New Research on Antioxidants

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Editors

NOVA

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CONTENTS

PREFACE

In biological systems, the normal processes of oxidation (plus a minor contribution from ionizing radiation) produce highly reactive free radicals. These can readily react with and damage other molecules. In some cases the body uses free radicals to destroy foreign or unwanted objects, such as in an infection. However, in the wrong place, the body's own cells may become damaged. Should the damage occur to DNA, the result could be cancer. Antioxidants decrease the damage done to cells by reducing oxidants before they can damage the cell. Virtually all studies of mammals have concluded that a restricted calorie diet extends the lifespan of mammals by as much as 100%. This remarkable finding suggests that food is actually more damaging than smoking. As food produces free radicals (oxidants) when metabolized, antioxidant-rich diets are thought to stave off the effects of aging significantly better than diets lacking in antioxidants. The reduced levels of free radicals, resulting from a reduction in their production by metabolism, is thought to be a major cause of the success of caloric restriction in increasing life span.

Antioxidants consist of a group of vitamins including vitamin C, vitamin E, selenium and carotenoids, (such as beta-carotene, lycopene, and lutein). This new book brings together the latest research in this dynamic field.

Chapter I - Some therapy against leukaemia is based on the use of compounds able re-induce the differentiation program. The use of the active form of vitamin D, calcitriol $(1,25D_3)$, or trans-retinoic acid (ATRA) in monocytic or myeloid leukemic cell differentiation respectively has been taken into consideration in clinical therapy. They are natural compounds that are accepted at relatively high doses by the organism, but secondary effects can take place due to other functions different than regulating the differentiation program in cancerous cells. These secondary effects due to overloading of both, ATRA or $1,25D_3$, can be surpassed by co-treatment with compounds with antioxidant properties such as vitamin C, vitamin E, polyphenols, carotenoids or other antioxidants. In some cases, these compounds also have demonstrated anti-leukaemic activity. Thus, they use in clinical studies has been taking into consideration. In several cases, these natural compounds are unable to induce the differentiation program in leukemic cells but they enhance the effect of the activators of differentiation such as ATRA or $1,25D_3$. In the present chapter the authors review the effect of the use of natural antioxidant compounds on the treatment of leukemic cells and will try to define the probable common mechanism of action for this plethora of substances.

Chapter II - For more than half a century, numerous proposals have been advanced for the mode of action of carcinogens. This review presents a wide array of evidence that implicates oxidative stress (OS) in many aspects of oncology, including formation of reactive oxygen species (ROS) by the major classes of carcinogens (as well as minor ones), cancer stages, oncogene activation, aging, genetic and infectious illnesses, nutrition, and the role of antioxidants (AOs). Although diverse origins pertain, including both endogenous and exogenous agents, ROS are frequently generated by redox cycling via electron transfer (ET) groups, e.g., quinones (or phenolic precursors), metal complexes (or complexors), aromatic nitro compounds (or reduced products), and conjugated imines (or iminium species). The authors believe it is not coincidental that these functionalities are often found in carcinogens or their metabolites. The pervasive aspects of DNA binding by ultimate carcinogens, and mutations caused by ROS are treated. Often, ROS are implicated in more conventional rationales, such as oncogenes. A multifaceted approach to mechanisms appears to be the most logical.

The OS unifying theme represents an approach, which is able to rationalize the diverse data associated with tumorigenesis. Because this theoretical framework aids in the understanding of cancer initiation, it can serve as a useful tool in combating cancer, particularly in relation to prevention by antioxidants.

Chapter III - *Astragali Radix,* a main component in many Chinese herbal medicines, was shown to have antioxidant effects on lipid peroxidation. Afrormosin, calycosin and odoratin as isoflavones in *Astragali Radix* were isolated and identified as antioxidant components on lipid peroxidation. Biochnin A, daidzein and genistein as other isoflavones also were shown to have antioxidant effects on lipid peroxidation. These results demonstrated that *Astragali Radix,* afrormosin, calycosin, formononetin and odoratin as isoflavones in *Astragali Radix*, biochanin A, daidzein and genistein as other isoflavones have antioxidant effects on oxidative stress.

Chapter IV - Protection against free radical-initiated oxidative damage has long been recognized as the most important biological function of vitamin E. However, the mechanism by which vitamin E exerts its antioxidant function *in vivo* has yet to be delineated. Recent findings that dietary vitamin E reduces the rates of mitochondrial superoxide generation and levels of labile iron provide a rational explanation as to how the vitamin may exert its antioxidant function at the tissue level. Superoxide is a key precursor for other reactive oxygen/nitrogen species (ROS/RNS), and is capable of releasing iron from its protein complexes. The labile or available form of iron has the potential to catalyze the formation of reactive hydroxyl radicals. Superoxide can also react readily with nitric oxide to form peroxynitrite. Both hydroxyl radicals and peroxynitrite have potential to initiate oxidative damage to essential biomolecules. Thus, by reducing available superoxide, dietary vitamin E may reduce available hydroxyl radicals and peroxynitrite, and thus attenuate oxidative damage. Furthermore, by reducing the levels of ROS/RNS, vitamin E may modulate the activation and/or expression of redox-sensitive biological response modifiers, and, thereby, mediate the cellular events leading to the onset of cardiovascular, cancer, aging and other degenerative diseases.

Chapter V - This chapter is aimed to underline the increasing importance that natural antioxidants have been gaining in the last years. Antioxidants are naturally present in many foods, so that they can be seen as potential recovery sources: oilseeds, nuts, cereals, legumes, vegetables, fruits, herbs, spices and teas. Besides these, antioxidants are often present in food

processing by-products and wastes, so that the employment of low-cost industrial wastes could greatly reduce the production costs and increase the margin profit of the products. The introductive section summarises the classes of antioxidant compounds (mainly focusing on phenolic compounds), their potential food and no-food applications, and the main problems you have to account for when recovering antioxidants from residual sources, such as selection of a suitable agriculture by-product, choice and optimisation of the extraction procedure, analytical characterisation and evaluation of antioxidant activity of the obtained extracts, evaluation of potential applications of the isolated substances.

The second part of the chapter presents an experimental work dealing with recovery of phenolic compounds from wine-making wastes through a simple solvent extraction process. Trials were carried out in order to evaluate the feasibility of using different by-products (grape stalks, grape marcs before and after distillation), the influence of grape variety, of different sample pre-treatments, type of solvent, extraction temperature and time (extraction kinetics) on extracts yield and quality in terms of phenolics content and antioxidant power. Food applications of the obtained compounds to inhibit oil oxidation and to extend shelf-life of fresh fruits were also investigated.

Chapter VI - For the first time, the antioxidant activity of carnosine has been discovered in the pioneering experiments of Severin and co-workers. The results of these experiments are taking new twists in the context of current concept of mechanisms of free radical processes in excitable tissues.

Chapter VII - In analogy with p-phenylenediamine and N,N'-diphenyl-p-phenylenediamine it has been supposed that the degradation of all *N*-phenyl-*N*'-alkyl-p-phenylenediamine (PPD) antioxidants begins with amine N-H bonds cleavage and benzoquinonediimine intermediates are formed. Nevertheless, the results of non-isothermal DSC measurements of a series of PPD antioxidants indicate that the N-bonded alkyl carbon atom is crucial for their antioxidant effectiveness and the existence of aromatic ketimine structures may be supposed. It has been shown that IR spectra of commercial PPD antioxidant samples heated on air correspond to the double dehydrogenated ketimine structures with Phenyl-N=C double bond and not to their N,N´-dehydrogenated benzoquinonediimine counterparts. DFT quantum-chemical calculations of the optimized structures of a series of PPD antioxidants and of their possible dehydrogenation products have been used for IR spectra interpretation as well as for energy and electronic structure data evaluation. These results support the idea of preferential ketimine Ph-N=C structures formation (they are more stable than the corresponding quinonediimine structures) despite the tertiary carbon centered radicals formation in the first dehydrogenation step need not be energetically preferred over the nitrogen centered ones. The results of recent PPD degradation studies using DFT and semiempirical methods of quantum chemistry are compared.

Chapter VIII - *Identification, purification and characterization* of a new protein from the serum of patients with Eales' disease is presented. The protein was purified using preparative electrophoresis and HPLC. It resolved in α_2 globulin region. The purified protein had a retention time of 9.2 min in RP-HPLC. The molecular weight as determined by gel permeation chromatography was 88 kDa. Hence the protein is referred as 88 kDa protein. Periodic acid Schiff's staining revealed it as a glycoprotein. It was completely denatured above 70º C. Its isoelectric pH was 5. The protein was present in the vitreous and epiretinal membrane of Eales' patients and the blood of patients with systemic diseases like tuberculosis, leprosy and rheumatoid arthritis, but was absent in diabetic retinopathy and

healed vasculitis of Eales' disease, as in the latter there is no inflammation. Hence 88 kDa protein is an acute phase protein produced in inflammatory conditions. The protein of different sources and diseases was immunologically the same.

Chapter IX - α-Ketoglutarate (α-KG) is a Krebs cycle intermediate. Ornithine α-ketoglutarate (OKG) is a salt formed from one molecule of α-ketoglutarate and two molecules of ornithine, which is a urea cycle intermediate. The authors investigated the effects of α-KG as well as OKG against ammonia induced oxidative stress in rats. Administration of ammonium acetate significantly elevated the levels of lipid peroxides and depleted the antioxidants (superoxide dismutase, catalase, glutathione peroxidase and reduced glutathione). α-KG as well as OKG positively modulated these changes. Thus it can be concluded that α-KG as well as OKG would exert their antihyperammonemic effects by positively modulating the lipid peroxides and antioxidants.

In the other study, the authors investigated the effects of α-KG on biochemical circadian rhythms during N-nitrosodiethylamine (NDEA) induced hepatocarcinogenesis in rats. NDEA caused a delay in acrophase of thiobarbituric acid reactive substances (TBARS) and antioxidants and an increase in mesor values of TBARS and decrease in mesor values of antioxidants. Administration of α-ketoglutarate reversed the circadian rhythm alterations caused by NDEA treatment. It can be suggested that α-KG would exert its chemopreventive effect by positively modulating the circadian rhythms of lipid peroxides and antioxidants.

Chapter X - The antioxidant content of a number of prophylactic and treatment preparations of the "Vision" company was determined. These were found to lie in the range $(7.5 - 1.7) \cdot 10^{-3}$ mole/kg, which is close to the antioxidant content of dry medicinal plants.

Chapter I

ROLE OF ANTIOXIDANTS IN THE THERAPY AGAINST LEUKEMIA

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ABSTRACT

Some therapy against leukaemia is based on the use of compounds able reinduce the differentiation program. The use of the active form of vitamin D, calcitriol ($1,25D_3$), or trans-retinoic acid (ATRA) in monocytic or myeloid leukemic cell differentiation respectively has been taken into consideration in clinical therapy. They are natural compounds that are accepted at relatively high doses by the organism, but secondary effects can take place due to other functions different than regulating the differentiation program in cancerous cells. These secondary effects due to overloading of both, ATRA or $1,25D_3$, can be surpassed by co-treatment with compounds with antioxidant properties such as vitamin C, vitamin E, polyphenols, carotenoids or other antioxidants. In some cases, these compounds also have demonstrated anti-leukaemic activity. Thus, they use in clinical studies has been taking into consideration. In several cases, these natural compounds are unable to induce the differentiation program in leukemic cells but they enhance the effect of the activators of differentiation such as ATRA or $1,25D_3$. In the present chapter we review the effect of the use of natural antioxidant compounds on the treatment of leukemic cells and will try to define the probable common mechanism of action for this plethora of substances.

INTRODUCTION

Leukaemia can be defined as an haematopoietic stem cells malignant neoplasia that initiates in the bone marrow but that finaly invades blood and other organs causing the death of the organism. Although they are known with the same name, there are different types of leukaemia depending depending on the lineage of the compromised cells (myeloid or lymphoid) and of the mature state of the leukemic cell (acute or chronic). If the leukemic cell stops the differentiation program at a early state in the development of the haematopoiesis, it is an acute leukaemia but if leukemic cells stops at the latest steps in the differentiation program, we are treating a chronic leukaemia.

Between the different kinds of leukaemia, acute myeloid leukaemia (AML) is at the moment, the most common form of leukaemia being the most frequent cause of death. It can be specifically defined as a clonal disorder characterized by the accumulation of abnormally differentiated myeloid cells that are not mature. However, due to the different steps and lineages in the maturation of myeloid cells, several different types of AML has been described (Table I).

Table I. FAB Classification of AML

Morphological Features of AML	
Name	Morphological characteristics
Acute myelocytic leukemia (M1)	Very undifferentiated cells, very low cytoplasmic granules, some are promyelocytes
Acute myelocytic leukemia (M2)	Predominance of granulated blasts, small number of monocytoid cells, evidences of differentiation beyond promyelocytic stage
Acute promyelocytic leukemia (M3)	Predominance of typically hypergranular promyelocytes, cells have large basophilic and eosinophilic granules
Acute myelomonocytic leukemia (M4)	Precursors of monocytes and granulocytes, serum lysozyme elevated
Acute monoblastic leukemia (M5)	Large monoblasts showing abundant agranular cytoplasm that may be vacuolated and basophilic
Erythroleukemia (M6)	Predominance of megaloblastoid red cell precursors, some myeloid blasts can also be seen, presence of multinucleated red cell precursors
Megakaryocytic leukemia (M7)	Variable morphology, megakaryocytic features may not be seen with light microscopy

The most well known causes of AML are multiple genetic defects, which often involve either translocations or mutations within oncogenes. These genes can be critical components of signalling pathways that regulate proliferation, differentiation, and apoptosis.

Treatment of AML has not suffer significant changes in the past years. Anthracyclines and cytarabine are still the mainstays of first-line therapy. Novel chemotherapeutic agents, such as temozolomide, an oral alkylating agent, and troxacitabine, a novel nucleotide analogue, have been studied, but it is unclear whether they are better than conventional therapy. In addition to this, most AML patients are intrinsically resistant to chemotherapy. In fact, most patients die either after relapse or from the side effects associated with the treatment.

Future strategies to fight against AML must be developed to identify the proteins that are involved in the process of leukemogenesis and, thus, to understand the mechanisms by which the leukaemia develops. The advances made in the elucidation of the mechanisms involved in leukemogenesis and the molecular alterations underlying the pathogenesis of AML have enabled the development and use of new therapies directed to specific molecular targets. One important think to understand is that a unique genetic abnormality may not be sufficient to promote leukemogenesis. The cooperation or the interaction between various regulatory pathways are frequent events in the development of leukaemia (Delgado, et al., 2000; Hayakawa, et al., 2000; Mizuki, et al., 2000; Stirewalt, Meshinchi & Radich, 2003). Therefore, if multiple defects are required, targeting one of these multiple genetic abnormalities may not be sufficient to reverse leukemogenesis, but this reversion may be enhanced by simultaneously targeting several different pathways within the same AML clone.

Small-molecule inhibitors of signalling pathways are being currently developed for the treatment of AML. These compounds are typically targeted against pathways that have been activated by mutation. However, to date only modest clinical results have been obtained. The causes are diverse: in some cases, the target molecule is inhibited for an inadequate duration, and in other cases, the clinical effect does not show a relationship with the inhibition of the target. Although small-molecule inhibitors are a promise in the treatment of AML, this field of pharmacotherapy is still quite new and they are several obstacles to overcome. However, it is important to believe that the discovery and characterization of new molecular alterations in AML will help to develop novel therapies that will cure the majority of patients.

One of the prototypes for this approach is the treatment of acute promyelocytic leukaemia (APL, M3 status in table I) with all-trans retinoic acid (ATRA) (Tallman, et al., 2002). In this type of AML, the retinoic acid receptor fused to the promyelocytic leukaemia (PML) gene product. The appropriate treatment with ATRA has turned a poor-prognosis disease into one in which most cases are cured. AML, like APL, could be cured with the appropriate combination of chemotherapy with targeted therapy. Numerous investigations have been performed to elucidate the mechanisms and search for effective agents in the treatment of other types of AML. Now, we know that the molecular abnormalities in AML can be found both at the epigenetic level and at the genetic level. Many of these molecular pathways result in changes in gene transcription, which result in a blockade in differentiation of leukemic cells. Following to ATRA, several new agents have emerged, although their clinical effectiveness remains to be confirmed. Therefore, genetic therapy and a combination of different strategies will improve the treatment of AML.

The therapeutic targets in the treatment against leukaemia can be grouped in three levels: mutated proteins, adaptive non-mutational changes to survive after the initial mutational event, and cell surface antigens. Most of the therapies are based on the use of specific compounds against one specific target in different kind of leukaemia. Most of these therapies can be reviewed in (Lopez-Lluch, et al., 2008). The present work is focussed on the use of

antioxidants as co-inducers of the differentiation process in leukemic cells or as protective agents against side effects of chemotherapy.

TREATMENT AGAINST LEUKAEMIA WITH DIFFERENTIATION-INDUCING AGENTS

All trans retinoic acid (ATRA) is able to improve the outcome of acute promyelocytic leukaemia (APL) at a magnitude that is unparalleled in any other cancer. ATRA can induce a complete remission in nearly every newly diagnosed APL patient by inducing cell differentiation. Moreover, when combined with chemotherapy, ATRA doubles the cure rate compared to chemotherapy alone. Furthermore, ATRA has become the only standard transcription factor therapy in clinical practice, by specifically targeting the aberrant transcription factor that underlies the pathogenesis of this disease.

For these reasons, ATRA has been transformed into the most common differentiation agent used as therapeutic drug. The classic chromosome abnormality of APL is a translocation t(15;17)(q22;q21) resulting in the generation of fusion gene and protein PML-RARα, which plays a central role in APL pathogenesis. PML-RARα fusion protein forms a complex with retinoid x receptor (RXR), nuclear corepressors (N-CoR), Sin3A, and histone deacetylase (HDAC). This complex repress the transcription of target genes (Collins, 1998) and acts as a dominant negative factor on the retinoic acid-signalling pathway (Melnick & Licht, 1999) blocking the differentiation of myeloid cells. The mechanism of action of ATRA has been broadly described (FIGURE 1). When ATRA (pharmacological doses) binds to the RAR receptors it induces the degradation of PML-RARα protein through the ubiquitin-proteosome and caspases (Nervi, et al., 1998; Zhu, et al., 1999) leading to restoration of terminal differentiation of promyelocytes. Moreover, ATRA induces relocalization of PML in APL cells (Melnick & Licht, 1999), and a pharmacological dose (1 µM), CoR is dissociated from the repressive complex, whereas CoA (coactivator) is recruited to the complex (Collins, 1998). As a result, the repression of transcriptional activation of target genes is relieved and the differentiation of promyelocytic process is restored.

A synthetic retinoid, 4-[(5,6,7,8-tetrahydro-5.5.8.8-tetramethyl-2-naphthyl)-carbamoyl] benzoic acid, AM80 that is an specific RAR-α agonist, is effective in treating APL appearing after relapse from ATRA-induced complete remission. Among 24 APL patients who relapsed after ATRA therapy, 14 (58%) achieved a second complete remission with Am80 alone (Takeuchi, et al., 1998). Am80 has several potential advantages over ATRA: it is 10 times more potent than ATRA in inducing differentiation in vitro, and is markedly more stable.

Other differentiation-inducing agents have been studied as potential differentiation therapy of acute leukaemia. They can be divided into several different groups (Table II). Most of these agents have only been studied in vitro on only leukaemia cell lines, such as NB4, H-60, U937, K562, Kasumi-2, or in a primary leukaemia cells culture assay. Therefore, the results of clinical trials of their effectiveness are limited.
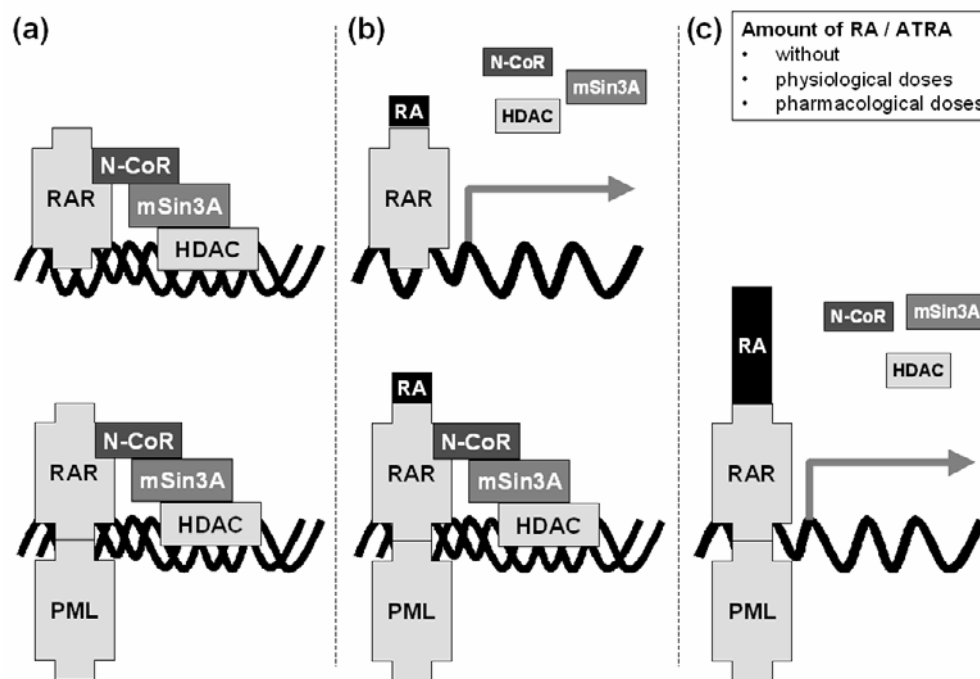
Figure 1. Mechanism of action of ATRA in differentiation of APL cells. The receptor of retinoids is complexed with several other proteins in DNA. In the case of the fusion protein between RAR and PML found in APL cells this complex is also found in the absence of retinoids. Physiological doses of ATRA induce the gene expression directed by RAR in normal cells but not in APL. However, the addition of pharmacological doses of ATRA can unblock the effect of PML in APL and induce gene expression restoring the differentiation pathway. From (Lopez-Lluch, et al., 2008) with permission.

Differentiation induction therapy has been already tested in myelodisplastic syndromes to ameliorate maturation defects and restore normal haematopoietic function. In a pilot study, the combination of ATRA, granulocyte colony-stimulating factor (G-CSF), erythropoietin (EPO) and α-tocopherol demonstrated the well tolerance, the normalization of neutrophils counts and improvement of platelets and red blood cells in a significant subgroup of patients (Ganser, et al., 1996). This study also demonstrates the importance of a combined therapy including antioxidants in the treatment of leukaemia.

However, the differentiation therapy of cancer remains as an only partially attained goal. During the last years, several studies have demonstrated that the combination of different natural compounds with antioxidant activity with leukaemia differentiation inducers such as ATRA or $1,25D_3$ enhance the effectiveness of these inducers. These combinations permit to decrease the dose of the inducers avoiding side effect such as the calcemic effect of $1,25D_3$ that preclude their use in the clinic. Even today, an active investigation on the effect of derivatives $1,25D_3$, on differentiation is being carried out. Then, another approach that may obviate this problem is to combine $1,25D_3$ or its derivatives with other agents that increase the antileukemic effects of low, non-toxic concentrations of vitamin D compounds. The following part of the chapter will review the experiments with these antioxidants and their mechanism of action on leukaemia. However, most of these studies have been developed in

vitro and the results only indicate good perspectives as promising agents in the combinatory therapy of leukaemia.

Table II. Inducers of differentiation in leukaemia therapy

Differentiation agents groups in leukaemia therapy	
Group	Compounds
Vitamin analogues	Retinoids and its derivatives
	Vitamin D and its derivatives
Cytokines	GM-CSF
	G-CSF
	Interferon
	TNF
Polar-aplanar compounds	Hexamethylene bisacetate
Inhibitors of DNA methylation	5-aza-2-deoxycitidine
cAMP analogues	8-Cl-cAMP
	db-cAMP
Chemotherapeutics compounds	Aclarubicin
	Cytosine arabinoside
	Hydroxyurea
	1-β-D-arabinofuranosylcytosine (Ara-C)
	Phorbol esters
Plant derived compounds	Meisoindigo
	Flavonoids
	Cotylenin A
	Tanshinone IIA
	Pyranocoumarins,
	Intermedeol
	Magnolialide

ANTIOXIDANTS IN LEUKAEMIA TREATMENT

It is known that vegetables are a good source of preventive agents against cancer. Plant foods show several potentially anticarcinogenic substances such as carotenoids, vitamin C, vitamin E, selenium, and dietary fibre. Between these compounds, vitamins are considered the most active compounds against carcinogenesis. Thus, the use of high doses of vitamins between which we can found several antioxidants such as vitamin E or vitamin C or combinations of several of them have been extensively studied in the treatment of several types of cancer (Potter & Steinmetz, 1996; Prasad, et al., 1999).

Several other substances from plants have also shown anticarcinogenic effects by different mechanisms. Most of the phytochemicals such as glucosinolates and indoles, thiocyanates and isothiocyanates, phenols, and coumarins can induce a faster elimination of carcinogenic compounds by inducing detoxifying phase II enzymes. Ascorbate and phenols can block the formation of carcinogens such as nitrosamines. Flavonoids and carotenoids can

disable the oxidative stress produced by some carcinogens acting as antioxidants. Moreover, carotenoids and other compounds can affect DNA synthesis and induce differentiation. Thus, plant foods in the diet results in a protective mechanism to reduce cancer risk by several different reasons (Potter & Steinmetz, 1996).

Next, we summarize the current knowledge about the role of different antioxidants in leukaemia therapy and in especial in their effect of therapies based on the induction of the differentiation program.

Ascorbate

Due to the absence of the last enzyme of its biosynthesis pathway, ascorbate is an essential nutrient in several animal species including humans (Chatterjee, 1978). Ascorbate can affect several essential processes in the organism being scurvy the most severe manifestation of ascorbate deprivation (Padh, 1990). Moreover, potential benefits of ascorbate in different pathological processes in humans such as prevention of cancer, cardiovascular risk and cataracts, damage of sperm cells and protection against cigarette smoke-dependent damage have demonstrated the influence of ascorbate on human health (reviewed in (Bendich & Langseth, 1995)). In fact, ascorbate is involved in many cellular functions including modulation of cell growth and differentiation, collagen, carnitine and norepinephrine biosynthesis, activation of hormones, oxygen radical scavenging, and regulation of transcription factors (Alcain & Buron, 1994; Frei, England & Ames, 1989; Gershoff, 1993; Lopez-Lluch, et al., 2001; Munoz, et al., 1997; Padh, 1990).

The use of ascorbate or some of its derivatives has been suggested for cancer therapy, especially against chemotherapy resistant tumour cells (Osmak, et al., 1997). Actually, ascorbate prolongs survival in cancer patients (Cameron & Pauling, 1976) probably by reducing the growth rate of tumoral cells as has been shown in cell cultures (Osmak, et al., 1997; Park, et al., 1980; Venugopal, et al., 1996) and animal models (Gruber, Tewfik & Tewfik, 1980; Pauling, et al., 1985). In vitro studies have demonstrate that near physiologic levels of ascorbate are able to suppress growth of bone marrow cells from patients with AML (Park, et al., 1980) while normal haematopoietic cells are not affected. However, although ascorbate is considered a model of antioxidant compound, the anticancerous effect of this antioxidant has been truly related to its prooxidant activity through the production of H_2O_2 since catalase avoids its growth inhibitory and even its toxic effect (Arakawa, et al., 1994; Nemoto, Otsuka & Arakawa, 1996; Sestili, et al., 1996).

