sepharose column profile

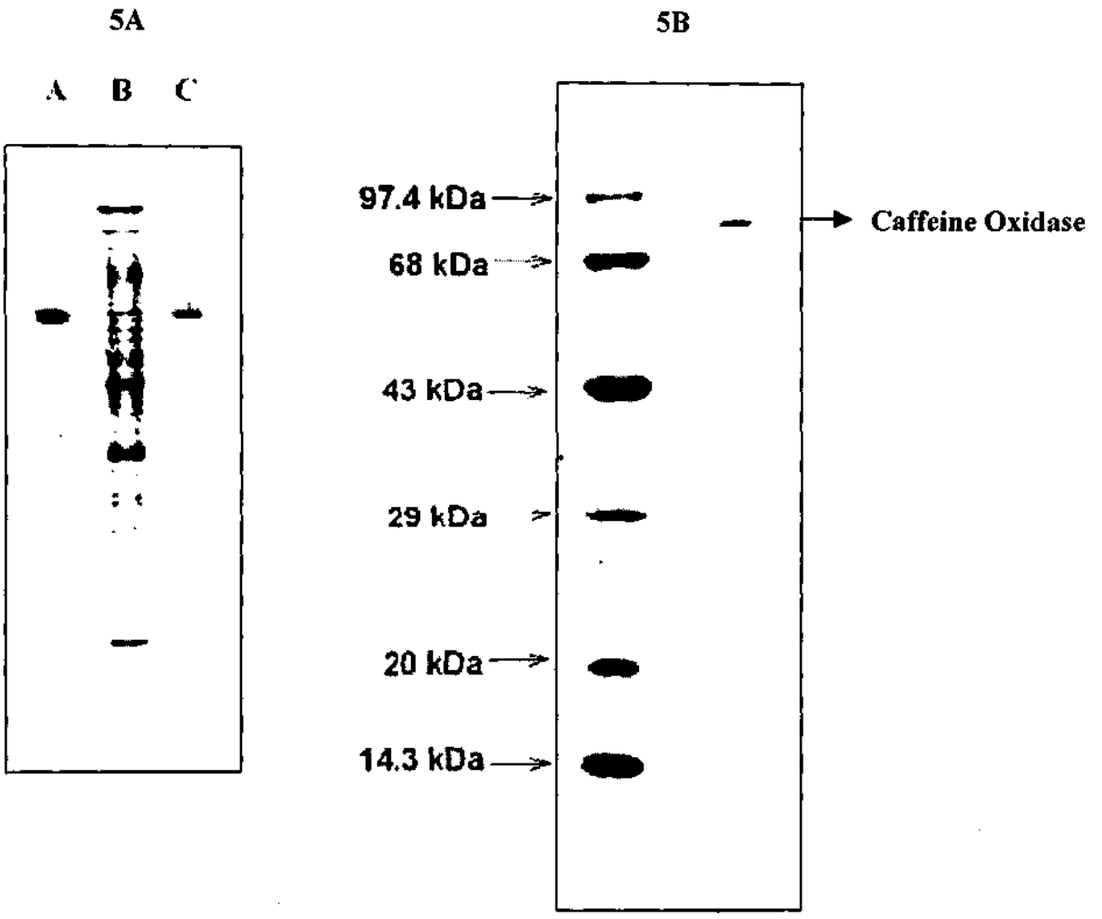


Fig. 5A: Native PAGE of the caffeine oxidase carried out under non-denaturing conditions. Lane A, enzyme activity staining of purified protein (4 μg); Lane B, crude protein (10 μg) and Lane C, purified protein (10 μg). Lane B and C stained by Coomassie Brilliant Blue.

Fig. 5B: SDS-PAGE of purified protein on 10% polyacrylamide gel. Lane A, Standard protein markers and Lane B, purified protein (20 μg) stained by Coomassie Brilliant Blue. Details are as described under methods.

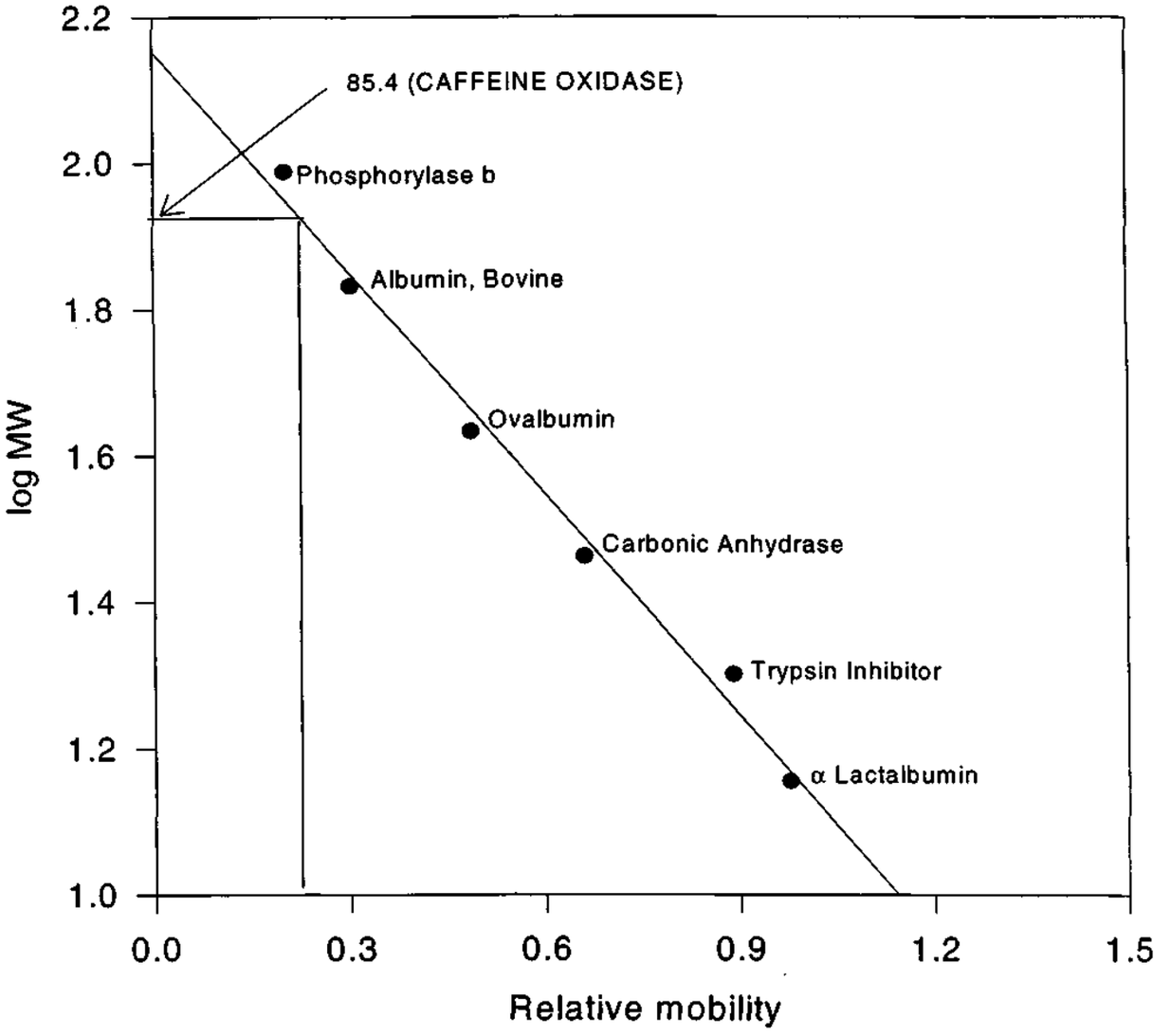


Fig. 5C: Molecular weight of pure caffeine oxidase by SDS-PAGE

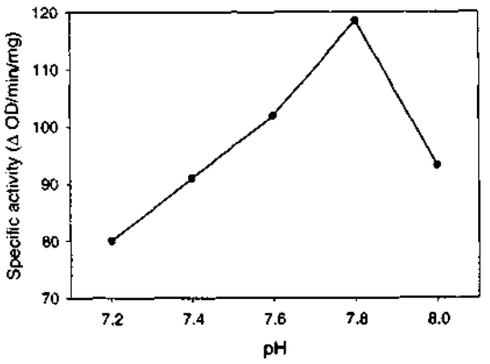


Fig. 6: Effect of pH on caffeine oxidase

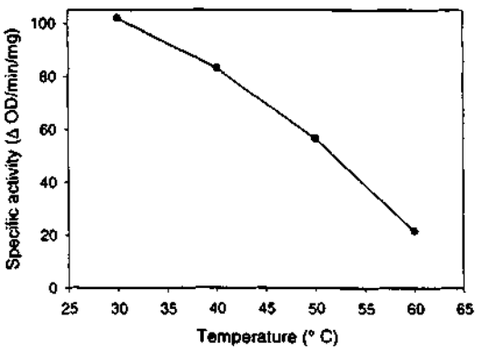


Fig. 7: Effect of temperature on caffeine oxidase activity

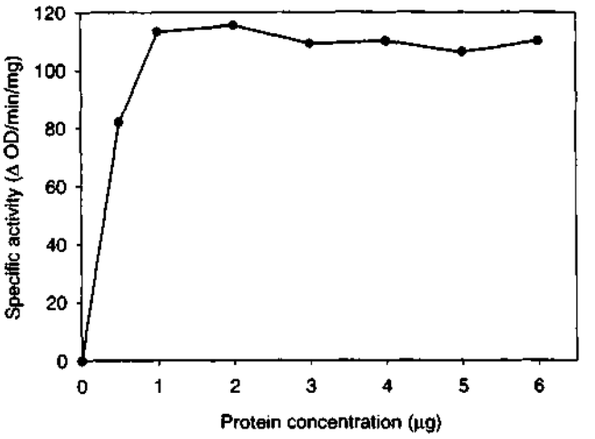


Fig. 8: Effect of protein concentration on caffeine oxidase activity

much increase in the fold purification (3.6) in the DEAE-cellulose column, 43% of the total activity was recovered (**Fig. 3**). After the DEAE-cellulose column, the active fractions were loaded on to phenylsepharose column previously equilibrated with phosphate buffer (0.05 M, pH 7.8 + 10% glycerol). The protein was eluted with phosphate buffer (0.005 M, pH 7.8 + 10% glycerol) as a sharp peak (**Fig. 4**). The fractions containing caffeine oxidase were pooled and concentrated by ultra filtration using Amicon PM 10 membrane filter. This yielded a homogenous protein (43.8 fold purification, 11.3% yield), as seen from the gel electrophoresis.

Purity and molecular mass

The PAGE of the pure enzyme obtained under non-denaturing conditions revealed a single band as shown in **Fig. 5 A** and enzyme activity staining confirmed that the enzyme had been purified to homogeneity. SDS-PAGE carried out under denaturing conditions also revealed a single band **Fig. 5B**. These results confirm that the enzyme has been purified to homogeneity. The subunit molecular mass of the enzyme was estimated to be ~85 kDa based on mobility relative to standard molecular weight markers (**Fig: 5C**).

Enzyme properties

Effect of pH: The effect of pH on caffeine oxidase was investigated over a range of 7.2 to 8.0. The optimum pH for the conversion of caffeine to 1,3,7-trimethyluric acid was found to be 7.8 (**Fig. 6**).

Effect of temperature: The activity of the enzyme was measured at various temperatures ranging between 30-60° C. The optimum temperature was found to be 30° C. The enzyme looses activity above 30° C (**Fig. 7**).

Effect of protein concentration: Caffeine oxidase activity was measured against various concentrations of protein at constant substrate and DCPIP/PES concentration. Under the standard assay conditions, it was noticed that, 2 μg of protein was sufficient to get the maximum activity (**Fig. 8**).

Effect of caffeine concentration on enzyme activity: Caffeine oxidase activity was measured against various concentrations of caffeine (0.04-1.5 mM) at constant enzyme (2.5 μg) and DCPIP/PES concentrations. Under the standard assay conditions, it was

Table 5: Activity of caffeine oxidase with various electron acceptors

Electron acceptor	Concentration (in mM)	nm	Specific activity[1] (μmols/min/mg)
DCPIP	0.04	600	3.9.0 ± 0.12
Cyt c	0.1	550	4.6 ± 0.6
NBT	0.1	540	0.04 ± 0.004
NAD^+	0.1	340	0.0
$NADP^+$	0.1	340	0.0
O_2	dissolved O_2	302	0.46 ± 0.05

[1]***Each assay contained purified enzyme (1.75 μg), caffeine(500 μM) and indicated amount of various electron acceptors. The other details are as mentioned in the text.***

Table 6: Effect of various compounds on caffeine oxidase

Inhibitors	% Inhibition[¶]
Caffeine alone	0.0
NaN_3(1mM)	2.1 ± 1.9
α-α'-Dipyridyl(1mM)	12.4 ± 2.5
H_2O_2(1mM)	24.0 ± 1.1
p-CMB(1mM)	0.0
N-Methylmaleimide(1mM)	6.0 ± 1.2
Iodoacetamide(1mM)	5.6 ± 1.6
O-Phenanthroline(0.35mM)	89.1 ± 0.9
Benzimidazole(1mM)	4.2 ± 3.0
Salicylic acid(1mM)	5.4 ± 3.4
Sodium Arsenite(1mM)	7.2 ± 2.7
Methanol(1mM)	18.9 ± 1.8

[¶]***The enzyme (1.75 μg) was preincubated with various compounds for 5 min at 30°C and then the assays was carried out as described under methods. In each assay 500μM of caffeine was used. 100% activity (0% inhibition) represents 3.9 μmol of DCPIP reduced per min per mg***

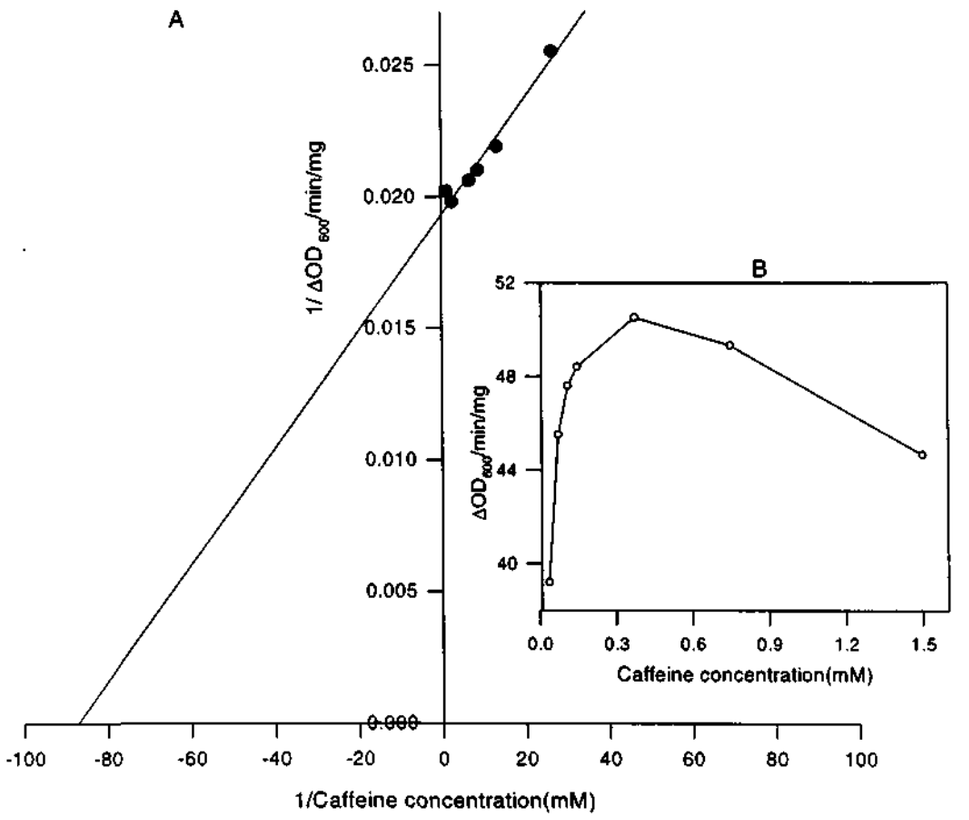


Fig. 9: Effect of caffeine on caffeine oxidase activity. A) Lineweaver-Burk representation and B) normal representation. The reaction mixture contained in a final volume of 1.0 ml, 0.05 M potassium phosphate pH 7.8, 40 μM DCPIP, 40 μM PES, 1.75 μg of caffeine oxidase and varying concentrations of caffeine (0.0375 to 1.5 mM).

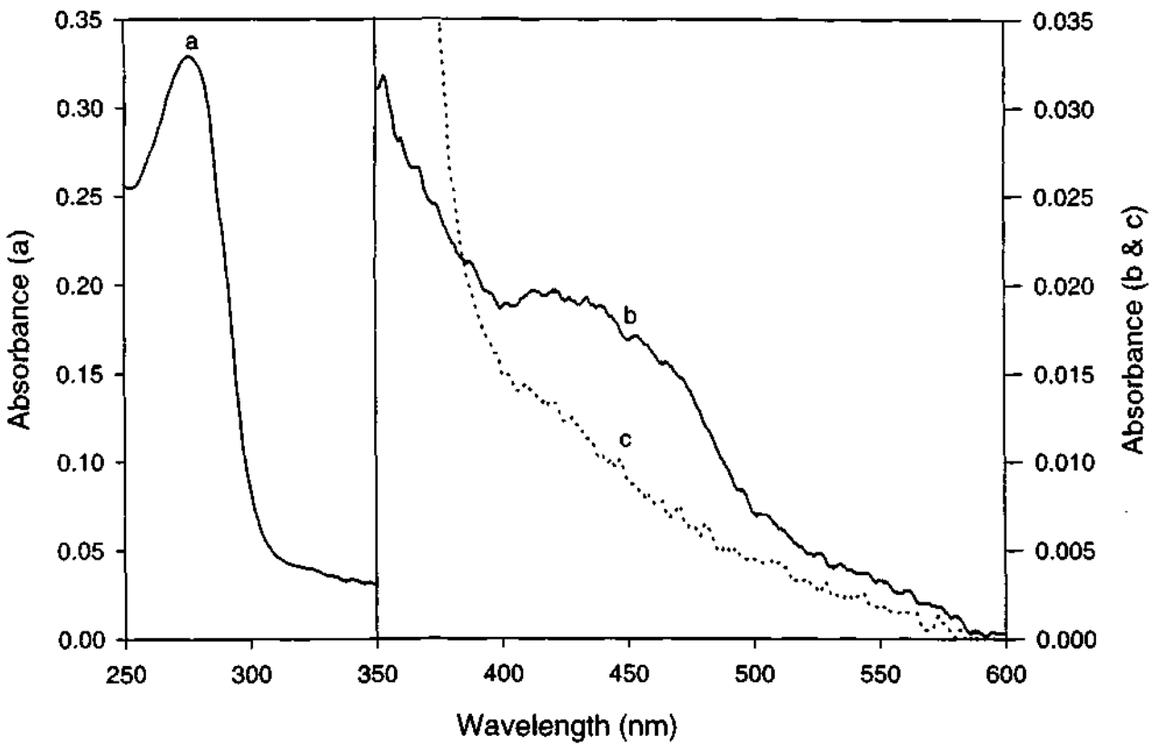


Fig. 10: Absorption spectra of the purified caffeine oxidase (b) oxidized form and (c) hydrosulfite reduced form

noticed that 0.375 mM of caffeine was sufficient to get the maximum activity (**Fig. 9, inset**). At higher concentrations of caffeine, the enzyme gets inactivated.