On the other hand, ascorbate is also able to stimulate the differentiation of several mesenchyme-derived cell types including osteoblasts (Franceschi & Iyer, 1992) also other cell types (Farquharson, et al., 1998; Lee, et al., 2003; Ragab, et al., 1998; Savini, et al., 2002; Takahashi, et al., 2003). For example, ascorbate increases the in vitro differentiation of epidermal keratinocytes (Savini, et al., 2002), it also differentiates embryonic stem cells to cardiac myocytes (Takahashi, et al., 2003) or affects the differentiation of embryonic brain cortical precursors into neurons and astrocytes (Lee, et al., 2003). The role of ascorbate in collagen synthesis has been associated to its essential activity on mesenchymal differentiation (Franceschi & Iyer, 1992). However, not all the effect of ascorbate on differentiation can be explained by its essential activity on collagen synthesis. Moreover, we have demonstrated that ascorbate has also shown cooperative activity with $1,25D_3$ in myelomonocytic cell differentiation to mature monocytes (Lopez-Lluch, et al., 2001; Lopez-Lluch, et al., 1998; Lopez-Lluch, et al., 2005; Quesada, et al., 1996).

Addition of physiological concentrations of ascorbate to HL-60 cells increased specific differentiation markers induced by 1,25D$_3$ (Lopez-Lluch, et al., 1995; Quesada, et al., 1996). Although ascorbate, as several other antioxidants, were ineffective in the absence of any other inducer, it increased by about a 50% the number of cells displaying differentiation-associated enzymes or cell surface markers in the presence of 1,25D$_3$. On the other hand, Kang et al., have shown that millimolar concentrations of L-ascorbic acid (LAA) induce the expression of the granulocytic-specific cell surface marker CD 66b in HL-60 cells (Kang, et al., 2003). In this case, catalase abolished and superoxide dismutase promoted LAA-induced differentiation of HL-60 indicating a role of H2O2 in this pro-differentiating activity of ascorbate. However, our studies have demonstrated that such these high doses of ascorbate induce apoptosis in the cells in agreement with other studies that report the same apoptotic effect at millimolar concentrations of ascorbate (Sakagami & Satoh, 1997). In that case, we consider that high concentrations of ascorbate do not increase differentiation in AML cells but induce cell death by apoptosis.

The mechanism by which ascorbate enhances the differentiation activity of calcitriol in leukemic cells remains to be completely elucidated. To date, we have found that ascorbate induces a long-term increase in cAMP levels in HL-60 cells (Lopez-Lluch, et al., 1998) and changes the early response to 1,25D$_3$ by modulating AP-1 transcription factor (Lopez-Lluch, et al., 2001). In most cases, ascorbate-dependent mechanism seems to be dependent on its activity as external acceptor of electrons through the plasma membrane redox system (Lopez-Lluch, et al., 1998; Lopez-Lluch, et al., 1995; Navas, Villalba & Cordoba, 1994). This system is modulated during TPA-induced differentiation in HL-60 cells (Burón, et al., 1993). It is also able to regulate the response to several hormonal factors such as growth factors or insulin (Brightman, et al., 1992; Crane, et al., 1982; Krieger-Brauer & Kather, 1995). Moreover, it can regulate the cytosolic redox balance and then, control intracellular signalling mechanisms during the early steps of the differentiation program. Thus, PMRS can be related to a localized rise of intracellular levels of H$_2$O$_2$ during the first times of incubation that would modulate the response to 1,25D$_3$ and by this mechanism increase the differentiation of cells. As we have already found, this increase if H2O2 can be accompanied by a decrease in superoxide (O$_2$$^{\bullet-}$) (Lopez-Lluch, et al., 2001) similarly to the effect of other compounds with higher depletion activity of O$_2$$^{\bullet-}$ that also induce differentiation of HL-60 cells (Satoh, Sakagami & Motohashi, 1997).

Ascorbate not only affects the differentiation program induced by 1,25D$_3$ in leukemic cells. Recently, imatinib, a BRC/ABL tyrosine kinase inhibitor have been shown as a differentiation inducers with remarkable clinical effects in Chronic Myelogenous Leukaemia (CML) although resistance to this compound appears in most of the blast crisis cases. In the case of resistance, addition of ascorbate together with imatinib to these cells partly restores sensitivity to imatinib. The mechanism of action of ascorbate in this case seems to be through the inhibition of the Nrf2-dependent transcription of detoxifying limiting enzymes such as gamma-glutamylcysteine synthetase (gamma-CGS) that is involved in GSH synthesis (Tarumoto, et al., 2004). Ascorbate seems to inhibit the Nrf2-DNA binding induced in imatinib-treated cells. Furthermore, very recently, ascorbate has shown also a remarkable stimulating activity on the Cytotoxic/cytostatic activity of N-salicylidene-L-glutamato diaqua copper(II) complex (CuC) against mice leukemia cells L1210. Although both, ascorbate and CuC were initially prepared as substances with antioxidant properties the combined treatment of L1210 cells induces strong oxidative stress and death of cells (Paulikova, et al., 2008).

Thus, it seems that ascorbate can exert different roles enhancing the therapy against leukaemia induced by different compounds.

Coenzyme Q

Coenzyme Q (CoQ) is the only endogenous antioxidant that is sintesized by all the aerobic organisms. This lipophilic compound locates into all the cell membranes. Due to its redox capability, CoQ is the only lipophilic intermediary in the electron transport chain in inner mitochondrial membrane. In plants, a similar compound, plastoquinone, exerts a similar function in the photosynthesis electron chain. But, CoQ also shows a very important role in the protection of membranes against oxidative damage by disrupting the lipid peroxidation chain or by recycling vitamin E or ascorbate (Fernandez-Ayala, et al., 2000). Moreover, the activity of CoQ in plasma membrane can prevent the activation of the neutral sphingomyelinase activity and then, release of ceramide and induction of cell death (Figure 2).

Very recently, the importance of CoQ as endogenous antioxidant and essential electron carrier in mitochondria have suggested its use as therapy target in cancer treatment. During chemotherapy, CoQ_{10} levels may change due to its role as key component of the antioxidant defence in cell membrane. A recent work have demonstrated that levels of CoQ_{10} in plasma in acute lymphoblastic leukaemia (ALL) patients increase around 2-3 times (Niklowitz, et al., 2007). This increase was accompanied by the increase of the reduced, and thus, active, form of the quinone. We have recently found a similar response of different cancer cell lines after the treatment with the chemoterapic camptothecin (Brea-Calvo, et al., 2006). Then, a natural response against chemotherapy based on CoQ synthesis is suggested.

Due to its important role in the prevention of cell death by oxidative damage, the CoQ-dependent activities can be used as targets for chemotherapy against leukaemia and other types of cancer. Inhibition of these oxidoreductases can increase ROS production in cells and affect cell cycle progression (Bello, et al., 2005). Moreover, short chain analogues of CoQ can also be used in leukaemia treatment because they affect mitochondrial activity and cell cycle progression by interfering with the activity of the mitochondrial electron transport chain (Fernandez-Ayala, et al., 2005). As example, another compound such as BHA (4-tert-buthyl-4-hydroxyanisole) is widely used as food additive and has shown cytotoxicity against U937 monocytic cells. BHA inhibits complex I and complex IV activities producing ATP depletion and activation of caspases inducing apoptosis (Okubo et al., 2004) then, it has been also suggested as putative anti-leukemic compound.

Vitamin E

Vitamin E lumps together a family of eight isomers consisting in four tocopherols and four tocotrienols: α, β, γ, and δ in each case. These compounds are essential components of the human diet since they are synthesized exclusively by photosynthetic organisms. Recently, they exert important and unique biological effects on nutrition and health other than antioxidant properties and are, therefore, now receiving increased attention.
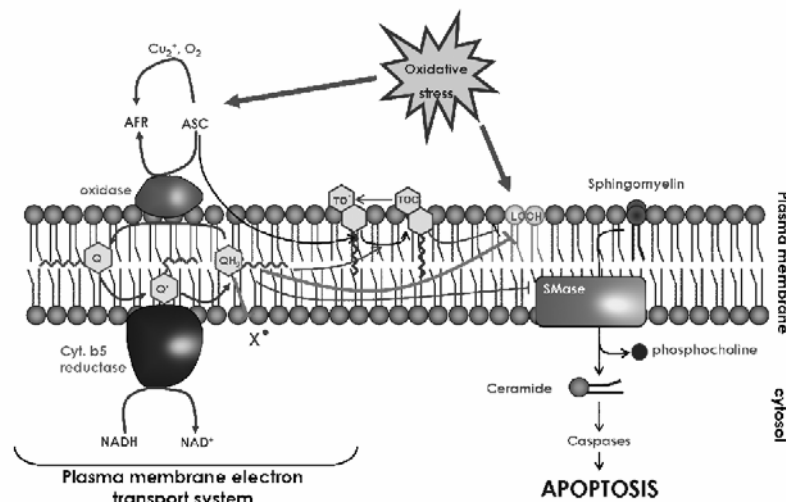
Figure 2. Role of Plasma Membrane Redox System (PMRS) in antioxidant protection of membranes. Cytochrome B5-reductase can maintain a coenzyme Q redox cycle in cell membranes, specially in plasma membrane that is involved in prevention of lipid peroxidation and maintenance of reduced forms of ascorbate and tocopherol. This system can avoid the release of ceramides to cytosol and prevent the apoptosis induced by activation of apoptosis by induction of caspase activity.

The effect of vitamin E on haematopoiesis and blood cell differentiation has been studied from long time. Early studies showed abnormal erythropoiesis in vitamin E deficient monkeys (Fitch, et al., 1980). However, contradictory results have been shown in mouse myeloid leukaemia cells (M1) were vitamin E induces the appearance of several differentiation markers whereas, as other antioxidants, it inhibited the dexamethasone-induced differentiation process (Sakagami, et al., 1981; Takenaga, Honma & Hozumi, 1981).

Like ascorbate, vitamin E, by itself does not strongly induce differentiation in leukaemia cells. In vitro studies have shown that only very high doses of α-tocopherol induce the appearance of myelomonocytic differentiation markers such as NBT-reducing activity and the expression of the integrin CD11b. This modest effect is probably due to the lipidic nature of this compound. In our hands, α-tocopherol was less effective than ascorbate on 1,25D$_3$–induced differentiation (unpublished results). However, a more soluble derivative of α-tocopherol such as vitamin E-succinate (VES) is able to partially induce significant monocytic differentiation in HL-60 cells by itself. Already in 1982, VES was established as the most effective form of vitamin E against cancer. VES stimulates HL-60 cells to produce superoxide radicals and to show non-specific esterase activity although does not change the expression of cell surface antigens (Turley, Sanders & Kline, 1992). Several further studies have demonstrate that alone or in combination with other dietary micronutrients, VES can be useful as an adjunct to standard cancer therapy increasing tumour response and even decreasing the toxicity of chemotherapics in normal cells (Prasad, et al., 2003). Moreover, other experiments have demonstrate that VES added to low levels of 1,25D3 increased the differentiation induced by this last compound (Sokoloski, et al., 1997).

More recently, hybrid compounds between α-tocopherol and retinoic acid (ATRA) have been produced and used in the treatment of leukaemia. Tretinoin tocoferil is a α-tocopherol ester of ATRA and is able to induce the granulocytic differentiation of the human promyelocytic leukaemia HL-60 cells. The inactivity of tretinoin tocoferil as teratogenic agent and its very low toxicity have suggested its use on myelomonocytic leukaemia (Makishima, et al., 1996). Furthermore, this compound also enhances cellular differentiation induced by sub-optimal concentrations of ATRA, dimethyl sulfoxide, phorbol esters and 1,25D3. In fact, the combination of tretinoin tocoferil and 1,25D3 may be useful for treating myelomonocytic leukaemia since it have enhanced the 1,25D3–dependent differentiation of several different human myelomonocytic cell lines such as HL-60, U937, ML-1, THP-1, P39/TSU, and P31/FUJ cells. (Makishima & Honma, 1997; Makishima, et al., 1996; Makishima, et al., 1998).

Another hybrid compound, in this case between 9-cis retinoic acid and α-tocopherol (9CTT), has shown properties as granulocytic differentiation inducer in the APL cells NB4 and HT-93 cells. However, its effect is weaker in non-APL leukemic cells such as HL-60 and U937 cells. In this case, in combination with $1,25D_3$, 9CTT produces a more than additive induction of differentiation in APL cells (Makishima, et al., 1998).

Recently, the other members of the vitamin E, tocotrienols have been taking into consideration in therapies against several diseases due to their powerful neuroprotective, anticancer and cholesterol-lowering properties often not exhibited by tocopherols. Tocotrienols are found in seed endosperm and are rarely found in vegetative tissues of plants. The structural difference between these compounds and tocopherols is the presence of three trans double bonds in the hydrocarbon tail in tocotrienols. Their use in the prevention and treatment of different cancer types are currently under study (Wada, et al., 2005). On the other hand, other vitamin E derived compounds, tocopherylquinones (TQ) that are the product of vitamin E oxidation have been suggested as a new class of alkylating electrophilic quinines. TQ can function as highly cytotoxic agents able to escape multidrug resistance in acute lymphoblastic leukaemia cell lines specially in the case of γ-TQ and δ-TQ whereas α-TQ does not induce cytotoxicity (Cornwell, et al., 1998).

Polyphenols

Polyphenols are a big group of compounds found in plants and uniquely characterize by the presence of more than one phenol group in the molecule. They are considerer the most abundant antioxidant in the diet although the diversity of their structures makes them different from other antioxidants. Moreover, the amount of different polyphenols found to date in plants and plant foods, several thousands, make them a complex family of compounds with very interesting therapeutic properties against cancer, cardiovascular diseases, inflammation and other diseases. Polyphenols are classified according to the nature of their carbon skeleton in: phenolic acids, flavonoids, stilbenes and lignans. Several compounds in each subgroup have been already studied in cancer and leukaemia therapy. In fact, the effect of polyphenols in leukemic cell differentiation is known from the late 90's and from there, several different compounds have been studied to determine the in vitro effect on different models of leukemic cell differentiation.

Phenolic acids are abundant in foods being caffeic acid and ferulic acid the most present. However, it seems that caffeic acid, ferulic acid and other phenolic acid such as chlorogenic acid do not increase the differentiation of HL-60 cells produced by $1,25D_3$. But, derived

compounds such as the more lipophilic ethyl of ferulic and caffeic acid are able to induce the differentiation of HL-60 cells by themselves or to enhance the maturation produced by vitamin $1,25D_3$ (Sokoloski, Shyam & Sartorelli, 1997).

One of the most known phenolic acids in leukaemia treatment is carnosic acid, a diterpene present in rosemary known to be a strong dietary antioxidant showing also antimutagenic and anticarcinogenic properties (Steiner, et al., 2001). This compound inhibits proliferation in leukemic cell lines such as HL-60 and U937 but cause only marginal induction of differentiation. However, when added to ATRA, TPA or $1,25D_3$-induced cells, carnosic acid substantially augments the differentiating effect of these compounds (Danilenko, Wang & Studzinski, 2001). This compound also synergistically potentiate the activity of $1,25D_3$ or its derivative Ro25-4020 in Balb/c mice bearing WEHI-3B D(-) murine myelomonocytic leukaemia delaying tumour appearance and reducing tumour size. Rosemary dry extract mixed with food produced a similar effect (Sharabani, et al., 2006). These results suggest that carnosic acid and derivatives of $1,25D_3$ can cooperate not only in *in vitro* assays but also show antileukemic activity *in vivo* and then, their combination should be evaluated in the treatment of myeloid leukaemia (Danilenko, et al., 2003).

Flavonoids are the most abundant polyphenols in the diet because they are present ubiquitously in plants. These compounds can be classified in several different classes. According to the degree of oxidation of the oxygen heterocycle we can find: flavones (luteolin, apigenin), flavonols (quercetin), isoflavones (genistein and daidzein), anthocyanins (red fruids pigments), flavanols (catechins), proanthocyanidins (polymeric flavanols) and flavanones (hesperidin, narigin) (Scalbert & Williamson, 2000).

As in most of the cases of polyphenols, most of these flavonoids have been also shown to present cancer preventive properties. In human colon carcinoma cells HT-29, flavonoids affects proliferation, differentiation and apoptosis with selectivity toward transformed cells (Wenzel, et al., 2000). Specific catechins have been proposed to optimise current chemotherapeutic protocols in leukaemia (Annabi, et al., 2006). Flavonoid-rich polyphenol fraction from pomegranate (Punica granatum) fruit exert antiproliferative and prodifferentiation activity on HL-60 cells in a similar way than in breast and prostate cancer cells (Kawaii & Lansky, 2004). The anthocyanin cyanidin-3-O-beta-glucopyranoside (Cy-g) is able to induce apoptosis in T-lymphoblastoid whereas in HL-60 promyelocytic cells Cy-g also induces differentiation to macrophage-like cells and granulocytes (Fimognari, et al., 2004). Furthermore, proanthocyanidins extracted from barley bran, prodelphinidin B-3, T1, T2, and T3, induce low levels of cell differentiation in HL-60 cells by themselves but potentiate ATRA-induced granulocytic and also butyrate-induced monocytic differentiation in HL-60 cells (Tamagawa, et al., 1998). Pycnogenol, a complex mixture of bioflavanoids and proanthocyanidins and organic acid already found in more that 500 dietary supplements, foods and beverages as is Pycnogenol, also have shown differentiation activities in leukemic cells (Huang, et al., 2005b).

Stilbenes, are phenol-based plant metabolites widely represented in nature. Among the stilbenes, the phytoalexin resveratrol have recently attracted attention for its different effects at the cell, tissue and physiological levels. Resveratrol inhibits growth of several myeloid leukaemia cell lines (HL-60, NB4, U937, THP-1, ML-1, Kasumi-1) and fresh samples from 17 patients with acute myeloid leukaemia. Moreover, resveratrol also induce the appearance of several differentiation markers depending on the cell line. Thus, in erithroleukemic K562 cells, resveratrol also induces a remarkable γ-globin synthesis, an erythroid differentiation

marker (Ragione, et al., 2003; Rodrigue, et al., 2001). It also enhances the expression of adhesion molecules (CD11a, CD11b, CD18, CD54) in each of the cell lines except in Kasumi-1. Moreover, resveratrol induces the production of superoxide in U937 cells. Resveratrol also synergistically increases the differentiation induced by ATRA (Asou, et al., 2002). Moreover, it also have shown inhibitory effect on the growth of AML cells suggesting that this compound can have a future role in the therapy of AML (Asou, et al., 2002; Estrov, et al., 2003).

Lignans are polyphenolic substances derived from phenylalanine via dimerization of substituted cinnamic alcohols. Lignans are present in foods such as flax, pumpkin and sesame seeds, rye, soybeans, broccoli, beans and some berries. However, although they have been identified in human plasma and urine, the exact dietary origin is unknown and probably, the dietary lignans are metabolised by the gut microflora and derived metabolites absorbed through the gut epithelium. Between lignans, magnolol (MG) and honokiol (HK), abundantly available in the medicinal plants Magnolia officinalis and M. obovata, have shown anti-inflammatory and anti-oxidant properties and also are able to synergistically enhance HL-60 cell differentiation initiated by low doses of $1,25D_3$ and ATRA (Fong, et al., 2005). Previously, other lignans such as Saucernetin-8 and –7 had shown antiproliferative and pro-differentiating activity in leukemic cells (Seo, et al., 2004a; Seo, et al., 2004b). Moreover, several other lignans and terpenoids found in extracts from different plants have been proposed as candidates as inducers of differentiation in HL-60 (Luyengi, et al., 1996; Umehara, et al., 1996).

Carotenoids

As precursors of retinoids, carotenoids also show activity as differentiation agents in leukemic cells. However, the induction of cell differentiation by carotenoids without (lutein) and with (β-carotene) ATRA activity suggests that these agents induce differentiation independently of the mechanism of action of ATRA (Gross, et al., 1997) although they induce granulocytic differentiation as ATRA. However,   -carotene is less potent in the induction of differentiation than ATRA and induce less differentiation at higher doses than ATRA (Biesalski & Schaffer, 1997). The carotenoid lycopene also functions as a very potent antioxidant able to affect several human cancer cells. As has been demonstrated with other antioxidants, the combination of low concentrations of lycopene or β -carotene with 1,25D3 synergistically affects cell proliferation and differentiation in the HL-60 promyelocytic leukaemia cell line (Amir, et al., 1999; Heber & Lu, 2002; Sokoloski, et al., 1997).

Moreover, other studies have demonstrated that the oxidation products of acyclic carotenoids other than lycopene induce apoptosis in leukemic cells (Nara, et al., 2001). Furthermore, it seems that a redox mechanism affecting the production of ROS and the oxidation of glutation is implicated in the cell cycle delay and apoptosis induction by β-carotene and derivatives (Palozza, et al., 2002).

Furthermore, several different carotenoids have been considered as agents able to revert the multidrug resistance found in several leukaemia and cancer (Gyemant, et al., 2006), then, the importance of these compounds in chemotherapy resistance seems to be clear.
Sesquiterpene Lactones

Sesquiterpenes are a class of terpenes consisting in three isoprene units. They can be acyclic of can contain one, two or three cycles. Terpenes and terpenoids are the primary

constituents in the essential oils of many types of plants and flowers. This is the reason why terpenes and sesquiterpene lactones are found in several extracts from plants. Several of these compounds have received considerable attention in pharmacological research in the treatment of inflammation or neoplasia.

The use of sesquiterpene lactones in the treatment of leukaemia has been studied since the early 70s and several different compounds have shown anti-leukemic activity by themselves or in combination with ATRA or 1,25D$_3$. For example, sesquiterpene lactones from extracts from *Tanacetum parthenium (L.)* or other Mexican-Indian medical plants are responsible of anti-inflammatory effects in vitro and enhance differentiation induced by 1,25D$_3$ in HL-60 cells (Kang, et al., 2002). In this extract, parthenolide was the most active compound whereas other sesquiterpenes were inactive depending on the modification of signal pathways during differentiation (see below). Costunolide, is another germacranolide sesquiterpene lactone that exists in several medicinal plants that also increase the degree of differentiation when combined with 1,25D$_3$ (Kim, et al., 2002). Another example can be found with helenalin, a cell-permeable pseudoguainolide sesquiterpene lactone, extracted from several plant species of the Asteraceae family. In this case, this compound is able to induce differentiation in HL-60 cells by itself (Kim, Oh & Kim, 2005).

SIGNALLING PATHWAYS AFFECTED BY ANTIOXIDANTS

The different nature of the above-described plethora of compounds and other antioxidant compounds able to modulate differentiation and even apoptosis in leukemic cells does not permit to obtain a clear picture of the molecular mechanism involved in their effect. In most cases, the antioxidant capacity of these compounds has been associated to its pro-differentiating activity, but, recently, most of these compounds have shown pro-oxidant activity in cell cultures. However, it seems that there are a few common pathways that are affected by most of these compounds.

The Role of the Cell Redox State

It is clear that one of the main activities of antioxidants compounds in differentiation may be based on the activity of these compounds as scavengers of reactive oxygen species (ROS). In fact, the very different chemical structure of polyphenols, carotenoids, VES, lipoic acid, ascorbate, hydroxyurea, dithiocarbamates, or ebselen that enhance the differentiation induced by ATRA or 1,25D$_3$ in leukemic cells, indicate that the only common property between them is the modulation of the intracellular redox state. However, it is not completely clear that the antioxidant activity of these compounds is the responsible factor in their potentiating effect but the prooxidant activity found in most of these compounds must be also considered. This last section will be focussed on the role of these "antioxidants" in differentiation and apoptosis in leukemic cells.

Several works have suggested that ROS can play a role in the differentiation program of different types of AML (Nagy, et al., 1993; Nagy, et al., 1995). On the other hand, decrease of GSH levels in cells have been related to the differentiation of the monocytic THP-1 cells induced by deferoxamine (Seo, et al., 2006). The topoisomerase inhibitor β-lapachone induces differentiation and apoptosis in various human cancer cells. This compound increases

hydrogen peroxide (H2O2) in human leukaemia HL-60 cells, an increase blocked by N-acetyl-L-cysteine (NAC), ascorbic acid, and α-tocopherol. This H_2O_2 increase found in HL-60, U937, and Molt-4 is due to the low level of GSH present in these cells. Then, it is not rare to think that the increase in ROS levels in these cells is responsible of the monocytic differentiation induction found with β-lapachone at lower concentrations. Moreover because this monocytic differentiation is suppressed by NAC (Chau, et al., 1998).

On the contrary, deregulation of ROS levels has been related to the leukemogenesis in blood cells. In fact, granulocyte colony-stimulating factor (G-CSF) stimulates the production of ROS in a mechanism dependent on the activation of Lyn, PI3K and Akt (Zhu, et al., 2006). Most of the acute myeloid leukaemia (AML) express the truncated G-CSF receptor and they produce more ROS than those cells showing a wild type receptor and addition of NAC diminish G-CSF induced ROS production and cell proliferation by inhibiting Akt activation. Furthermore, intracellular ascorbic acid is reduced in abnormal leukocyte states such as leukaemia (Barton & Roath, 1976). Intracellular ascorbate levels were reduced in chronic myeloid leukaemia (CML) and chronic lymphoid leukaemia (CLL) and in a significant population of patients with AML and ALL. Then, the presence of this antioxidant may be important in the regulation of intracellular pathways in these cells. Studies performed in mice, able to synthesize ascorbate whereas humans are not, demonstrate that vitamin C deficiency in leukaemia occurs during malignancy due to an enhanced rate of utilization of this compound (Bhattacharjee, et al., 1985). Then, the leukemogenesis is accompanied by the increase in ROS production. Therefore, ROS levels can have an important role in both leukemogenesis and normal haematopoiesis program.

Several works demonstrate that cells, including the leukemic cell line HL-60 cells, incorporate very high amounts of reduced ascorbic acid by the uptake of the oxidized form, dehydroascorbate through the facilitate hexose transporters (glucose transporters) and in a mechanism independent of intracellular GSH content (Guaiquil, et al., 1997; Vera, et al., 1995; Vera, et al., 1994; Wolf, 1996). High levels of reduced ascorbate in HL-60 cells protects cells against oxidative damage induced by high levels of H_2O_2, serum or glucose-free cultures or X radiation. Thus, intracellular levels of ascorbate are used by these cells mainly in protection against oxidative damage since ascorbate levels decrease after oxidative injury (Witenberg, et al., 1999a; Witenberg, et al., 1999b). Thus, ascorbate may be an important component of the cell machinery against oxidative injury and subsequent leukemogenesis.

However, the role of ROS and antioxidants in leukaemogenesis and differentiation seems to be contradictory in some cases. In fact, ROS are related with differentiation and antioxidants are scavengers of ROS and protect against leukaemogenesis but also enhance the differentiationprogram induced by several agents. Polyphenols are considered as antioxidants because in most cases, these compounds induce the antioxidant response of cells increasing enzymes that protects cells against oxidative injury. However, some reports have found that treatment of human myeloid leukemia cells with the polyphenols carnosic acid (Danilenko, Wang & Studzinski, 2001) or caffeic acid (Nardini, et al., 2001) results in the decrease of intracellular levels of ROS. On the contrary, our results on HL-60 cells have shown that carnosic acid, resveratrol, curcumin and even high levels of ascorbate increases H_2O_2 levels in HL-60 cells indicating that these compounds increase ROS during early phases of differentiation. In agreement with our results, Strasser et al., (Strasser, et al., 2005) have shown that curcumin 10 μM increases ROS species just after 1 h after administration in undifferentiated U937 cells. The induction of ROS levels just after treatment produces an

increase in GSH levels and further decrease in ROS levels after 24 h of incubation. This study demonstrate that the antioxidative effect of curcumin is preceded by an oxidative stimulus in a time and dose-dependent effect being the GSH induction a response of the cell to the oxidative insult (Strasser, et al., 2005). This cell response have been denominated as the hormetic response where mild oxidative injury causes an antioxidant response that increases the protection of the cell (Rattan, 1998).

This hormetic effect has been also found with other classic antioxidant compounds. In fact, α-tocopherol and hydrocoumarins have been also proposed as antimelanogenic compounds by affecting melanosome differentiation interrupting melanisation increasing the intracellular GSH content (Yamamura, Onishi & Nishiyama, 2002). Also, resveratrol, that have shown antiproliferative and apoptotic effect on several myeloid cell lines, increases GSH level after 24-48 h of exposure affecting also to the activity of glutathione reductase and peroxidase (Luzi, et al., 2004). Very recently, carotenoids have been shown to activate the antioxidant response element transcription system through a Nrf2-dependent mechanism in agreement with an hormetic effect (Ben-Dor, et al., 2005). But more interesting, the disturbance of cell cycle and apoptotic effect due to carotenoids seems to be dependent on the differentiation degree of the cell, being highly active against non-differentiated (leukemic) cells whereas they show no effect on differentiated or normal cells (Bodzioch, et al., 2005; Palozza, et al., 2003).

Another important effect of ROS in leukemic cells is the induction of apoptosis. However, the literature is not clear about the role of ROS in apoptosis in these cells. In some cases, the induction of the apoptotic pathway seems to be dependent on the antioxidant role of the compounds, although in other cases, the reduction of ROS levels avoids apoptosis in these cells. Our experience demonstrates that leukemic cell lines respond in a different way to antioxidant compounds depending mainly on cell concentration. In fact, in HL-60 cells, high concentrations of cells prevent the apoptosis found with high levels of ascorbate (0.5 mM). Moreover, the cell line also affects the in vitro experiment (unpublished results). For example, NB4 cells are less sensible to ascorbate-induced apoptosis than HL-60 cells. These in vitro experiments can highlight the importance of ROS on the apoptosis program in leukemic cells but do not explain the exact role of these compounds into the organism and the mechanism by which antioxidants affect apoptosis in these cells. Moreover, and in agreement with our results, a recent work have demonstrated that ascorbic acid at moderated concentrations can reduce cell growth and induce apoptosis in three different AML cell lines and also in leukemic cells from peripheral blood from patients (Park, et al., 2004). In this mechanism, the effect of ascorbate was prooxidant since it induced oxidation of GSH to GSSG, and accumulation of H_2O_2 in a dose-dependent fashion. This prooxidant activity of ascorbate was directly related to the induction of apoptosis since catalase completely abrogate this effect.

On the other hand, specific natural polyphenols such as the anthocyanin, cyanidin-3-rutinoside, have been shown to selectively kill leukemic cells by induction of oxidative stress (Feng, et al., 2007). Also, ethyl acetate extracts from three marine algae significantly increase ROS levels and induce apoptosis in U937 and HL-60 cells and addition of NAC to these cells blocks apoptosis suggesting that ROS are key mediators in apoptosis in these cells (Huang, et al., 2005a). New compounds in study for leukaemia treatment such as the farnesyltransferase inhibitor manumycin A, also induce apoptosis and increase nitric oxide and other ROS production in these cells, an effect that also was inhibited by the addition of NAC to these cells (She, et al., 2005). The same effect can be found when cells are treated with

homocysteine thiolactone where NAC, ascorbate, α-tocopherol and folate reduced the apoptosis induced by this agent (Huang, et al., 2002).

On the other hand, quercetin is also an antioxidant that acts as anticancer drug. This compound exerts its role by either diminishing or promoting ROS generation (antioxidant or pro-oxidant role). A recent study have demonstrated that addition of soluble antioxidants such as ascorbate, NAC or GSH increase whereas H_2O_2 attenuates the antiproliferative and apoptotic effect of quercetin on HL-60 cells indicating a role of the antioxidant function of this compound and the subsequent reduction of ROS on growth arrest and cell death (Chen, et al., 2004). However, in this work, the levels of ascorbate were too high (1 mM). At these levels, ascorbate exerts different effects on ROS since our results demonstrate that whereas cells show a dramatic reduction in superoxide anion (Lopez-Lluch, et al., 2001), H_2O_2 levels increases. Moreover, other agents that induce apoptosis in APL and also in AML such as arsenic trioxide are enhanced in their apoptotic effect by the addition of ascorbate suggesting a putative future role of both compounds in the treatment of AML (Bachleitner-Hofmann, et al., 2001).

In other cases, the use of nutritional antioxidants such as ascorbate and α-tocopherol has been proposed to avoid the appearance of secondary acute myelogenous leukaemia after etoposite (VP-16) treatment of other cancer (Kagan, et al., 1999). In this case, the antioxidants prevent the pro-oxidative activity of etoposide phenoxyl radical after one-electron oxidation of etoposide mainly by myeloperoxidase that is highly active in haematopoietic stem cells and other cell lines such as HL-60. Then, dietary antioxidants can minimize the genotoxic effect of radicals in bone marrow myelogenous progenitor cells after chemotherapy.

MAPK Pathway

The effect of vitamin E succinate (VES) on leukemic differentiation seems to be dependent on the induction of ERK during the first moments of treatment. In fact, VES-induced ERK phosphorylation is abolished by the ERK inhibitor, PD98059, which resulted in a remarkable prevention of VES-induced monocytic differentiation. This same effect has been found with several other antioxidants such as resveratrol, pyrrolidine dithiocarbamate or hydroxityrosol (Della Ragione, et al., 2002). However, the ERK is not the only factor affected by VES since inhibition of the ERK activity by PD98059 also diminished the VES-induced p21WAF1 protein expression, but is unable to change the phosphorylation state of the retinoblastoma protein (Lee, et al., 2002).

The effect induced by the combination of the polyphenol carnosic acid in the presence of $1,25D_3$ is associated with the increase in the activation of MAPK pathway affecting the Raf-mitogen-activated protein/ERK. This pathway also enhance the activation of and the binding of AP1 to its cognate DNA element in the promoter of the vitamin D receptor gene. Then, the potentiation due to carnosic acid can be due to the activation of this MAPK pathway (Danilenko, et al., 2003).

Although the mechanism of action of these compounds in MAPK activation is not clear several mechanisms are being proposed. Most of these phenolic compounds generally appear to be electrophiles. Then, they can activate MAPK pathway via an electrophilic-mediated stress response. This response will activate the bZIP transcription factor NRF2 that dimerizes with Mafs and binds to the antioxidant response element found in promoters (ARE). By this

mechanism, most of these compounds would induce many of the phase II drug metabolic enzymes such as thioredoxins, γGCS, HO-1 (Rushmore & Kong, 2002).

However, not all the polyphenols could affect in a similar way. In fact, it has been described that different polyphenols can affect antagonistic pathways and show different effect in the same cells. Hence, EGCG and curcumin produce opposite response in keratinocyte differentiation. Like carnosic acid in leukemic cells, the catechin EGCG alters MAPK cascade function activating involucrin gene transcription via Ras, MEKK1, MEK3, ERK1/2-p38delta cascade and then, targets AP1 and CAATT enhancer binding protein transcription factors. However, the turmeric curcumin, also able to potentiate differentiation in leukemic cells, antagonizes the EGCG-dependent response by interfering in this signalling pathway (Eckert, et al., 2006). Moreover, in pancreatic stellate cells, curcumin also inhibit the effect of PDGF by inhibiting MAP kinase pathway (ERK, JNK and p38) but not affecting NF-kB (Masamune, et al., 2006). Then, these results indicate that polyphenols can affect the same signal pathway in opposite ways in different cells.
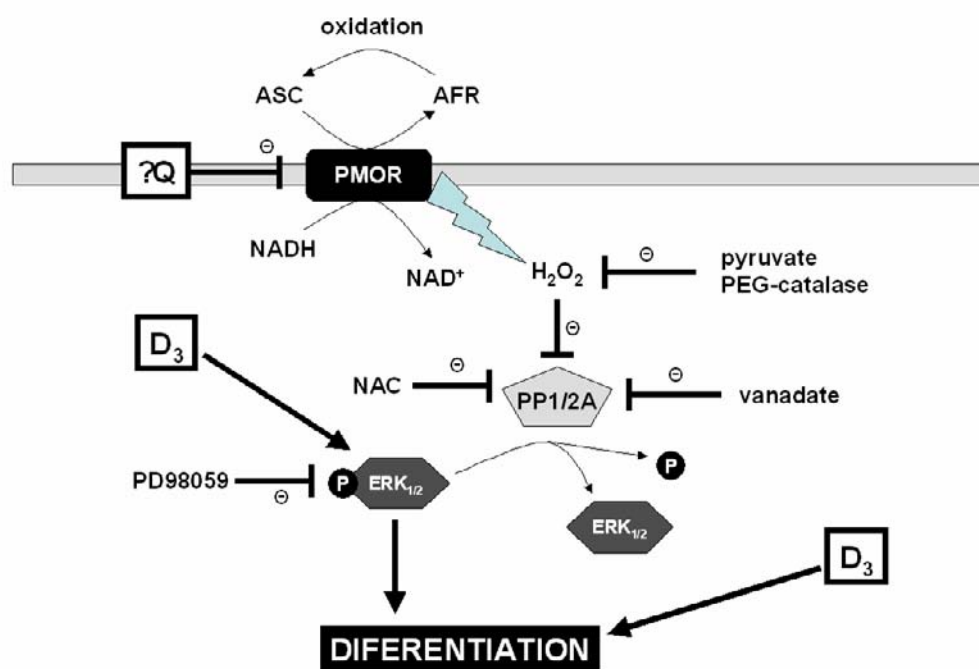
Figure 3. A putative role of plasma membrane redox system (PMRS) activated by ascorbate in the stimulation of differentiation by $1,25D_3$. The prooxidant activity of ascorbate or even other antioxidants with prooxidant capacity will increase H_2O_2 levels that will inhibit the phosphatase PP1/2A as other compounds do. This inhibition will permit the maintenance of the phosphorylation of ERK and the enhancement of the differentiation induced by $1,25D_3$. From (Lopez-Lluch, et al., 2008) with permission.

The same ERK-dependent mechanism has been reported for sesquiterpene lactones such as costunolide that potentiates $1,25D_3$-induced cell differentiation predominantly into monocytes. In this case, inhibitors for PKC, PI3-K, and ERK markedly inhibit differentiation induced by costunolide and $1,25D_3$ (Kim, et al., 2002).

Ascorbic acid enhances the activation of the Rac1 and p38 induced by arsenic trioxide in leukemic cells. The effect of ascorbate in this pathway can be related to its antiapoptotic effect in this treatment since inhibition of p38 potentiates arsenic-dependent apoptosis (Verma, et al., 2002). Then, p38 in this case acts and a negative regulator of arsenic trioxide-induced apoptosis and will increase malignant cell growth. Other authors indicate that ascorbate can induce the ERK/MAPK pathway in AML cell lines by a Raf1 dependent mechanism (Park, et al., 2005). Moreover, in neuronal cells such as the PC12 cell line, a lipophylic derivative of ascorbate, 6-octa-AA-2G, that is rapidly hydrolysed to ascorbate into cells, enhances neurite differentiation by activation of P44 and P42 ERK (Zhou, Tai & Yamamoto, 2003). As downstream effector, ascorbate increases the activity of MAPK-activated protein kinase 2. On the other hand, inhibition of ERK pathway reduces ascorbate-dependent apoptosis and inhibition of cell growth in AML cell lines. Our work demonstrate that ascorbate can increase differentiation in $1,25D_3$-induced HL-60 cells. Recent results demonstrate that the mechanism of ascorbate depends on H2O2 since catalase or pyruvate, known scavengers of H_2O_2, abrogate the effect of ascorbate (unpublished results). Probably, the activity of ascorbate as electron acceptor in the PMRS will induce the production of H_2O_2 in cells. In fact, PMRS have been linked to the regulation of hormone-dependent intracellular signalling in most cases depending on the H_2O_2 levels (Brightman, et al., 1992; Crane, et al., 1982; Goldenberg, Crane & Morre, 1978). This activity will inhibit the activity of the PP1/2A phosphatase and, by this mechanism, will maintain the activity of the ERK pathway (FIGURE 3). However, this mechanism must not be implicated in VES-induced activation since α-tocopherol seems to act by activating PPA2 causing dephosphorylation of PKC and, probably, dephosphorylation and then, activation of other proteins.

The Role of Cyclooxygenase and The Inflammatory Pathway

Most of the antioxidants studied to far show anti-inflammatory properties. Ascorbate and polyphenols such as resveratrol, curcumin, or quercetin and α-tocopherol affects COX activity. Moreover, known COX inhibitors such as aspirin or indomethacin also enhance differentiation induced by $1,25D_3$ in HL-60 cells (Lopez-Lluch, et al., 2005).

In most cases, these compounds inhibit the inflammatory mechanisms of cells affecting both, enzymatic activity and pro-inflammatory gene transcription. We have shown that ascorbate increases cAMP levels in $1,25D_3$-induced cells. Our work has demonstrate that the increase in cAMP levels in the cells during the differentiation process is a key component in the enhancing effect of ascorbate (Lopez-Lluch, et al., 1998). Although part of the effect of ascorbate can be explained by its activity on the PMRS (Lopez-Lluch, et al., 1998), its known effect as anti-inflammatory agent and the cAMP increase found with other known COX inhibitors, suggest that inhibition of COX during early phases of differentiation induction can increase the response to, at least, $1,25D_3$ (Lopez-Lluch, et al., 2005). Our results agree with the ascorbate-dependent downregulation of the expression of COX-2, probably by inhibition of NF-kB activity, found in HL-60 cells (Han, et al., 2004)

On the other hand, not all the studies have shown cooperative activity of antioxidants on leukaemia therapy. In fact, several antioxidants have shown inhibitory effect of differentiation of mouse myeloid cells (M1) induced by the glucocorticoid synthetic compound dexamethasone (Takenaga, Honma & Hozumi, 1981). In this case, the effect of the antioxidants was higher as higher the inhibition of PGE2 synthesis. This effect was also found

in the case of ascorbate enhancing effect on $1,25D_3$ differentiation although in this case, the antioxidant effect increased differentiation (Lopez-Lluch, et al., 2005).

The Role of NF-κB

Another important common mechanism in antioxidants is the inhibition of NF-*k*B. Near all the above-indicated antioxidant compounds show inhibitory activity against the NF-κB pathway. However, the role of NF-κB in differentiation of leukemic cells seems to be controversial because there are contradictory studies about its role. For example, the inhibition of NF-κB was present as a key factor in differentiation since antisense treatment against the RelA subunit of NF-κB in HL-60 cells increases differentiation in these cells (Sokoloski, et al., 1997). However, the differentiation induced by $1,25D_3$ in NB4 cells was shown to be mediated by phosphorylation and proteolytic cleavage of IκB followed by translocation of NF-κB to the nucleus (Berry, Clark & Meckling-Gill, 2002). Also, the differentiation induced by ATRA is linked to the activation of NF-κB although the inactivation of the transcription factor does not affect granulocytic differentiation (Mathieu, et al., 2005).

Besides that, it seems that most of the antioxidants that have shown cooperation with ATRA or $1,25D_3$ are inhibitors of NF-κB translocation to the nucleus although they seems to act through different mechanisms. VES reduces the nuclear content of NF-κB in both, the presence or the absence of $1,25D_3$ (Sokoloski, et al., 1997). The same effect has been found when curcumin, a polyphenol with potent antitumour properties and enhancer of differentiation in leukemic cells, is added to $1,25D_3$ (Sokoloski, Shyam & Sartorelli, 1997). Curcumin also reduced NF-κB activity in nuclear extracts of HL-60 cells in both, the presence or absence of $1,25D_3$, indicating that this regulation is not important by itself in non-stimulated cells but when $1,25D_3$ is present, the inhibition of NF-κB activity can enhance the differentiation program. In fact, curcumin inhibits I*κ*B kinase, (IκBK), then inhibiting NF-κB activation and several oncogenes responding to this factor (Lin, 2004).

Other polyphenol, resveratrol, also affect NF-κB in AML cells. Fresh AML cells and AML cell lines OCIM2 and OCI/AML3 proliferate after incubation with IL-1β, however, the addition of resveratrol inhibits the proliferation of these cells in a dose-dependent effect arresting then at the S phase (Estrov, et al., 2003). The mechanism of action of IL-1β is dependent on NF-κB activation and the presence of resveratrol suppressed the activation of nuclear NF-κB. Moreover, exposure of U937 to resveratrol suppress the binding activity of NF-κB (Asou, et al., 2002).

Parthenolide, a sesquiterpene lactone, also enhances $1,25D_3$-induced differentiation in HL-60 cells via inhibition of NF-κB activity although partenolide is unable to activate cell differentiation by itself (Kang, et al., 2002). In the case of the sesquiterpene lactone costunolide, the enhanced levels of cell differentiation found when combined with $1,25D_3$ closely correlate with the inhibitory levels of NF-κB-binding activity. (Kim, et al., 2002).Very importantly, santonin, another sesquiterpene lactone without inhibitory activity on NF-κB is unable to enhance $1,25D_3$-induced cell differentiation.

Although it has been not demonstrated in the case of ascorbate, NF-κB can be also affected by ascorbate since it also suppress the activation of NF-κB under some circumstances such as TNF-α activation. In this case, intracellular ascorbate inhibits TNF-α-

induced activation of NF-κB in several cell lines including leukemic cells U937 and HL-60. The mechanism of action of ascorbate was the inhibition of nuclear translocation of NF-κB, the reporter transcription of this expression factor and phosphorylation of its inhibitor IKB. It seems that ascorbate can affect NIK (NF-κB inducing kinase) and IκB-kinase (IKK) activity (Carcamo, et al., 2002). Moreover, L-ascorbic acid also represses the constitutive activation of NF-κB in HL-60 cells by suppression of the binding of the p65/p50 heterodimer NF-κB to DBA through inhibition of the degradation of IκB-α and prevention of the nuclear translocation of p65. This inhibitory effect was dependent on GSH levels and generation of H_2O_2 levels, indicating a prooxidative role of ascorbate in this effect (Han, et al., 2004).

Then, it seems clear that, at least in the monocytic differentiation induced by $1,25D_3$, inhibition of NF-κB seems to be a key factor. Most of the leukemic cells show high levels of NF-κB activity that would be involved in the inhibition of the genomic pathway of cellular differentiation when induced by $1,25D_3$. In fact, tumour necrosis factor-α (TNF-α) is a known activator of NF-κB and suppress the vitamin D response element (VDRE)-promoter activity. These antioxidants, ascorbate or partenolide, are able to suppress the inhibitory activity of TNF-α and permit the activation of the differentiation process even in the presence of TNF-α.

However, not all the antioxidants able to increase differentiation induced by D3 have been shown to decrease NF-κB activity. Although we can not discard different effects due to differences in the dose used in the experiments, β-carotene treatments at 10 μM have demonstrated increase in NF-κB-DNA binding at the same time that β-carotene delays cell cycle progression and induced apoptosis (Palozza, et al., 2003). However,

The Role of cAMP

The role of cAMP in myeloid differentiation is known from early 70s (Chaplinski & Niedel, 1982) and the potentiation between intracellular cAMP-elevating agents and inducers of leukemic cell differentiation seems to be clear (Fontana, Munoz & Durham, 1985). However, experiments performed in murine erythroleukemic cells have shown that cAMP-increasing agents inhibits cell differentiation probably indicating differences between murine and human haematopoiesis (Sherman, Shafman & Kufe, 1986).

We have found that the enhancement of differentiation induced by ascorbate in HL-60 cells treated with $1,25D_3$ depends on the increase of cAMP (Lopez-Lluch, et al., 1998). The importance of cAMP in monocytic differentiation induced by $1,25D_3$ was highlighted by the potentiation found with known inducers of cAMP pathway such as isobutylmethylxanthine, forskolin and PGE_2. Moreover, we also found that ascorbate inhibits activity of COX in these cells and that inhibitors of this enzyme such as ibuprofen and aspirin also increase cAMP and enhance differentiation in $1,25D_3$-induced cells (Lopez-Lluch, et al., 2005). Then, it is clear that cAMP is an important intermediate in the monocytic differentiation program.

The same role of cAMP have been found in the induction of granulocytic differentiation with ATRA (Yang, Chao & Shaio, 1998). In fact, ATRA induces a marked increase in cAMP within few minutes after treatment (Zhao, et al., 2004). In APL cells, addition of cAMP derivatives or the increase of its levels in cells by treatment with theophilline or other agents also help the ATRA-induced granulocytic pathway (Guillemin, et al., 2002; Zhu, et al., 2002). The activation of the differentiation program by cAMP is related to the release of the RAR subunit of the RAR/RXR heterodimer or the PML-RAR complex found in APL depending on

the PKA activity. It is known that RXR can dimerize with VDR and activate the $1,25D_3$–induced genomic activation and then, activate the differentiation of leukemic cells. Thus, the release of RXR rom RAR/RXR heterodimer permits higher levels of free RXR to bind to VDR in $1,25D_3$-induced cells.

A similar mechanism has been suggested for other phytochemicals. For example, the polyphenol carnosic acid is able to increase VDR and RXR expression and then, greatly enhance the differentiation induced by both $1,25D_3$ and ATRA (Danilenko, et al., 2003; Danilenko, Wang & Studzinski, 2001). However, apart of our papers about the effect of ascorbate on cAMP levels, no information is found on the role of other antioxidants in cAMP-depending pathway in leukemic cells.

ROLE OF ANTIOXIDANTS IN PROTECTION AGAINST CHEMOTHERAPY-INDUCED DAMAGE

Oxidative stress seems to play a very important role in the carcinogenic process (Klaunig & Kamendulis, 2004). Reactive oxygen species (ROS) can elicit multiple effects by mediating carcinogen activation, causing DNA damage, and interfering with the repair machinery of the damaged DNA (Jackson & Loeb, 2001). In addition, ROS levels increases in preneoplastic cells during their promotion stage. This seems to induce the growth of initiated cells, and to play a role during cell cycle progression either by further damage the unstable genetic material or by altering the growth of tumour cells (Behrend, Henderson & Zwacka, 2003; Denda, et al., 1993). Thus, tumour cells continually undergo high and persistent oxidative stress by ROS although this stress does not appear to be enough to induce cell death. On the contrary, as an hormetic effect, pleneoplastic cells show increased cell resistance to ROS because the cell antioxidant mechanisms are induced. Thus, they shown high levels of internal antioxidants such as coenzyme Q (CoQ) and are also able to accumulate high levels of ascorbate and reduced glutathione. These high levels of endogenous antioxidants may increase the chemotherapeutic resistance of the cells.

Recently we have shown that anticancer drugs such as camptothecin (CPT), a topoisomerase I inhibitor that induces oxidative stress, also increases endogenous CoQ synthesis and membrane levels (Brea-Calvo, et al., 2006). It has been demonstrated that CPT but also other anticancer drugs -such as etoposide, doxorubicin, and methotrexate- induces a significant increase in ROS production (Sanchez-Alcazar, et al., 2003). Thus, as trained cells, the up-regulation of CoQ synthesis is induced in an attempt to protect them against the oxidative stress generated after exposure to anticancer drugs. Moreover, following the exposure to radiation or chemotherapy, surviving cells also greatly increase cholesterol synthesis acquiring a state of cytoresistance. CoQ and cholesterol share the first part of their respective synthesis chain being FPP a common substrate for CoQ and cholesterol biosynthesis. If CoQ and/or cholesterol synthesis are blocked, the acquired cytoresistance diminishes and cells remain sensitive to the damaging agents. Therefore, statins, a class of hypolipidemic agents, which inhibit the HMG-CoA reductase, and thus, inhibit both cholesterol and CoQ synthesis could be appropriate candidates for the treatment of AML. In fact, it has already being showed that exposure to lovastatin sensitise both AML cell lines and primary AML cell cultures to apoptosis (Dimitroulakos, et al., 1999; Wong, et al., 2002).

However, in the case of the treatment of leukaemia or other cancer with chemotherapics the sensibility of normal cells to the anti-tumoural agent must be also be taken into consideration specially in the treatment of leukaemia in childs. Thus, appart of its effect on cell differentiation in most of the cases as cofactors of an active differentiation agent, antioxidants also are important factors in the protection of the whole organism against damage induced by potent chemotherapic agents used against leukaemia such as anthracyclines. Treatment with coenzyme Q_{10} in anthracycline-treated children against acyte lymphoblastic leukaemia or non-Hodgkin lymphoma have demonstrated a protective effect of cardiac activity (Iarussi, et al., 1994). In fact, further experiments have demonstrate that not only tumoural cells but the whole human organism activates protective mechanism based on coenzyme Q_{10} production after chemotherapy. In children treated against ALL, the level of plasma CoQ_{10} increases near three times just after chemotherapy. At the same time, the active reduced form of CoQ_{10} increases in plasma indicating a more active antioxidant mechanism (Niklowitz, et al., 2007). Then, it is clear that the organism, including tumoural cells, respond against oxidative damage induced by chemotherapy increasing endogenous antioxidant mechanisms. In cancer cells, the balance between the protection of these mechanism and the strength of the anti-cancer drug will move the result towards cell death or survival.

CONCLUDING REMARKS

Independently of the antioxidant or prooxidant mechanism used by these antioxidants, it is clear that their activity in cell differentiation or apoptosis open an important field of research to find a common mechanism able to increase the effectiveness of antileukemic pharmacological compounds. In fact, currently, phase I to III trials are demonstrating that the use of phytochemicals and other compounds with antioxidant activity is important in the treatment of leukaemia and other cancers (as published in the National Cancer Institute web page (www.cancer.gov). For example, curcumin and ubiquinone are currently studied as agents in the treatment of Myelodysplastic Syndromes, known as preleukemic syndromes. Long time ago Prasad et al., (Prasad, et al., 1999), already proposed that supplements with multiple antioxidant vitamin together with diet and lifestyle modifications may improve the efficacy of standard and experimental cancer therapies. The present work has tried to highlight the different and complex mechanisms used by compounds considered as antioxidants in the therapy against leukaemia. The identification of the main pathway implicated in their reinforcement of differentiation mechanism will help in the design of more specific and effective compounds in the therapy of leukaemia by reestablishment of the differentiation pathway.

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Chapter II

MECHANISM OF TUMORIGENESIS: FOCUS ON OXIDATIVE STRESS, ELECTRON TRANSFER AND ANTIOXIDANTS

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ABSTRACT

For more than half a century, numerous proposals have been advanced for the mode of action of carcinogens. This review presents a wide array of evidence that implicates oxidative stress (OS) in many aspects of oncology, including formation of reactive oxygen species (ROS) by the major classes of carcinogens (as well as minor ones), cancer stages, oncogene activation, aging, genetic and infectious illnesses, nutrition, and the role of antioxidants (AOs). Although diverse origins pertain, including both endogenous and exogenous agents, ROS are frequently generated by redox cycling via electron transfer (ET) groups, e.g., quinones (or phenolic precursors), metal complexes (or complexors), aromatic nitro compounds (or reduced products), and conjugated imines (or iminium species). We believe it is not coincidental that these functionalities are often found in carcinogens or their metabolites. The pervasive aspects of DNA binding by ultimate carcinogens, and mutations caused by ROS are treated. Often, ROS are implicated in more conventional rationales, such as oncogenes. A multifaceted approach to mechanisms appears to be the most logical.

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The OS unifying theme represents an approach, which is able to rationalize the diverse data associated with tumorigenesis. Because this theoretical framework aids in the understanding of cancer initiation, it can serve as a useful tool in combating cancer, particularly in relation to prevention by antioxidants.

INTRODUCTION

After heart disease, cancer is the most common cause of death in the United States [1]. Although many body organs may be involved, the majority of cases occur in the lung, alimentary canal, and breast. Various disciplines come into play in the multifaceted attack on the enigmatic origins of cancer. Huge sums of money have been invested in research and treatment. Elucidation of how cancer arises should contribute to our ability to prevent and cure. This review is primarily concerned with evidence that bolsters oxidative stress (OS) as a unifying theme of carcinogenesis. Beginning roughly a half-century ago and continuing into the present, the OS theory has arisen from research in numerous laboratories. It is intriguing that this thesis has found successful application to most of the major classes of carcinogens, as well as minor ones, various drug classes, enzymes, hormones, and toxins. Since a broad approach is taken which is not in depth, only representative references are included, in an update of the earlier review [1] which includes numerous structures.