Activity with various electron acceptors: Caffeine oxidase activity was determined aerobically. The assay mixture in a total volume of 1 ml of phosphate buffer (pH 7.8, 0.05 M) contained caffeine (0.5 mM), protein (1.75 μg) and one of the electron acceptors listed in **Table 5**. Activity was determined by measuring absorbance changes at the wavelengths indicated and the results are summarized in **Table 5**.

Kinetic parameters: Michaelis-Menten constant (k_m) value was determined for caffeine. Data were obtained by Lineweaver-Burk plots, using duplicate assays at five subsaturating concentrations. The k_m value calculated for caffeine was 11. 4 μM (**Fig. 9**).

Inhibitor studies: Various potential inhibitors were tested against caffeine oxidase activity of the purified enzyme (**Table 6**). Caffeine oxidase activity was inhibited by metal-chelating agent α,α'-bipyridyl and O-Phenanthroline, and the thiol reagent idoacetamide. The other inhibitors like H_2O_2, benzimidazole, salicylic acid, sodium arsenite, sodium azide and methanol inhibited the caffeine oxidase activity but not above 40%.

Spectral characteristics: Absorption spectra of the native and hydrosulfate reduced forms of the enzyme are shown in **Fig. 10**. The native enzyme exhibited absorption maxima at 280 and 450 nm. Reduction of the enzyme resulted in the elimination of the absorption maxima at 450 nm. The spectral characteristics suggest that the enzyme is an iron containing flavoprotein.

Substrate specificity: Substrate specificity of the purified caffeine oxidase was examined with a variety of 1-, 3-, and 7-substituted xanthines including xanthine, hypoxanthine, and adenine (**Table 3**). The activity was assayed by DCPIP reduction, in the presence of PES, and expressed relative to caffeine since maximum activity was noticed with this substrate. The caffeine oxidase accepted substituted xanthines with substitutions at the 1-, 3-, and 7-positions as substrates (**Table 3**). Theobromine (3,7-dimethylxanthine) with N-1H replaced by various groups such as alkyl, hydroxyethyl, benzyl, 5-oxohexyl, allyl, propargyl, and butenyl served as good substrates whereas theophylline (1,3-dimethylxanthine) analogues with N-7H replaced with alkyl substitution higher than methyl for example, ethyl, propyl, and butyl, were not accepted by the enzyme as substrates. This could be due to the

proximity of the bulky substituents to the C-8 position. Replacement of N-3 methyl in caffeine with higher alkyl group as in 3-propyl-1,7-dimethylxanthine (1,7-dimethylenprofylline) served to be a good substrate for caffeine oxidase. HPLC analysis indicated the formation of C-8 oxidized compounds (data not shown).

DISCUSSION

Chemical synthesis of 1,3,7-trimethyluric acid involves a lengthy procedure with stringent experimental conditions. There are many reports on the synthesis of 8-oxopurines and one of the limitations of these methods is that, most of the purines are insoluble in organic solvents, and hence they should be protected as acetyl or trityl groups to increase the solubility and hence the number of steps involved in the synthesis increases. The methods developed for the chemical synthesis of these compounds are not satisfactory since they involve the usage of expensive reagents, yields are varying and suitable for compounds soluble in organic solvents (27,28), but not for water-soluble ones.

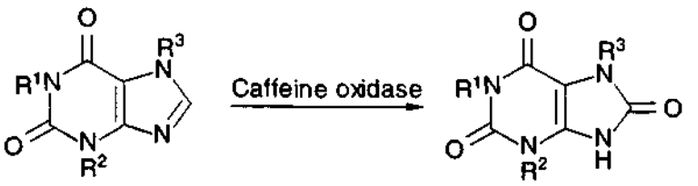
In the present study we have prepared novel uric acid analogues by using a mixed culture consortium consisting of strains belonging to the genera *Klebsiella* and *Rhodococcus*. It has been reported that, this mixed culture consortium efficiently carries out C-8 oxidation of caffeine, theobromine, theophylline and paraxanthine (45,46). Recently it has also been shown that, this mixed culture consortium has broad substrate specificity and it accepts 1-, 3-, and 7- substituted xanthines and converts them to their corresponding 8-oxo derivatives (46). In fact, mixed microbial cultures are used with success in the microbial synthesis of vitamins, antibiotics, dehalogenation of organochlorine insecticides, degradation of benzene, chlorinated biphenyls, alkaloid cocaine, etc. Substituted theobromines were used as substrates, where N-1 H is replaced by various groups such as alkyl, hydroxy ethyl, benzyl, 5-oxohexyl, 2-oxopropyl, allyl, propargyl, and butenyl. This mixed culture consortium quantitatively converts all the substituted theobromines into corresponding C-8-oxidized compounds. The compounds were fully characterized by various spectral analyses and as far as we know, these uric acids are hitherto not known. Earlier it was reported that, the optimal size of alkyl substituent at N-1 position is pentyl and higher than pentyl group at N-1 position in

xanthine will not be accepted as a substrate (46). However, pentoxifylline with a $-(CH_2)_4COCH_3$ substitution at N-1 position is readily accepted by this mixed culture and is quantitatively converted into corresponding C-8 oxidized product.

Enzymatic hydroxylation at the C-8 position of xanthine derivatives is effected either by a mixed function oxidase or xanthine oxidase (63). We have noticed that, cell-free extract prepared from the mixed culture readily carries out C-8 oxidation of various substituted xanthines in the presence of PES (phenazine ethosulfate) (data not presented). So it is quite possible that, the C-8 oxidation is mediated by xanthine oxidase. However, xanthine oxidases isolated from various microbial sources have never been shown to accept trimethyl- and dimethylxanthines as substrates (64,65). The exceptional ability of the mixed culture to convert 1,3,7-trimethylxanthine and various 1-, 3-, and 7- substituted xanthines to their corresponding 8-oxo derivatives in quantitative yields clearly distinguishes this xanthine oxidase from the previously reported xanthine oxidases from various microbial sources. Hence the caffeine oxidase from the mixed culture appears to be unique and novel. Its substrate specificity is distinctly different from that of xanthine oxidase. As far as we know, such an enzyme has never been reported so far and hence it was of interest to purify caffeine oxidase and characterize to whatever extent that was possible.

Xanthine oxidase plays a vital role in carrying out oxidation of various purines and aza compounds (50). The xanthine oxidase isolated from various sources accepts xanthine and monomethylxanthine, but it has never been shown to accept caffeine (1,3,7-trimethylxanthine), theobromine or theophylline (dimethylxanthines) as substrates. We report here a simple procedure for the purification of a novel enzyme, caffeine oxidase from caffeine induced mixed culture consisting of strains belonging to the genera *Klebsiella* and *Rhodococcus*.

The present study, for the first time, reports the C-8 oxidation of caffeine to 1,3,7-trimethyluric acid and this reaction is mediated by caffeine oxidase, an enzyme that appears to be hitherto not known. Enzymatic hydroxylation at C-8 position of xanthine derivatives is effected either by a mixed function oxidase or xanthine oxidase. Such a transformation is known to take place in the mammalian system and the reaction is



Caffeine: $R^1=R^2=R^3=CH_3$

Theobromine: $R^1=H$, $R^2=R^3=CH_3$

Theophylline: $R^1= R^2=CH_3$, $R^3=H$

Fig. 12: C-8 oxidation of caffeine, theobromine and theophylline to the corresponding uric acids by caffeine oxidase.

catalyzed by the hepatic cytochrome P450 system (53,54,67). In the present study, it has been noticed that, the cell free extract prepared from mixed culture cells grown on caffeine is devoid of cytochrome P450. We have demonstrated that, the C-8 oxidation of caffeine to 1,3,7-trimethyluric acid does not require any reduced pyridine nucleotide but requires an artificial electron acceptor such as PES. From our results it is abundantly clear that, the transformation of caffeine into 1,3,7-trimethyluric acid is not mediated by cytochrome P450 system. So we feel it is reasonable to conclude that, if the C-8 oxidation of caffeine and related compounds is catalyzed by a xanthine oxidase, then it must be a unique xanthine oxidase having properties significantly different from what has been reported earlier for various xanthine oxidases isolated from different sources. Since caffeine is the most preferred substrate, we have named it as caffeine oxidase. It is to be noted here that, xanthine oxidase does not accept caffeine as the substrate.

Cell-free extract prepared from caffeine grown mixed culture cells (36 h) was shown to convert caffeine, theobromine and theophylline and other 1-, 3-, and 7-substituted theobromines (**Table 1**) to their respective 8-oxo compounds in the presence of PES, an electron acceptor (**Fig. 12**). The metabolic conversion of these xanthines to their corresponding uric acids was confirmed by performing large scale incubations as described under methods and isolating the uric acids as reported earlier (45,46), and comparing their HPLC profile as well as mass spectral data with the authentic samples (46). The rates of C-8 oxidation with caffeine, theobromine and theophylline were 0.31, 0.16 and 0.05 μmol per minute per mg protein. Glucose grown mixed culture cells failed to bring about C-8 oxidation suggesting the inducible nature of caffeine oxidase.

A simple, two step procedure involving ion exchange and hydrophobic column chromatographies was employed to purify caffeine oxidase to homogeneity. A typical purification profile of the enzyme is shown in **Table 4**. Although considerable activity was lost during the purification procedure, the specific activity of the enzyme was significantly enhanced after the phenyl sepharose column chromatography. The enzyme was purified about 44 fold from the crude extract with a yield of 11.4%.

The purified enzyme, upon PAGE carried out under non-denaturing conditions, showed only one protein band, which coincided with the band of enzyme activity (**Fig.**

5A). SDS-PAGE of the purified enzyme showed a single protein band and the subunit molecular mass of the enzyme was estimated to be approx. 85 kDa based on mobility relative to standard proteins (**Fig. 5B**). The subunit molecular mass of caffeine oxidase is close to the value reported for xanthine oxidase from *Arthrobacter* (64), but differs from xanthine oxidase from human liver (52). The spectrum of the purified caffeine oxidase exhibited absorption maxima at 280 and 450 nm and minimum at 410 nm. Reduction of the enzyme with sodium hydrosulfite eliminated the absorption maximum at 450 nm (**Fig. 10**). The spectral characteristics are similar to those of xanthine oxidases (64-66). The purified enzyme shows maximum activity at 30° C and it loses nearly 20, 50 and 80% of the original activity at 40, 50 and 60° C, respectively. Caffeine oxidase has a pH optimum of 7.8. It loses significant amount of activity below pH 7.0 and above pH 8.0.

The effect of caffeine concentration on the enzyme activity is given in **Fig. 9** and it follows a normal Michaelis-Menten kinetics. The kinetic analysis revealed that caffeine oxidase has an apparent K_m of 11.4 μM for caffeine.

Cytochrome c and DCPIP have been shown to be effective electron acceptors for caffeine oxidase (**Table 5**). However, NAD^+ and $NADP^+$ did not serve as electron acceptors like xanthine oxidase from *Arthrobacter* (64) and *Enterobacter cloacae* (65). Ferricyanide, NBT and O_2 served as poor electron acceptors for caffeine oxidase unlike xanthine oxidase from *Arthrobacter* (64). The DCPIP reduction assay was used to find out the effect of several compounds on caffeine oxidase (**Table 3**).

It was noticed that, among the compounds tested, O-phenanthroline was shown to be the most potent inhibitor whereas H_2O_2, bipyridyl and methanol produced only marginal inhibition. Compounds such as PCMB, iodoacetamide, N-methylmaleimide, salicylate and sodium arsenite, which are the known inhibitors of xanthine oxidase (54), did not inhibit caffeine oxidase. Our results suggest that a metal center may be involved in the active site or is necessary for stability of the enzyme. Certainly, there are some similarities between caffeine oxidase and xanthine oxidase isolated so far, particularly, the absorption spectrum, suitability of electron acceptors. etc. However, it differs from xanthine oxidases in the fact that, known inhibitors of xanthine oxidase fail to inhibit caffeine oxidase. Also molecular

mass of some of the xanthine oxidases isolated appears to be different from what has been determined for caffeine oxidase.

All the xanthine oxidases (54) isolated from different sources so far have never been shown to accept caffeine (1,3,7-trimethylxanthine) or dimethylxanthine (theobromine or theophylline) as substrates. The most preferred substrate for xanthine oxidase is xanthine. However, xanthine oxidase isolated from cow's milk was shown to accept 1-methylxanthine as substrate, converting it to 1-methyluric acid. Caffeine oxidase purified from mixed culture exhibited wide substrate specificity, as it accepted various substituted xanthines with substitution at 1-, 3-, and 7- positions. Theobromine (3,7-dimethylxanthine) with N-1H replaced by various groups such as alkyl, hydroxyethyl, benzyl, 5-oxohexyl, allyl, propargyl, and butenyl served as good substrates whereas theophylline (1,3-dimethylxanthine) analogues with N-7H replaced with alkyl substitution higher than methyl for example ethyl, propyl, and butyl, were not accepted by the enzyme as substrates. This could be due to the proximity of the bulky substituents to the C-8 position. Replacement of N-3 methyl in caffeine with higher alkyl groups as in 3-propyl-1,7-dimethylxanthine (1,7-dimethylenprofylline) served to be a good substrate for caffeine oxidase. We have demonstrated quantitative conversion of all the substrates presented in **Table 3** to their respective 8-oxo compounds (uric acids) using resting cells of mixed culture grown on caffeine. It is known that, the time required for the quantitative conversion is different for each substrate and 8-oxo compounds formed in each case do not inhibit caffeine oxidase. The substrate specificity is one of the most significant differences between caffeine oxidase and various xanthine oxidases isolated so far. In fact, the exceptional ability of the caffeine oxidase from mixed culture to convert caffeine and various 1-, 3-, and 7- substituted xanthines to their corresponding 8-oxo derivatives in quantitative yields clearly distinguishes this caffeine oxidase from the previously reported xanthine oxidase isolated from various microbial and mammalian sources.

In conclusion, we have purified caffeine oxidase to homogeneity and this enzyme carries out C-8 oxidation of various substituted xanthines. The distinguishing features of this enzyme are its wide substrate specificity and not being inhibited by the known inhibitors of xanthine oxidase. The caffeine oxidase used as an efficient reagent to carryout

the synthesis of 8-oxo derivatives of various alkylxanthines merits attention because of the numerous applications of these compounds in the formulations of drugs and cosmetics. Such 8-oxo derivatives are not easy to prepare by conventional chemical synthesis methods.

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CHAPTER 3: PART B

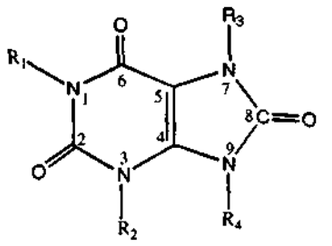
Efficient Scavenging of Hydroxyl Radicals and Inhibition of Lipid Peroxidation by Novel Analogues of 1,3,7-Trimethyluric Acid

INTRODUCTION

Oxygen-containing free radicals are the causative agents in aging, cancer, heart diseases and inflammation (1-4). These radicals interact with membrane lipids and initiate the chain reaction of lipid peroxidation leading to tissue damage. It is interesting to note that, in the biological system there is an in-built defense mechanism against free radical-induced deleterious effects through the scavenging effects of various endogenous antioxidants. One of the effective endogenous antioxidants, which participate in the prevention of oxidative damage in humans, is uric acid (5). It has been shown that uric acid and related analogues at concentrations similar to its physiological levels, suppress oxidative degradation of low density lipoprotein (LDL) components (6), inhibit oxygen free radical-induced DNA damage (7), function as efficient antioxidant and free radical scavengers (5,8,9) and protect erythrocyte membranes from lipid peroxidation (10,11). The antioxidant activity of uric acid has been attributed to its ready formation of urate anion radical through one electron oxidation and 8-oxo group seems to be necessary for the stabilization of the urate anion radical (8,12). In fact it has been suggested that 8-oxo group is an important functional moiety for urates' antioxidant property (8,12). Studies have indicated that, methylation at certain nitrogens in uric acid can enhance, as well as retard, the antioxidant property of uric acid (13). Recent reports indicate that purine molecules such as xanthine, caffeine, theophylline and theobromine without the 8-oxo group lack antioxidant activity and hence these molecules do not possess any significant protective effects towards LDL constituents (6).