Of the numerous theories that have been advanced, OS is the most comprehensive, and has stood the test of time. It can rationalize and correlate most aspects associated with carcinogenesis [1]: 1) generation of reactive oxygen species (ROS) from the main classes of carcinogens, in addition to miscellaneous ones; 2) activated oxygen as a universal factor in oncology; 3) DNA cleavage and base oxidation; 4) association with the three major stages of carcinogenesis; 5) relationship to mutagenesis; 6) oncogene activation; 7) tobacco effect; 8) dietary impact; 9) aging factor; 10) association with illness, such as inflammation; 11) correlation with certain hereditary illnesses; 12) estrogen involvement; 13) probability of second cancers; 14) Haddow paradox; 15) beneficial influence of antioxidants (AOs); 16) application to a wide range of other physiologically active substances.

TENETS OF OS THEORY

Electron transfer (ET) and (OS) have been implicated in the actions of drugs and toxins, such as, carcinogens [1], anti-infective agents [2], anticancer drugs [3], reproductive toxins [4], nephrotoxins [5], hepatotoxins [6], nerve toxins [7], cardiovascular toxins [8], mitochondrial toxins [9], abused drugs [10] and various others, in addition to human illnesses [11]. The preponderance of bioactive substances or their metabolites incorporate ET functionalities, which, we believe, play important roles in physiological responses [1]. These main groups include quinones (or phenolic precursors), metal complexes (or complexors), aromatic nitro compounds (or reduced derivatives), and conjugated imines (or iminium species). In vivo redox cycling with oxygen can occur giving rise to OS through generation of ROS, as discussed in the Metabolism section. In some cases, ET results in interference with

normal electron transport chains, e.g., respiration. Alternatively, ROS can arise in some instances by non-ET avenues.

Generally, active entities possessing ET groups display reduction potentials in the physiologically responsive range, i.e., more positive than – 0.5 V. However, a correlation between reduction potential and activity is not always observed since important roles are played by other factors, such as, solubility, metabolism, diffusion, adsorption, site binding, cell permeability, and stereochemistry. Reduction potential is influenced by various factors including conformation, which can differ in vitro vs. in vivo. Hence, electrochemistry, which has enjoyed relatively little attention, can provide valuable insight into the mode of action. Our theoretical framework incorporates several features common to most carcinogens:

- Binding to DNA by alkylation or complexation
- Existence of an ET entity present in the parent carcinogen or frequently in a metabolite
- Formation of ROS usually by ET involving oxygen
- ROS generation in close proximity to DNA giving rise to mutation, apparently by strand cleavage and base oxidation

There is a plethora of experimental evidence supporting the OS theoretical framework, including generation of the common ROS, lipoperoxidation, degradation products of oxidation, depletion of AOs, effect of exogenous AOs, DNA oxidation and cleavage products, as well as electrochemical data. This comprehensive, unifying mechanism is in keeping with the frequent observations that many ET substances display a variety of activities, e.g., multiple drug properties, as well as toxic effects.

Although our focus is on this theory, it should be emphasized that bioactivity is quite complicated. Other well-supported, general proposals include the action of oncogenes. Evidence indicates that OS plays a role in a number of the alternative hypotheses. The most likely scenario is complementarity entailing a multifaceted approach.

CHRONOLOGY

The fundamental framework of the OS theory was laid down by four groups in the 1950s [1]. It is remarkable that the essential features still provide a useful base even though the experimental, supporting evidence at the time was quite limited. The unifying theme is in marked contrast to the approach, still prevalent, that each type of cancer has a different cause. Several corollaries were advanced, including protection by antioxidants and application to anticancer agents and aging. Though little attention was paid to the theory in the ensuing ten to fifteen years, a surge of activity began in the 1970s. Despite the mounting volume of research, the OS theory was largely ignored by most oncologists until recent years.

STAGES IN CARCINOGENESIS

Carcinogenesis is a complex process characterized by three major stages: initiation, promotion, and progression [1]. Complete carcinogens are both initiators and promoters,

whereas incomplete carcinogens require promoters to produce malignant transformations. Most carcinogens must be converted metabolically into an active form, i.e., transformation from a pro-carcinogen into an ultimate carcinogen. Our review is primarily concerned with initiation, the first stage, which is the most studied and best understood. Interaction of the ultimate carcinogen with DNA results in irreversible alterations, i.e., mutations. Many investigators have pointed out an unmistakable correlation between carcinogenesis and mutagenesis, indicating that the former entails a type of mutation. Extensive evidence is documented which indicates that ROS species are pervasive mutagens. Numerous antecedent studies have demonstrated that ROS participate in all three stages. In addition to the modes addressed in this review, ROS can also interfere with cell signaling by altering protein kinase cascades and transcription factors, ultimately leading to tumor development [12].

Promotion, which follows initiation, is a multi-stage, often reversible, process of gene activation. There is convincing evidence that cellular oxidation states, i.e., the relative levels of ROS, AO defense entities, and radical scavengers, can promote initiated cells to neoplastic growth. Investigations dealing with involvement of ROS have continued into more recent years. One of the most powerful tumor promoters is phorbol myristate acetate. It is well established that its activation is accompanied by appearance of ROS, including SO and hydrogen peroxide, as well as hydroxyl radicals and lipid peroxidation. Phagocytic stimulation also plays a role.

It is not surprising that cancer cells, which display no specialized function within the body, appear to represent a simpler type. Reversion to unicellular behavior and to a fetal phenotype have been discussed. Another major difference with normal cells concerns telomerase. Cellular immortality, a hallmark of cancer, involves this enzyme at various stages along the continuum of multi-stage carcinogenesis. The catalyst rebuilds telomeres, the termini on chromosomes. Telomerase may have potential as an early biomarker or, perhaps, as a player in the control of cancer.

METABOLISM [1]

1. Generation of ROS

As evolution took place in the presence of steadily increasing amounts of atmospheric oxygen, life forms adopted numerous mechanisms to defend against, as well as utilize, the reactivity of the molecule. The marked activity can be attributed to the propensity of molecular oxygen to form various radical species. The formation of ROS is guided by external and internal agents, e.g., irradiation, exogenous chemicals, and endogenous entities, e.g., phagocytes and enzymes, such as cytochrome P450 mono-oxygenases (CYP450). Likewise, creation of ROS can be ebbed or reversed in the presence of certain agents, e.g., AOs and various enzymes, including superoxide dismutase (SOD), glutathione reductase (GSH) and catalase (CAT). Aerobic life, as we know it, might be viewed as a contest between the formation and deactivation of ROS. In this light, OS can be defined as a state wherein this process becomes unbalanced.

CYP450, a chief enzyme in many oxidative processes, manipulates molecular oxygen in order to mono-oxygenate a targeted substrate. The oxidation can be helpful to the organism,

leading to detoxification and excretion. However, as in the case of aromatic hydrocarbons, carbon tetrachloride and others, CYP450 oxidative metabolites can be harmful.

Molecular oxygen often undergoes a single electron reduction to form the superoxide radical anion (SO). In vivo, SO can be converted to various oxidative species, including: peroxides and various oxy radicals, namely hydroxyl, peroxyl, and alkoxyl. Such entities, including SO itself, can cause several internal anomalies, including: enzyme inactivation, lipid and protein peroxidation, and DNA oxidation. SO is usually neutralized by enzymatic conversion to the less reactive, non-radical hydrogen peroxide via SOD. Hydrogen peroxide, can be Fenton catalyzed to form one of the most powerful ROS, the hydroxyl radical. The Fenton reaction is part of a net transformation called the Haber-Weiss reaction, whereby superoxide and molecular oxygen undergo redox cycling with a catalyst, usually iron. Processes like these compel living things to limit the presence of catalytic entities like metals. CAT keeps the level of hydrogen peroxide in check via reactions that form water and molecular oxygen. Also, GSH can act in a variety of ways to combat radical formation. Many other species, including metals, metal chelators, cofactors, antioxidants, singlet oxygen, and enzymes are involved in these redox transformations.

Numerous studies have indicated a link between oxidative processes, especially those done on or in the vicinity of DNA, and incidence of cancer. A common end result of OS is DNA scission and DNA base oxidation, particularly to 8-hydroxy-2-deoxyguanosine (8-OHdG).

2. Oxidation of Phenols

Oxidation, often catalyzed by CYP450, is one of the most important reactions in biochemistry. This is a common pathway in drug transformations resulting in activation, toxicity and detoxification. The pathway for phenol involves formation of hydroquinone, catechol, o- and p- quinones, and semiquinones. Redox cycling can come about from electron donation by semiquinones to molecular oxygen to form superoxide. Various ROS can arise from such a cycle, many of which will be discussed throughout this review. Biologically active quinones have also been known to bind to various macromolecules.

3. Reduction of Nitro Aromatics

The nitro compounds, both benzenoid and heterocyclic, comprise a diverse category in pharmacology, often accompanied by high toxicity. The reaction avenues entailing reductases have been extensively explored. A common sequence, involves the following products: nitroso derivative, radicals, hydroxylamine, and primary amine.

Redox cycling with generation of ROS can occur with participation of nitroso, oxy radical, hydroxylamine, and oxygen. Electrochemical studies demonstrate that generally nitroso displays a more positive reduction potential than nitro. Binding to biopolymers can occur with nitroso and hydroxylamine.

4. Oxidation of Aromatic Amines

Microsomal oxidases are capable of performing these oxidations. In the case of pri-amines, the initial process is N-hydroxylation. The metabolites are essentially in reverse order of those arising from $ArNO_2$.

5. Imine and Iminium Formation

Properly conjugated imines and iminium ions can function as ET species, although this category is not as well recognized as others. Catalyzed oxidation of tertiary amines in vivo leads to these functionalities. In addition, imines are formed from uncatalyzed condensation of primary amines, e.g., in protein or amino acids, with carbonyl compounds. Familiar examples in this class are paraquat and flavins.

RADIATION

Involvement of ROS in carcinogenesis was first established for radiation and then extended to other classes [1]. Recent years have witnessed continuing interest, including use of AOs for skin protection against ultraviolet (UV) rays. The following discussion is divided between UV and more powerful radiation.

Extracellularly generated hydrogen peroxide mediates UV-induced activation of the epidermal growth factor receptor, whereas intracellular production of ROS is not sufficient for receptor activation [13]. UV filters are mainly used to guard against the harmful effects of solar rays [14]. However, increased sunscreen use is associated with more skin cancers, particularly melanoma. Evidence shows that skin penetration by UV filters result in ROS levels above those produced by UV alone. Benzo[a]pyrene synergistically enhances production of hydrogen peroxide by UV in cultured cells [15]. Treatment with catalase and other scavengers of ROS decreased the level of the peroxide in the system. Resulting DNA damage is believed to play a role in cancer. The epoxide metabolite of the polynuclear hydrocarbon is involved, along with UV, in generation of ROS that leads to enhanced amounts of 8-OHdG [16]. Cell signaling pathways initiated by UV radiation are believed to contribute to promotion and progression in skin tumors [17]. Studies with AOs and caspase inhibitors suggest that ROS activate caspase-8, supporting a ROS-mediated apoptotic mechanism for enhanced UV-induced inflammation. Results argue for an important role for UV-generated ROS as mediators of a signaling pathway that culminates in apoptosis [18]. Obesity in mice is associated with greater susceptibility to UV-induced OS and, thus, may be a risk factor for resultant skin diseases [19]. The role of visible light has been largely ignored [20]. Data indicate that this weaker type of radiation in the blue region can lead to ROS that contribute to tumorigenesis via oxidative attack on DNA.

The large amount of attention devoted to protection against UV exposure by AOs is partly a reflection of the skin cancer problem. In a recent review, the role of ROS and AOs was summarized [21]. ROS, generated in large amounts, are involved in all steps in tumorigenesis. Supplementation with AOs can inhibit the insults. Many AOs, reflecting

beneficial effects, have been investigated including the following: silymarin [22], genistein [23], green tea [24], grape seed proanthocyanidins [25], Hibiscus rosa simensis [26], botanical AOs [27], broccoli sprouts [28], cycloartenol [29], and dietary factors (β-carotene, vitamin D analogs, vitamin C and vitamin E) [30]. Since CD11b+ cells appear to be the major source of OS from UV exposure, reduction in infiltration by these cells may be an effective strategy for reduction of solar-induced skin disorders mediated by ROS [31].

Various studies address higher energy radiation in relation to OS in cancer. Mice bearing cancer cells exhibited elevated levels of ROS in thymocytes on irradiation, consistent with the OS hypothesis [32]. Ellagic acid increased OS in cancer cells on radiation, thus increasing cytotoxic effects [33]. A study concentrated on apoptotic death associated with membrane damage via ROS [34]. A review deals with exposure to radiation in relation to ROS and cell signaling [35]. Protection against oxidative damage by high-energy radiations was observed with various AOs including melatonin [36,37] and pycnogenol [38]. An AO connection was proposed for increased efficacy of proteasome inhibitors in radiation therapy [39]. An increase in iron and decrease in AO vitamins are involved in the mechanism of oxidative damage by total body irradiation [40]. Prevention by radical scavengers of DNA repair represents measures that reduce the onset of radiation-induced genomic instability [41]. Increasing the Glu peroxidase/SOD ratio may be a strategy for lowering the risks of radiation pneumonites [42]. Photodynamic therapy (PDT) is a promising cancer treatment that employs a combination of visible light and photosensitizers in order to induce apoptosis in cancer cells [43]. Findings indicate that both ROS and NO play a part in the apoptosis process.

REACTIVE OXYGEN SPECIES

Various peroxides have long been associated with carcinogenesis, including cancer from exogenous application [1]. Examples are hydrogen peroxide, benzoyl peroxide and tert-butyl hydroperoxide. AOs exert protective effects.

Since 2000 much additional work is available on this topic. ROS and tumor biology are intertwined in a complex web, making it difficult to understand which came first [44]. Evidence suggests that transformed cells use ROS signals to drive proliferation and other events required for tumor progression. This confers a state of increased basal OS, vulnerable to chemotherapeutic agents. It appears that cancer cells may die by the same systems they require. Oxygen radical production was higher in patients with sporadic colorectal cancer, in comparison with controls, which indicates that ROS may play a role in the etiology [45]. Literature is reviewed on ROS and lung tumorigenesis by mutant K-ras [46]. ROS in low concentrations possess important functions in inter-and intra-cellular signaling [47]. Senescence, apoptosis and cell death can also be modulated by these species, depending upon concentration. The role of ROS in apoptosis is discussed with implications for cancer therapy [48]. Lipid peroxidation appears to be a major source of endogenous DNA damage leading to cancer in humans [49,50]. A number of risk/protective factors exist for renal cell carcinoma including obesity, hypertension, diabetes, smoking, antioxidants, and oral contraceptives [51]. Lipid peroxidation is an important mechanism in renal carcinogenesis. Existing evidence supports cross-talk between lipid peroxidation and other pathways.

HALOALKANES (CCL₄)

CCl$_4$ and other haloalkanes (HAs) are widely used industrial solvents. The carcinogenicity and toxicity associated with CCl$_4$ exposure is well established [1]. Many HAs, including CCl$_4$, are procarcinogens that become activated via a sequence of redox reactions whereby CYP450 acts as the principal catalyst in the dehalogenation. In a study involving ^{18}O, several oxidative paths and oxidative species were verified. The isotope was present in large amounts in lipids and macromolecules, indicating oxidation. Considerable evidence supports a mechanistic path entailing electron uptake followed by chloride expulsion forming the trichloromethyl radical that combines with oxygen to yield a peroxyl radical. Subsequent steps yield various ROS. Possible means of DNA attack are addressed elsewhere [1]. In relation to the effects on exogenous and endogenous antioxidants, vitamin E decreased the level of liver injury, and the fruit antioxidant, schisandrin B, also protected, presumably by maintaining the GSH/GSSG ratio. Depletion of SOD has also been noted.

Other research [1] involving HAs dealt with their effects on enzyme signaling, tissue repair proteins and oncogenes, including induction of human proto-oncogenes in transgenic mice. Studies with CHCl$_3$ and CBr$_4$ indicate activity resembling that of CCl$_4$. BrCH$_2$CH$_2$Br and CCl$_4$ were synergistic.

QUINONES

In a review on the role of quinones in toxicology, part of the treatment concerns their involvement with cancer [52]. Among the metabolic precursors are benzene, PAHs, and estrogens. The usual toxic pathways are discussed, namely, OS and alkylation of DNA and proteins. One of the most commonly mutated tumor suppressor genes is p53 [53]. PAH o-quinones generate ROS through redox cycling, which causes p53 inactivation. The bulk of the mutations were not random, but involved guanosine as indicated by formation of 8-OHdG. Semiquinone radicals in cigarette smoke contribute to the carcinogenicity [54]. Of the precursors, methyl-substituted dihydroxybenzenes were more toxic than hydroquinone or catechol. Other toxic pathways derive from the multitude of ROS generators present in the smoke. The quinones formed from PCBs undergo Michael addition with DNA to form adducts which contributes to the carcinogenic process [55]. Characterization and quantitative determinations were carried out on the adducts. Naphthaquinones are present in the environment and in vivo as metabolites of naphthalene [56]. The toxicity of various substituted derivatives correlated with amount of redox cycling and thiol depletion, as well as the degree of thiol alkylation. Since 2, 3- and 1, 8-naphthalenediols do not form quinones, their cytotoxicity is much lower than the diols that do.

PHENOLS

Hydroquinone is a powerful human carcinogen to which people are exposed [57]. The nephrocarcinogenesis appears to be mediated by a GSH adduct formed by interaction with the

quinone metabolite. Catechol induces stronger DNA damage than hydroquinone [58]. The difference in NADH-mediated redox properties contributes to the different carcinogenicities. Chlorophenol is a probable human carcinogen which is a precursor of the benzoquinone electrophile that reacts covalently with biopolymers [59]. Results suggest that direct reaction of phenoxy radicals with DNA contributes to peroxidase- driven carcinogenicity. Ochratoxin A is a mycotoxin implicated in human kidney cancer [60]. Ease of oxidation to the hydroquinone-quinone couple was correlated with that of p-chlorophenol. The genotoxicity of ochratoxin A was addressed based on bioactivation to the quinone followed by covalent adduction to DNA [61]. Formation of DNA lesions was investigated with chlorinated catechols derived from pentachlorophenol [62]. Lesion formation decreased in the order: 4-Cl > 4, 5-diCl > 3, 4, 5-triCl. Pentachlorophenol is a possible human carcinogen [63]. In a study of DNA interaction, the adduct is a dichlorobenzoquinone nucleoside in which 2 chlorines have been displaced. Tumor promotion by the food AO 2,6-di-tert-butylphenol is well documented [64]. Considerable evidence supports involvement of the quinone methide metabolite by DNA adduction. Hydroxylation of the *tert*-butyl group produced a more reactive electrophile. Tamoxifen, is used for treatment of breast cancer and as a cancer prevention agent [65]. However, the drug causes increased cancer risk and leads to DNA adducts. Various bioactivation pathways have been proposed for the mode of action involving carbocation, o-quinone and quinone methide. In a study of PCB toxicity, oxygenated metabolites were capable of multiple adverse effects [66]. Inhibition of gap junctional intercellular communication represents a novel mode of action.

AROMATIC HYDROCARBONS

Exposure to monocyclic and polycyclic aromatic hydrocarbons (PAHs) is intimately linked to cancer incidence. These chemicals are commonly found in industrial settings, diesel exhaust, foods and cigarette smoke. The literature based on the ET-ROS approach was reviewed earlier [1]. Relevant material is also present in the Quinones and Phenols sections.

BENZENOID

Benzene is believed to act as a carcinogenic agent through phenolic and quinoidal metabolites [67]. 1,4-Hydroquinone displayed attributes against topoisomerase, including DNA cleavage, similar to those of 1,4-benzoquinone. Two quinone methide metabolites of the phenolic AO butylated hydroxytoluene may be tumor promoters [68]. The metabolites are strongly electrophilic and undergo Michael additions with protein. The modified proteins are implicated in various aspects of tumorigenesis. Glu S-transferase may interact with the quinone methide, thus decreasing cellular protection from oxidants [69].

NAPHTHALENE

Lung injury occurs by naphthalene in the air via P450 metabolic activation [70]. The compound is a respiratory carcinogen [71]. Oxidative metabolism results in formation of the 1,2- and 1,4- diols, as well as the corresponding quinones, which have been implicated in tumorigenesis via a role in DNA damage.

PAHs

Various members of this class are powerful carcinogens, which are activated by P450 enzymes via oxidation to metabolites which form DNA adducts [72]. The important pathways include one-electron oxidation, epoxide generation, and o-quinone formation [73]. Data show that 8-OHdG production is dependent on Cu-catalyzed redox cycling of the o-quinones to produce ROS [74]. Catalase suppressed formation of lesions [75]. Iron can also be involved. Scavenging experiments pointed to participation of hydroxyl radical. A review addresses PAH photochemistry and photo toxicity [76]. The effects of co-existing biologically relevant molecules and ions were studied in relation to the toxic effects of PAHs [77].

Benz[a]anthracene produced oxidative DNA damage via dihydroxy radicals [78]. The catechol derivative, capable of redox cycling, was also identified. An alternate oxidative route for DNA adduct formation entails conversion to the hydroxymethyl groups of 7, 12-dimethylbenz[a]anthracene [79]. A subsequent step comprises transformation to the electrophilc, DNA alkylating sulfate esters. It should be noted that DNA alkylation could result in generation of ROS [1].

The potent pyrene class has been the object of considerable attention. The dihydrodiol form of benzo[a]pyrene requires further metabolic activation to exert its carcinogenic effects [80]. The diolepoxides are the ultimate carcinogenic species [81]. Stereochemistry of the DNA adducts was investigated. DNA binding studies provided further evidence that P450 catalyzed formation of epoxide in the fjord region of dibenzo[a, 2]pyrene is a critical step in carcinogenesis [82], resulting in about 98% depurinating adducts, mainly derived from one-electron oxidation [83]. Only one depurinating diol epoxide adduct was formed. Tumorigenicity, mutation and DNA adduct data indicate a major role for Ade adducts in tumor initiation. Smoking may cause mutations by formation of PAH o-quinones, e.g., from benzo[a]pyrene [84]. The resultant ROS cause DNA mutations and 8-OHdG formation.

In investigations of other PAHs, a comparison of cancer induction by fjord and bay region diol epoxides of benzo[c]chrysene showed that the ones from the fjord region are more carcinogenic than those from the bay region [85]. Major metabolites of dibenzo[cmno] chrysene were found to be dihydrodiol and phenolic derivatives [86]. Benzo [ghi] perylene, a PAH without a classic bay region, yielded a 3, 4-epoxide that plays an important role as the ultimate mutagenic metabolite [87].

AROMATIC NITRO COMPOUNDS

Reductive metabolism leading to toxic substances, namely, nitroso and hydroxylamine derivatives, is described in the metabolism section.

Simple members, such as o-and p- nitrotolune, demonstrate carcinogenic potential [88]. The *ortho-* isomer gave clear evidence for cancer at multilple sites in rats and mice. The *para-* isomer gave a weaker response, i.e., some evidence or equivocal or none. The authors suggested differences in metabolism as responsible for the observed results. In a study of nitroanilines, a tumorigenic effect was observed mainly in the dinitro types that are classed as alkylators [89]. Both the nitro and amino groups possess carcinogenic potential. For metabolism of 2-nitroanisole, human hepatic microsomes yielded 2-nitrophenol and dihydoxynitrobenzenes [90]. The oxidative conversions are attributed to CYP450 enzymes.

The risk to human health from air pollutants is an important issue [91]. Many mutagenic and carcinogenic compounds, such as polycyclic aromatic hydrocarbons and nitrated-derivatives, are the main constituents of these pollutants. Analyses have indicated that 1-nitropyrene and 1,3-, 1,6- and 1,8-dinitropyrene are major nitroarene components of airborne, surface soil and diesel exhaust particles (DEP). Diesel engine emission appears to be a principal source of contaminants and DEP and its crude extracts are often used as model compounds for the investigation of air pollutants. The relationship between chemical carcinogenesis by polycyclic hydrocarbons, such as benzo[a]pyrene, and drug metabolizing enzymes has been extensively studied. The induction of drug metabolizing enzymes and the activation of procarcinogens have been also examined. Since nitrated polycyclic aromatic hydrocarbons are widespread environmental contaminants, it can be assumed that humans will ordinarily be exposed to them. The conclusion was drawn that induction of CYP450 may catalyze the genotoxic activation of DEP. Diesel exhaust is suspected to contribute to lung cancer. 3-Nitrobenzanthrone (3-NBA) exists in diesel exhaust particulate matter, coal-burning-derived particles, surface soil, and river sediments [92]. Mutagenicity is at the same level as that of 1,8-dinitropyrene, which is one of the strongest mutagens, and a carcinogen to rats. 3-NBA forms multiple DNA adducts in vitro and in experimental animals. It is a lung carcinogen to rats and is suspected as a human carcinogen. Oxidative DNA damage may play an important role in the carcinogenic process of 3-NBA in addition to DNA adduct formation. The N-hydroxylamino metabolite caused Cu-mediated DNA damage, indicating involvement of hydrogen peroxide. Nitropyrenes (NPs) present in fuel emissions are mutagenic and carcinogenic [93]. Nitro reduction of 1-NP leads to OS, which then produces, increased 8-OHdG via ROS. These events may well play a part in carcinogenesis. Impaired DNA repair enzymes could also be a factor. Adduct formation following nitro reduction is believed to be a major factor in NP-mediated DNA damage [94]. 1-Nitropyrene facilitates Cu(II)-mediated DNA insult in the presence of NADH. Catalase and a Cu(I) chelator attenuate the DNA damage indicating involvement of hydroperoxide and Cu(I). The authors designated a DNA-Cu-hydroperoxide complex as the main reactive species. However, we note that the data may be rationalized by involvement of Cu(I) as a Fenton catalyst. Hence, oxidative DNA attack and DNA adduct formation may play an important part in the carcinogenesis. The position of the nitro substituent determines the relative tumor activity [95]. Human breast cancer cells can activate DNA. Articles deal with DNA damage induced by dinitropyrenes [96,97], 2,7-dinitrofluorene [98], and 6-nitrochrysene [99,100]. The comparative tumorigenicity of 6-

nitrochrysene and its metabolites was ascertained [101]. A review outlines carcinogenicity and mutagenicity of various nitro aromatic compounds, including monocyclic, polycyclic and heterocyclic types [102]. Dietary silymarin suppresses rat carcinogenesis by 4-nitroquinoline-1-oxide [103] (see antioxidants).

AROMATIC AMINES

1. Hydrocarbon Types

Prominent examples are naphthylamines, benzidine, aminobiphenyl, and aminofluorene. Metabolic activation leading to mutagenesis and carcinogenesis has been reviewed [1]. 2,6-Dimethylaniline is a rodent carcinogen and a possible human carcinogen. Metabolism leads not only to the usual hydroxylamine product, but also, mainly, to a phenolic derivative [104]. Subsequent conversions might lead to iminoquinones. Various alkylanilines were metabolically converted to electrophilic intermediates, which bind covalently to DNA [105]. The adducts are formed in the bladder which is a common target for carcinogenic arylamines. DNA adducts were identified after formation from the hydroxylamine metabolites of diverse arylamines [106]. 4-Aminobiphenyl is found in a large number of hair dyes [107]. A study indicated elevated levels of DNA adducts from the amine in people using hair dye frequently. Aromatic amines initiate bladder cancer in humans with inflammation/infection playing an important role [108]. ROS and RNS were generated from benzidine under a variety of conditions. A novel metabolite, an azobenzidine, was identified. GSH hindered oxidative metabolism. DNA binding studies were also performed. Lactoperoxidase-catalyzed activation of carcinogenic hydrocarbon and heterocyclic amines was investigated [109]. Two of the products formed were hydrazo and azo derivatives. The level of binding to DNA for various of these amines was ascertained. A QSAR study was carried out for hydrocarbon and heterocyclic amines involving nitrenium ion selectivity with mutagenicity and carcinogenicity [110]. Since oxidative metabolism generates toxic metabolites of aromatic amines, reductive toxification was employed as a countering measure [111]. Human reductase was used for this purpose.

2. Heterocyclic Types (Cooked Foods)

Heterocyclic aromatic amines (HAAs) are generated in overheated foods [112]. The compounds are mutagens and suspected human carcinogens. Most prevalent types are imidazoquinoxaline and imidazopyridine. The metabolic, activating reactions are similar to those of arylamines involving N-oxidation. The end result is formation of DNA adducts via nitrenium ions. More detail is available in an earlier review [1]. Molecular dynamics of a food carcinogen-DNA adduct indicate hindered nucleotide incorporation [113]. A study was made of the formation of mutagenic HAAs from creatinine [114]. A DNA adduct formed with aminophenylnorharman may be responsible for the comutagenic action of norharman with aniline [115].

Other metabolic pathways have been reported including a role for ROS [1]. This may involve redox cycling of the hydroxylamine and nitroso derivatives. A common DNA oxidation product, 8-OHdG, was detected. Endogenous and exogenous AOs are known to be helpful.

ALKYLATING AGENTS

This class falls both in the cancer and anticancer areas [1]. Representative chemical types include epoxides, nitrogen mustards, aziridines, alkyl sulfonates and nitrosoureas. The primary mechanism entails attack on base sites of DNA. Evidence indicates that these agents are capable of generating ROS, followed by oxidation and chain cleavage of DNA. Possible mode of ROS formation is discussed.

ALKENES

Mechanism for this class has been rather well delineated involving metabolic epoxidation followed by DNA alkylation. The process is accompanied by generation of ROS (see alkylating agents).

Styrene

Sterochemical aspects of biotransformations involving R and S forms of styrene oxides were examined [116]. Metabolism results in unequal amounts of two isomers which differ in their mutagenic and genotoxic activity. Inhibition of CYP-450 metabolism of mice in vivo produced cell replication rates in lungs exposed to styrene comparable to that of controls [117]. The findings support a role for oxidative metabolites in toxicity. In a study of styrene oxide genotoxicity, the influence of genetic polymorphisms of enzymes involved in DNA repair depended on exposure dose [118]. The major metabolite styrene oxide and the minor but potent, metabolite 4-vinylphenol cause similar toxicities. There is decrease in GSH in mouse liver and lungs, apparently due to ROS generated by the metabolites [119]. The OS is reversible.

Other Monosubstituted Alkenes

Acrylonitrile, an industrial monomer, produced increased incidence of tumors in rats, as well as increased production of 8-OHdG [120]. ESR showed formation of a nitrogen-centered radical from the monomer in the presence of hydrogen peroxide and Cu(II), which may be involved in DNA damage. The investigation was aimed at exploring toxic avenues other than via epoxide formation. Although most attention has been directed toward DNA alkylation by toxic alkenes, recent work has involved protein substrates. For example, acrylonitrile undergoes selective covalent binding to Cys of rat liver carbonic anhydrase [121]. This

interaction may aid in prevention of adduction to more critical molecules, namely, DNA. In addition to formation of ROS, other mechanisms may play a role in the toxicity of acrylonitrile, namely, covalent binding to macromolecules, GSH depletion, and cyanide formation from the epoxide [122]. The report contains information on covalent binding to Glu S-transferase. Acrylonitrile is a suspected carcinogen derived from cooked foods [123]. In line with other alkenes, metabolism forms the epoxide glycidamide. Another reaction entails conjugate addition by N-terminal valine in Hb. The epoxide is a mutagenic metabolite [124]. Adducts with thymidine and cytidine were identified.

Polyhalogenated Alkenes

A 2004 review discusses the literature on cancer risk in humans by trichloroethylene [125]. Most of the studies showed increased incidence for various types of cancer. In the reactions of trichloroethylene and its epoxide with CYP450 enzymes, inactivation occurred and sites of modification were determined [126]. An investigation of cytotoxicity of tri-and pentachloroethylene on epidermal keratinocytes revealed elevation of lipid peroxidation and decline in AO enzyme activities [127]. Vitamin E afforded protection against the OS. The reaction of tetrachloroethylene oxide with albumin was investigated [128]. The reactivity of tribromoethylene oxide was compared to that of related chlorinated epoxides [129].

Conjugated Dienes

1,3-Butadiene is a carcinogen and mutagen that acts via oxidized metabolites that react with DNA [130]. The deoxyuridine adducts are highly mutagenic lesions that may contribute to carcinogenicity. The diepoxy metabolite is a carcinogen and mutagen [131]. The toxicity is attributed to interaction with DNA and crosslinking. The crosslinked products from deoxyguanosine were characterized. The diepoxy compound, the ultimate carcinogen, causes point mutations, deletions and chromosomal aberrations [132]. The major crosslink involves guanine. Higher exposure produced mainly monoalkylated lesions. A similar study was reported [133]. Like butadiene, the polymer monomer isoprene, a carcinogen, is transformed in vivo to epoxides that form adducts with macromolecules [134]. Guanine adducts from monoepoxides were identified. An epoxide, a major mutagenic metabolite of the monomer chloroprene, was reacted with various nucleosides, and the resultant adducts were characterized [135].

N-NITROSO COMPOUNDS

Metabolic pathways leading to tumorigenic effects are delineated [1]. Oxidative events appear to play a role during metabolism, which may contribute to initiation of cancer [136]. Attention has centered on α-hydroxylation followed by C-N cleavage of the N, N-dialkyl precursor [137-139]. A CYP450 catalyst is the most efficient [137]. One of the substrates studied is N- nitrosonornicotine, the most abundant member of the class in tobacco [138].

Diazonium ions arise from cleavage of the hydroxylated product [139, 140]. In some cases, toxic glyoxal can also be produced [140]. An electrophile, which binds to DNA, is involved [141, 142]. A study of the effect on DNA by some members revealed depurination, deamination and formation of a novel nucleobase [143].

HYDRAZINES

Humans are exposed to various hydrazines, many of which are carcinogenic and teratogenic [1]. Their activity apparently arises from reactive intermediates, including carbon radicals. The metabolic route for $RNHNH_2$ entails dehydrogenation to an azo product which on hydrogen atom abstraction yields an azo radical. On subsequent decomposition, a carbon radical results (see CCl_4). 1,2-Disubstituted hydrazines can serve as carbocation precursors by oxidative conversion to azoxy intermediates. Studies have noted DNA alkylation, as well as other DNA lesions, perhaps via the carbocation and carbon radical-O_2 interactions to form ROS. ET, involving CYP450, seems to be a major player leading to DNA strand scission by radicals. Free radical scavengers alleviated the deleterious effects. Hydrazine activity is reportedly aided by catalytic metals, whereas •OH scavengers and SOD proved protective. Increases in lipid soluble fluorophores, indicators of OS, were observed in liver mitochondria which were alleviated by vitamin E. Hydroxylamine and superoxide are also suspected players in the bioactivation. GSH and SOD suppressed mutation in certain cases. Hydralazine adversely affects GSH, and induces lipid peroxidation. Isoniazid, an antitubercular hydrazine, generates DNA lesions via ROS. CAT acted as an inhibitor. Other research concerning hydrazines implicates genetic factors, as well as tumor suppressor genes and oncogenes.

ALIPHATIC ALDEHYDES

It is well known that formaldehyde and ROS are cytotoxic, as well as potentialy carcinogenic [144]. The combination resulted in formation of DNA-protein crosslinks, accompanied by synergistic increase of cellular ROS and cell death. In long-term studies, formaldehyde and acetaldehyde produced an increase in malignant tumors and showed specific carcinogenic effects on various organs and tissues [145]. Prior reports deal with ROS production from formaldehyde [144] and acetaldehyde [146].

ABUSED DRUGS

Alcohol

In a treatment of breast cancer etiology, there is association with various mechanisms, including mutagenesis by acetaldehyde, perturbation of estrogen metabolism, induction of oxidative damage and involvement of one-carbon metabolism [147]. Generated ROS may cause damge leading to DNA and protein sdducts. Acetaldehyde, formed in elevated amounts

is mutagenic and carcinogenic [148]. It induces ROS and activates various procarcinogens. A casual relationship has been established between excess alcohol consumption and cancers of many organs [149]. Among the various mechanistic contributors are ROS and RNS. In a review of alcohol and cancer, metabolic acetaldehyde is designated as predominantly responsible for alcohol associated carcinogenesis [150]. Part of its role entails enhanced production of free radicals, activation of various procarcinogens and binding to DNA, as well as proteins. The induction of CYP450 results in increased levels of ROS and in activation of various carcinogens [151]. In a 2005 review of some aspects of pathogenetic mechanisms in alcohol carcinogenesis, part of the discussion entailed the role of alcohol metabolism [152]. A symposium dealt with the role of alcohol metabolism [153]. Part of the discussion addressed participation of free radicals and macromolecular adducts. Aspects included in a 2005 symposium on mechanism included production of acetaldehyde, induction of CYP450 and associated OS, conversion of procarcinogens to active forms, accumulation of iron and subsequent OS, and various cell signaling events [154]. The implications of DNA adducts from metabolic alcohol are addressed [155]. The most-studied adduct is the one from deoxygunosine. The genotoxic effects may be partly related to adducts containing DNA-protein crosslinks and DNA interstrand crosslinks. A recent book chapter organizes the literature on alcohol mechanisms in relation to metabolism, cell signaling, toxicity, addiction, prevention, therapy, and beneficial effects [146]. Various metabolites play important roles in the carcinogenic responses. The initial 1-hydroxyethyl radical is associated with lipid peroxidation and alkylation of DNA bases. Cellular AOs can interact with these radicals resulting in their destruction. Most attention has been centered on the intermediate acetaldehyde as a key participant. Metabolism yields the acetyl radical which has the potential of forming peracetic acid, a precursor of the hydroxyl radical. Side products in the C_4 category may participate. Considerable literature is devoted to adducts arising from DNA, some involving crosslinks.

Marijuana

In many societies, marijuana (THC) is the second commonly smoked substance after tobacco [156]. Regular smoking causes widespread airway inflammation and injury, in addition to dysregulated growth of epithelial cells that may be precursors to lung cancer. THC may contribute to these insults through its ability to augment OS. Marijuana smoke has deleterious consequences on airway epithelial cell energetics, including mitochondrial operation [157]. A recent review points to ET-ROS involvement for many mitochondrial toxins [9]. Despite considerable evidence, there is a common perception that marijuana poses no danger and has little adverse effect on the lung [157]. A 2006 report states that an association exists between marijuana smoking and lung cancer based on molecular, cellular and histopathological findings [158]. The induction and regulation of carcinogen-metabolizing enzyme CYP1A1 by THC has implications for the drug as a cancer risk factor [159]. Some of the pulmonary consequences of habitual marijuana smoking appear to be similar to those of regular tobacco use [160]. Cannabinoids are immune modulators that affect intracellular signaling pathways, such as epigenetic ones associated with cell proliferation [161].

TOBACCO

Cancer caused by cigarette smoking is one of the most prevalent. A large variety of carcinogens is present in the smoke, including nicotine and others addressed in a review [10]. The genotoxic effects of myosmine, a tobacco alkaloid, were determined in a human esophageal adenocarcinoma cell line [162]. Involvement of nitrosation and OS has been discussed [10,2]. Smoking can promote inflammation-associated adenoma formation in the mouse colon [163]. Data indicate a link between 5-lipoxgenase and carcinogenesis. Evidently, smoking induces expression of the enzyme, which plays a role in inflammation. A study highlights the occurrence of lipid peroxidaton in environmental tobacco smoke exposure, and possible protective effects of selenium and vitamin E [164]. Use of chewing tobacco, which has increased in recent years, is associated with cancer of various body components [165]. Evidence indicates that the cytotoxic effects are mediated through generation of ROS.

METALS

General

Epidemiological evidence points to the high incidence of respiratory tract cancers in workers exposed to Cr(IV) [166]. Other metal ions that are human carcinogens include Ni, Be, As and Cd. Considerable evidence exists that OS may be a common pathway for metals in which a correlation exists among generation of ROS, DNA damage, tumor promotion and apoptosis. Evidence indicates that metals in tobacco smoke play a leading role in lung carcinogenesis [167]. The review addresses the mechanistic role of metals, as well as DNA damage and radical participation.

It is relevant that the carcinogenic heavier metals generally possess reduction potentials amenable to ET in the biological domain [1]. This is in keeping with the greater positive charge (electron attractive) and the larger atomic surface for radical delocalization.

Chromium

A study was made of oxidative mechanisms of the ionic forms of Cr [168]. Much evidence indicates that Cr(VI) induces OS, DNA damage, apoptosis, altered gene expression and lipid peroxidation. Cr(VI) demonstrates significantly higher toxicity than Cr(III). The Cr(III) form which can be oxidized in vivo to the higher, more dangerous valence state must be considered a potential carcinogen [169]. This has implications for increased use of Cr(III) complexes as dietary supplements. Assays on Cr-exposed workers showed a higher index of DNA damage [170]. Data suggest that oxidative damage is a participating mechanism in dichromate carcinogenesis, mutagenesis and toxic effects [171]. A comparison was made of hepatocyte toxic mechanisms for chromate and arsenite [172]. Cr(VI) cytotoxicity was associated with ROS, lipid peroxidation and loss of mitochondrial membrane potential, which were prevented by AOs, catalase and radical scavengers. GSH was depleted. In contrast, arsenite cytotoxicity involved much less OS, but decline in mitochondrial membrane potential

did occur. As is a human carcinogen, as well as a strong, dose-dependent mutagen [173]. The roles of ROS and RNS in mediating the genotoxic response are presented. AOs may be a useful interventional treatment in reducing the deleterious effects. As, Ni, Cd and Co are human carcinogens that not only induce OS, but also interfere with nucleotide and base excision repair [174]. Ability to inactivate DNA repair was compared for various valence states of As.

Iron

Partly because of the widespread use of iron supplements, there is not general recognition of the toxic effects of the metal. Excess hepatic Fe may be both directly and indirectly carcinogenic [175]. Levels of lipid hydroperoxides, malonaldehyde and 8-OHdG increased. The effect of free Fe appears to be mediated by ROS and oxidative damage that are mutagenic and carcinogenic.

Lead

This metal and its compounds, which play an important part in industry, are suspected human carcinogens [176]. Lead is reported to increase the amount of lipid peroxidation. Experimental results strongly indicate a protective effect by vitamin C and silymarin against the toxic effects of Pb.

Selenium

At dietary levels Se may prevent the development of many types of cancers [177]. However, at high concentrations Se compounds can be cytotoxic or perhaps carcinogenic, with possible involvement of OS. Sodium selenite induces DNA damage including strand breaks and base damage. The various aspects of pro-oxidant toxicity are discussed.

ASBESTOS

Data indicate that asbestos fibers induced various ROS, such as superoxide, hydrogen peroxide and hydroxyl radicals [178]. Several studies report formation of ROS from phagocytic cells exposed to various types of asbestos fibers. ROS are generated not only from cells, but also by the fiber itself. Superoxide anions arose from metal ions on the peripheral surface of asbestos fibers, notably ferrous ion, by ET reaction to molecular oxygen. Several other studies have implicated superoxide, hydrogen peroxide, and hydroxyl radicals by either the Fenton or Haber-Weiss reaction catalyzed by iron. Studies emphasize the importance of iron in asbestos-induced hydroxyl radical formation, lipid peroxidation and DNA single strand breaks. The role of ROS in asbestos-induced cytotoxicity, mutation and gene induction has been studied for many years. The effects of antioxidants on mesothelial cell remain

controversial at the moment. Exposure to asbestos is associated with chronic pulmonary diseases and cancer of the lung, pleura, and peritoneum [179]. The mechanism of fiber carcinogenesis is far from clear and is likely to be complex, depending on fiber dimensions, surface properties, and physical durability. The induction of ROS and RNS plays an important role in fiber genotoxicity. The βigH3, a secreted protein induced by the transforming growth factor-β and essential for cell adhesion, is downregulated in asbestos-induced tumorigenic human bronchial epithelial cells. Intriguing epidemiologic data from Turkey imply that predisposition may play an important role in determining individual susceptibility to mineral fiber carcinogenesis and to the development of mesothelioma. In villages built with stones mined from the nearby natural caves [180,181] at least 50% of deaths are caused by malignant mesothelioma. Closer observation revealed that mesotheliomas only occurred in certain homes and not in others even though all homes contained similar amounts of erionite. Further analysis of pedigrees of families who lived in homes in which cancers occurred showed that these mesotheliomas seemed to be inherited in an autosomal dominant pattern. Approximately 50% of descendents of affected parents developed mesotheliomas. When members of unaffected families married into affected families, 50% of their descendents also developed the condition. Clearly, genetics is a key factor because mesotheliomas do not develop in non-affected families regardless of environmental exposure. It is well accepted that asbestos fibers are the cause of virtually all cases of human malignant mesothelioma [182]. It is also known that all asbestos types, including chrysotile and amphiboles, have been shown in epidemiological and toxicological studies to be fully capable of inducing the tumors. In addition to heavy asbestos exposure, milder exposure can also induce tumor. In a study of fiber features, thin ones should be included in the list of fiber types contributing to the induction of human malignant mesothelioma. A study was made of tumor suppressor gene alterations in patients with malignant mesothelioma due to environmental asbestos exposure in Turkey [183]. Particle stimulated chemiluminescence (CL) production by human polymorphonuclear leucocytes (PMN) has been utilized to evaluate the pathogenicity of mineral and glass fibers with the understanding that ROS production as measured by CL is etiopathogenically related to fiber toxicity [184]. Findings indicate that neither the total number, nor the specific range of fiber dimension solely determines CL production. As a consequence, other physicochemical factors, such as the surface reactive characteristics of milled fibers, may be more closely related to CL production by PMN.

MISCELLANEOUS CARCINOGENS

1. Inorganic Agents

Inorganic ROS, such as hydrogen peroxide, are treated in another section. One can regard active oxygen as a universal carcinogen.

A. Ozone

Studies reveal a number of metabolic products in the ROS category, including organic peroxides, hydrogen peroxide, ozonides, hydroxyl radical and singlet oxygen, some of which

result in oxidation of DNA [1]. Ascorbate, GSH, SOD and CAT act as AOs in inhibition of ozone insult.

SOD-overexpressing mice [185] are resistanr to ozone-induced tissue injury and increases in tumor necrosis factor-alpha [186-188], apparently via AO action. A mixture of ozone and nitrogen dioxide resulted in elevated levels of the AO Mn SOD [188].

B. Potassium Bromate

This inorganic oxidant, used as a food additive, displays cancer-inducing properties [1]. Enhanced lipid peroxidation is associated with oxidative DNA damage.

There is increased hydrogen peroxide and reduction in AO enzymes, including GSH [189]. *Nigella sativa* (black cumin) amelioriates events associated with carcinogenesis, apparently through diminution of OS. Coumarin is beneficial in protecting against $KBrO_3^-$ mediated OS and tumor promotion [190]. Renal cell tumors were significantly increased in rats given this oxidant [191]. DNA damage resulted in 8-OHdG. The mechanism for cancer risk in humans likely involves OS. Evidence indicates that bromate is a mutagenic carcinogen operating via ROS [192]. However, certain thresholds of OS are required in order for damaging oxidation to occur [193]. A study indicates that the mechanism of DNA damage differs from the usual type of OS, e.g., involving the hydroxyl radical [194]. In a recent review, research strategy is outlined for ascertaining the bromate mechanism [195].

C. Nitric Oxide

Expression of the alpha-subunit of hypoxia inducible factor (HIF-1 alpha) occurred in oral carcinoma accompanied by induction of NO synthases (NOS) [196]. Accumulation of HIF-1 alpha was prevented by inhibition of NOS or by the AOs ascorbic acid and N-acetlycysteine. Apparently radicals are involved in the mechanism. There is enhanced lipid peroxidation and decrease in AOs [197]. NO-donating aspirin is a promising agent against cancer [198]. The action mode entailed GSH depletion and generation of OS (peroxides and OS) leading to apoptosis of cancer cells. Brain tumor cells may protect themselves against NO cytotoxicity by overinducing SOD protein [199]. NO exhibits many roles in vivo including lethality, e.g., by inhibiting caspase-mediated apoptosis [200].

2. Nitroalkanes

This class receives less attention than the aromatic counterpart [1]. 2-Nitropropane undergoes enzyme-catalyzed oxidation in the presence of hydrogen peroxide resulting in formation of superoxide. Generation of 8-OHdG apparently is a response to OS. Tetranitromethane, an oxidizer in propellants and explosives, is carcinogenic [201]. It is a new type of carcinogen that induces oxidative DNA damage via involvement of tyrosine.

3. Azo Dyes

Toxicity of hair dyes and food coloring has attracted attention [1]. Of possible modes of action, one comprises metabolism to tumorigenic pri-amines. Also, the parent azo compound

might operate in ET with generation of ROS. In SAR studies, there is correlation between carcinogenicity and radical production. Several members were found to elicit liver peroxidation.

4. Polychlorinated Biphenyls (PCBs)

PCBs, which are implicated in a variety of toxic effects, exhibited high carcinogenic potency [1]. As indicated in the sections on quinones, an oxidative pathway leads to a diphenoquinone derivative which possesses a reduction potential favorable for in vivo ET. In addition to earlier reports, recent evidence provides support for ET-ROS-OS. PCBs are complete carcinogens and tumor promoters in the liver [202]. Oxidative stress from increased generation of ROS, such as superoxide, is implicated in the toxicity [203]. Evidence in 2006 suggests involvement of ROS, redox metals and CYP enzymes in induction of tumor cell death and DNA damage [204]. These toxins are known to alter mammalian AO defense systems [205]. There is a major decrease in GSH status, as well as of AO enzymes.

5. Gossypol

This substance, present in cottonseed oil, contains many ET functionalities in the parent and its metabolites [1]. Gossypol possesses a reduction potential conducive to redox cycling. ROS are produced which compromise the AO defense systems and attack DNA. Gossypolone and its imine derivative exhibit cytotoxicity, which was decreased in the presence of the AOs mannitol and catalase [206].

6. Anticancer Agents

A baffling paradox enunciated by Haddow in the early years states that, generally, agents that are carcinogenic are also antineoplastic, and vice-versa [1]. Well known examples are radiation and nitrogen mustards. If the premise is valid that both actions involve OS, then a reasonable rationale, advanced in 1959 and developed further in 1986, exists. Also relevant is the observation that the incidences of second cancers increase after treatment of primary ones with antitumor drugs. Of course, specificity would be important in connection with the greater sensitivity of tumors to ROS and preferential binding to cancer cells. Other mechanistic approaches appear plausible, e.g., interference with DNA or enzyme action, but these could also entail OS.

7. Estrogens

Estrogens play a role as carcinogens in mammary and female reproductive tissues [207]. Metabolism entails formation of ROS, alterations of intracellular redox status, and oxidative DNA damage. Estradiol induces an increase in sensitivity to oxidative DNA damage through

an estrogen-receptor mediated mechanism. Other investigations reveal involvement of o-quinone metabolites, which can generate ROS via redox cycling [4]. A 2003 report reinforces the thesis that OS plays a crucial role in estrogen-induced carcinogenesis. Related material is presented in the phenols and quinones sections.

8. Quinolones

This class is a known liver carcinogen in rats and also exhibits mutagenicit [1]. A possible mechanistic pathway from metabolism comprises metal complex formation followed by redox cycling with ROS formation [2].

OTHER FACTORS

The ET-ROS-OS approach may also be applicable in many of these topics.

ONCOGENES

Reports reveal a major role of OS in the activation of oncogenes [1]. Protection by AOs is in accord with a role by ROS with subsequent mutation of DNA. Thus, the popular oncogene aspect can be incorporated within the ROS-OS framework.

GENETICS

A hereditary disposition, which varies between individuals, appears significant for some cancers, such as, the breast type in females [1]. The oncogene aspect may be a key factor (see above).

AGING

The relation between aging and increased risk of cancer is well established [1]. The risk increases after age 50, particularly for males. A plausible rationale consists in part of continuing exposure to ROS with resulting DNA damage.

HEREDITARY ILLNESSES

Certain illnesses with genetic components are accompanied by increasd risk of cancer, including Bloom's syndrome, Down's syndrome and Fanconi's anemia [1]. In all cases, the condition comprises elevation in ROS and decrease in AO defenses.

CHRONIC INFLAMMATION AND INFECTION

Much evidence exists for involvement of chronic inflammation as a cancer risk factor [1]. Examples in the infection category include Heliobacter pylori, Schistosoma harmatobium, hepatitis virus and liver flukes. Increased levels of ROS and RNS are reported.

VIRUSES

OS occupies a niche in the viral category [1]. This condition has been observed during HIV infection, which could be part of the pathogenesis. Toxicity by ROS and RNS may be part of the mechanism of tissue injury in many viral infections.

There appears to be a direct link between hepatitis C proteins and OS, as well as carcinogenesis [208]. Extracts of leaves from various vegetables had anti-tumor and radical scavenging activity [209]. There was a correlation between the two effects. In a study of hepatitis B virus replication, OS increased after treatment with interferon-gamma-1-beta, and hydrogen peroxide [210]. The AO N-acetylcysteine inhibited the antiviral effect.

PEROXISOME PROLIFERATION

Various hepatocarcinogens produce increases in peroxisomes [1]. Generated excess hydrogen peroxide inflicts DNA damage (see ROS section).

NUTRITION

Carcinogens in foods can occur naturally, as additives, or from storage and cooking [1]. There are indications that red meat and fats may be related to colon cancer. The colon is high in iron (ET agent) content. Unsaturated fats readily undergo peroxidation. Dietary caloric restriction reduces cancer risk, presumably by lower production of ROS in mitochondria.

A diet rich in fish oil containing polyunsaturated fatty acids (PUFA) attenuated tumor growth [211]. The AO effect is in marked contrast to the usual lipid peroxidation and OS for the PUFA class. Controlled clinical trials involving possible health benefits of fish-oils often gave conflicting results. Early stages of cancer were inhibited by rectifying poor levels of AOs [212]. The incidence of prostate cancer was reduced by correction of low AO intake. In contrast, irreversible precancerous lesions, established common cancers, including those of chronic smokers, did not respond favorably to AO therapy.

ANTIOXIDANTS

The crucial roles of vitamins C and E in nutrition have long been recognized [1]. More than fifty years ago, lack of vitamin C was associated with various problems of aging. At that

time, a number of investigators suggested higher intakes of the vitamin. The use of AOs (mainly vitamin C, E and beta carotene) for prevention of illnesses of aging, e.g., cancer, stroke and heart attacks, has attracted an abundance of recent attention in research and news media. These findings are based on the hypothesis that OS is an important contributor to the distresses commonly associated with the aging process.

The supporting evidence for AO effectiveness has been characterized as overwhelming. However, there is disagreement concerning proper supplementation for the general public. Some advise waiting until more information is available, whereas others feel that supplementation should immediately be a part of the daily diet. Also, debate continues with regard to the preferred form of the protective agents, namely, tablets or fruits and vegetables. The marked increase in laboratory work has been augmented in recent years by epidemiological studies. Generally, the population statistics indicate a beneficial influence on a variety of aging illnesses. For a smaller number of cases, adverse effects have been reported. Also a recent report (see below) presents a different view on AO effectiveness. The ensuing reports discuss literature on the AO topic. Focus is on the more recent findings. There is also treatment of AOs in other portions of this chapter.