Some of the mono-, di-, and trimethyluric acids and their corresponding xanthines have been examined earlier for their preventive effect on lipid peroxidation (11). One of the compounds which has a high potency in the prevention of hydrogen peroxide-induced lipid peroxidation in human erythrocyte membranes is 1,3,7-trimethyluric acid (11). This observation has prompted us to prepare analogues of 1,3,7-trimethyluric acid and evaluate their activity. We chose to prepare analogues of 1,3,7-trimethyluric acid with N-1 methyl replaced by various groups mainly because N-1 position is not in proximity to the 8-oxo group, an important functional moiety of urates (8,12). Hence, any bulky substitution at N-1 position would not affect the formation of urate radical. It is known that, some of the

Table 1: Chemical Structures of 1,3,7-Trimethyluric Acid Analogues (IX-XVIII), Uric acid (I) and Methyluric acids (II-VIII)



Compounds	R_1	R_2	R_3	R_4
Uric acid (I)	-H	-H	-H	-H
1-Methyluric acid (II)	$-CH_3$	-H	-H	-H
3-Methyluric acid (III)	-H	$-CH_3$	-H	-H
3,7-Dimethyluric acid (IV)	-H	$-CH_3$	$-CH_3$	-H
1,3-Dimethyluric acid (V)	$-CH_3$	$-CH_3$	-H	-H
1,7-Dimethyluric acid (VI)	$-CH_3$	-H	$-CH_3$	-H
1,3,7-Trimethyluric acid (VII)	$-CH_3$	$-CH_3$	$-CH_3$	-H
1,3,7,9-Tetramethyluric acid (VIII)	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$
1-Ethyl-3,7-dimethyluric acid (IX)	CH_3CH_2-	$-CH_3$	$-CH_3$	-H
1-Propyl-3,7-dimethyluric acid (X)	$CH_3CH_2CH_2-$	$-CH_3$	$-CH_3$	-H
1-Butyl-3,7-dimethyluric acid (XI)	$CH_3CH_2CH_2CH_2-$	$-CH_3$	$-CH_3$	-H
1-Pentyl-3,7-dimethyluric acid (XII)	$CH_3CH_2CH_2CH_2CH_2-$	$-CH_3$	$-CH_3$	-H
1-(β-hydroxyethyl)-3,7-dimethyluric acid(XIII)	$-CH_2\ CH_2OH$	$-CH_3$	$-CH_3$	-H
1-Benzyl-3,7-dimethyluric acid (XIV)	$Ph-CH_2-$	$-CH_3$	$-CH_3$	-H
1-(2′-Oxopropyl)-3,7-dimethyluric acid (XV)	$CH_3CO\ CH_2-$	$-CH_3$	$-CH_3$	-H
1-Allyl-3,7-dimethyluric acid (XVI)	$CH_2=CH-CH_2-$	$-CH_3$	$-CH_3$	-H
1-Propargyl-3,7-dimethyluric acid (XVII)	$CH\equiv C-CH_2-$	$-CH_3$	$-CH_3$	-H
1-Butenyl-3,7-dimethyluric acid (XVIII)	$CH_2=CH-\ CH_2CH_2-$	$-CH_3$	$-CH_3$	-H

long chain-substituted uric acids exhibit better inhibitory activity against oxygen radical-induced lipid peroxidation compared to known antioxidants such as α-tocopherol (14).

In the present investigation, we have prepared analogues of 1,3,7-trimethyluric acid with N-1 methyl group replaced by alkyl, hydroxyethyl, benzyl, 2-oxopropyl, allyl, propargyl and butenyl groups (IX-XVIII, **Table 1**) (details of their preparation and characterization are presented in the previous section). These analogues (IX-XVIII) were used as test compounds to evaluate their protective potential against lipid peroxidation and the ability to scavenge oxygen free radicals. These properties are compared with that observed from uric acid and methyluric acids (I-VIII, **Table 1**). The present study has provided some information regarding the relationship between the relative potencies of various analogues and their structures. In addition, some of the analogues prepared in the present study are significantly more potent as hydroxyl radical scavengers than many of the uric acid derivatives tested so far.

METHODS AND MATERIALS

Chemicals

Uric acid (I), 1-methyluric acid (II), 3-methyluric acid (III), 3,7-dimethyluric acid (IV), 1,3-dimethyluric acid (V), 1,7-dimethyluric acid (VI), 1,3,7-trimethyluric acid (VII), 2-thiobarbituric acid (TBA), Phenazine methosulfate (PMS), Nitroblue tetrazolium (NBT), β-Nicotinamide adenine dinucleotide (reduced form, NADH), 1,1-diphenyl-2-picrylhydrazyl (DPPH), Tris and 2-deoxyribose were obtained from Sigma Chemical Company, St. Louis, MO., USA. 2,2'-Azo-bis(2-amindinopropane) hydrochloride (AAPH) was obtained from Aldrich Chemical Co. (St. Louis, MO). All other materials were from standard suppliers and of analytical grade.

Crocin was isolated from saffron by water/methanol extraction, which was initially repeatedly extracted with diethyl ether to eliminate possible interfering substances as reported earlier (15). The extract containing crocin was diluted with 10 mM phosphate buffer (pH 7.4), and estimated using an extinction coefficient 89,000 M^{-1} cm^{-1} reported for crocin in aqueous solution (16).

Synthesis of N-1 substituted theobromines

Substituted theobromines such as 1-ethyl-3,7-dimethylxanthine, 1-propyl-3,7-dimethylxanthine, 1-butyl-3,7-dimethylxanthine, 1-pentyl-3,7-dimethylxanthine, 1-(β-hydroxyethyl)-3,7-dimethylxanthine, 1-benzyl-3,7-dimethylxanthine, 1-(2′-oxopropyl)-3,7-dimethylxanthine, 1-allyl-3,7-dimethylxanthine, 1-propargyl-3,7-dimethylxanthine and 1-butenyl-3,7-dimethylxanthine were prepared as described in chapter 3: part A (17). The yields varied from 45-83% and the compounds were purified by column chromatography (18).

Preparation of various substituted uric acids (IX-XXII)

A bacterial consortium consisting of strains belonging to the genera *Klebsiella* and *Rhodococcus* quantitatively converts various N-1 substituted theobromines to their respective 8-oxo derivatives (**Table 1**). The details of this microbial process have been described in chapter 3: part A (18,19). The isolated yields varied from 95-97% and the compounds were passed through a small pad of silica gel, eluted with $CHCl_3$–CH_3OH (95:5, v/v) to obtain pure 8-oxo compounds. Purity was determined (by HPLC and TLC) and characterized as reported earlier (19).

Lipid peroxidation in erythrocyte membranes

Human erythrocyte membranes were prepared as reported earlier (5). Human blood collected in heparinised tubes was centrifuged at 2000 × g for 20 min at 0° C. The plasma and buffy coats were removed by aspiration. Erythrocytes were washed twice with isotonic phosphate buffered saline, pH 7.4 (10 mM phosphate, pH 7.4, 152 mM sodium chloride) and lysed in cold phosphate buffer (10 mM, pH 7.4). The membranes were centrifuged at 20,000 × g for 40 min at 0° C, washed thrice with phosphate buffer (10 mM, pH 7.4) and resuspended in the same buffer. The membranes were oxidized with tert-butylhydroperoxide as reported earlier (5). The extent of peroxidation was assayed by measuring thiobarbituric acid (TBA)-reactive substances (TBARS) in membranes as described (20). Briefly, to 1 ml membrane suspension (2 mg) with or without test compounds (I-XVIII, 500 μM, final concentration), tert-butylhydroperoxide (1 mM) was added and incubated at 37° C for 15 min. The reaction was stopped by the addition of 3.0

ml of stopper solution [15% trichloroacetic acid (w/v) + 0.375 % TBA (w/v) + 0.125 M HCl + 0.6 mM BHT], heated in a boiling water bath for 30 min, cooled, centrifuged and the supernatant was used to quantify TBARS photometrically at 532 nm. The results are expressed as percentage inhibition, which represents the degree of protection by the test compounds against peroxidation of erythrocyte membranes induced by tert-butylhydroperoxide.

Protein concentration was estimated by the method of Lowry et al. (21).

Measuring hydroxyl radical scavenging

Hydroxyl radicals were generated by a mixture of ascorbic acid, H_2O_2 and Fe^{+2}-EDTA and estimated using the 2-deoxyribose method (22) with appropriate controls (23). Briefly, the assay mixture contained 2-deoxyribose (2.8 mM), ferrous iron solution (20 μM), EDTA (100 μM) and one of the test compounds (I-XVIII, 500 μM) in a total volume of 1.2 ml of potassium phosphate buffer (10 mM, pH 7.4). The test compounds were dissolved in phosphate buffer, 10 mM, pH 7.4. The ferrous iron solution and EDTA were premixed before they were added to the assay mixture. The reaction was initiated by the addition of a mixture of H_2O_2 (1.42 mM) and ascorbate (100 μM). The values given in the parenthesis represent final concentrations. The mixture was incubated at 37° C for 30 min. At the end of the incubation period, 1.0 ml of 1% (w/v) TBA in sodium hydroxide (50 mM) and 1ml of TCA (2.8%, w/v) were added and the mixture was heated for 20 min in a boiling water bath, cooled and absorbance at 532 nm was measured which corresponds to the deoxyribose damage. The results are expressed as percentage inhibition by the test compounds.

The test compounds which exhibited good activity, the assay was repeated at different concentrations (10 μM-500 μM). The reciprocal absorption values obtained for different concentrations were plotted against the concentrations of the test compounds, and from the graph the second order rate constants were calculated as described by Halliwell et al., (22) assuming that 2-deoxyribose reacts with hydroxyl radical with a rate constant of $3.1 \times 10^9\ M^{-1}\ S^{-1}$.

Superoxide anion radical scavenging activity

Superoxide anion radical scavenging activity of uric acid analogues (I-XVIII) was determined by monitoring the competition kinetics of Nitroblue Tetrazolium (NBT) reduction by superoxide anion generated using PMS-NADH system (non-enzymatically) (24) in phosphate buffer (50 mM, pH 7.8). Test compounds (500 μM), NBT (50 μM), Phenazin Methosulfate (PMS) (10 μM) in Tris-HCl buffer (16mM, pH 8.0) was preincubated for 1 min at room temperature in 1 ml cuvette. Superoxide anion radical was generated by adding NADH (78 μM) and the reduction of NBT was recorded for 2 min at 560 nm. The results were expressed as % inhibition of NBT reduction.

Crocin bleaching assay for peroxyl radical scavenging activity

The reactivity of uric acid and uric acid analogues with peroxyl radicals was measured by competition kinetics of crocin bleaching in the presence of peroxyl radicals generated by thermal decomposition of an azo compound (15). The test was carried out at 40° C in phosphate buffer (10 mM, pH 7.4) containing crocin (10 μM) and test compounds (10 μM) in a total volume of 1 ml. The peroxyl radicals were generated by adding AAPH (10 mM) and the rate of crocin bleaching was recorded at 440 nm in a thermostated spectrophotometer. The bleaching rate was linear 1.5 min after the addition of AAPH and the rate from 2 to 5 min was used for calculations. The results were expressed as % inhibition of crocin bleaching by peroxyl radical.

Scavenging of DPPH stable free radical

Scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH), a stable nitrogen-centered free radical, was measured as described elsewhere (25). Briefly, the reaction was started by mixing test compounds (100 μM) with DPPH (100 μM) in 1 ml cuvette and the time course of the optical density change was determined at 517 nm for 30 min (all dissolved in ethanol). DPPH radical scavenging activity was expressed as ΔOD at 517 nm per unit time (decrease in the absorption of DPPH at 517 nm).

Heme-catalyzed peroxidation of analogues of 1,3,7-trimethyluric acid (IX-XVIII)

Heme-catalyzed peroxidation was carried out as reported earlier (26). The assay mixture contained hemoglobin (8 μM), one of the test compounds (IX-XVIII, 100 μM) and

Table 2: The Hydroxyl Radical Scavenging Ability of Various Uric Acid Derivatives in the Presence and Absence of EDTA in Hydroxyl Radical Generating System (I – XVIII)

Compounds (500 μM)	Inhibition (%)[a]	Inhibition (%)[b]
Uric acid (I)	50.1 ± 0.1*	29.2 ± 1.0**
1-Methyluric acid (II)	56.0 ± 5.0*	n.d[c]
3-Methyluric acid (III)	n.d[c]	n.d[c]
3,7-Dimethyluric acid (IV)	52.1 ± 5.2*	24.6 ± 1.6**
1,3-Dimethyluric acid (V)	51.4 ± 2.6*	32.2 ± 1.9**
1,7-Dimethyluric acid (VI)	56.4 ± 4.0*	34.0 ± 3.2**
1,3,7-Trimethyluric acid (VII)	49.6 ± 4.1*	25.7 ± 1.5**
1,3,7,9-Tetramethyluric acid (VIII)	53.8 ± 6.2* (29.0 ± 0.7)**	17.8 ± 2.0**
1-Ethyl-3,7-dimethyluric acid (IX)	58.6 ± 4.8*	49.0 ± 5.4*
1-Propyl-3,7-dimethyluric acid (X)	60.1 ± 4.0* (26.5 ± 0.9)**	27.6 ± 3.0**
1-Butyl-3,7-dimethyluric acid (XI)	66.1 ± 0.9* (30.4 ± 0.6)**	45.8 ± 3.5*
1-Pentyl-3,7-dimethyluric acid (XII)	55.7 ± 4.2*	42.5 ± 4.5*
1-(β-hydroxyethyl)-3,7-dimethyluric acid(XIII)	52.6 ± 6.0*	26.8 ± 3.2**
1-Benzyl-3,7-dimethyluric acid (XIV)	68.4 ± 6.0* (32.0 ± 0.7)**	n.d[c]
1-(2′-Oxopropyl)-3,7-dimethyluric acid (XV)	52.8 ± 5.1*	32.6 ± 3.2*
1-Allyl-3,7-dimethyluric acid (XVI)	49.1 ± 6.6*	13.3 ± 1.6
1-Propargyl-3,7-dimethyluric acid (XVII)	71.5 ± 5.8* (35.1 ± 0.7)*	21.1 ± 1.8**
1-Butenyl-3,7-dimethyluric acid (XVIII)	73.6 ± 9.1* (43.2 ± 1.5)*	28.4 ± 3.0**

[a] Iron-EDTA mixture was added in hydroxyl radical generating system. [b] Iron ion alone added in hydroxyl radical generating system. [c]not determined. The values presented in the parenthesis are the % inhibition of deoxyribose degradation by corresponding xanthines. The values represent the mean ± S.D., (n=3). Data was analyzed by one-way ANOVA and means are compared with control by using Student's t-test. Significant differences from the control (0 % inhibition) are P < 0.001(*) and P < 0.05 (**).

phosphate buffer (50 mM, pH 7.4) in a total volume of 0.9 ml. The reaction was initiated by the addition of H_2O_2 (3 mM, 0.1 ml) and followed at room temperature by noting the change in absorbencies at the appropriate maximum for each compound (see **Table 8**). Assays were also carried out at pH 8.0.

Statistical analysis

Results are expressed as means ± S.D.; significant differences between antioxidant treated groups and control (no addition) were determined by using one-way ANOVA followed by Student's *t*-test; $P \leq 0.05$ was considered to be statistically significant. The respective levels of significance are given in the tables.

RESULTS

Hydroxyl radical scavenging activity:

In the present study, several hitherto unknown water-soluble analogues of 1,3,7-trimethyluric acid (IX-XVIII, **Table 1**) were prepared following a novel microbial method (16) [details in chapter 3: part A] and their antioxidant property was evaluated. It was noticed that, many of the analogues prepared are far better hydroxyl radical scavengers than uric acid (I) and known methyluric acids tested (II-VIII, **Table 2**). Among the analogues tested, 1-butenyl- (XVIII), 1-propargyl- (XVII) and 1-benzyl-3, 7-dimethyluric acids (XIV) exhibited high hydroxyl radical scavenging property (**Table 2**). In contrast, the corresponding xanthines viz.1-butenyl-3,7-dimethylxanthine, 1-propargyl-3,7-dimethylxanthine, and 1-benzyl-3,7-dimethylxanthine at 500 µM concentration showed markedly less hydroxyl radical scavenging activity (**Table 2**, values presented in parenthesis). These xanthines showed nearly 50% of the activity that was observed with the corresponding 8-oxo compounds. The size of the alkyl substituent at the N-1 position of various analogues of 1,3,7-trimethyluric acid appears to affect the hydroxyl radical scavenging property since there is a gradual increase in potency when the N-1 methyl is replaced by ethyl, propyl, and butyl as in compounds IX, X and XI, respectively (**Table 2**). The optimal size of the alkyl substituent at the N-1 position appears to be butyl for hydroxyl radical scavenging activity. However, the activity can be further enhanced when

Table 3: Comparison of the Second Order Rate Constants of Analogues of 1,3,7-Trimethyluric Acid, Uric Acid, Dimethylsulfoxide and Mannitol

Test compounds	Second Order Rate Constant ($M^{-1} S^{-1}$)
1-Propyl-3,7-dimethyluric acid (X)	$2.1\text{-}3.1 \times 10^{10*}$
1-Butyl-3,7-dimethyluric acid (XI)	$2.6\text{-}3.7 \times 10^{10*}$
1-Benzyl-3,7-dimethyluric acid (XIV)	$2.4\text{-}3.7 \times 10^{10*}$
1-Propargyl-3,7-dimethyluric acid (XVII)	$2.3\text{-}3.7 \times 10^{10*}$
1-Butenyl-3,7-dimethyluric acid (XVIII)	$3.2\text{-}6.7 \times 10^{10*}$
Uric acid (I)	$1.4\text{-}1.6 \times 10^{10*}$
Dimethylsulfoxide	$1.3\text{-}1.6 \times 10^{10*}$
Mannitol	$1.9\text{-}2.5 \times 10^{9}$

The second order rate constant is calculated from the slope of the line from the plot of concentration of test compounds against 1/ absorbance (k = slope $\times k_{DR} \times [DR] \times A^0$), where A^0 is the absorbance, measured in the absence of hydroxyl radical scavenger, $k_{DR} = 3.1 \times 10^9\ M^{-1}\ S^{-1}$, derived from pulse radiolysis studies (22) and [DR] = 2.8 mM. * denote $p < 0.001$, when compared with mannitol using student's t-test.