Oxidative stress is now recognized to be associated with more than 200 diseases, as well as with the normal aging process. A compelling amount of evidence has led to the "oxidative hypothesis" [213]. Yet studies have concluded that vitamin E supplementation does not prevent cancer or major cardiovascular events. A similar situation exists for diabetes, in that despite the undeniable presence of substantial oxidative stress, attempts to treat the disease by supplementation with AOs have failed to produce any significant improvements. Research demonstrates that modest induction of the catalytic AOs SOD and catalase by extracts of five widely studied medicinal plants may be a much more effective approach than supplementation with AOs (such as vitamins C and E) that can, at best, stoichiometrically scavenge a very small fraction of total oxidant production. SOD is a tumor suppressor protein that increases the dismutation rate of superoxide and inhibits cancer cells growth in vitro as well as in xenografts in mice [214]. In overexpression investigations CuZnSOD and MnSOD were similarly effective at suppressing cancer cell growth. Findings indicate that the removal of tumor produces ROS, which then aggravate metastatic tumor growth by activating several growth factors [215]. PEG-catalase can effectively prevent this metastatic tumor growth by detoxifying the ROS.

A considerable amount of research has been devoted to flavonoids. Studies suggest that diets rich in polyphenols, such as flavonoids, may lead to a reduced risk of gastrointestinal cancers [216,217]. The results highlight multiple routes in which dietary derived flavonols may exert beneficial effects in the gastrointestinal tract, including reaction with nitrous acid which protects against toxic N-nitrosoamine formation, as well as cell-signaling events [218]. A SAR study was performed concerning the anticancer activity of phenolic antioxidants against breast cancer cells and a spontaneous mammary tumor [219]. Berries contain a diverse range of phytochemicals with biological properties such as AO and anticancer [220-223]. Important components are various phenolics. Berry extracts inhibited cell proliferation in various cancer cell lines. The antioxidant activity of bioflavonoids suggests their potential to block cancer growth and inhibit tumor metastasis [224,225]. Propolis, a flavonoid-containing material collected by honeybees, is effective in suppressing lung tumorigenesis induced by nitrosoamine in mice [226,227]. Data show that green tea polyphenols stimulate the transcription of detoxifying enzymes through the AO-reponsive element [228], in addition

to other pharmacological effects [229]. Signaling pathways may involve mitogen-activated protein kinases. A study demonstrates that tumeric or curcumin, polyphenolic AO, administered in the diet or applied as paint may have chemopreventive effect on oral precancerous lesions [230]. Tocopherol may exert direct beneficial effects in the gastrointestinal tract and their return to the gastrointestinal tract by the liver through the bile may be physiologically advantageous [231].

In many cases, cancer therapy produces ROS, which attack healthy cells and tissues, consequently leading to further damage and unintentional side effects. These adverse effects may be decreased by antioxidants, but it must be assumed that any antioxidant found to reduce toxicity of tumor therapy on healthy tissue has the potential to decrease effectiveness of cancer therapy on malignant cells. Vitamin C belongs to the few vitamins for which evidence exists for a protective role against some types of cancer, and it functions as a potent antioxidant, protecting mitochondria and other cell structures from oxidative cell injury [232]. Data demonstrate that nonprotein thiols suppress matrix metalloproteinase activation and function, and introduce the prospect for their use in chemopreventive application for cancer [233]. There is strong evidence that supranutritional levels of selenium have various health benefits in humans, particularly the prevention of several types of cancer [234], including lung, colon-rectal cancers. In use of selenite in cancer therapy, apparently apoptosis is induced through OS. A nitroxide AO dramatically delays the onset of thymic lymphomas in mice, and reduces oxidative damage [235]. The likely mechanism of action is reduction in oxidative stress. Garlic-administered rats experienced a significant reduction in lipid peroxidation with simultaneous elevation in antioxidant levels [236]. The presence of organosulfur, such as diallyl disulfide [237], compounds could have decreased lipid peroxidation and increased antioxidant levels in rats with hepatocarcinogesis induced by N-nitrosodiethylamine [236]. There is a review of the fundamental chemistry of tocopherols relevant to their AO action [238]. Included are differences among the tocopherols in relation to interaction with the important chemical species in lipid peroxidation. Results indicate that ginseng extract, an antitumor agent, exhibit effective AO activity in both lipid and aqueous mediums by both chelation of metal ions and scavenging of free radicals [239,240]. The tumor suppressor p53 promotes the expression of many antioxidant genes that prevent apoptosis [241]. In low cellular stress, low concentrations of p53 induce the expression of antioxidant genes. Diminished levels of β-carotene, β-tocopherol, SOD and glutathione peroxidase were observed in brain tumor patients as compared to controls [242]. Maliganant tumors also showed a relative decrease in antioxidant levels as compared to benign tumors. Comparison of brain tumors suggests an inverse relationship between AO level and grades of malignancy. Marked decrease in AO levels may have a role in genesis of OS in brain tumors. The degree of decline in AO levels may indicate severity of malignancy in brain tumors. OS leading to DNA insult is associated with carcinogenesis. A survey indicates that ingestion of antioxidants may be associated with reduced level of DNA damage, albeit the effect is lower than previously expected [243]. Increasing incidents of skin cancer are being reported from many countries where majority of the population is Caucasian [244]. Approximately 1.3 million cases of basal-cell or squamous-cell cancers are diagnosed annually in the USA alone. Among all the cancers, a skin cancer is believed to be one of the most preventable and/or curable cancer types. The most serious form of skin cancer is melanoma. ROS, such as superoxide, hydrogen peroxide and singlet oxygen, are believed to play a major role in many pathological conditions including skin cancer. A 2001 review [244] addresses the use of AOs

in the chemoprevention of this type of cancer. Prostate adenocarcinoma cell lines have reduced levels of AO enzyme expression. AO enzyme levels were lower in patients with prostatic neoplasia than in benign, suggesting that OS is an early event in carcinogenesis. A body of evidence suggests a role for AOs in the fight against prostatic cancer [245]. Results suggest that tamoxifen treatment is the most effective during co-administration of the AOs riboflavin, niacin and CoQ10 in terms of mitochondrial antioxidant and antitumor activity [246-248].

One must use caution in distinguishing between anticancer agents used for prevention and those used to combat tumors already established [1]. For example, some time ago, vitamin C was claimed to produce measurable benefits in the treatment of cancer. However, evidence indicates that it must never be regarded as an alternative to proven, conventional methods of cancer treatment since its efficacy is still unproven. On the other hand, sufficient evidence has now accumulated to conclude that this AO, as inhibitor, is linked to a reduced risk of virtually all cancers [1].

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Chapter III

ANTIOXIDANT EFFECTS OF ASTRAGALI RADIX OXIDATIVE STRESS

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ABSTRACT

Astragali Radix, a main component in many Chinese herbal medicines, was shown to have antioxidant effects on lipid peroxidation. Afrormosin, calycosin and odoratin as isoflavones in *Astragali Radix* were isolated and identified as antioxidant components on lipid peroxidation. Biochnin A, daidzein and genistein as other isoflavones also were shown to have antioxidant effects on lipid peroxidation. These results demonstrated that *Astragali Radix,* afrormosin, calycosin, formononetin and odoratin as isoflavones in *Astragali Radix*, biochanin A, daidzein and genistein as other isoflavones have antioxidant effects on oxidative stress.

Keywords: Antioxidant effect, *Astragali Radix*, isoflavone, oxidative stress

I. INTRODUCTION

It has been suggested that various neurological disease (particularly those accompanying aging, the cranial nerves, etc.) involve oxidative stress. Some of these diseases have been successfully controlled with traditional herbal medicine. It has been demonstrated that Huang-chi-kuei-chin-wu-wu-tang, a Chinese herbal medicine, has ameliorating effects for patients with abnormal sensations and pain in the legs in neuropathy such as SMON (subacute myelo-

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optico neuropathy) [1]. Huang-chi-kuei-chin-wu-wu-tang was shown to have an inhibitory effect on lipid peroxidation (LPO) by iron *in vitro* [2]. It has been demonstrated that reactive oxygen (ROS) generated by copper produces LPO of the erythrocyte membrane in a manner similar to iron. Huang-chi-kuei-chin-wu-wu-tang is a combination of five medicinal plants including *Astragali Radix, Cinnamomi Cortex, Paeniae Radix, Zigiberis Rhizoma* and *Zizphi Fructus*. *Astragali Radix* is a main medicinal plant in Huang-chi-kuei-chin-wu-wu-tang. It was investigated whether *Astragali Radix* has antioxidant effects on LPO of mouse brain homogenate by copper *in vitro*.

Toda *et al.* have already reported that methanol extracts of 20 different crude drugs showed antioxidant activities by using the evaluation method on the air oxidation of linoleic acid [3]. The presented investigation showed the isolation and identification of antioxidant components in *Astragali Radix* by using the evaluation method on the air oxidation of linoleic acid.

Flavonoids, such as flavonols (kaempferol and querecetin), Flavones (luteolin and rutin) and flavanones (hesperidin and taxifolin) have antioxidant effects [4,5]. It was investigated whether isoflavones of *Astragali Radix*, afrormosin, calycosin, formononetin and odoratin have antioxidant effects on LPO by ROS *in vitro*. It was investigated whether other isoflavones, biochanin A, daizdein, and genistein have antioxidant effects on LPO by ROS *in vitro*.
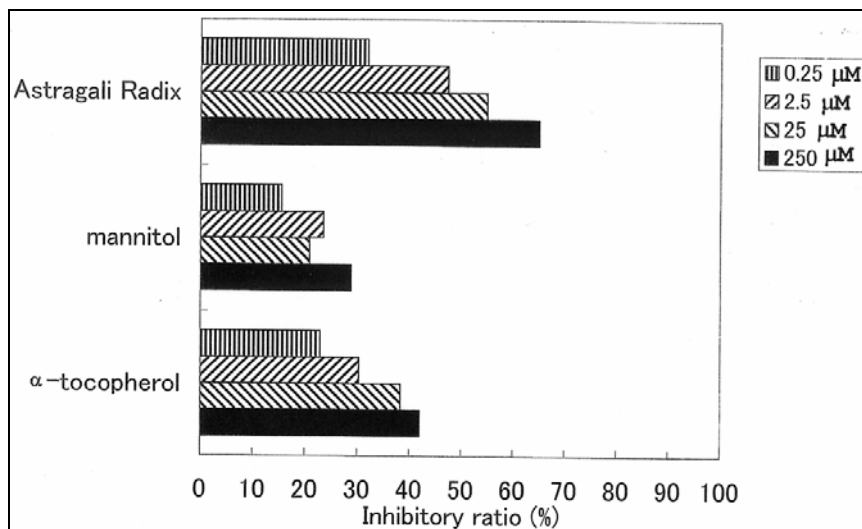
Figure 1. Antioxidant effects of *Astragali Radix.*

II. ANTIOXIDANT EFFECTS OF
ASTRAGALI RADIX ON OXIDATIVE STRESS [6]

Figure 1 shows an antioxidant effect of *Astragali Radix* on LPO of mice brain homogenate by copper *in vitro*. The results showed that *Astragali Radix* has antioxidant effect

on LPO as mannitol and α-tocopherol. Toda *et al.* have reported that LPO by copper are inhibited by Huang-chi-kuei-chin-wu-wu-tang [7].

Astragali Radix has been used as a diuretic or a tonic in Oriental medicine. It contains sucrose, astragaloside, formononetin, calycosin, (3R)7,2'-hdihydroxy-5',6'-dimethoxy-isoflavan-7-*o*-β-D-glucoside, β-sitosterol, palmitic acid and daucosterol [8,9]. It has been demonstrated that total saponine fraction of *Astragali Radix* have activities in scavenging ROS [10].

From these findings, the present results suggest that *Astragali Radix* has antioxidant effects on oxidative stress as LPO.
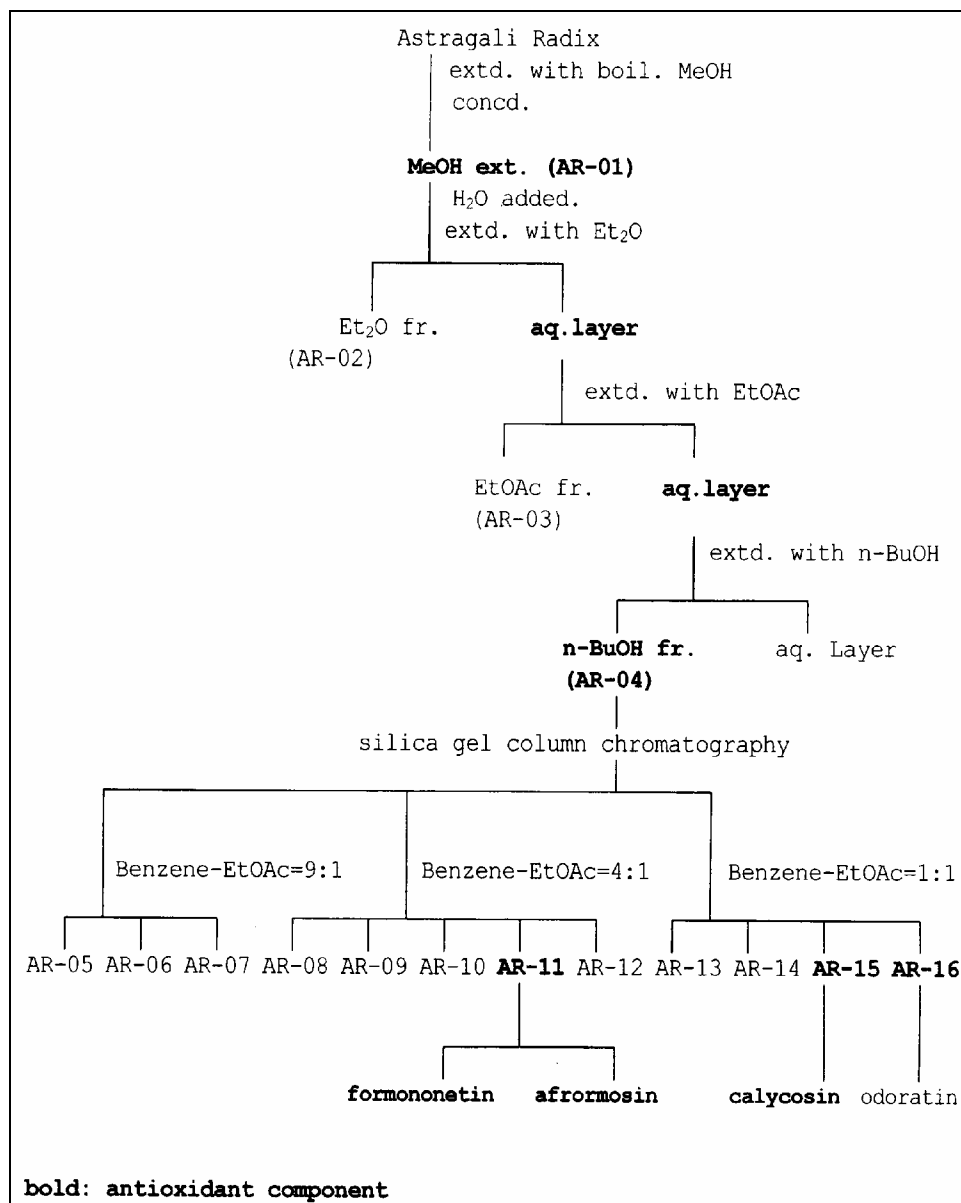
Figure 2. Isolation of antioxidant component in *Astragali Radix.*

III. ISOLATION OF ANTIOXIDANT COMPONENTS ASTRAGALI RADIX ON OXIDATIVE STRESS [11]

The isolation and identification of active components in antioxidative methanol extract of *Astragali Radix* was carried out by using our evaluation method on the air oxidation of linoleic acid. As shown in Figure 2, the inhibitory ratios of the methanol extract added to linoleic acid at 0.1 % concentration was low, whereas that of ether extract (AR-2) was high. Thus the ether extract (AR-02) was fractionated repeatedly by silica gel column chromatography with benzene-ethyl acetate mixture as the eluent. The fractions were monitored by thin layer chromatography and measurements of inhibitory ratios on the air oxidation of linoleic acid were made when they were added at 0.1 % concentration. The active fractions (AR-11, 15 and 16) were purified by recrystallization. Then, formononetin, afrormosin, calycosin and odoratin in the active fraction (AR-11, 15 and 16) were identified on the basis of melting points and spectral comparisons as shown in Figure 3. Afrormosin, calycosin and odoratin were active, but formononetin had no activity. Inhibitory ratios of these three active components were superior and similar to dl-α-tocopherol.
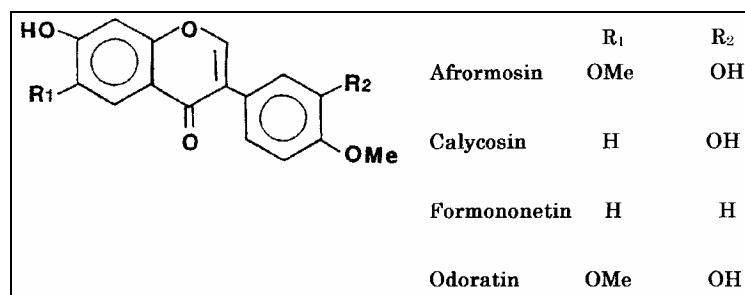
Figure 3. Chemical structures of isoflavones in *Astragali Radix.*

The relationship between inhibitory ratios and the added concentration of tested samples was examined. All the active samples tested showed dose-dependent antioxidant effects on the air oxidation of linoleic acid. Therefore, the main antioxidant components in *Astragli Radix* were isoflavones, afrormosin, calycosin and odratin.

It was demonstrated that other isoflavones and genistein and diadzein, have antioxidant activities [12]. Afrormosin, calycosin and odoratin have never been shown to have antioxadant activities. It was concluded that such components in *Astragali Radix* prevented LPO.

IV. ANTIOXIDANT EFFECTS OF ISOFLAVONES IN ASTRAGALI RADIX ON OXIDATIVE STRESS [13]

Figure 4 shows antioxidant effects of four isoflavones of *Astragali Radix*, afrormosin, calycosin, formononetin and odoratin, on LPO by the interaction of Fe^{2+}- and hydrogen peroxide *in vitro*. The results showed that calycosin and formononetin have the antioxidant

effects on LPO. As shown in Figure 6, these four tested isoflavones have a methoxy group at 4' position. These results demonstrated that hydroxyl groups at the 7 and 3' positions on isoflavone, which have a methoxy group at the 4' position, are necessary to inhibit such LPO. Although ordoratin has hydroxyl groups at the 7 and 3' positions, it has a methoxy group at the 6 position. Calycosin had an antioxidative effect, but it did not exhibit a methoxy group at the 6 position.
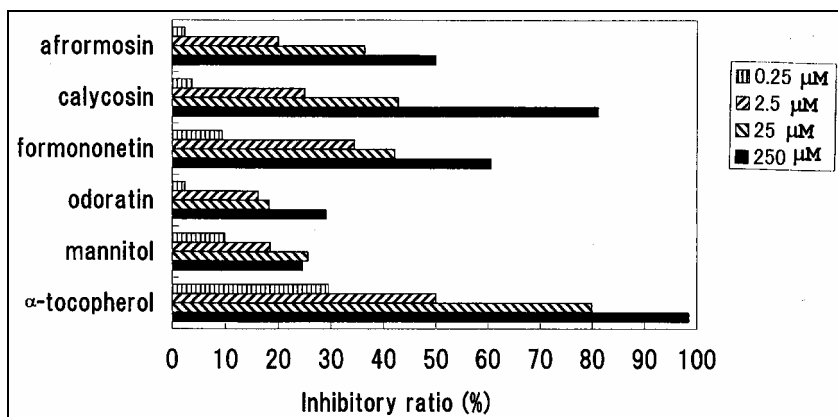
Figure 4. Antioxidant effects of isoflavones in *Astragali Radix.*

It seemed that calycosin and formononetin have antioxidant effects because they have no methoxy group at 6 position. Wei *et al.* [14] demonstrated that the antioxidant properties of isoflavones are structurally related, and the hydroxy group at 4' position is crucial in 12-O-tetradecanoylphorbol-13-acetate-acitivated HL-60 cells and superoxide anion-generation by xanthine-xanthine oxidase. However, the tested isoflavones in the present experiment have a methoxy group at the 4' position.

From these results, it was concluded that the antioxidant effects of isoflavones, which have a methoxy group at the 4' position, are derived from hydroxyl group at the 7 and 4' positions. However, it seemed that isoflavones, which have a methoxy group at the 6 and 4' position, have no antioxidant properties.

V. ANTIOXIDANT EFFECTS OF OTHER ISOFLAVONES ON OXIDATIVE STRESS [15]

Figure 5 shows antioxidant effects of other isoflavones, biochanin A, daidzein, genistein and formononetin, on LPOs by the interaction of heamoglobin and hydrogen peroxide *in vitro*, following the previous section. The results showed that these four isoflavones have antioxidant effects on LPO. Especially, genistein was the strongest in four isoflavones. It was demonstrated that the antioxidative activity of a flavonoid is determined by its reactivity as a hydrogen- or electron-donating agent, its reactivity with other antioxidants and the transtion metal-chelating potential. The structural features imparting the greatest antioxidant activity were deduced from the following studies: the *meta* 5,7-dihyroxy arrangement of transition

metal ions to the flavonoid is the 4-keto group in the C ring and 5-hydroxy group in the A ring [5].
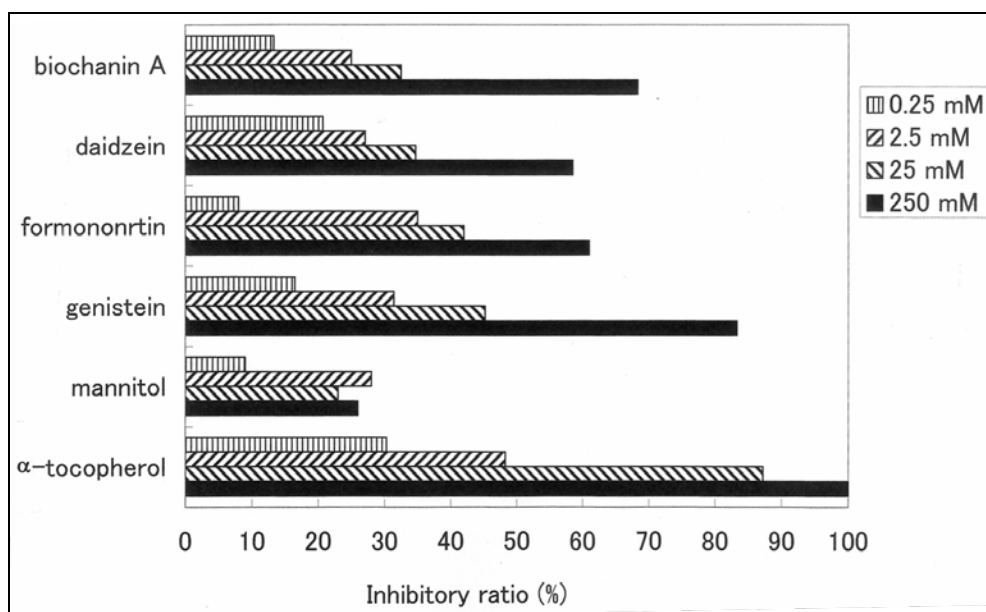
Figure 5. Antioxidant effects of other isoflavones.

As shown in Figure 6, biochanin A and genistein have *meta* 5,7-dihydroxy arrangements in the A ring, and have a 4-keto group in the C ring and 5-hydroxy group in the A ring. Daidzein and genistein have 4'-hydroxy group in the B ring. However, formononetin had antioxidant effects on LPOs, though it has no *meta* 5,7-dihydroxy arrangements in the A ring, and 4'-hydroxy group in the B ring.
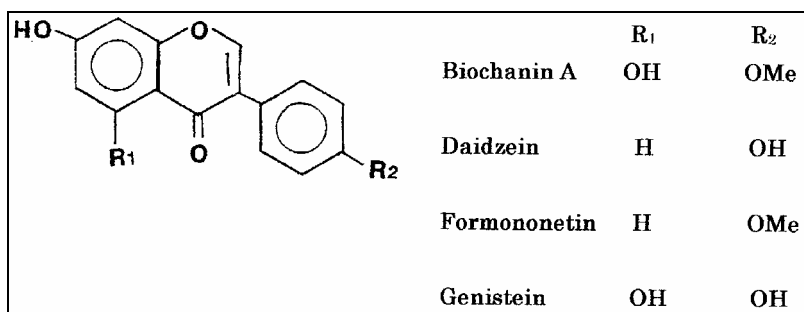
Figure 6. Chemical structures of other isoflavones.

These results demonstrated that the differences in antioxidant effects of isoflavones are dependent on the relation between the chemical structures and ROS.

Yase has shown that Huang-chi-kuei-chin-wu-wu-tang has ameliorating effects for patients with abnormal sensations and pain in the legs in neuropathy such as SMON [1]. Huang-chi-kuei-chin-wu-wu-tang has been recorded in Chinkuei yao lueh that it is used for treating paralysis in the legs and arms, generalized pain, and weak pulse. The complains of

SMON are similar to those in records in Chinkuei yao lueh. The record in Chinkuei yao lueh demonstrated that Huang-chi-kuei-chin-wu-wu-tang is an useful traditional medicine for SMON. Toda *et al.* has shown that Huang-chi-kuei-chin-wu-wu-tang has an antioxidant effect on LPO derived from the interaction of Fe^{3+} and 8-hyrdoxyquinoline [2]. 8-hydroxyquinoline is a homologue of chinoform, which is causally related to the pathogenic process of SMON. Huang-chi-kuei-chin-wu-wu-tang, and *Astragali Radix* as a main component in Huang-chi-kuei-chin-wu-wu-tang were shown to have antioxidant effects on LPO by copper *in vitro* [6,7]. It has been shown that ROS derived from iron and copper induce various oxidative stresses [16]. The presented results demonstrated that OKGT, and *Astragali Radix* have antioxidant effects on oxidative stress. Afrormosin, calycosin and odoratin were shown to be antioxidative components in *Astragali Radix* by using the evaluation method on the air oxidation of linoleic acid [11]. *Astragali Radix* has been recorded in Shen Nung Pen Tsao Ching. *Astragali Radix* has been used as a medicinal herb and food for health care. The presented result also demonstrated that *Astragali Radix* has been used as a medicinal herb and food for health care. Calycosin ,an isoflavone of *Astragali Radix*, were shown to have antioxidant effect on lipid peroxidation by the interaction of Fe^{2+} and hydrogen peroxide *in vitro* [13]. Biochnin A, daidzein and genistein, other isoflavones, were shown to have antioxidant effects on lipid peroxidation by the interaction of Fe^{2+} and hydrogen peroxide *in vitro* [15]. Insoflavones have used as anti-aging drugs. Also it has been called a phytoestrogen. ROS has been shown to induce an oxidative stress such as aging. The presented results also demonstrated that isoflavones have an anti-aging effect [5]. These results demonstrated that *Astragali Radix*, afrormosin, calycosin, formononetin and odoratin as isoflavones in *Astragali Radix*, Biochanin A and genistein as other isoflavones have antioxidant effects on oxidative stress *in vitro* (Table 1).