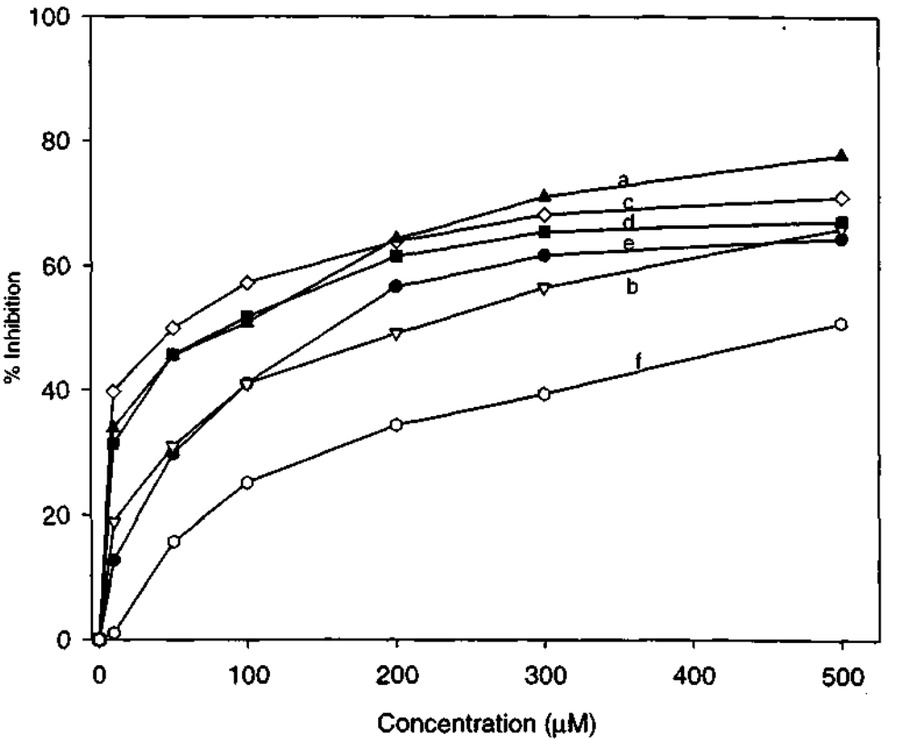


Fig. 1: Effect of a) 1-butenyl-3,7-dimethyluric acid (XVIII), b) butyl-3,7-dimethyluric acid (XI), c) 1-propargyl-3,7-dimethyluric acid (XVII), d) 1-benzyl-3,7-dimethyluric acid (XIV), e) 1-propyl-3,7-dimethyluric acid (X), and f) uric acid (I) on the inhibition of deoxyribose degradation. Each point represents the mean of three experiments.

the butyl group is replaced by a substituent of similar size with unsaturated entity such as butenyl (XVIII, **Table 2**).

The rate constant for the reaction of a scavenger molecule with hydroxyl radical was determined for test compounds which exhibited high activity. These compounds (X, XI, XIV, XVII, XVIII) inhibit the degradation of deoxyribose in a dose dependent manner (**Fig. 1**). Some of the analogues used in this study were shown to be more efficient as hydroxyl radical scavengers than uric acid (I) at all the concentrations tested (10 μM-500 μM, **Fig. 1**). In fact, 200 μM concentration of uric acid scavenged hydroxyl radicals to an extent comparable to 10 μM concentration of analogues XVIII, XVII and XIV (**Fig. 1**). From the graph, with concentrations of the test compounds on x-axis and reciprocal absorption values obtained for different concentrations of test compounds used on y-axis, the second order rate constants (Ks) for reaction of the test compounds with hydroxyl radicals were calculated as reported earlier (22). In a similar way, the rate constants for established scavengers of hydroxyl radical such as mannitol, dimethylsulfoxide and uric acid (I) were determined and the values obtained for them matched well with the reported values (22). The second order rate constant (Ks) for some of the test compounds with hydroxyl radicals using deoxyribose assay are presented in **Table 3**. The competition plots for mannitol gave values in the range 1.9-2.5 $\times 10^9$ M^{-1} S^{-1}, for dimethylsulfoxide values in the range 1.3-1.6 $\times 10^{10}$ M^{-1} S^{-1} and for uric acid values in the range 1.4-1.6 $\times 10^{10}$ M^{-1} S^{-1} (**Table 3**). These results clearly suggest that, some of the analogues of 1,3,7-trimethyluric acid are good scavengers of hydroxyl radicals, with effectiveness better than mannitol (**Table 3**).

When the deoxyribose assay was performed in the absence of EDTA (**Table 2**), the inhibition of hydroxyl radical mediated degradation of deoxyribose was considerably less as compared to assays carried out in the presence of iron-EDTA mixture in hydroxyl radical generating system. This suggests that, test compounds (IX-XVIII) chelate with iron and protect the target molecule, deoxyribose. However, more experiments have to be carried out to substantiate the iron chelation property of these compounds, although the

Table 4: Inhibitory Effect of Test Compounds (I-XVIII) on tert-butylhydroperoxide (1 mM)-Induced Lipid Peroxidation in Human Erythrocyte Membranes

Compounds (500 μM)	Inhibition (%)
Uric acid (I)	$71.9 \pm 1.0^{*}$
1-Methyluric acid (II)	$66.4 \pm 1.2^{*}$
3-Methyluric acid (III)	$57.7 \pm 0.7^{*}$
3,7-Dimethyluric acid (IV)	$60.8 \pm 3.4^{*}$
1,3-Dimethyluric acid (V)	$77.0 \pm 0.5^{*}$
1,7-Dimethyluric acid (VI)	$77.7 \pm 1.9^{*}$
1,3,7-Trimethyluric acid (VII)	$56.5 \pm 6.0^{*}$
1,3,7,9-Tetramethyluric acid (VIII)	$28.0 \pm 1.0^{**}$
1-Ethyl-3,7-dimethyluric acid (IX)	$61.5 \pm 7.0^{*}$
1-Propyl-3,7-dimethyluric acid (X)	$59.5 \pm 2.5^{*}$
1-Butyl-3,7-dimethyluric acid (XI)	$55.9 \pm 2.2^{*}$
1-Pentyl-3,7-dimethyluric acid (XII)	$30.8 \pm 1.8^{**}$
1-(β-hydroxyethyl)-3,7-dimethyluric acid(XIII)	$46.9 \pm 1.1^{*}$
1-Benzyl-3,7-dimethyluric acid (XIV)	$62.6 \pm 0.2^{*}$
1-(2′-Oxopropyl)-3,7-dimethyluric acid (XV)	$22.4 \pm 1.8^{**}$
1-Allyl-3,7-dimethyluric acid (XVI)	$53.2 \pm 1.5^{*}$
1-Propargyl-3,7-dimethyluric acid (XVII)	$55.4 \pm 0.7^{*}$
1-Butenyl-3,7-dimethyluric acid (XVIII)	$57.4 \pm 1.6^{*}$

The values represent the mean ± S.D., (n=3). Data was analyzed by one-way ANOVA and means are compared with control by using Student's t-test. Significant differences from the control (0 % inhibition) are $P < 0.001$(*) and $P < 0.05$ (**).

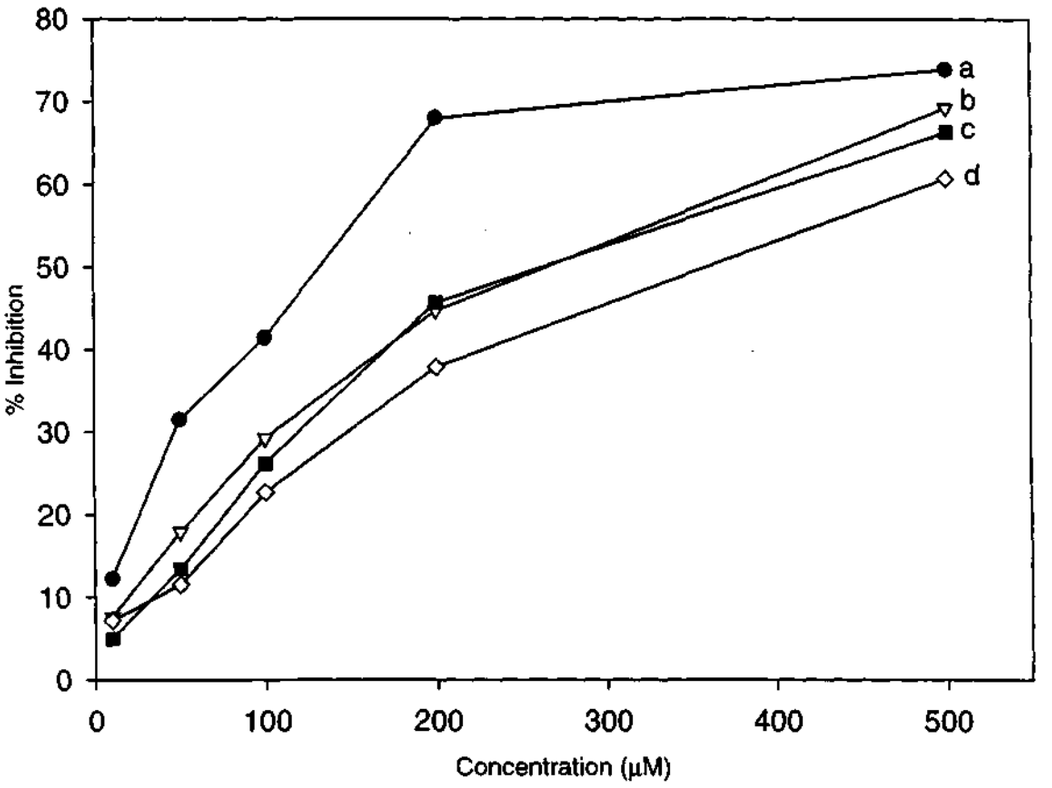


Fig. 2: Effect of different concentration of test compounds a) uric acid (I), b) 1-ethyl-3,7-dimethyluric acid (IX), c) 1-propyl-3,7-dimethyluric acid (X), and d) 1-benzyl-3,7-dimethyluric acid (XIV) on tert-butylhydroperoxide-induced lipid peroxidation in human erythrocyte membranes. Each point represents the mean of three experiments.

Table 5: DPPH stable free radical scavenging activity of uric acid (I), dimethyluric acids (IV-VI), trimethyluric acid (VII), tetramethyluric acid (VIII) and 1,3,7-trimethyluric acid analogues (IX-XVIII)

Compound (100 μM)	ΔOD at 517nm for 30 min
Uric acid (I)	0.7601
3,7-Dimethyluric acid (IV)	0.2847
1,3-Dimethyluric acid (V)	1.0110
1,7-Dimethyluric acid (VI)	0.0449
1,3,7-Trimethyluric acid (VII)	0.2839
1,3,7,9-Tetramethyluric acid (VIII)	0.1614
1-Ethyl-3,7-dimethyluric acid (IX)	0.2850
1-Propyl-3,7-dimethyluric acid (X)	0.3178
1-Butyl-3,7-dimethyluric acid (XI)	0.3134
1-Pentyl-3,7-dimethyluric acid (XII)	0.2623
1-Benzyl-3,7-dimethyluric acid (XIV)	0.3924
1-(2′-Oxopropyl)-3,7-dimethyluric acid (XV)	0.2454
1-Propargyl-3,7-dimethyluric acid (XVII)	0.2916
1-Butenyl-3,7-dimethyluric acid (XVIII)	0.3031
Xanthine	0.0000
Caffeine	0.0000
Glutathione	0.1682
BHT	0.2484

All the 1-,3-, and 7- substituted theobromines and other methyl xanthines failed interact with DPPH stable free radical at 100 μM concentration

property of uric acid to form co-ordination complexes with iron ions has already been reported (9).

Inhibition of tert-butylhydroperoxide-induced lipid peroxidation in human erythrocyte membranes:

The analogues of 1,3,7-trimethyluric acid (IX-XVIII) were also tested for their ability to protect tert-butylhydroperoxide-induced lipid peroxidation in human erythrocyte membranes. It was noticed that, among the analogues (IX-XVIII) tested, excepting compound XII and XV, all others suppressed the lipid peroxidation to a significant extent although these compounds are marginally less potent than 1,3-dimethyl, 1,7-dimethyluric acids (V, VI) and uric acid (I) (**Table 4**). However, they are as potent as 3-methyl, 3,7-dimethyl and 1,3,7-trimethyluric acids (**Table 4**). The more lipophilic 1,3,7,9-tetramethyluric acid (VIII) showed significantly less activity than uric acid (I) (**Table 4**). The inhibitory effect of different concentrations of some of the test compounds on lipid peroxidation in erythrocyte membranes is shown in **Fig. 2**. These studies clearly indicate that, the test compounds inhibit lipid peroxidation in human erythrocyte membranes in a dose dependent manner.

DPPH stable free radical scavenging activity:

The stable free radical 2,2-diphenyl-1-picryl-hydrazyl (DPPH) is a useful reagent to investigate the radical scavenging activity of any compound (antioxidant). In this reaction, DPPH abstracts a hydrogen atom from the compound (donor) and this reaction involves a color change from violet to yellow that can easily be monitored at 517 nm. The capacity of uric acid and its analogues to scavenge the DPPH stable radical were tested and results are summarized in **Table 5**. These analogues interact with DPPH stable radical in a time dependent manner although at slower rate than uric acid, but the rate of interaction is significantly higher than BHT and glutathione. At 100 μM concentration, 1,3-dimethyluric acid removes DPPH radical almost completely within 5 min. At the same concentration, uric acid interacts with DPPH radical slowly and at the end of 30 min, it removes 76% of DPPH. However, other uric acid analogues interact with DPPH radical much slower than uric acid and 1,7-dimethyluric acid is a very poor DPPH radical scavenger at 100 μM concentration (**Table 5**).

Table 6: Peroxyl radical scavenging ability of uric acid (I) and various uric acid derivatives (VII-XVIII) by crocin bleaching assay

Addition of Peroxyl Radical Scavengers (10 μM)	Inhibition (%)
Uric acid (I)	82.1 ± 3.9
1,3,7-Trimethyluric acid (VII)	84.5 ± 1.3
1,3,7,9-Tetramethyluric acid (VIII)	58.8 ± 3.7
1-Ethyl-3,7-dimethyluric acid (IX)	74.8 ± 2.3
1-Propyl-3,7-dimethyluric acid (X)	74.0 ± 1.8
1-Butyl-3,7-dimethyluric acid (XI)	72.8 ± 2.4
1-Pentyl-3,7-dimethyluric acid (XII)	70.6 ± 2.6
1-(β-hydroxyethyl)-3,7-dimethyluric acid (XIII)	78.8 ± 1.2
1-Benzyl-3,7-dimethyluric acid (XIV)	66.4 ± 3.0
1-(2-Oxopropyl)-3,7-dimethyluric acid (XV)	75.7 ± 1.5
1-Propargyl-3,7-dimethyluric acid (XVII)	65.6 ± 3.1
1-Butenyl-3,7-dimethyluric acid (XVIII)	76.2 ± 2.1

The values represent the mean ± S.D., (n=3). Data was analyzed by one-way ANOVA and means are compared by using Student's t-test. Significant differences from the control (0 % inhibition) is $P < 0.001$.

Difference between antioxidant treated and control were determined by the least significance difference test with significance defined at $p < 0.05$.

Table 7: Superoxide anion radical scavenging ability of uric acid (I) and various uric acid derivatives (VII-XVIII) by NBT reduction assay

Compounds (500μM)	Inhibition (%)
Uric acid (I)	09.48 ± 1.0
1-Methyluric acid (II)	15.30 ± 3.0
3,7- Dimethyluric acid (IV)	14.03 ± 0.3
1,3- Dimethyluric acid (V)	09.58 ± 2.0
1,7- Dimethyluric acid (VI)	08.36 ± 0.7
1,3,7-Trimethyluric acid (VII)	09.88 ± 2.0
1,3,7,9-Tetramethyluric acid (VIII)	12.68 ± 2.2
1-Ethyl-3,7-dimethyluric acid (IX)	27.20 ± 0.2
1-Propyl-3,7- dimethyluric acid (X)	34.06 ± 1.4
1-Butyl-3,7- dimethyluric acid (XI)	15.94 ± 2.0
1-Pentyl-3,7- dimethyluric acid (XII)	15.63± 0.8
1-Benzyl-3,7- dimethyluric acid (XIV)	40.62 ± 1.8
1-(2-Oxoproyl)-3,7- dimethyluric acid (XV)	14.48 ± 3.5
1-Propargyl-3,7- dimethyluric acid (XVII)	19.53 ± 5.1
1-Butenyl-3,7- dimethyluric acid (XVIII)	22.75 ± 3.9

Superoxide anion radical was generated by using NADH-PMS (non-enzymatically)

Table 8: Heme Catalyzed Oxidation of Uric acid (I) and Various Analogues of 1,3,7-Trimethyluric Acid (VII – XVIII)

Compounds	nm	Change in absorbance		
		0 min	1 min	5 min
Uric acid (I)	292	1.200	0.900	0.660
1,3,7-Trimethyluric acid (VII)	300	1.145	0.620	0.010
1,3,7,9-Tetramethyluric acid (VIII)	292	0.850	0.630	0.600
1-Ethyl-3,7-dimethyluric acid (IX)	300	1.040	0.770	-0.030
1-Propyl-3,7-dimethyluric acid (X)	300	1.250	0.840	0.060
1-Butyl-3,7-dimethyluric acid (XI)	300	1.120	0.590	0.090
1-Pentyl-3,7-dimethyluric acid (XII)	292	0.660	0.232	0.070
1-(β-hydroxyethyl)-3,7-dimethyluric acid (XIII)	300	0.950	0.619	0.012
1-Benzyl-3,7-dimethyluric acid (XIV)	300	1.130	0.735	-0.040
1-(2-Oxopropyl)-3,7-dimethyluric acid (XV)	292	0.640	0.340	0.175
1-Allyl-3,7-dimethyluric acid (XVI)	300	0.800	0.056	-0.090
1-Propargyl-3,7-dimethyluric acid (XVII)	300	1.040	0.600	0.040
1-Butenyl-3,7-dimethyluric acid (XVIII)	300	1.160	0.820	0.099

The peroxidation was initiated by the addition of H_2O_2 (3 mM) and followed by noting the change in absorbencies at the appropriate λ_{max} for each compound at room temperature.