Table 1. Antioxidant effects of *Astragali Radix* and isoflavones in *Astragali Radix* on oxidative stress *in vitro*

Oxidative stress	Antioxidant
LPO by copper	Astragali Radix
LPO by air oxidation	isoflavone in *Astragali Radix* (afrormosin, calycosin, odoratin)
LPO by Fe^{2+} and hydrogen peroxide	isoflavone in *Astragali Radix* (calycosin, formononetin)
LPO by Fe^{2+} and hydrogen peroxide	other isoflavone (biochanin A, daidzein genistein)

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Chapter IV

ROLE OF VITAMIN E IN CELLULAR ANTIOXIDANT DEFENSE - A NEW PERSPECTIVE

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ABSTRACT

Protection against free radical-initiated oxidative damage has long been recognized as the most important biological function of vitamin E. However, the mechanism by which vitamin E exerts its antioxidant function *in vivo* has yet to be delineated. Recent findings that dietary vitamin E reduces the rates of mitochondrial superoxide generation and levels of labile iron provide a rational explanation as to how the vitamin may exert its antioxidant function at the tissue level. Superoxide is a key precursor for other reactive oxygen/nitrogen species (ROS/RNS), and is capable of releasing iron from its protein complexes. The labile or available form of iron has the potential to catalyze the formation of reactive hydroxyl radicals. Superoxide can also react readily with nitric oxide to form peroxynitrite. Both hydroxyl radicals and peroxynitrite have potential to initiate oxidative damage to essential biomolecules. Thus, by reducing available superoxide, dietary vitamin E may reduce available hydroxyl radicals and peroxynitrite, and thus attenuate oxidative damage. Furthermore, by reducing the levels of ROS/RNS, vitamin E may modulate the activation and/or expression of redox-sensitive biological response modifiers, and, thereby, mediate the cellular events leading to the onset of cardiovascular, cancer, aging and other degenerative diseases.

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I. INTRODUCTION

Vitamin E is the term referring to all tocol and tocotrienol derivatives qualitatively exhibiting the biological activity of RRR-α-tocopherol. The term "tocopherols" is the generic description for all mono-, di- and trimethyl tocols and tocotrienols, and is not synonymous with "vitamin E." There are four tocopherols (tocos) and four tocotrienols that occur naturally, differing in the number and position of methyl groups on the chroman ring. The eight tocopherol compounds isolated from plant sources have a 6-chromanol ring and a phytyl side-chain [1, 2]. Tocotrienols have a similar structure as their corresponding tocopherols, except their side chains contain three double bonds at the 3', 7' and 11' positions. Additionally, synthetic vitamin E, either in free or ester form (e.g., α-tocopheryl acetate and tocopheryl succinate), is available commercially. The naturally occurring α-tocopherol, formerly known as d-α-tocopherol, is now designated as RRR-α-tocopherol. The synthetic α-tocopherol, which consists of eight stereoisomers [3, 4], previously named dl-α-tocopherol or 2DL,4'DL,8'DL-tocopherol, is now called all-rac-α-tocopherol.

Vitamin E was discovered over 80 years ago as a lipid-soluble substance in lettuce and wheat necessary for the prevention of fetal death and resorption in rats that had been fed a rancid lard diet [5] Subsequently, a number of species-dependent and organ-specific deficiency symptoms of vitamin E, such as liver necrosis in rats and pigs, erythrocyte hemolysis in rats and chicken, and white muscle disease in calves, sheep, mice and mink, were also reported [6]. However, the need of vitamin E for humans was questioned due to a) the lack of a definite clinical deficiency syndrome attributable to its deficiency, b) absence of a widespread deficiency in humans, and c) difficulty of inducing a deficiency in healthy adults [7]. In the late 1960s, the need of vitamin E for humans was recognized in connection with studies of premature infants [8]. Secondary vitamin E deficiency was subsequently observed in patients with a variety of fat malabsorption conditions, such as abetalipoproteinemia, chronic cholestatic hepatobiliary disorder and cystic fibrosis [8, 9]. Conclusive evidence of the essentiality of vitamin E for humans was obtained from patients with familiar isolated vitamin E deficiency or atoxia with isolated vitamin E deficiencyin the 1990s. These patients have no malabsorption syndrome, but have neurological dysfunctions and extremely low serum vitamin E [10-12]. The very low vitamin E status of these patients is attributable to their absence of α-tocopherol transfer protein (α-TTP) resulting from the mutation of its gene located at chromosome 8q13 [13]. α-TTP, a cytosolic protein with high affinity to RRR-α-tocopherol, is required for the secretion of tocopherol into lipoproteins and facilitates its return to the liver.

With the recognition of a role of free radicals in the pathogenesis of cancer, cardiovascular disease and other degenerative diseases, and their possible prevention by antioxidants, there has been a renewed and expanded interest in vitamin E during the past decade. The scientific rationale and a number of epidemiological data and retrospective studies are supportive that increase intake of vitamin E is associated with a reduced risk of degenerative diseases. However, recent prospective, randomized, placebo-controlled trials have failed to verify a consistent benefit [14-30]. Based on the available scientific evidence, the U.S. Food and Drug Administration did not approve the health claims associated with vitamin E intake and risk of cancer or cardiovascular disease. Similarly, the recent Panel on Dietary Antioxidants of the U.S. Food Nutrition Board did not recommend an increase in its

daily allowance. In addition to the inconclusive findings from prospective placebo-controlled trials, a lack of understanding on the mode of action of vitamin E at the tissue level is also responsible. The large amount of information accumulated in recent years has provided a better understanding of the role of vitamin E in cellular functions. This article focuses on the possible mechanism by which vitamin E exerts its antioxidant defense at the tissue level.

II. FREE RADICAL-INDUCED OXIDATIVE DAMAGE

Even under normal cellular environment, a large variety of conditions are capable of initiating or enhancing oxidative stress. These conditions include inadequate intake of antioxidants, high intake of oxidizing or oxidizable compounds, exposure to noxious chemical or physical agents, strenuous physical activities, injury and wounds, and certain hereditary disorders [31]. The harmful effects resulting from oxidative stress are attributable to the reaction of ROS/RNS with cellular components with resultant degradation and/or inactivation of essential cellular constituents [32-36].

Superoxide, hydrogen peroxide, nitric oxide and peroxyl radicals are the most significant ROS/RNS generated in aerobic environment [37-39]. Among ROS/RNS, peroxy radicals derived from polyunsaturated fatty acids has special significance due to its involvement in lipid peroxidation, the most common indicator of free radical processes in living systems. Cell membranes, which contain relative high proportion of polyunsaturated fatty acids, are more susceptible to free radical-induced lipid peroxidation. Free radical-induced lipid peroxidation has been implicated as a critical initiating event leading to cell injury or organ degeneration [33-36]. The process of lipid peroxidation (or autoxidation) may continue until all unsaturated fatty acids are oxidized or peroxyl radicals are scavenged by an antioxidant or by self-quenching. Vitamin E is the most important antioxidant/free radical scavenger protects against peroxidative damage to membrane lipids.

III. ANTIOXIDANT FUNCTION OF VITAMIN E

The antioxidant property of tocopherols was first reported by Olcott and Emerson in 1937 [40]. The antioxidant activity of tocopherols is attributed to their ability to scavenge or react with peroxyl radicals more rapidly than can polyunsaturated fatty acids. Also, tocopherols can inhibit oxidation reactiond induced by the electronically excited singlet oxygen, and can react with hydroxyl, perhydroxy, superoxide and nitric oxide [1, 41-43]. The antioxidant activity of tocopherols is determined by their chemical reactivity with molecular oxygen, superoxide radicals, peroxyl radicals, or other radicals, or by their ability to inhibit autoxidation of fats and oils. The chemical structures of the tocopherols and tocotrienols support a hydrogen-donating power in the order α>ß>γ>δ [1, 44,45]. The presence of electron-releasing substituents in positions *ortho-* and/or *para-*to the hydroxy function increases the electron density of the active centers that facilitate the homolytic fission of the O-H bond, increase the stability of the phenoxy radical and improve the reactivity with peroxy radicals. The relative antioxidant activity of tocopherols *in vitro*, however, varies considerably depending upon the experimental conditions and the assessment method employed [1, 44-46].

Vitamin E is the most important lipid-soluble chain-breaking antioxidant in plasma, red cells and tissues and plays an essential role in maintaining the integrity of biological membranes [1, 2, 47, 48]. Vitamin E can react more rapidly with peroxy radicals several orders of magnitude faster than with acyl lipids, and thus prevents free radixal-induced peroxidative tissue damage [1, 45, 47]. One tocopherol molecule can protect up to 10^3 - 10^8 polyunsaturated fatty acid molecules at low peroxide levels, and a small ratio of α-tocopherol to polyunsaturated fatty acid molecules (1:1,500) in the red cell membrane is sufficient to interrupt the free radical chain reactions. The antioxidant role of vitamin E *in vivo* is supported by the findings that synthetic antioxidants can prevent or lessen certain vitamin E-deficient symptoms in experimental animals, and that increased production of peroxidation products, such as malondialdehyde, ethane, pentane and isoprostane, are found in vitamin E-depleted animals [1, 2, 47, 49].

While a number of biochemical abnormalities are associated with vitamin E deficiency and vitamin E has long been recognized as the most important lipid-soluble chain-breaking antioxidant, the mechanism by which vitamin E functions as an *in vivo* antioxidant or prevents various metabolic and pathological lesions is not yet clear. The difficult in establishing its mode of action at the tissue level is partly due to the presence of vitamin E regeneration systems and functionally interdependent antioxidant systems [2, 32, 50]. A number of compounds including ascorbic acid, glutathione (GSH), lipoic acid and ubiquinol, for example, are involved in the regeneration or restoration of vitamin E [51-55]. Also, by reducing hydroperoxide formed, seleno-enzyme GSH peroxidase and its metabolically related enzymes augment the function of vitamin E against peroxidative damage to membrane lipids [50, 56-58]. The ability of the GSH peroxidase system to respond to oxidative stress adaptively or compensatively may be responsible for the lack of significant accumulation of lipid hydroperoxides in the tissues of vitamin E-deficient and other oxidatively stressed animals [31, 32, 59-61]. By augmenting the overall antioxidant defense, the GSH peroxidase system also complements the tocopherol regeneration systems in preserving vitamin E.

Since not all the biological effects can be satisfactorily explained based on its antioxidant property, a number of other functions of vitamin E have been suggested [2, 62, 63]. A mediating role of vitamin E in mitochondrial superoxide generation proposed recently [64-66] provides a plausible explanation as to how the vitamin may exerts its action at the tissue level.

IV. VITAMIN E AND ROS/RNS

Mitochondrion, which utilizes over 85% of oxygen, is the major generation site of superoxide, which plays a central role in the formation of ROS/RNS [38, 39, 67]. Mitochondrion has the highest concentration of vitamin E [68], and disruption of mitochondrial structure is one of the earliest events in the skeletal muscle of vitamin E-deficient animals [69]. Also, *de novo* synthesis of xanthine oxidase, which catalyzes superoxide generation, is markedly increased in the skeletal muscle of vitamin E-deficient rabbits [70], and its activity is significantly elevated in the liver of vitamin E-deficient rats [71]. These findings suggest an increased superoxide production during vitamin E deficiency and a role of superoxide in the pathogenesis of vitamin E-deficiency. Subsequent studies have shown that dietary vitamin E dose-dependently reduced the rate of mitochondrial superoxide

generation in the tissues of rats and mice [64, 72]. The mechanism by which dietary vitamin E reduces mitochondrial superoxide generation, however, has yet to be delineated. Vitamin E may reduce the level and production via one or more of the following mechanisms: a) as an integral part of membrane structure vitamin E serves to maintain mitochondrial integrity and stability and thus prevents or decreases electron leakage, b) as a biological modifier vitamin E mediates the superoxide generation systems, such as NADPH oxidase and xanthine oxidase, directly, and c) as a free radical scavenger vitamin E scavenges superoxide generated [64, 66]. However, whether this role of vitamin E is secondary to its antioxidant function or not remains to be elucidated [65].

Superoxide and its dismutation product, hydrogen peroxide, are chemically not very reactive. However, they may be converted to highly reactive hydroxyl radicals in the presence of transition metal ions [73-75]. Also, superoxide can react readily with nitric oxide to form peroxynitrite [37]. Peroxynitrite and hydroxyl radicals are the most reactive free radicals that may occur in biological systems. Additionally, transitional metal ions can catalyze the decomposition of lipid hydroperoxides and initiate lipid peroxidation [75-77].

The cellular labile or available form of iron, normally associated with low molecule mass, has the potential to participate in redox cycling and catalyze the formation of hydroxyl radical from superoxide and/or hydrogen peroxide [73-75]. The vast majority of transition metals, such as iron and copper, are normally transported and stored as protein complexes, and are not available to catalyze the formation of the hydroxyl radical. However, several compounds, including superoxide and hydrogem peroxide, may release iron from its protein complexes [77-82]. The state and levels of available iron can be modified by oxidants or reductants acting on cell iron sources, such as ferritin and transferrin.

Dietary vitamin E has been shown to alter iron metabolism and attenuate iron-induced oxidative damage [83, 84], although the mechanism involved has yet to be delineated. The data obtained from recent studies shows that supplementation of vitamin E results in a dose-dependently decrease of the levels of labile iron in tissues of both male and female rats [85]. As vitamin E may mediate the levels and/or generation of superoxide [64, 72], and superoxide may release iron from protein complexes [77-82], it is suggestive that dietary vitamin E may protect against oxidative stress by attenuating the release of iron from protein complexes. By reducing superoxide, vitamin E not only reduces levels of ROS/RNS, but also limits the release of iron from its protein complex. In collaboration with higher rates of mitochondrial superoxide generation and higher levels of labile iron, higher levels of oxidation products were found in the tissues of rats fed a low vitamin E diet [85]. Additionally, transgenic mice over-expressing Mn-superoxide dismutase had lower tissue levels of labile iron and oxidation products, while Mn-superoxide knockout mice had higher tissue levels of labile iron and oxidation products [86]. These findings support the view that superoxide plays an importanr role in initiating oxidative tissue damage, and suggest that vitamin E exerts its antioxidant function by limiting the generation and/or levels of superoxide and related ROS/RNS [64-66].

The findings that dietary vitamin E dose-dependently reduced the rate of mitochondrial superoxide generation, as well as levels of labile iron and lipid peroxidation products observed provide a rational explanation as to how vitamin E may exert its antioxidant function at the tissue level. The antioxidant function of vitamin E can be partly attributable to its ability to limit the generation and/or level of superoxide. By reducing the generation and/or levels of superoxide vitamin E not only reduces the levels of harmful free radicals including peroxynitrite, but also limits the release of iron from its protein complex. Also, by

reducing superoxide and available labile iron, the possibility of hydroxyl radical formation is also reduced. Thus, vitamin E may prevent oxidative damage or exert its antioxidant function at the tissue level by i) directly reacting with oxidant or scavenging free radicals, and ii) down-regulating mitochondrial superoxide generation, which in turn reduces both the formation of reactive peroxylnitrite and hydroxyl radicals.

V. POSSIBLE MECHANISM BY WHICH VITAMIN E FUNCTIONS AS AN ANTIOXIDANT AT THE TISSUE LEVEL

Regulation of signal transduction and gene expression is a multifaceted process involving ligands, receptors, and second messengers that trigger cascades of protein kinases and phosphatases and propagate the signal to the nucleus to alter gene expression. Increasing evidence suggests that ROS/RNS play an important role in cell death induced by many different stimuli, and that the expression and activation of a number of vital biological modifiers are closely related to oxidative stress status [87-92]. Redox-based regulatory pathways provide additional means of gating signal transduction, and redox-based regulation of gene expression emerges as a fundamental regulatory mechanism in living cells. The intracellular production of ROS/RNS seems to be vital importance in mediating cell proliferation, differentiation, apoptosis, necrosis, vascular hyperglycermia, platelet adhesion/aggregation, thrombosis, tumor angiogenesis, and other important cellular events [93-96]. For example, c-Jun N-terminal kinase or stress-activated protein kinase, an important member of the mitogen-activated protein kinase superfamily, and its signaling pathway is a key modulator in cell death mediated by ROS and RNS [97].

Recent advances in molecular biology and genomic techniques have led to the discovery of novel vitamin E-sensitive genes and signal transduction pathways [63, 98-100]. RRR-α-tocopherol, for example, has been shown to regulate key cell signaling functions, protein kinase C activity and vascular smooth muscle cell growth by mechanisms unrelated to its antioxidant [99, 101]. Also, RRR-α-tocopherol may modulate the expression of the hepatic collagen α1 gene, α-TTP gene, α-tropomysin gene and collagenase gene, and diminishe adhesion molecule, collagenase and scavenger receptor expression and increases connective tissue growth factor expression [63, 99]. While the mechanism by which vitamin E mediates cell-signaling functions is not yet clear, alteration of redox state or homeostasis may at least partly be responsible [65]. Changes in the pattern of gene expression through redox-sensitive regulatory transcription factors are crucial components of the machinery that determines cellular responses to oxidative conditions [87-93, 102]. Thus, by reducing the generation and/or levels of superoxide and related ROS/RNS, dietary vitamin E may modulate the activation and/or expression redox-sensitive biological response modifiers, and may thereby attenuates the cellular events leading to the onset of cardiovascular, cancer, aging and neurodegenerative diseases [103-110]. However, whether this mediating role of vitamin E in the cell-signaling events is independent of or secondary to antioxidant function or changes in redox state remains to be elucidated.

VI. CONCLUSION

While vitamin E has long been recognized as the most important lipid-soluble antioxidant, the mechanism by which vitamin E exerts its antioxidant function at the tissue level remains to be elucidated. Information accumulated over the past decade has provided a better understanding on the possible mode of action of the vitamin as an antioxidant at the tissue levels. The ability of dietary vitamin E to mediate the levels and/or generation of superoxide provides a rational explanation as to how the vitamin may function as an antioxidant. By reducing available superoxide and labile iron, dietary vitamin E may reduce the possibility of forming reactive hydroxyl radicals and peroxynitrite, and thus protect against oxidative damage. Also, by reducing superoxide and related ROS/RNS, dietary vitamin E may modulate the activation and/or expression of redox-sensitive biological response modifiers, and thereby attenuates the cellular events leading to the onset of cardiovascular, cancer, aging and other degenerative diseases.

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Chapter V

NATURAL ANTIOXIDANTS FROM AGRO-FOOD BY PRODUCTS: AN EXPERIMENTAL APPROACH FOR RECOVERY OF PHENOLICS FROM WINE-MAKING BY-PRODUCTS

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ABSTRACT

This chapter is aimed to underline the increasing importance that natural antioxidants have been gaining in the last years. Antioxidants are naturally present in many foods, so that they can be seen as potential recovery sources: oilseeds, nuts, cereals, legumes, vegetables, fruits, herbs, spices and teas. Besides these, antioxidants are often present in food processing by-products and wastes, so that the employment of low-cost industrial wastes could greatly reduce the production costs and increase the margin profit of the products. The introductive section summarises the classes of antioxidant compounds (mainly focusing on phenolic compounds), their potential food and no-food applications, and the main problems you have to account for when recovering antioxidants from residual sources, such as selection of a suitable agriculture by-product, choice and optimisation of the extraction procedure, analytical characterisation and evaluation of antioxidant activity of the obtained extracts, evaluation of potential applications of the isolated substances.

The second part of the chapter presents an experimental work dealing with recovery of phenolic compounds from wine-making wastes through a simple solvent extraction process. Trials were carried out in order to evaluate the feasibility of using different by-products (grape stalks, grape marcs before and after distillation), the influence of grape variety, of different sample pre-treatments, type of solvent, extraction temperature and time (extraction kinetics) on extracts yield and quality in terms of phenolics content and

antioxidant power. Food applications of the obtained compounds to inhibit oil oxidation and to extend shelf-life of fresh fruits were also investigated.

INTRODUCTION

The importance of the antioxidants contained in foods is well appreciated for both preserving the foods themselves (especially fats, oil and fat containing food products), for preventing deterioration of other oxidisible goods, such as cosmetics, pharmaceuticals and plastics, and supplying essential antioxidants *in vivo*. Since synthetic antioxidants, such as BHA and BHT, have restricted use in foods due to their toxicological effects on various species and suspected carcinogenic potential, the search of natural and safe antioxidants, especially of plant origin, has greatly increased in recent years. This, together with the fact that antioxidants are naturally present in many foods (oilseeds, nuts, cereals, legumes, vegetables, fruits, herbs, spices, teas and meat) [65], explains why scientific literature about natural antioxidants has been proliferated so much.

When dealing with recovery of natural antioxidants, different aspects and problems should be considered and faced off: choice of a suitable extraction source; classes of compounds you are interested in for extraction; optimisation of the extraction procedure; chemical analysis of the extracts and evaluation of their antioxidant power; application of the extracts.

This chapter aims to summarize all these aspects, underlining the great opportunity of exploiting agricultural and industrial wastes for recovery of antioxidants (in particular phenols), and presenting a case-study related to the extraction of phenolic compounds from wine-making wastes [90, 93].

Classes of Natural Antioxidants

Natural antioxidant definition includes different chemical compounds, such as tocopherols, carotenoids, phenolic compounds, amino acids, peptides, protein hydrolysates, phytates, phospholipids, vitamins and enzymes [82].

Among the most important groups of natural antioxidants are the tocopherols, flavonoids and phenolic acids.

Tocols

Tocols can be classified as either tocols or tocotrienols (Figure 1); within each of these two classes there are four isomers (α, β, γ, δ), making a total of eight tocols.

Tocols can act as antioxidant by two primary mechanisms: a chain-breaking electron donor mechanism and a chain-breaking acceptor mechanism. The second is the major and includes singlet oxygen scavenging or quenching. The antioxidant power (AOP) is strongly concentration dependent, but at high concentration a pro-oxidative effect can be observed. Tocols are very stable with respect to heat.
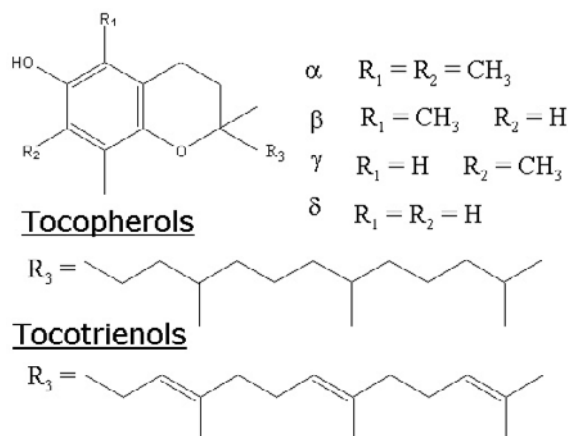
Figure 1. Chemical structure of tocols.

Carotenoids

Like tocopherols, carotenoids (Figure 2) are also effective singlet oxygen quencher, with the rate of quenching depending on the number of conjugated double bonds: a conjugated chain with seven or fewer double bonds is not able to delocalize the unpaired electrons gained from the singlet oxygen.
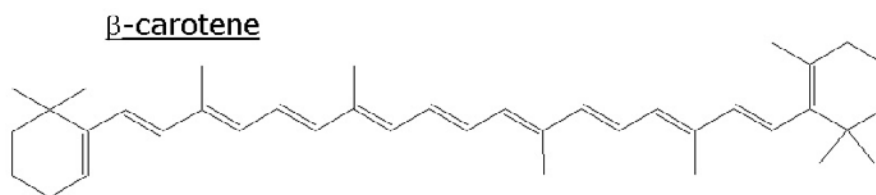
Figure 2. Chemical structure of a carotenoid.

Phenolic Compounds

Phenolic compounds are ubiquitous in plants, where they play an important role in growth and reproduction, providing protection against pathogens and predators, besides contributing towards the colour and sensory characteristics of fruits and vegetables [8]. Structurally, phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl substituents, and range from simple phenolic molecules to highly polymerised compounds, even though the group is often referred to as "polyphenols". Most phenols naturally occur as conjugates with mono- and polysaccharides, linked to one or more of the phenolic groups, and may also occur as ester and methyl ester derivatives. They can be categorised into the following classes: simple phenolics, benzoquinones (C_6); hydroxybenzoic acids (C_6-C_1); acetophenones, phenylaceteic acids (C_6-C_2); hydroxycinnamic acids, phenylpropanoids (C_6-C_3); naphtoquinones (C_6-C_4); xanthones (C_6-C_1-C_6); stilbenes, anthraquinones (C_6-C_2-C_6); flavonoids, isoflavonoids (C_6-C_3-C_6); lignans, neolignans [(C_6-C_3)$_2$]; biflavanoids [(C_6-C_3-C_6)$_2$]; lignins [(C_6-C_3)$_n$]; condensed tannins (proanthocyanidins or flavolans [(C_6-C_3-C_6)$_n$]). Figure 3 reports the structure of only some phenolic compounds, as an example.
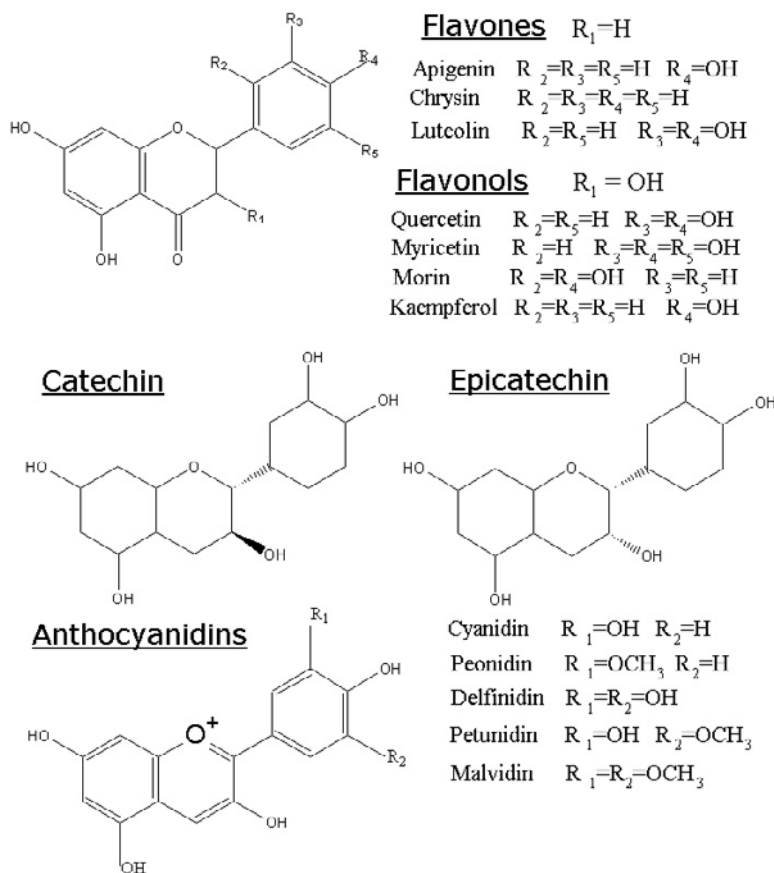
Figure 3. Chemical structure of some phenolic compounds.

The antioxidant activity of phenolic compounds is due to their ability to scavenge free radicals, donate hydrogen atoms or electron, or chelate metal cations, with a strong structure-activity relationship [8, 43, 48, 52, 75].

Amino Acids, Peptides

Amino acids and peptides (in particular histidine-containing peptides) are typical metal-chelating agents frequently present in foods and found in abundance in protein hydrolysates [82].

Phytates, phospholipids, vitamins and enzymes

Phytates are strongly negatively charged compounds, the antioxidant activity of which is presumably due to chelation of prooxidant metal ions [82].

Phospholipids are obtained as by-products of oil-refining (especially from soybean) and are known to affect lipid oxidation.

Fat-soluble vitamins, such as vitamin E and vitamin A, and water soluble vitamins such as vitamin C as well as β-carotene (vitamin A precursor) are known to exert antioxidant activity [104].

Enzyme antioxidants include superoxide dismutase, catalase, glutathione peroxidase and reductase, glutathione-5-transferase and phenol oxidase [108].

Natural Antioxidants from Residual Sources

Literature is so rich of examples of recovery of antioxidant compounds from natural sources, that it is not possible to report here a complete list of them. This abundance of literature is due to the fact that antioxidants are naturally present in many foods, so that they can be seen as potential sources of natural antioxidants: oilseeds, nuts, cereals, legumes, vegetables, fruits, herbs, spices, teas, meats, trees and the different parts of the plants such as leaves, roots, hulls, sprouts and seeds [65, 82].