Peroxyl radical and scavenging activity:

The peroxyl radical scavenging ability of uric acid and its analogues were determined by the competition kinetics of crocin bleaching and results are summarized in **Table 6**. Uric acid appeared as a potent peroxyl radical scavenger at micromolar concentration (10 μM). Among the compounds tested, tetramethyluric acid is least active (**Table 6**). All the new analogues of 1,3,7-trimethyluric acid are good peroxyl radical scavengers at micromolar concentration (10 μM). The inhibition of peroxyl radical assisted crocin bleaching by analogues of 1,3,7-trimethyluric acid ranges from 65% to 79% (**Table 6**) at 10 μM concentration.

Superoxide anion radical scavenging by uric acid analogues:

Superoxide anion radical scavenging ability of uric acid (I), 1-methyluric acid (II), dimethyluric acids (IV-V), tetramethyluric acid (VIII), trimethyluric acid (VII) and its analogues (IX-XVIII) were studied by the competition kinetics of NBT reduction (**Table 7**). Superoxide anion radical was generated by using PMS-NADH (non-enzymatic) system. Uric acid, monomethyl, dimethyl and trimethyluric acids appeared as poor superoxide anion radical scavengers (**Table 7**). However, some of the analogues of 1,3,7-trimethyluric acid viz, 1-ethyl, 1-propyl, 1-benzyl and 1-Butenyl-3,7-trimethyluric acids are better superoxide anion radical scavengers (**Table 7**) at 500 μM concentration.

Heme catalyzed degradation of uric acid analogues:

In the present study it was demonstrated that, excepting 1,3,7,9-tetramethyluric acid (VIII), all other analogues of 1,3,7-trimethyluric acid were oxidized by hydrogen peroxide in the presence of hemoglobin. In fact, they were oxidized comparatively faster than uric acid and 50% oxidation required 1-2 min (**Table 8**). There was no change in the rate of degradation when the assays were carried out at pH 8.0.

DISCUSSION

It was reported earlier that 1,3,7-trimethyluric acid has high potency in the prevention of lipid peroxidation (11). In the present study we have prepared several hitherto unknown water-soluble analogues of 1,3,7- trimethyluric acid (IX-XVIII, **Table 1**)

with a view to find compounds having better antioxidant property than 1,3,7-trimethyluric acid (VII), uric acid (I) and various methyluric acids (II-VII, **Table 1**). Earlier studies have shown that, 1,3,7-trimethylxanthine (caffeine) at mM concentration efficiently scavenges hydroxyl radical (27-29). However, at lower concentrations caffeine and some of the methylxanthines either exhibit low potency in the prevention of lipid peroxidation (11) or not able to suppress oxidative degradation of LDL components (6). Interestingly, 8-oxocaffeine (1,3,7-trimethyluric acid) is a good radical scavenger and a potent antioxidant in model system (11). In fact, earlier studies have established that uric acid and its methylated analogues act as free radical scavengers (5-11). These studies clearly indicate that the 8-oxo group of urates is an important functional moiety responsible for their high hydroxyl radical scavenging and antioxidant properties. The present study substantiates the earlier findings regarding the role of 8-oxo group of urates in their radical scavenging property. It was noticed that, some of the analogues of 1,3,7-trimethyluric acid tested viz. 1-butenyl- (XVIII), 1-propargyl- (XVII) and 1-benzyl-3,7-dimethyluric acid (XIV) exhibited far better hydroxyl radical scavenging property than uric acid (I) and methyluric acids tested (II-VIII, **Table 2**). The corresponding xanthines (without the 8-oxo group) showed significantly less activity (Table 2, values presented in parenthesis). It was also noticed that, the size and nature of the alkyl substituent at N-1 position appear to affect the hydroxyl radical scavenging property of the analogues of 1,3,7-trimethyluric acid (**Table 2**).

It is known that 1,3,7-trimethyluric acid (VII) reacts with stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) at significantly reduced rate compared to uric acid whereas 1,3,7,9-tetramethyluric acid (VIII) reacted with DPPH at rates that were barely detectable (13). So it was suggested that, urates to have maximum reactivity with DPPH and probably with other free radicals, the hydrogen at N_7 must be available to react. If either the hydrogen at N_7 or the hydrogen at N_3 are not present, the reactivity of the urates is reduced to less than 0.1 of that of uric acid (13). In the present investigation, the reaction between DPPH and various analogues of 1,3,7-trimethyluric acid was studied by following the decrease in absorbance of DPPH at 517 nm. It was noticed that, these analogues reacted with DPPH at initial rates (**Table 5**) which are similar to the rates reported earlier

for 7-methyl-, 3,7-dimethyl-, and 1,3,7-trimethyluric acids (13). It appears that, the reactivity of analogues of 1,3,7-trimethyluric acid (IX-XVIII) with DPPH does not reflect on their ability to scavenge hydroxyl radicals as evidenced by the fact that, many of the analogues tested (**Table 2**) are potent hydroxyl radical scavengers. Even 1,3,7,9-tetramethyluric acid (VIII) exhibited good activity (**Table 2**).

Methylated analogues of uric acid have been shown to inhibit hydrogen peroxide induced lipid peroxidation of human erythrocyte membranes *in vitro* (11). In the present study it was noticed that, excepting compound XII and XV (**Table 4**), all other analogues of 1,3,7-trimethyluric acid tested suppressed tert-butylhydroperoxide-induced lipid peroxidation of human erythrocyte membranes to a significant extent. Some of our results are in line with the reported studies on the effect of uric acid and its analogues on oxidation of human low density lipoprotein (LDL) *in vitro* (6). These studies have indicated that, more lipophilic methyluric acids are less active compared to uric acid (6). Earlier it was reported that, alkoxyl radicals seem to be the main initiating species responsible for lipid peroxidation in tert-butylhydroperoxide-treated erythrocytes (30). However, later it was demonstrated that, both peroxyl and alkoxyl radicals are produced by reaction of tert-butylhydroperoxide with rat liver microsomes and infact, more peroxyl radicals are formed than alkoxyl radicals (31). Our results show that uric acid (I) and some of the methyluric acids (II, V, and VI) are better inhibitors of tert-butylhydroperoxide-induced lipid peroxidation in human erythrocyte membranes than the analogues of 1,3,7-trimethyluric acid (IX-XVIII, **Table 4**). We have also noticed that uric acid (I) and 1,3,7-trimethyluric acid (VII) are better peroxyl radical scavengers than the analogues of 1,3,7-trimethyluric acid (IX-XVIII, **Table 6**). Interestingly, many of the analogues of 1,3,7-trimethyluric acid prepared are significantly more potent hydroxyl radical scavengers than uric acid (I) and known methyluric acids (II-VIII, **Table 2**). These results clearly suggest that antioxidants act differently toward the hydroxyl and peroxyl radicals.

It has been suggested that, uric acid (I) is a very poor superoxide anion radical scavenger at 500 μM concentration (32). Consistent with the earlier reports we have observed that, uric acid and methylated uric acids are poor superoxide anion radical scavengers. However, the new analogues of 1,3,7-trimethyluric acid are marginally better

superoxide anion radical scavengers than the uric acid and methyluric acids (**Table 7**). Xanthine, methylxanthines and 1-, 3-, and 7- substituted theobromines are not superoxide anion radical scavengers.

The present study has also observed that, excepting compound VIII (**Table 8**), all other analogues of 1,3,7-trimethyluric acid were oxidized by H_2O_2 in the presence of hematin suggesting their ability to protect hemoglobin and erythrocyte membranes against oxidative damage. Heme-catalyzed oxidation of uric acid and methyluric acids has been reported earlier (26,33-35). It has also been reported earlier that, tetramethyluric acid (VIII) is virtually unaffected by the methemoglobin-peroxide system (26). In fact it has been suggested that, any purine with an unsubstituted N-7 or N-9 position and an 8-oxo substituent is susceptible to peroxidation in the presence of hemoprotein and peroxide, and the oxidation is initiated by dehydrogenation of the purine at N-9 catalyzed by a heme-peroxide complex (26). It appears that, the antioxidant property of tetramethyluric acid (VIII) does not depend on hydrogen at N-9. It is known that 8-oxo group is an important functional moiety for urates' antioxidant activity (6,8,12). So it is quite possible that, unsubstituted N-9 position may enhance the antioxidant activity in these compounds. This may be the reason why 1,3,7-methyluric acid (VII) inhibited tert-butylhydroperoxide-induced lipid peroxidation of human erythrocyte membranes to a greater extent than 1,3,7,9-tetramethyluric acid (VIII) (**Table 4**). Further studies have to be carried out to ascertain the significance of hydrogen at N-9 in the antioxidant property of urates.

It was suggested that, for a compound to exhibit powerful antioxidant activity, it should have the ability to deactivate radicals by giving up electrons and also should be lipophilic in order to protect biological membranes against lipid peroxidation (8,14). In fact to be a very effective antioxidant, uric acid derivative should contain both lipophlic and hydrophilic molecular fractions so that it can exert a site-specific antioxidant protection against reactive oxygen species. In the present study we have noticed that, some of the analogues of 1,3,7-trimethyluric acid such as 1-butenyl-3,7-dimethyluric acid (XVIII), 1-propargyl-3,7-dimethyluric acid (XVII), 1-benzyl-3,7-dimethyluric acid (XIV) and 1-butyl-3,7-dimethyluric acid (XI) not only very efficiently scavenge hydroxyl radicals but also display protective effects towards tert-butylhyroperoxide-induced lipid

peroxidation *in vitro*. One of the important criteria for a good antioxidant is that, either it should get degraded after its interaction with the reactive species or it should get converted back to its original form. If the antioxidant radical does not undergo these processes, then it will acts as a prooxidant and which is as dangerous as free radicals. All the new analogues of 1,3,7-trimethyluric acid undergo degradation during heme catalyzed peroxidation, and in fact, degradation is much faster than uric acid. This supports the view that, these compounds have significant antioxidant property. It is quite possible that some of these analogues may have biological usefulness and hence could find applications in the formulation of antioxidants. However, further studies will be required to ascertain their antioxidant property *in vivo*.

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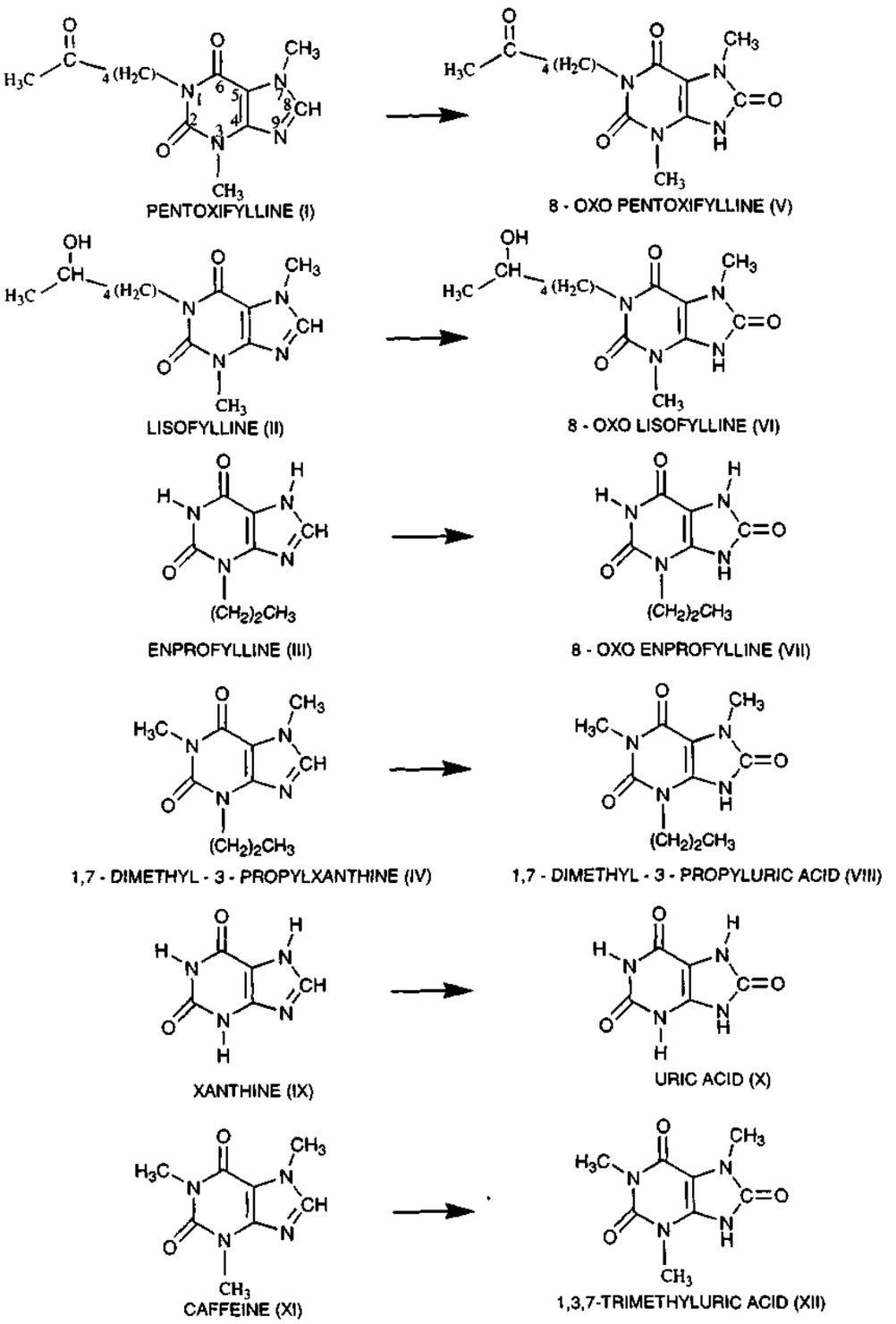
CHAPTER 3: PART C

Antioxidant and Radical Scavenging Properties of 8-Oxo Derivatives of Xanthine Drugs, Pentoxifylline and Lisofylline

INTRODUCTION

Caffeine analogues such as pentoxifylline [I, 1-(5'-oxohexyl)-3,7-dimethylxanthine] and lisofylline [II, 1-(5'-hydroxyhexyl)-3,7-dimethylxanthine] are used as drugs (1-3). Recent studies carried out using numerous purine analogues led to the selection of enprofylline (3-propylxanthine, III) as a drug with bronchodilator property (4). Pentoxifylline (I) is widely used in the treatment of cerebrovascular and peripheral vascular diseases (1-3). Recent studies indicate that, pentoxifylline has antiatherogenic and antithrombatic properties (5). Oxidative modification of plasma low density lipoproteins (LDL) within the vessel wall is believed to play a role in the progression of atherosclerosis (6). Damage to the vascular system during inflammation is caused, in part by the recruitment and adhesion of neutrophils to the endothelium and their release of destructive reactive molecules (7). Both pentoxifylline (I) and lisofylline (II) are known to possess anti-inflammatory properties (8) which are probably related to their ability to suppress oxygen radical production or scavenge reactive oxygen species (9,10). It has been suggested that, pentoxifylline (I) reduce the cellular expression of TNF-α, an activator of neutrophils, via the inhibition of phosphodiestrase activity, thereby it inhibits the production of free radicals (8-10). Lisofylline (II) is an important metabolite of pentoxifylline in humans, a potent inhibitor of inflammatory lipid mediator phosphatidic acid formation, stimulated by IL-1β and TNF-α, *in vivo* (11). Ability of pentoxifylline (I) to scavenge hydroxyl radicals has been demonstrated earlier (12). Although substituted xanthines such as pentoxifylline (I) and lisofylline (II) are used as drugs, the corresponding 8-oxo derivatives have never been prepared and tested for their biological and pharmacological properties, including antioxidative potential and ability to scavenge reactive oxygen species. The non-availability of the 8-oxo derivatives of these drugs has prevented their biological evaluation. It is quite possible that these drugs could get transformed to their corresponding 8-oxo derivatives *in vivo* which may be partly responsible for their biological activity. Caffeine, theophylline and theobromine are known to get metabolized to their corresponding 8-oxo derivatives in the mammalian system (13,14). These metabolites (methyluric acids) are known to inhibit lipid peroxidation in

Fig.1: Chemical structures of pentoxifylline, lisofylline, enprofylline, 1,7-dimethyl-3-propylxanthine, xanthine, caffeine and their corresponding 8-oxo derivatives.



human erythrocyte membranes *in vitro* and function as free radical scavengers (15,16), suggesting their antioxidant effects *in vivo*. In fact, caffeine, theophylline and theobromine exhibit significantly less antioxidant and radical scavenging activity than the corresponding molecules with the 8-oxo group, indicating that the 8-oxo-group of uric acid is an important functional moiety responsible for their high antioxidant and radical scavenging properties (17,18).

In the present investigation, we have prepared for the first time 8-oxopentoxifylline [V, 1-(5′-oxohexyl)-3,7-dimethyluric acid], racemic 8-oxolisofylline [VI, 1-(5′-hydroxyhexyl)-3,7-dimethyluric acid] and 8-oxoenprofylline (VII, 3-propyluric acid) following a novel microbial method (19,20). To increase the lipophilicity of enprofylline (III), we have substituted N-1 and N-7 hydrogens with methyl groups and the resulting compound viz. 1,7-dimethyl-3-propylxanthine (1,7-dimethylenprofylline, IV) was also microbially converted to 1,7-dimethyl-3-propyluric acid (VIII, 8-oxo derivative of IV). All the 8-oxo compounds prepared (V-VIII, **Fig. 1**) were tested for their ability to scavenge hydroxyl and peroxyl radicals. These 8-oxo compounds were also tested for their protective potential against lipid peroxidation. These properties are compared with those of the corresponding xanthines (I-IV, **Fig. 1**). The present study provides additional information in understanding the mechanism of action of some of these drugs.

MATERIALS AND METHODS

Chemicals

Uric acid, 2-Thiobarbituric acid, 2-Deoxyribose, Phenazine methosulfate (PMS), Nitroblue tetrazolium (NBT), β-Nicotinamide adenine dinucleotide (reduced form, NADH), Tris and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma Chemical Company, St. Louis, MO., USA. 2,2'-Azo-bis(2-amindinopropane) hydrochloride (AAPH) was obtained from Aldrich Chemical Co. (St. Louis, MO). Enprofylline (3-propylxanthine) was a generous gift from Dr. Hans Jurgen Fedrsel, Astra Production chemicals AB, Sweden. Pentoxifylline was prepared using theobromine and alkyl halide as reported earlier (21). 3-Propyl-1,7-dimethylxanthine was prepared

following the published procedure (22). Crocin was isolated from saffron by water/methanol extraction (details are given in chapter 3: part B). All other materials procured from standard suppliers and were of analytical grade.

Preparation of 8-oxopentoxifylline, 8-oxoenprofylline and 3-propyl-1,7-dimethyluric acid

A bacterial consortium consisting of strains belonging to the genera *Klebsiella* and *Rhodococcus* quantitatively convert pentoxifylline, enprofylline (3-propylxanthine) and 3-propyl-1,7-dimethylxanthine to their respective 8-oxo derivatives (**Fig. 1**, substituted uric acids). The details of this microbial process have been described in chapter 3: part A (19,20). The 8-oxoderivatives prepared were characterized by various spectral analyses (details are given in chapter 3: part A).

Preparation of 8-oxolisofylline [1-(5'-hydroxyhexyl)-3,7-dimethyluric acid]

Sodium borohydride ($NaBH_4$) reduction of 8-oxopentoxifylline yielded racemic 8-oxolisofylline. 5 mmole of 8-oxopentoxifylline was dissolved in dry methanol and cooled to 0° C and $NaBH_4$ (5 mmole) was added to it and stirred for 3 hrs. The reaction was quenched by adding water and extracted with chloroform (2 × 50 ml). The organic layer was dried over anhydrous sodium sulfate and solvent removed under vacuum. The residue was purified by column chromatography over silica gel column and the compound was eluted with chloroform:methanol (95:5). The compound was characterized by various spectral analyses.

M.p.: 122-123° C.

IR (nujol) γ_{max} **(cm^{-1})**: 3300 (-OH), 1640, 1680 (-NC=O).

^{1}HNMR (300 MHz, $CDCl_3$): δ(ppm) 1.45-1.73 (6H, m, $-CH_2-CH_2-CH_2$), 1.19 (3H, d, CH_3-CHOH-), 3.5 (3H, s, $-NCH_3$), 3.7 (1H, m, -CHOH), 3.8 (3H, s, $-NCH_3$), 4.0 (2H, t, J=7.2, $-N-CH_2-$).

^{13}C NMR (100 MHz, $CDCl_3$): δ 20.8, 25.41, 27.3, 29.8, 33.5, 40.6, 43.0, 57.5, 99.4, 141.4, 148, 151,155.

Mass spectra: m/z 296 (M^+), 196 (M^+ -$C_6H_{12}O$, base peak), 153 (M^+ -$C_6H_{12}O$-CO-CH_3), 125 (M^+ -$C_6H_{12}O$-2CO-CH_3), 97 (M^+ -$C_6H_{12}O$-2CO-CH_3-N_2), 82 (M^+ -$C_6H_{12}O$-2CO-2CH_3-N_2).

Measuring hydroxyl radical scavenging

Hydroxyl radicals were generated by a mixture of ascorbic acid, H_2O_2 and Fe^{2+}-EDTA and estimated using the 2-deoxyribose method (23). Each assay contained 2-deoxyribose (2.8 mM), ferrous iron solution (20 μM), EDTA (100 μM) and one of the test compounds (500 μM) in a total volume of 1.2 ml of phosphate buffer (10 mM, pH 7.4). The ferrous iron solution and EDTA were premixed before they were added to the assay mixture. The reaction was initiated by the addition of a mixture of H_2O_2 (1.42 mM) and ascorbate (100 μM), and incubated at 37° C for 30 min. Then 1 ml of thiobarbituric acid (1%, w/v) in NaOH (50 mM) and 1 ml of TCA (2.8%, w/v) were added, boiled for 20 min, cooled and the absorbance measured at 532 nm. The results are expressed as % inhibition by test compounds. The assay was repeated at different concentrations (10-500 μM) of test compounds (V-VIII, and uric acid). Reciprocal absorption values obtained for different concentrations were plotted against the concentrations of the test compounds and from the graph the second order rate constants for the reaction of test compounds with hydroxyl radical were calculated, assuming that deoxyribose reacts with hydroxyl radical with a rate constant of 3.1×10^9 M^{-1} S^{-1} (23). In a similar way, the rate constants for established scavengers of hydroxyl radical such as mannitol and dimethylsulfoxide were determined.

Measuring peroxyl radical scavenging

Peroxyl radical scavenging activity of test compounds was measured by competition kinetics of crocin bleaching in the presence of peroxyl radicals generated by thermal decomposition of an azo compound as reported earlier (24,25). The test was carried out at 40° C in phosphate buffer (10 mM, pH 7.4) containing crocin (10 μM) and increasing concentrations (0-50 μM) of test compounds in a total volume of 1 ml. The peroxyl radicals were generated by adding AAPH (10 mM) and the rate of crocin bleaching was recorded at 440 nm in a thermostated spectrophotometer. The bleaching rate was linear 1.5 min after the addition of AAPH and the rate from 2 to 5 min was used for

calculations. Bleaching rates were plotted as Vb/Va versus [A]/[C], according to the equation Vb/Va = 1 + Ka/Kc. [A]/[C], where Vb is the basal bleaching rate of crocin in the absence of scavenger, Va is the bleaching rate of crocin in the presence of scavenger, [C] and [A] are the concentrations of crocin and test compounds (scavengers), respectively. Ka and Kc are the rate constants for the reaction of the peroxyl radical with scavengers and crocin, respectively. This plot gives a straight line intersecting the ordinate, with a slope of Ka/Kc.

Scavenging of DPPH stable free radical

Scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH), a stable nitrogen-centered free radical was measured as described elsewhere (26). Briefly, the reaction was started by mixing test compounds (I-XII, 100 μM) with DPPH (100 μM) in a 1 ml cuvette and the time course of the optical density change was determined at 517 nm for 30 min (all dissolved in ethanol). DPPH radical scavenging activity was expressed as ΔOD at 517 nm per unit time (decrease in the absorption of DPPH at 517 nm).

Superoxide anion radical scavenging activity

Superoxide anion radical scavenging activity of uric acid (X) and test compounds (V-IX) were determined by monitoring the competition kinetics of Nitroblue Tetrazolium (NBT) reduction by superoxide anion generated by PMS-NADH system (non-enzymatically) (27) in phosphate buffer (50 mM, pH 7.8). Test compounds (500 μM), NBT (50 μM) and Phenazin Methosulfate (PMS) (10 μM) in Tris-HCl buffer (16mM, pH 8.0) was preincubated for 1 min at room temperature in a 1 ml cuvette. Superoxide anion radical was generated by adding NADH (78 μM) and the reduction of NBT was recorded for 2 min at 560 nm. The results were expressed as % inhibition of NBT reduction.

Heme-catalyzed peroxidation of 8-oxo derivatives of pentoxifylline, lisofylline, enprofylline and 1,7-dimethylenprofylline

Heme-catalyzed peroxidation was carried out as reported earlier (28). The assay mixture contained hemoglobin (8 μM), one of the test compounds (V-IX, 100 μM) and phosphate buffer (50 mM, pH 7.4) in a total volume of 0.9 ml. The reaction was initiated by the addition of H_2O_2 (3 mM, 0.1 ml) and followed at room temperature by noting the

Table 1: The hydroxyl radical scavenging ability of pentoxifylline, lisofylline, 1,7-dimethylenprofylline and their corresponding 8-oxo derivatives (uric acids).

Test compounds (500μM)	Inhibition of (%)[a]	Inhibition of (%)[b]
Pentoxifylline (I)	31.0 ± 1.3	n.d[c]
Lisofylline (II)	42.0 ± 1.0	n.d[c]
Enprofylline (III)	50.7 ± 2.3	n.d[c]
1,7-dimethylenprofylline (IV)	43.1 ± 3.8	n.d[c]
8-oxopentoxifylline (V)	72.2 ± 0.5	32.6 ± 3.2
8-oxolisofylline (VI)	58.2 ± 7.1	15.9 ± 3.0
8-oxoenprofylline (VII)	53.6 ± 0.7	16.3 ± 3.0
1,7-dimethyl-8-oxoenprofylline (VIII)	52.1 ± 1.3	15.2 ± 5.9
Uric Acid (X)	50.1 ± 0.1	27.2 ± 3.5

[a] Iron-EDTA mixture was added in hydroxyl radical generating system. [b] Iron ion alone added in hydroxyl radical generating system. [c]not determined. The values represent the mean ± S.D., (n = 3). Data was analyzed by one-way ANOVA and means are compared with control by using Student's t-test. Significant differences from the control (0 % inhibition), P < 0.05.

Table 2: Comparison of the second order rate constants of of uric acid, 8-oxopentoxifylline, 8-oxolisofylline, 8-oxoenprofylline and 8-oxo-1,7-dimethylenprofylline dimethylsulfoxide and mannitol

Test compounds	Second Order Rate Constant ($M^{-1} S^{-1}$)
1-(5-Oxohexyl)-3,7-dimethyluric acid (V)	$1.6\text{-}4.2 \times 10^{10}$
1-(5-hydrohexyl)-3,7-dimethyluric acid (VI)	$1.5\text{-}2.8 \times 10^{10}$
3-Propyluric acid (VII)	$1.4\text{-}1.9 \times 10^{10}$
3-propyl-1,7-dimethyluric acid (VIII)	$1.4\text{-}1.7 \times 10^{10}$
Uric acid (X)	$1.4\text{-}1.6 \times 10^{10*}$
Dimethylsulfoxide	$1.3\text{-}1.6 \times 10^{10*}$
Mannitol	$1.9\text{-}2.5 \times 10^{9}$

The second order rate constant is calculated from the slope of the line from the plot of concentration of test compounds against 1/ absorbance (k = slope $\times k_{DR} \times [DR] \times A^0$), where A^0 is the absorbance, measured in the absence of hydroxyl radical scavenger, $k_{DR} = 3.1 \times 10^9\ M^{-1}\ S^{-1}$, derived from pulse radiolysis studies (23) and [DR] = 2.8 mM. * denote $p < 0.001$, when compared with mannitol using student's t-test.

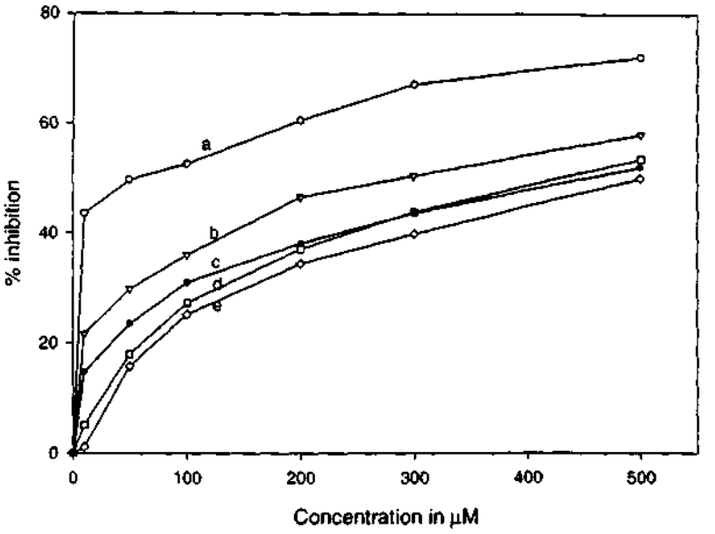


Fig.2: Effect of various concentrations of test compounds a) 8-oxopentoxifylline, b) 8-oxolisofylline, c) 1,7-dimethyl-8-oxoenprofylline, d) 8-oxoenprofylline and e) uric acid on the deoxyribose degradation. The results are expressed as % inhibition of deoxyribose degradation. The experimental details are mentioned in the text.

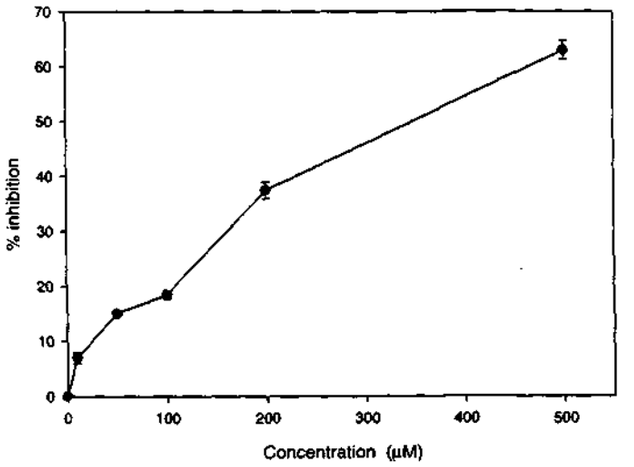


Fig.3: Effect of different concentration of 1,7-dimethyl-8-oxoenprofylline on t-butylhydroperoxide-induced lipid peroxidation in human erythrocyte membranes. The results are expressed as % inhibition of lipid peroxidation. The experimental details are mentioned in the text.

change in absorbencies at the appropriate maximum for each compound (see **Table 7**). Assays were also carried out at pH 8.0.

Lipid peroxidation in human erythrocyte membranes

Effect of test compounds on tert-butylhydroperoxide-induced lipid peroxidation in human erythrocyte membranes was assayed as reported earlier (15,29). Washed erythrocyte membranes were prepared and processed as reported earlier (29). The peroxidation of lipids in erythrocyte membranes was carried out in total volume of 1.0 ml containing protein (2.0 mg), with or without the test compounds. The reaction was started by the addition of tert-butylhydroperoxide (1 mM) and incubated at 37° C for 15 min. The extent of peroxidation was assayed by measuring thiobarbituric acid-reactive substances (TBARS) in membranes as described earlier (30). The results are expressed as % inhibition, which represents the degree of protection by the test compounds against lipid peroxidation in erythrocyte membranes.

Protein was estimated by the method of Lowry et al. (31).

RESULTS

Hydroxyl radical scavenging activity:

In the present study we have prepared 8-oxopentoxifylline (V), 8-oxolisofylline (VI), 8-oxoenprofylline (VII) and 1,7-dimethyl-8-oxoenprofylline (VIII). All the 8-oxo compounds prepared are water soluble and stable in aqueous medium. The present study demonstrates for the first time that V and VI are significantly better hydroxyl radical scavengers than the parent drugs (I and II, **Table I**), whereas VII and VIII show marginally better activity than the corresponding xanthines (III and IV, **Table 1**). It has been noticed that 8-oxopentoxifylline (V) is a much better scavenger of hydroxyl radicals than 8-oxolisofylline (VI) at all concentrations tested (**Fig. 2**). All the 8-oxo compounds tested (V-VIII) inhibit the degradation of deoxyribose in a dose dependent manner and compounds V and VI are very efficient at lower concentrations (10-50 μM, **Fig. 2**). In fact, 8-oxo compounds (V-VIII) exhibits better activity than the physiological antioxidant, uric acid (29) at all the concentrations tested (**Fig. 2**).

Table 3: Inhibitory effect of test compounds on tert-butylhydroperoxide (1 mM)-induced lipid peroxidation in human erythrocyte membranes.

Test compounds (500 μM)	Inhibition (%)*
Pentoxifylline (I)	10.5 ± 0.5
Enprofylline (III)	14.2 ± 2.6
1,7-dimethylenprofylline (IV)	23.7 ± 4.7
8-oxopentoxifylline (V)	41.4 ± 2.4
8-oxolisofylline (VI)	51.3 ± 0.8
8-oxoenprofylline (VII)	11.8 ± 1.8
1,7-dimethyl 8-oxoenprofylline (VIII)	62.9 ± 1.7

****Results are expressed as % inhibition of lipid peroxidation. the values represent the mean ± s.d of triplicate experiments.***

Table 4: DPPH stable free radical scavenging activity of uric acid, trimethyluric acid, 8-oxopentoxifylline, 8-oxolisofylline, BHT and glutathione

Compound (100 μM)	ΔOD at 517nm for 30 min
Control*	1.0093
Uric acid (X)	0.7601
1,3,7-Trimethyluric acid (XII)	0.2839
8-oxopentoxifylline (V)	0.2786
8-oxolisofylline (VI)	0.3226
Xanthine (IX)	0.0000
Caffeine (XI)	0.0000
Glutathione	0.1682
BHT	0.2484

Xanthine, caffeine, pentoxifylline and lisofylline are failed interact with DPPH stable free radical at 100 μM concentration.

****DPPH alone, without any scavenger molecules***

The rate constant for the reaction of a scavenger molecule with hydroxyl radical was determined for test compounds which exhibited high activity, and they were found to inhibit the degradation of deoxyribose in a dose dependent manner (**Fig. 2**). From the graph with concentrations of the test compounds on x-axis and reciprocal absorption values obtained for different concentrations of test compounds used on y-axis, the second order rate constants (Ks) for reaction of the test compounds with hydroxyl radicals were calculated as reported earlier (23). In a similar way the rate constants for established scavengers of hydroxyl radical such as mannitol, dimethylsulfoxide and uric acid were determined, and the values obtained for them compared well with the reported values (23). The second order rate constant (Ks) for the test compounds with hydroxyl radicals using deoxyribose assay are presented in **Table 2**. It was observed that 8-oxopentoxifylline (V) show high hydroxyl radical scavenging property (Ks, 1.6-4.2 $\times$ 10^{10} $M^{-1}S^{-1}$) with an effectiveness comparable to that of dimethylsulfoxide (Ks, 1.4-1.6 $\times$ 10^{10} $M^{-1}S^{-1}$) and better than mannitol (Ks, 1.9-2.5 $\times$ 10^{9} $M^{-1}S^{-1}$).

When the deoxyribose assay was performed in the absence of EDTA (**Table 1**), the inhibition of hydroxyl radical mediated degradation of deoxyribose was considerably less, when compared to assays carried out in the presence of iron-EDTA mixture in hydroxyl radical generating system. This suggests that test compounds (V-IX) chelate with iron and protect the target molecule, deoxyribose.

Inhibition of tert-butylhydroperoxide-induced lipid peroxidation in human erythrocyte membranes:

The 8-oxo analogues of pentoxifylline, lisofylline, enprofylline, and 1,7-dimethylenprofylline were tested for their ability to protect tert-butylhydroperoxide-induced lipid peroxidation in human erythrocyte membranes *in vitro*. The present study has shown that compounds V, VI and VIII significantly suppress t-butylhydroperoxide-induced lipid peroxidation in human erythrocyte membranes, whereas compounds I, III and VII show markedly less activity (**Table 3**). It was noticed that 8-oxoenprofylline is a poor inhibitor of lipid peroxidation in human erythrocyte membranes (**Table 3**) However, 3-propyl-1,7-dimethyluric acid (VIII) shows very good activity as the inhibitor of lipid

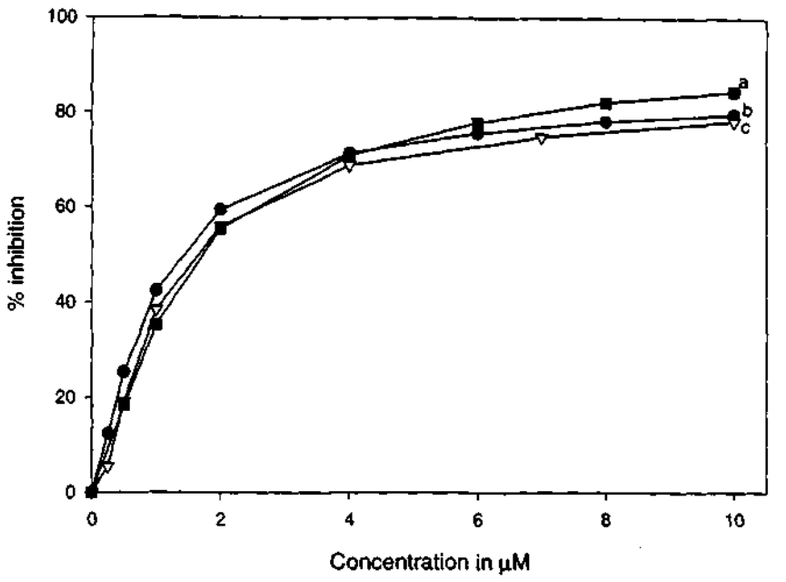


Fig. 4: Effect of different concentrations of test compounds a) uric acid, b) 8-oxopentoxifylline, and c) 8-oxolisofylline on crocin bleaching by peroxyl radical. The results are expressed as % inhibition of crocin bleaching. The experimental details are mentioned in the text.

Table 5: IC_{50} values for uric acid, 8-oxopentoxifylline and 8-oxolisofylline for the inhibition of peroxyl radical-mediated reaction

Compound	IC_{50}	K_{rel}
Uric acid (X)	1.9 ± 0.05	4.5 ± 1.0
8-oxopentoxifylline (V)	1.8 ± 0.08	4.1 ± 0.5
8-oxopentoxifylline (VI)	2.2 ± 0.13	3.8 ± 0.3

Xanthine, pentoxifylline and lisofylline failed to scavenge peroxyl radicals

The values represent the mean ± s.d of triplicate experiments.

peroxidation (**Table 3**). The inhibitory effects at different concentrations of VIII, on lipid peroxidation by a fixed dose of t-butylhydroperoxide are shown to be dose dependent (**Fig. 3**).

DPPH stable free radical scavenging activity:

The stable free radical 2,2-diphenyl-1-picryl-hydrazyl (DPPH) is a useful reagent to investigate the radical scavenging activity of any compound (antioxidant). The reaction mechanism consists of abstracting a hydrogen atom from a antioxidant donor to give diphenylpicrylhydrazine and a antioxidant radical. The reaction involves a color change from violet to yellow that can easily be monitored by measuring the decrease in absorbance at 517 nm. In the present investigation, the reaction between DPPH and 8-oxopentoxifylline (V) and 8-oxolisofylline (VI) was studied by following the decrease in absorbance of DPPH at 517 nm. The ability of compound V, VI, X and XII to scavenge the DPPH stable radical are summarized in **Table 4**. It was noticed that these analogues reacted with DPPH at initial rates (data not shown) similar to the rates reported earlier for 1,3,7-trimethyluric acids (32). At 100 μM concentration, uric acid interacts with DPPH radical significantly faster than 1,3,7-trimethyluric acid, 8-oxopentoxifylline and 8-oxolisofylline, and at the end of 30 min it removes ~76% of DPPH. However, 1,3,7-trimethyluric acid, 8-oxopentoxifylline and 8-oxolisofylline interact with DPPH radical much slower than uric acid, whereas corresponding xanthines failed to scavenge DPPH radical at 100 μM concentration.

Peroxyl radical and scavenging activity:

The peroxyl radical scavenging capacity of uric acid (X), 8-oxopentoxifylline (V) and 8-oxolisofylline (VI) were determined by crocin bleaching assay and results are summarized in **Table 5**. It was observed that V and VI are good peroxyl radical scavengers with an IC_{50} values of 1.8 ± 0.08 μM and 2.2 ± 0.13 μM, respectively (**Table 5**). It appears that both V and VI are as efficient peroxyl radical scavengers as uric acid (IC_{50}, 1.9 ± 0.05 μM) at all concentrations tested (**Fig. 4**). However, pentoxifylline (I), lisofylline (II) and xanthine (IX) failed to scavenge peroxyl radical, indicating the importance of 8-oxo group in urates for their antioxidant and radical scavenging properties. Relative rate constant

Table 6: Superoxide anion radical scavenging ability of uric acid, 8-oxopentoxifylline, 8-oxolisofylline, 8-oxoenprofylline and 8-oxo-1,7-dimethylenprofylline, by NBT reduction assay

Compounds (500 µM)	Inhibition (%)
Uric acid (X)	09.48 ± 1.0
1-(5-Oxohexyl)-3,7-dimethyluric acid (V)	17.33± 2.9
1-(5-Hydroxyhexyl)- 3,7-dimethyluric acid (VI)	22.96 ± 3.4
3-Propyluric acid (VII)	17.73 ± 5.3
3-Propyl-1,7-dimethyluric acid (VIII)	18.85 ± 5.4

Superoxide anion radical was generated by using NADH-PMS (non-enzymatically). Corresponding xanthines are failed scavenge superoxide anion radical.

Table 7: Heme Catalyzed Oxidation of Uric acid (I), 1,3,7-Trimethyluric Acid, 8-oxopentoxifylline, 8-oxolisofylline, 8-oxoenprofylline and 8-oxo-1,7-dimethylenprofylline

Compounds	nm	Change in absorbance		
		0 min	1 min	5 min
Uric acid (X)	292	1.200	0.900	0.660
1,3,7-Trimethyluric acid (XII)	300	1.145	0.620	0.010
1-(5-Oxohexyl)-3,7-dimethyluric acid (V)	300	1.050	0.584	0.030
1-(5-Hydroxyhexyl)- 3,7-dimethyluric acid (VI)	300	1.020	0.681	0.000
3-Propyluric acid (VII)	285	0.600	0.590	0.590
3-Propyl-1,7-dimethyluric acid (VIII)	300	1.030	0.609	-0.070

The peroxidation was initiated by the addition of H_2O_2 (3 mM) and followed by noting the change in absorbencies at the appropriate λ_{max} for each compound at room temperature.

ratios indicate that, uric acid, 8-oxopentoxifylline and 8-oxolisofylline are efficient peroxyl radical scavengers (**Table 5**).

Superoxide anion radical scavenging by 8-oxo derivatives of pentoxifylline, lisofylline, enprofylline and 1,7-dimethyl enprofylline:

Superoxide anion radical scavenging ability of uric acid (X), 8-oxopentoxifylline (V), 8-oxolisofylline (VI), 8-oxoenprofylline (VII) and 1,7-dimethyl-8-oxoenprofylline (VIII) were studied by the competition kinetics of NBT reduction (**Table 6**). Superoxide anion radical was generated by using PMS-NADH (non-enzymatic) system. These studies indicated that uric acid (X) is a poor superoxide anion radical scavenger (**Table 6**) and similar observations have been made earlier (33). However, compound V, VI, VII, and VIII, are marginally better superoxide anion radical scavengers (**Table 6**).

Heme catalyzed degradation of 8-oxo derivatives of pentoxifylline, lisofylline, enprofylline and 1,7-dimethyl enprofylline:

In the present study we have demonstrated that, 8-oxopentoxifylline (V), 8-oxolisofylline (VI), and 8-oxo-1,7-dimethylenprofylline (VIII) are oxidized by hydrogen peroxide in the presence of hemoglobin. In fact, they are oxidized comparatively much faster than uric acid (X) and 50% oxidation required 1-2 min (**Table 7**). However, 3-propyluric acid (VII) failed to undergo oxidation during the experimental period. There was no change in the rate of degradation when the assays were carried out at pH 8.0.

DISCUSSION

Reactive oxygen species produced by activated polymorphonuclear leukocytes appear to be responsible for tissue damage in a number of inflammatory conditions (34). Earlier studies have indicated that xanthine drug, pentoxifylline (I) reduces oxygen radical production and protects against tissue damage *in vivo* by the action of its metabolites (35). In fact, both pentoxifylline (I) and lisofylline (II) are known for their anti-inflammatory properties (8,9). It is also known that I inhibits hydroxyl radical-mediated degradation of deoxyribose (12).

The present study demonstrates for the first time that V and VI are significantly better hydroxyl radical scavengers than the parent drugs (I and II, **Table I**), whereas VII and VIII show marginally better activity than the corresponding xanthines (III and IV, **Table 1**). It has been noticed that 8-oxopentoxifylline (V) is a much better scavenger of hydroxyl radicals than 8-oxolisofylline (VI) at all concentrations tested (**Fig.2**). This could be possibly due to the fact that racemic 8-oxolisofylline (VI) was used in this study. It is known that R-isomer of lisofylline is biologically more active than its optical antipode (36), and it is quite possible that one of the stereoisomer could be more active than the other isomer and recemic compound. All the 8-oxo compounds tested (V-VIII) inhibit the degradation of deoxyribose in a dose dependent manner and compounds V and VI are very efficient at lower concentrations (10-50 μM, **Fig. 2**). It is interesting to note that 8-oxo compounds (V-VIII) exhibit better activity than the physiological antioxidant uric acid (29), at all concentrations tested (**Fig. 2**). In fact, 500 μM concentration of uric acid scavenged hydroxyl radicals to an extent comparable to 50 μM concentration of 8-oxopentoxifylline (V, **Fig. 2**) indicating that V is a more potent scavenger of hydroxyl radicals than uric acid. It has been reported earlier that uric acid and related analogues function as efficient antioxidant and free radical scavengers (17,29,37,38), and protect erythrocyte membranes from lipid peroxidation (15,16,38).

It was observed that 8-oxopentoxifylline (V) shows high hydroxyl radical scavenging property (Ks, 1.6-4.2 $\times 10^{10} M^{-1}S^{-1}$) with an effectiveness comparable to that of dimethylsulfoxide (Ks, 1.4-1.6 $\times 10^{10} M^{-1}S^{-1}$) and better than mannitol (Ks, 1.9-2.5 $\times 10^{9}$ $M^{-1}S^{-1}$). The rate constants obtained for dimethylsulfoxide and mannitol compared well with the reported values (23). The second order rate constant (Ks) reported earlier (7) for pentoxifylline (I) with hydroxyl radicals indicates that V is a more effective hydroxyl radical scavenger than is I. Similar observation was also made earlier with allopurinol and oxypurinol (39). Oxypurinol, a major metabolite of allopurinol is a better hydroxyl radical scavenger than is allopurinol (39). One cannot rule out the possibility that metabolism of pentoxifylline *in vivo* could lead to the formation of its 8-oxo derivative (V)

Earlier studies have shown that 1,3-dimethyluric acid and 1,3,7-trimethyluric acid have high potency in prevention of hydrogen peroxide induced lipid peroxidation in human erythrocyte membranes *in vitro* (15). In fact, V and VI are analogues of 1,3,7-trimethyluric acid where N-1 position is substituted with 5-oxohexyl/5-hydroxyhexyl group. The present study has shown that compounds V, VI and VIII significantly suppress t-butylhydroperoxide-induced lipid peroxidation in human erythrocyte membranes, whereas compounds I, III and VII show markedly less activity (**Table 3**). The antioxidative effect of compounds V, VI and VIII is evidenced by their ability to scavenge hydroxyl radicals very efficiently (**Table 1**). The ability of uric acid to inhibit lipid peroxidation in erythrocyte membranes is known (15). However, it is interesting to note that when N-3 hydrogen in uric acid is substituted by a propyl group as in 3-propyluric acid (8-oxoenpropylline, VII), the compound very poorly protected erythrocyte membranes from lipid peroxidation (**Table 3**). Surprisingly, 3-propyl-1,7-dimethyluric acid (VIII) shows very good activity as the inhibitor of lipid peroxidation (**Table 3**). The inhibitory effects at different concentrations of VIII on lipid peroxidation, by a fixed dose of tert-butylhydroperoxide are shown to be dose dependent (**Fig. 3**). It is interesting to note that VII and VIII which differ in methyl substitution at N-1 and N-7 positions, exhibit significant difference in potency in preventing lipid peroxidation in erythrocyte membranes *in vitro* (**Table 3**). This appears to be contrary to what has been suggested earlier that a methyl group at the N-7 position in a xanthine molecule would adversely affect the antioxidant activity (40). It is quite possible that the structural variations could change the lipophilicity, and inhibition of lipid peroxidation seems to be related to the lipophilicity of the compounds tested.

Earlier studies have demonstrated that uric acid has the ability to scavenge peroxyl radicals (41). Peroxyl radicals are highly reactive and can give rise to hydroperoxides and lead to lipid peroxidation (42). In the present study, we have analyzed the peroxyl radical scavenging ability of 8-oxopentoxifylline (V) and 8-oxolisofylline (VI) by competition kinetics of crocin bleaching. It was observed that V and VI are good peroxyl radical scavengers with an IC_{50} values of 1.8 ± 0.08 μM and 2.2 ± 0.13 μM, respectively (**Table**

5). It appears that both V and VI are as efficient peroxyl radical scavengers as uric acid (IC_{50}, 1.9 ± 0.05 μM) at all concentrations tested (**Fig. 4**). The high rate constant ratio indicates that, compound V and VI are potent peroxyl radical scavengers. However, pentoxifylline (I), lisofylline (II) and xanthine failed to scavenge peroxyl radical indicating the importance of 8-oxo group in urates, for their antioxidant and radical scavenging properties.

It is known that 8-oxocaffeine (1,3,7-trimethyluric acid) reacts with stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) at a significantly reduced rate compared to uric acid (32). So it was suggested that urates to have maximum reactivity with DPPH and probably with other free radicals, the hydrogen at N_7 must be available to react. If the hydrogen at N_7 or the hydrogen at N_3 are not present, the reactivity of the urates is reduced to less than 0.1, of that of uric acid (32). In the present investigation, the reaction between DPPH and 8-oxopentoxifylline and 8-oxolisofylline were studied and it was noticed that, these analogues reacted with DPPH at initial rates (data not shown) similar to the rates reported earlier for 1,3,7-trimethyluric acids (32). It appears that the reactivity of 8-oxopentoxifylline and 8-oxolisofylline with DPPH does not reflect on their ability to scavenge hydroxyl or peroxyl radicals, as evidenced by the fact that 8-oxopentoxifylline and 8-oxolisofylline tested (**Table 1 and 5**) are potent hydroxyl and peroxyl radical scavengers.

The available evidence suggests that, uric acid is a poor superoxide anion radical scavenger (33,). In fact, xanthines are inactive towards the superoxide anion radicals. The pulse radiolysis studies indicated that, pentoxifylline does not interact with superoxide radicals (9,12). In the present study, we have observed that uric acid, 8-oxopentoxifylline 8-oxolisofylline, 3-Propyluric acid and 3-Propyl-1,7-dimethyluric acids are poor superoxide anion radical scavengers and this is consistent with earlier reports. However, we have noticed that compounds V-VIII are much better superoxide anion radical scavengers than uric acid (**Table 6**), whereas the corresponding xanthines (I-IV) are not superoxide anion radical scavengers.

The present study has also observed that excepting 3-propyluric acid (**Table 7**), all other analogues of uric acid were oxidized by H_2O_2 in the presence of hematin suggesting their ability to protect hemoglobin and erythrocyte membranes. Heme-catalyzed oxidation of uric acid and methyluric acids has been reported earlier (28). Earlier it has been shown that, pentoxifylline interacts with hydroxyl radical and undergoes oxidative degradation (9). Our results suggest that 8-oxopentoxifylline and 8-oxolisofylline readily interact with radicals and undergo rapid degradation without forming a prooxidant.

Earlier studies have established that the 8-oxo group of urates is an important functional moiety responsible for high hydroxyl radical scavenging and antioxidant properties (17,18). It is quite possible that pentoxifylline (I) and lisofylline (II) could get converted to their corresponding 8-oxoderivatives *in vivo* as one of their metabolites. This is supported by the fact that caffeine, theophylline and theobromine get metabolized in the mammalian system to their corresponding 8-oxo derivatives (13,14) and these metabolites are known to inhibit lipid peroxidation in human erythrocyte membranes *in vitro* (15,16). The observation made in the present investigation are that 8-oxopentoxifylline (V) and 8-oxolisofylline (VI) are much better hydroxyl and peroxyl radical scavengers and more potent inhibitors of lipid peroxidation than the parent drugs (I and II). This suggests the possibility that the beneficial effects of pentoxifylline (I) and lisofylline (II), particularly their anti-inflammatory and antioxidant properties, are exerted more through their 8-oxo derivatives rather than the parent compounds.

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CHAPTER 3: PART D

Specific Inhibition of Mammalian 15-Lipoxygenase by 8-Oxo Derivatives of Pentoxifylline and Lisofylline

INTRODUCTION

Xanthine derivatives, viz, pentoxifylline and lisofylline are extensively used as drugs in many types of disorders (1-3). These xanthine derivatives are vasodilators, inhibit platelet aggregation and thromboxane A2 synthesis, inhibit phagocyte function (e.g. decrease the release of free radicals), increase prostacyclin synthesis from endoperoxides, reduce blood viscosity through increasing erythrocyte flexibility, decrease erythrocyte aggregation and reduce plasma fibrinogen concentration, and may be neuroprotective. Based on all the above mentioned properties, pentoxifylline and its analogues are extensively used in the treatment of cerebrovascular, peripheral vascular diseases and during acute ischaemic stroke (1-3). Recent studies have indicated the antiatherogenic and antithrombatic properties of pentoxifylline (4).

Oxidative modification of plasma low density lipoproteins (LDL) within the vessel wall is believed to play a role in the progression of atherosclerosis (5). Oxidation of Arachidonic acid (AA) is responsible for the initiation of atherosclerosis, and 15-lipoxygenase mediates this oxidative reaction in LDL (6). Arachidonic acid (AA) metabolites are also important mediators of a variety of physiological processes and inflammatory reactions. In addition, alterations in AA metabolism may potentially mediate key steps in certain neoplastic processes (7-9). Lipoxygenase mediated metabolism of arachidonic acid occurs in specific blood cell types and epithelial tissues and is activated during inflammation and tissue injury. The lipoxygenases are a structurally related family of non-heme iron dioxygenases that function in the production of fatty acid hydroperoxides. Three lipoxygenases have been identified and cloned in humans (10-13). They oxygenate arachidonic acid in different positions along the carbon chain and form the corresponding 5*S*-, 12*S*-, or 15*S*-hydroperoxides (hydroperoxyeicosatetraenoic acids, HPETEs). These three enzymes are known mainly from the blood cell types in which they are strongly expressed and these are the 5*S*-lipoxygenase of leukocytes, the 12*S*-lipoxygenase of platelets, and the 15*S*-lipoxygenase of reticulocytes, eosinophils, and macrophages (14,15).

Oxidative modification of plasma lipoproteins within the vessel wall is presumably an extracellular event, which has been implicated in the progression of atherosclerosis

((16). The concept that, plasma lipoproteins infiltrate into the arterial wall at sites of endothelial injury/dysfunction, decorate extracellular matrix and become modified is not new (17) but mechanisms responsible for generation of the modifying agent are emerging. Based on observations that, oxidized lipoproteins are involved in regulation of chemokines (18) and adhesion molecules (19). It is known that, antioxidant agents such as probucol (20,21), vitamins E and C (22,23) and butylated hydroxytoluene (BHT) (24) are effective at limiting atherosclerotic lesion progression. Recent reports suggest that, arachidonate 15-lipoxygenase (15-LOX) is involved in lipoprotein oxidation (25) and the oxidative products may be important in the regulation of proatherogenic molecules. Arachidonate 15-lipoxygenase is a lipid-peroxidizing enzyme, which is present in atherosclerotic lesions. Investigators have found stereospecific hydroxy fatty acids that can be ascribed to 15-LOX activity in extracts of vascular atherosclerotic tissue from animals (26) and humans (27). Hiltunen *et al.* have observed 15-LOX protein and mRNA in early stages of atherosclerosis in animals (28), while others have colocalized 15-LOX mRNA with epitopes of modified LDL in macrophage-rich areas of rabbit and human lesions (25,29). *In vitro* studies have shown that, non-specific inhibitors of lipoxygenase such as ETYA, piriprost and A64077 can ablate macrophage-mediated oxidation of LDL *in vitro* (30). It has also been shown that, inhibition of 15-LOX with PD146176, a specific inhibitor of 15-LOX lacking antioxidant properties, can attenuate the development of dietary cholesterol-induced atherosclerotic lesions in the rabbit (31).

Pentoxifylline and lisofylline were extensively used in the treatment of peripheral vascular diseas,. Which also has antiatherogenic and antithrombatic properties (4). It has been shown that, pentoxifylline inhibits laser-induced thrombus formation and atherosclerotic plaque development in animal models (4). However, the exact mechanism is not known. In the earlier chapter we have shown that, 8-oxo derivatives of pentoxifylline and lisofylline are more potent antioxidants and radical scavengers than their parent drugs. The effects of pentoxifylline and lisofylline on 15-lipoxygenase have not been studied so far. In the present study, for the first time we have shown that, 8-oxo derivatives of pentoxifylline and lisofylline are specific inhibitors of mammalian reticulocyte type 15-lipoxygenase.

MATERIALS AND METHODS

Chemicals

Linoleic acid and Nordihydroguaiaretic acid (NDGA) were obtained from Sigma Chemical Company, St. Louis, MO., USA. Pentoxifylline, lisofylline, 8-oxopentoxifylline and 8-oxolisofylline were prepared as described in chapter 3: part A and C. Preparation of COX-1 and COX-2 were described in chapter 2: part F. Potato 5-lipoxygenase and soybean 15-lipoxygenase were generous gifts from Prof. Reddanna, Central University of Hyderabad, India.

Preparation of rabbit reticulocyte 15-lipoxygenase (15-LOX)

The rabbit reticulocyte 15-LOX was partially purified from reticulocytes of rabbit with bleeding-induced anemia as reported previously (32). 15-LOX was prepared from the lysate of a reticulocyte-rich blood cell suspension, which was further subjected to ammonium sulfate fractionation. The 15-LOX thus obtained was used for the inhibition studies.

15-LOX assay

Polarographic method:

The enzyme activity of rabbit reticulocyte 15-LOX was measured by the rate of oxygen uptake during hydroperoxide formation of linoleic acid in aqueous solution. The oxygen uptake was polarographically measured using a Clark type oxygen electrode (YSI model-5300 biological oxygen monitor) as described earlier (33). In the inhibition studies of pentoxifylline, lisofylline and their 8-oxo derivatives on the rabbit reticulocyte 15-LOX, the enzyme was preincubated (1 min) with pentoxifylline, lisofylline, 8-oxopentoxyfylline, 8-oxolisofylline (0-500 μM, final concentration) or a known LOX inhibitor, NDGA (0-50 μM in ethanol) in a total volume of 2.9 ml of phosphate buffer (100 mM, pH 7.4) at room temperature. The reaction was started by the addition of linoleic acid (280 μM) in ethanol (10 μl). The inhibitors were added as highly concentrated DMSO stock solution so that the solvent concentration is always kept below 1%. At these concentrations, DMSO did not inhibit the LOX activity significantly. The reaction was carried out at room temperature

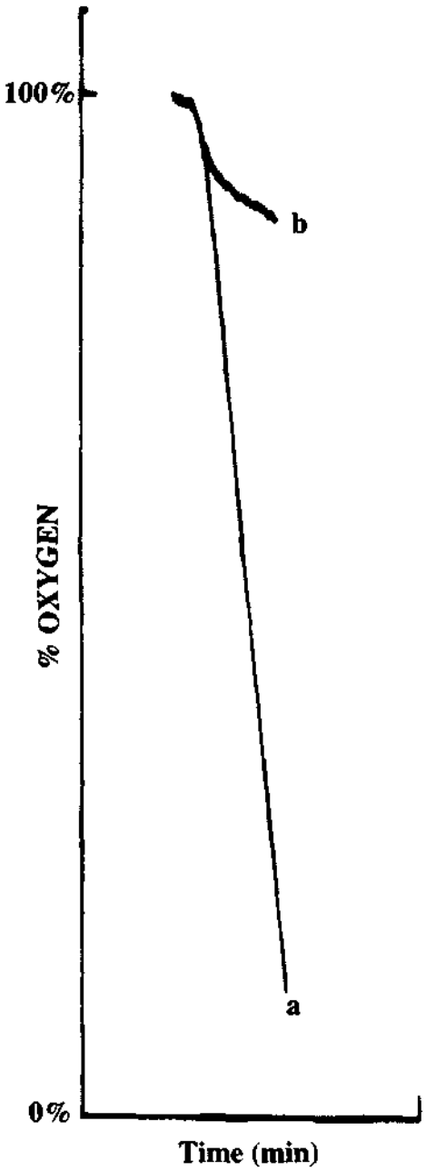

Fig. 1: Typical polarographic recording of 15-lipoxygenase activity
a) without inhibitor
b) with inhibitor (8-oxo lisofylline (250 μM)

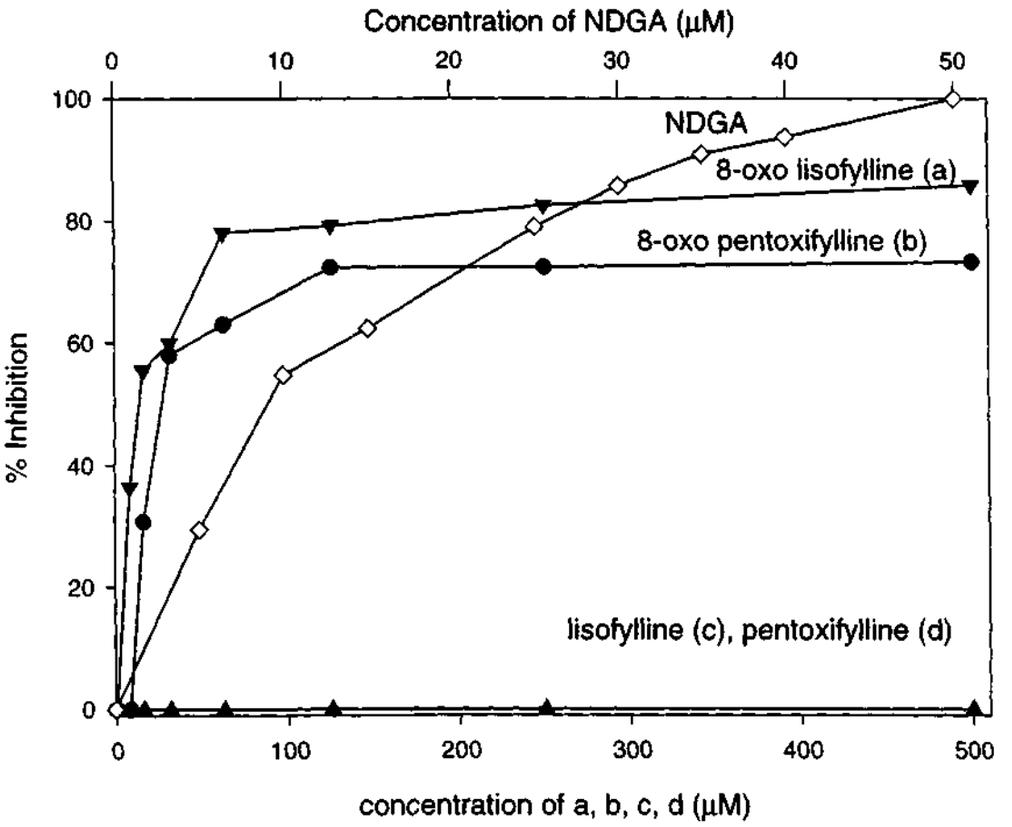

Fig. 2: Effect of 8-oxolisofylline (a), 8-oxopentoxifylline (b), lisofylline (c), pentoxifylline (d) and NDGA on rabbit reticulocyte 15-lipoxygenase. Each plot represents the mean ± SD of 3 experiments.

Table 1: Inhibition of rabbit reticulocyte type 15-lipoxygenase by NDGA, 8-oxopentoxifylline and 8-oxolisofylline

Compounds	IC_{50} (μM)[¶]
8-oxopentoxifylline	52.9
8-oxolisofylline	12.9
NDGA	9.5

[¶]Inhibition studies were carried out by polarographically

Table 2: Inhibition of rabbit reticulocyte type 15-lipoxygenase by 8-oxopentoxifylline and 8-oxolisofylline

Compounds	%Inhibition[¶]
8-oxopentoxifylline	
250 μM	58.3
500 μM	100
8-oxolisofylline	
250 μM	52.5
500 μM	98.9

[¶]Inhibition studies were carried out by spectrophotometrically

and the decrease in O_2 concentration was recorded. Since lipoxygenases are oxygen consuming enzymes, the rate of decrease in O_2 concentration is taken as a measure of enzyme activity. Typical polarographic recording of lipoxygenase activity is presented in **Fig. 1** in the presence and absence of inhibitor. The enzyme activity is calculated using the following equation and the activity is expressed in units/ml. One unit is defined as μmoles of oxygen consumed/min.

$$\text{LOX activity} = \frac{(Vol_T) \times (O_2/ml) \times \text{Slope/min}}{\text{Sensitivity factor} \times \text{Vol. Enz}}$$

Where,

Vol_T = Total volume of the reaction mixture

O_2/ml = Concentration of O_2 under standard atmospheric conditions

Slope/min = Maximum slope generated/minute

Vol. Enz = Volume of enzyme taken for the reaction mixture in ml

Spectrophotometric method:

Enzyme activity was also measured spectrophotometrically by recording the formation of conjugated hydroperoxide at 234 nm (34). For the inhibition studies of pentoxifylline, lisofylline and their 8-oxo derivatives on 15-LOX, the enzyme was preincubated with inhibitors (250 and 500 μM, final concentration) in a total volume of 1 ml at room temperature in phosphate buffer (100 mM, pH 7.4). The reaction was started by the addition of linoleic acid (280 μM) in ethanol (10 μl) and change in the absorbance at 234 nm was recorded for 1 min. Results are expressed as % inhibition of LOX activity.

RESULTS AND DISCUSSION

As shown in **Fig. 2**, both 8-oxo derivatives of pentoxifylline and lisofylline inhibited reticulocyte-type 15-lipoxygenase in a dose dependent manner with an IC_{50} value of 59.2 and 12.9 μM, respectively (**Table 1**). At 125-500 μM concentrations of 8-oxopentoxifylline and 8-oxolisofylline inhibited 73 and 87%, respectively as measured by the polarographic method (**Fig. 2**) and almost completely abolished according to spectrophotometric method (**Table 2**). However, potato 5-LOX, soybean 15-LOX,

Table 3: Effect of 8-oxo pentoxifylline and 8-oxo lisofylline on potato 5-LOX, soybean 15-LOX, rabbit reticulocyte 15-LOX, ram seminal vesicle COX-1 and human recombinant COX-2 at 500 μM concentration

Enzyme	Compound (500 μM)	% inhibition[¶]
5-LOX	8-oxo pentoxifylline	0.0
(Potato)	8-oxo lisofylline	0.0
15-LOX	8-oxo pentoxifylline	0.0
(Soybean)	8-oxo lisofylline	2.9
15-LOX	8-oxo pentoxifylline	81.4
(Rabbit reticulocyte)	8-oxo lisofylline	85.7
COX-1	8-oxo pentoxifylline	0.0
(Ram seminal vesicle)	8-oxo lisofylline	0.0
COX-2	8-oxo pentoxifylline	0.0
(Human recombinant)	8-oxo lisofylline	0.0

[¶]Inhibition studies were carried out by polarographically

mammalian COX-1 and COX-2 were almost unaffected by of 8-oxopentoxifylline and 8-oxolisofylline even at 1 mM concentration (**Table 3**). It is interesting to note that, both pentoxifylline and lisofylline completely failed to inhibit all the enzymes tested including rabbit reticulocyte 15-LOX. NDGA, a known inhibitor of LOX, inhibited almost completely at 50 μM concentration and inhibition is concentration dependent with an IC_{50} value of 9.5 μM for rabbit reticulocyte 15-LOX (**Table 1**). NDGA is an antioxidant and is well known non-specific LOX inhibitor (35).

Results presented in the earlier chapters clearly indicate that, both 8-oxo derivatives of pentoxifylline and lisofylline are significantly better radical scavengers and antioxidants than the parent drugs in all tested concentrations. Studies presented in this chapter section have shown that 8-oxo derivatives of pentoxifylline and lisofylline are specific inhibitors of rabbit reticulocyte 15-LOX. However, these 8-oxo compounds failed to inhibit soybean 15 LOX. Although 15-LOX from soybean is not identical with the mammalian 15-LOX, there exists a good correlation with these two enzymes regarding the inhibitory activity (36). Hence, soybean 15-LOX is commonly used in assays to study the inhibition of 15-LOX by various compounds. In the present study it was noticed that, 8-oxo derivatives of pentoxifylline and lisofylline failed to inhibit soybean 15 LOX although mammalian 15-LOX was inhibited to a significant extent.

It is known that, the oxidation of LDL by 15-LOX is responsible for the development of atherosclerosis *in vivo* and the putative role of 15-LOX in atherosclerosis has been discussed in great detail (6,37,38). However, at present, the role of 15-LOX inhibitors as possible antiatherosclerotic agents is still mostly a matter of conjecture, although some recent results with both antioxidant and nonantioxidant inhibitors of 15-LOX (39-41) seems to be promising. Recent studies also suggested that, LOX inhibitors are promising new class of chemopreventive agents (42).

Available evidences clearly suggested that, Pentoxifylline possess hemorheological actions that improve the microcirculation (43). It is a phosphodiesterase inhibitor that can cause vasodilation in some vessels by endothelium-dependent and independent mechanisms (44). Pentoxifylline also inhibits production of platelet activating factor, reduces platelet aggregation, increases erythrocyte membrane fluidity, and reduces the

production of inflammatory cytokines, particularly tumor necrosis factor, by phagocytes and vascular endothelium (45-48). Recent reports suggest that, pentoxifylline is also having antiatherogenic and antithrobatic properties (4). It has also been shown that, pentoxifylline inhibits laser-induced thrombus formation and atherosclerotic plaque development in animal models (4). Lisofylline, an important metabolite of pentoxifylline in humans *in vivo*, is a potent inhibitor of inflammatory lipid mediator, phosphatidic acid formation, stimulated by IL-1β and TNF-α, *in vivo* (49). However, so far it is not known the involvement of 8-oxopentoxifylline and 8-oxolisofylline in the observed pharmacological properties of pentoxifylline and lisofylline. Our results suggest that, 8-oxopentoxifylline and 8-oxolisofylline may be involved in the observed anti-inflammatory and antiatherogenic properties observed with pentoxifylline and lisofylline. This is possibly achieved by inhibiting an atherogenic enzyme, 15-LOX. Our results also suggest that, 8-oxolisofylline is more potent than the 8-oxopentoxifylline. It is interesting to note that, these compounds are specific inhibitors of mammalian 15-LOX whereas soybean 15-LOX is unaffected.

Since 8-oxopentoxifylline and 8-oxolisofylline are antioxidants and specific 15-LOX inhibitors, they can be used as antiatherosclerotic as well as anticancer drugs. However, further studies are required to understand the metabolism and mechanism of action of these compounds *in vivo*.

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