On the other hand, the processing of foods results in the production of by-products that are rich sources of bioactive compounds, including phenolic compounds which are also responsible for the adverse impact of many food industries. In industrial wastewaters, in fact, these compounds considerably increase biochemical and chemical oxygen demands, with detrimental effects on the flora and fauna of discharge zones, while in solid residues used as fertilizers, they may inhibit germination properties. Since in the last few years diminishing the environmental impact of industrial wastes has been a subject of increasing concern, recovery of phenolic compounds (which can be considered as high-added-value by-products for their antioxidant properties) from low-cost industrial wastes could be a great opportunity to reduce the production costs, increase the margin profit of the products and valorise the wastes reducing their polluting character.

Among the many investigated residual sources of antioxidants we can remember: cereal hulls, dried fruit hulls, peel and seeds of several fruits, the by-products of the olive industry, shrimp shell waste, and protein hydrolysates [8, 52].

Application of Natural Antioxidants

Phenolic compounds and antioxidants in general, have been reported to exert favourable effects on human health such as protection against cardiovascular disease, anti-inflammatory activity and anti-carcinogenic effects [23, 35, 38, 41, 83, 100, 103]. The pharmacological actions of phenolic antioxidants stem mainly from their free radical scavenging and metal chelating properties as well as their effects on cell signalling pathways and on gene expression. That's why traditionally, natural antioxidant extracts have been thought for a medical use, but since there are still many uncertainties about their effective bioavailability and metabolism [7, 34], it appears really interesting and promising their application in food systems.

Some studies have investigated the addition of natural antioxidants to vegetables oils to inhibit oxidation during storage [9, 13, 96] or during frying [42, 47]. Natural antioxidants have also been added to inhibit lipid oxidation in meat [10, 80] and biscuits [72], to preserve the endogenous antioxidant system of fish muscle [59], or to preserve fresh beef meat colour by incorporating them into packaging polyethylene films [51].The prerequisite is, of course, that the added natural compounds do not alter either the flavour or aroma of the food.

Furthermore, natural antioxidants are often used as ingredients or additives of both human and pet foods and of cosmetic products (body creams, sun protection creams, etc.).

Recovery of Natural Antioxidants

Different technologies are available for the extraction of secondary metabolites, such as phenolic compounds, from plant material [94]: solvent extraction, steam extraction, supercritical extraction [33, 56, 86, 105], high pressure extraction [30].

Solvent-extraction is the most commonly performed procedure but, at present unambiguous data on the methods and conditions for extraction are available and sometimes contradictory, particularly if different raw materials are considered. Moreover, results are difficult to compare even because phenols are measured in different ways and sometimes only the phenolic content of the final extracts is reported, but not the total yield. The aim of an extraction process should be, of course, to provide for the maximum yield of substances and of the highest quality (concentration of target compounds and antioxidant power of the extracts). However, the choice of operating parameters is often not motivated, even because many researches are simply aimed to analyse the phenolic content. Spigno et al. [90] tried to compare and summarize the different extraction procedures used for antioxidant recovery from different natural sources, in terms of phenols yield and content in the final extracts, and in terms of the optimisation of some process parameters. The variables to account for in process optimisation are many, so that it is not easy to optimise all them together, and often literature lacks of systematic approaches to optimise the process in order to maximise the extract yield, purity and antioxidant power of the obtained extracts [4, 52, 99]. Beside the process parameters, one must also take into account the difficulties related to the analysis of the extracts, and to the evaluation of the antioxidant power.

First of all the raw material should be selected on the basis of its potential antioxidant content, of its availability in sufficient amount, and of course of its cheapness.

Storage conditions, mincing and degreasing are among the most investigated pre-treatments of raw materials. For storage purpose, the sample can be dried, frozen or vacuum packed, or exposed to a combination of these techniques. In any case, the conditions employed should be as mild as possible to avoid oxidation, thermal degradation and other chemical and biochemical changes in the sample. Reduction of particle size should increase the superficial area available for mass transfer and, then, increase extraction yield, while degreasing might help removing interfering compounds and increasing extract purity.

Both extraction yield and antioxidant activity of extracts are strongly dependent on the solvent, due to the different antioxidant potential of compounds with different polarity. Ethyl-acetate and aqueous methanol or alcohols are often the solvents of choice for recovery of a wide range of phenolics from different sample types. Ethanol and water are the most widely employed solvents for hygienic and abundance reasons, respectively, but they are not selective and bring inevitably to the concomitant extraction of other substances. Anyway, depending on the starting substrate and on the target compounds, selection of the optimal solvent providing maximum antioxidant activity and yield should be achieved after accurate review of the available literature, and by means of comparative studies [52, 99]. Working in an inert atmosphere and away from light, would greatly prevent oxidation of phenolics, and

addition of antioxidant compounds such as ascorbic acid and SO_2 has been proposed for the same purpose [81].

Temperature and time are two other key process variables in solvent extraction. From a pure mass transfer point of view, temperature increase favours extraction by increasing solubility and diffusion coefficient of any substance. On the other hand, temperature affects the compound stability due to chemical and enzymatic degradation, losses by volatilization or thermal decomposition. In addition to thermal decomposition, phenols can react with other plant components, impeding their extraction, and prolonged exposure at moderate temperatures can also cause phenolic degradation [52]. There are also opposite results showing a positive effect of temperature on antioxidant activity of natural phenols [63]. A detailed kinetic investigation should be required for selection of the best time/temperature combination.

Ultrasound and microwave assisted solvent extractions (UAE and MAE) have also been successfully applied for the extraction of bioactive principle from plant materials. UAE has become a good alternative to classical extraction methods due to its high efficiency, low energy and water consumption (no reflux or refrigeration are needed). The enhancement on extraction is attributed to the disruption of the cell walls, reduction of the particle size and the enhancement on the mass transfer of the cell content to the solvent caused by the collapse of the bubble produced by cavitation [77, 97, 103]. MAE is based upon the selective and rapid localized heating of moisture in the sample by microwaves. Due to the localized heating, pressure builds up within the cells of the sample, leading to a fast transfer of the compounds from the cells into the extracting solvent. MAE can globally reduce solvent amount, and/or enhance extraction efficiency, and reduce working time [31, 57, 68, 78].

The final extracts must then be stored before their employment, investigating the stability and shelf-life as a function of storage conditions, where temperature, light and atmosphere are again the major factors influencing antioxidant activity.

Characterization of Antioxidant Extracts

In order to assess the efficiency of the adopted extraction procedure and the influence of the varied parameters, evaluation and quantification of both phenolic compounds and antioxidant power are needed.

Chemical analysis can be qualitative and/or quantitative. Unless a specific target substance is looked for, it is almost impossible to get a complete characterization of all the extracted phenols, because it is very difficult to possess the standards for the thousands of known phenols, even if, of course, depending on the natural source, this number can be greatly narrowed. Chromatographic methods (high performance liquid chromatography, gas chromatography eventually in combination with mass spectrometry) are the best methods for phenols identification and quantification because generally free of interference, but, as already underlined, they require appropriate standards [4, 76]. On the contrary, traditional methods have relied on direct measurement of absorption of radiation in the ultraviolet (for example 320 nm for cinnamic acids, 360 nm for flavonols, 280 nm for other phenols, 520 nm for anthocyanins) or, more commonly, on colorimetric methods using Folin-Ciocalteu reagent, which, however, is not specific for phenols because it reacts with any reducing substance undergoing interferences with many other compounds such as sugars and proteins

[24]. Another limit of these methods is that a commonly occurring phenolic depending on the analysed class must be selected (often gallic acid for total phenols, caffeic acid for cinnamic acids, quercetin for flavonols, malvidin for anthocyanidins, and so on), so that results are expressed in terms of molar equivalents, and this can be a problem particularly where there is not a single class of phenolics predominating. The spectrophotometric methods usually overestimate the phenols content compared to chromatographic methods. On the other hand, when a process optimisation study is being carried out, determination of total phenols, or of some classes of phenols, can be enough, and colorimetric analyses are decidedly more time and cost saving than chromatographic ones.

As it concerns the evaluation of antioxidant activity, although there is a great multiplicity of used methods, there are no approved, standardised methods [25, 66, 67, 95]. Since several rapid screening test methods and *in vitro* antioxidant protocols have been published and used, the data obtained by different researchers are extremely difficult to compare and interpret, due to the variability of experimental conditions and differences in physichochemical properties of oxidizable substrates. Mechanisms of antioxidants action can be multiple and they become more complex in real foods and biological systems respect to model systems; therefore valid evaluation of antioxidant activity should require the use of several different assay methods. From a comparison based on simplicity, instrumentation required, biological relevance, action mechanism, and application on lipophilic and/or hydrophilic substrates, three methods were proposed as the best ones [67]: the ORAC assay (oxygen radical absorbance capacity), the TEAC (trolox equivalent antioxidant capacity) or other ABTS radical based assays; and the Folin-Ciocalteu antioxidant capacity or total phenolic assay. The important thing is, whichever method is chosen, to bear in mind all its associated limits and disadvantages.

A CASE STUDY: RECOVERY OF PHENOLIC COMPOUNDS FROM WINE-MAKING BY-PRODUCTS

Grapes are one of the world's largest fruit crops, and even wine-making wastes such as marc (the residue after pressing for white wines or vinification for red wines) and stalks, are rich in phenols.

Most of the published references concerning antioxidant recovery from grape started from fresh raw material, rather than from wastes, as is the case for works which utilized directly whole grape [2, 73], grape seeds or skins [1, 11, 37, 56, 64, 81, 88]. However, the number of papers related to the use, or potential use of wine-making wastes, such as grape marc or stalks, has been increasing in the last years [14, 17, 18, 29, 36, 44, 53, 60, 89, 98].

Almost all the cited works used a solvent-extraction procedure [90], where the choice of operating parameters was not always motivated, and only a few studies were aimed to parameters optimisation. The most investigated factors up to now have been: crushing and drying pre-treatments; type of solvent; solvent/sample ratio; time and temperature of extraction.

As it concerns sample pre-treatments, degreasing was applied to grape seeds, which contain about a 13-20% lipids, without comparing results obtained from un-degreased samples [11, 36, 37]. As already commented, reduction of particle size should increase the superficial area available for mass transfer and, then, increase extraction yield. However, size

obtained after powdering of the sample was not always specified. Some authors [14] reported a higher extraction of phenolic compounds by acting on crushed than on uncrushed marc, but crushing details were omitted. Other authors [60] wrote that grinding of grape seeds could shorten the extraction time but did not increase the yield of proanthocyanidins, and, furthermore, caused a significant increase in the extraction of undesired concomitant components, so they used entire grape seeds, such as [3]. In [61] it was investigated the effect of different particle size in a continuous phenol extraction, and they concluded that a higher amount of total polyphenols was obtained with the lower flow rate, sample amount and particle size. Actually, the authors expressed the yield as an index of mg phenols l^{-1} h (the area under the polyphenols concentration curve as a function of time), but if these values are transformed into the yield of polyphenols (mg phenols/mg of sample), that is to say taking into account the solvent flow rate, very similar results for the different tested particle size of 0.5 and 5 mm are obtained.

The effect of the solvent/sample ratio has been investigated by [61] and by other authors for different raw materials [15, 32]: the higher the ratio, the higher the total amount of solids obtained, despite the solvent used, according to mass transfer principles.

Type of solvent has been the most investigated factor. Ethylacetate was reported as one of the best solvent for extraction of polyphenols from grape seeds (it is capable of selectively extracting proanthocyanidins) and water addition up to a certain level (10%) increased proanthocyanidins yield because of increased permeability of grape seeds, but beyond this level significant amount of concomitant substances were extracted [60]. On the other hand, alcoholic solvents have been commonly employed to extract phenolics from natural sources: they give quite high yield of total extract even though they are not highly selective for phenols. Particularly, mixtures of alcohols and water have revealed to be more efficient in extracting phenolic constituents than the corresponding mono-component solvent system [61, 105]. Regarding grape and wine-making wastes, influence of water content of alcohols has been studied only for extraction from seeds [3, 106]. Ethanol (a dietary alcohol) may be preferable than methanol in view of a food application of the extracts. Furthermore, being a polar solvent, it effectively extracts flavonoids and their glycosides, catecols and tannins from raw plant materials [12], but solubility of these compounds can be enhanced using a mixed solvent over a limited compositional range [15].

Time and temperature of extraction are important parameter to be optimised even in order to minimise energy cost of the process. Many authors agree in the fact that an increase in the working temperature favours extraction enhancing both the solubility of solute and the diffusion coefficient, but also that beyond a certain value phenolic compounds can be denatured [62, 105]. More contradictory are the data available for extraction length: some authors chose quite short extraction times [14, 62, 106]; other quite long times [37, 44, 60, 61].

Since, generally, we have noticed that literature lacks of systematic approaches to optimise the process in order to maximise the extract yield, purity and antioxidant power of the obtained extracts, the aims of our studies were to:

Investigate extraction of antioxidants from two wine-making wastes, marc and stems. Unlike marc, stalks have never been studied for this purpose. For better comparison marc and stalks were collected from the same grape variety and vintage. Then, possibility of using distilled marc, and influence of grape variety and vintage were also investigated.

Simplify the extraction procedure. On the basis of published researches, a solvent extraction method was selected and the influence of some process parameters on the final extract yield, phenolic content and antioxidant power was evaluated: degreasing pre-treatment of the material, type of solvent (ethylacetate, ethanol, and aqueous ethanol), extraction time and temperature; freeze-drying of extracts.

Investigate food application of extracts: inhibition of oil oxidation, and extension of fresh strawberry fruits shelf-life were considered.

Materials and Methods

Materials

Stalks and marc (by Barbera red grape) were kindly provided by a wine-making factory in Piacenza (northern Italy). Stalks were collected after the operation of pressing/destemming, while marc was collected after devatting. Both the materials were oven dried at 60°C up to a moisture content of about 2-4% (determined by dry weight in oven at 105°C until constant weight) and milled through a 2 mm sieve (final powder size ≤ 2 mm). Marcs by mixed red and white grapes were collected before and after distillation from a distillery in Pedemont (northern Italy).

All chemicals were of analytical grade. β-carotene, linoleic acid, BHA, Tween 40, gallic acid, caffeic acid and Trolox® were supplied by Fluka. Folin-Ciocalteu reagent was purchased from Merck, quercetin, ABTS and PVPP (polyvinylpolypyrrolidone) from Sigma-Aldrich Chemie GmbH.

Extraction of Phenolic Constituents

In case of degreasing pre-treatment, samples were degreased in a Soxhlet apparatus with hexane for 6 h in the case of stalks, and up to 24 h in the case of marc.

Dried and milled sample was extracted with the solvent in a thermostatic rotary shaker at 80 rpm (Infors AG, CH-4103 Bottmingen/Switzerland) with a 4/1 (v/w) ratio solvent/sample (wet weight of oven dried marc). The liquid extract was separated from solids by centrifugation (5350g for 5 min, ALC 4237R centrifuge). In the first set of trials, the liquid phase was then dried over anhydrous Na_2SO_4, and solvent was removed by evaporation under reduced pressure at 40°C. Petroleum ether was added to the concentrated (in ratio 5:1) to form a precipitate which was separated by centrifugation [60, 64]. The residue (crude extract) was dissolved in ethanol for freeze-storage and analysis.

In the second set of trials the liquid phase was directly analysed and freeze-dried, repeating the analyses on freeze-dried samples in order to assess any influence of this operation.

The first trials were aimed to the evaluation of suitability of marc or stalks as phenols sources, of ethanol or ethylacetate:water/9:1 as solvent, 5 or 24h as extraction time, 28°C or 60°C as extraction temperature. All the 32 resulting combinations (4 parameters, each one made varying on two levels) were performed at least in triplicates.

The mixture ethylacetate:water was selected since being reported as one of the best solvent for extraction of polyphenols from grape seeds [60], because it preferentially extracts those phenols that are readily dissolved in the lipid fraction of the food, because its low

boiling point facilitates its removal and reuse and, finally, because any possible residue is scarcely toxic since at levels around mg/l it is a typical component of fermented drinks [14]. Ethanol was chosen since alcohols are the most used solvents in antioxidants extraction works and, furthermore, it is the natural solvent of these compounds in the wine-making process. 28°C was selected to simulate a room temperature, 60°C was chosen as the upper limit for hot extraction since it is reported that polyphenols are heat sensible [15]. The extracts were characterised for total phenols content, and antioxidant power (β-carotene-linoleate system).

The next set of experiments was aimed to optimise the extraction of phenolic compounds from grape marc investigating extraction kinetics (from 1 to 24 h) at 45°C and 60°C, and the effect of water addition to ethanol on phenols yield and quality of extracts (phenols concentration and composition, and antioxidant power). All the extraction trials were carried out in triplicate.

For each phenolic class, yield and extract concentration were calculated as:

Yield (%): g $_{phenols}$ / 100 g $_{dried\ marc\ (dry\ weight)}$
Phenols content or Purity (%):g $_{phenols}$ / 100 g $_{freeze\text{-}dried\ extract}$
Total extract yield (%): g $_{freeze\text{-}dried\ extract}$ / 100 g $_{dried\ marc\ (dry\ weight)}$

Chemical Analyses

Total Phenols
Total phenols were determined according with two different methods:

1) Folin-Ciocalteu [74]. This method was used since it is the one adopted in almost all the published works about natural antioxidant recovery, being considered the best method for total phenolics (including tannins) determination [22].
2) Direct reading of the absorbance of the sample at 280nm [74]. This a faster procedure based on the absorbance of the aromatic ring. Most catechins have a maximum absorption at around 280nm, (+)-catechin is reported to be the major catechin monomer in all grape skins and total phenols and tannins contents are reported to be highly correlated with absorbance at 280nm [106].

In both cases total phenols were expressed as gallic acid equivalents (GAE) by means of calibration curves with standard gallic acid.

Tannins
The applied analytical method (acid butanol assay) is based on the ability of monomer and condensed 3-4, flavandiols to oxidise in acid and alcoholic medium at high temperature to give coloured procyanidins. Tannins were calculated by comparison with a standardised oligomeric procyanidin solution [74].

Percentage of tannins on total phenols was estimated by the polyvinylpolypirrolidone (PVPP) method [49] which is based on the fact that PVPP binds tannins. 1 ml of distilled water is added to 100 mg PVPP and vortexed with 1 ml of extract. The mixture is kept at 4°C for 15 min, vortexed again and centrifuged. The supernatant has only simple phenolics other

than tannins, so the difference between total phenols before and after the PVPP treatment gives the tannin fraction.

Anthocyanins

Free anthocyanins and anthocyanins combined with tannins were measured through a chemical method based on their specific properties of bleaching by SO_2, and calculated by comparison with a standardised anthocyanin solution [74].

Cinnamic acids and flavonols

Cinnamic acids were determined by reading absorbance of the sample at 320nm and expressed as caffeic acid equivalents (CAE) thorugh a calibration curve [19].

Flavonols were determined by reading absorbance of the sample at 370nm and expressed as quercetin equivalents (QE) through a calibration curve [19].

Antioxidant power

For the first set of experiments the antioxidant activity of the extracts was evaluated in a β-carotene-linoleate system [37]. 0.2 mg of β-carotene, 20 mg of linoleic acid and 200 mg of Tween-40 were mixed in 0.5 ml of chloroform. After removing of chloroform at 40°C under vacuum, the mixture was diluted with 50 ml of oxygenated water and well mixed. Aliquots of this emulsion (5 ml) were transferred into different test tubes containing 0.2 ml of extracts in ethanol (200 ppm) or 0.2 ml of ethanol (control), or 0.2 ml of BHA (200 ppm; GRAS regulations limit BHA to 200ppm of the fat or oil content of the food product). Absorbances of all the samples were read at 470 nm (holding the samples at 50°C) at 15 min intervals. All determinations were carried out in triplicate. An emulsion prepared as above without β-carotene served as blank. The antioxidant activity index (AAI) was evaluated in terms of bleaching of the β-carotene and calculated as:

$$AAI = \frac{(\Delta A_E - \Delta A_C)}{(\Delta A_B - \Delta A_C)} * 100$$

where ΔA_B is the variation in absorbance (from time 0 to 150') of the sample containing BHA (with assigned AAI = 100); ΔA_E that of the sample containing the extract; ΔA_C that of the blank (with assigned AAI = 0).

For the second set of experiments antioxidant power of the freeze dried extracts was assessed according to the ABTS assay [70], which is based on the ability of antioxidants to interact with the radical ABTS decreasing its absorbance at 734nm. Antioxidant power (AOP) was calculated as percentage inhibition:

$$AOP = \% \; Inhibition = \left(\frac{A_{Blank_{t=6}} - A_{Extract_{t=6}}}{A_{ABTS_{t=0}}} \right) * 100$$

where A_{Blank} was the value of absorbance for the blank (ethanol), $A_{Extract}$ was the absorbance of the extract (dissolved in ethanol), t indicates the time (in minutes) at which absorbance was read.

AOP was also converted into Trolox® equivalents antioxidant activity (TEAC) by a calibration curve obtained with standard Trolox® (1-15µM final concentration in the cuvette). TEAC is the ratio of mM Trolox® to mM phenols in the extract (as GAE).

Food Applications

Inhibition of oil oxidation and extension of shelf-life of fresh strawberry fruits were investigated as potential food applications of the extracts.

As it concerns prevention of oil oxidation, some preliminary tests were carried out according to the *Shaal Oven Test* (or accelerated resistance test) which simply consists in monitoring the increase of peroxides value (PV) [5] in an oil sample kept in a thermostatic oven at 60°C. The addition of antioxidants should avoid or, at least, delay, the peroxides formation. Sunflower oil was bought at a local market. As suggested by [9], calculated amounts of the extracts were mixed with 4 ml of absolute ethanol and added to 25g of oil, and alcohol was evaporated during stirring. Oil samples (25g), without and with antioxidants (200ppm BHA or 200ppm extract) were placed in open flasks and PV measurements were done every two days according to the AOCS method Cd 23-93 [20].

As it concerns extension of strawberries shelf-life, the antifungal activity of phenolic extracts against *Botrytis cinerea* was assessed. Fungi *B. cinerea* were isolated from diseased berries on PDA (potato dextrose agar) by the single spore procedure. Inoculated plates were held at 27°C for 7 days. The cultures were transferred to PDA slants and maintained at 4°C until use. The antifungal assay was determined on PDA plates amended with different extract concentrations. The plates were seeded with 6mm diameter mycelial plugs cut from the edge of 4-day-old *B. cinerea* plates. Plates in four replicates were used for each treatment, and the inoculated plates were incubated in the dark at 27°C. Growth inhibition was calculated as the percentage of inhibition of radial growth relative to the control.

Statistical Analysis

The results reported in this chapter are the averages of at least three replicates. Significant variables were calculated, subjecting results to a linear regression, using SPSS statistical program version 11.5 at a confidence level superior to 95% (*P<0.05*). Difference between means was evaluated by Tukey's post-hoc test. The same SPSS software was used to calculate correlation coefficients (R) to determine the existence of any relationship between antioxidant power and phenols concentration.

For statistical analysis all the percent data were transformed into arcsin values.

Results and Discussion

The influence of starting material (marc or stalks) on final phenols yield (evaluated as GAE by direct absorbance reading at 280nm) was not statistically significant (Figure 4a). However, it must be said that ethanol extraction from grape stalks gave a crude extract that was partly ethanol and partly water soluble, that is to say two different extracts were obtained. Considering separately the two stalks extracts, marc gave always statistically higher GAE recovery. On the contrary, phenols content of extracts was higher for stalks extracts (Figure 4b). This could be due to the fact that stalks are a lignocellulosic material, whose main

components lignin, cellulose and hemicelluloses can be separated only after strong acid and alkaline hydrolyses [92].
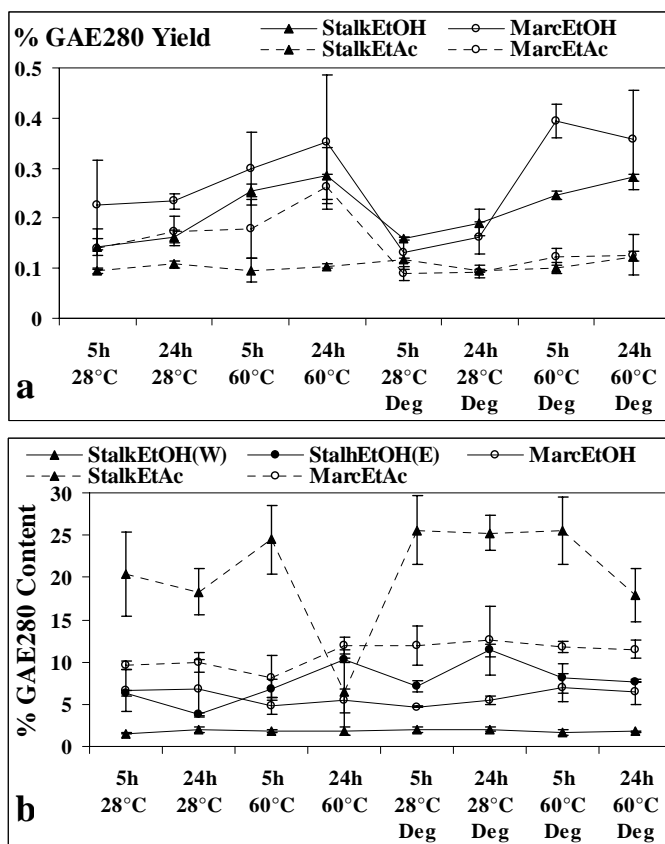
Figure 4. Phenols yield (a) and content (b) of extracts obtained with ethanol (EtOH) and ethylacetate (EtAc) from both grape marc and stalks (Deg: degreased sample; E: ethanol soluble extract; W: water soluble extract). Error bars indicate ± s.d.

The influence of the other investigated process parameters was then evaluated for marc and stalks separately.

Degreasing did not seem a useful pre-treatment because it reduced GAE yield and improved only slightly the phenols content of stalks-extracts.

Type of solvent was always highly significant. Extract purity was higher by using ethylacetate, particularly with stalks and, in fact, it is reported that the use of methanol, ethanol, acetone and their mixtures with water in different proportions generally yields a significant co-extraction of concomitant substances, which makes the procedure of extract purification more difficult and decreases the yield of target antioxidants [60], while ethylacetate exhibits significant selectivity in respect of natural products. On the other hand ethanol allowed for higher yields.

There was no significant difference between 5 and 24h, but this was not in agreement with some literature results. It was found out that in water-extracts made of grape the yield of polyphenols gently increased with the time, while in the case of alcohol-extracts it strongly

increased with the longer time of extraction [44]. Other authors [60] reported that the kinetics curves of proanthocyanidins yield were of parabolic shape with the initial part being linear (up to 8h), whereas their second parts showed a slower increase and an asymptotic ending. In most of the cited references the effect of time was not an investigated factor, with a length of extraction varying from less than 1 h to 48 h.

Temperature was strongly influent on GAE yields for both marc and stalks, but not on extract purity, probably because temperature increase favoured extraction by increasing solubility and diffusion coefficient of any compounds, not only of antioxidants.

The AAI ranged from 40 to 80 with no influence of solvent, time, temperature and degreasing, while stalks extracts showed a slightly higher power than marc ones. Correlation between AAI and GAE concentration was positive and significant ($P<0.01$), but weak (Pearson correlation coefficient of 0.42). Positive and variable coefficients (0.23-0.96) were reported also by other authors [37, 58]. In general, even using other assays for the antioxidant power, it is observed that the protection linearly increases with the antioxidants concentration up to a certain value, above which the increase is slower or absent. Furthermore, the degree of correlation depends on the class of compounds and is generally higher for total polyphenols than for anthocyanins, flavonoids or flavonols [26, 27, 55, 69]. The used β-carotene test is simple and can give a screening evaluation of the antioxidant power, but it gives poorly reproducible results (due to variations in β-carotene bleaching reaction), it is not specific (being subject to interference from oxidation and reducing agents in crude extracts), and linoleic acid is not representative of typical food lipids.

GAE yield and content data reported in Figure 4 could have been underestimated for two reasons. First of all samples could not be concentrated to a powder, but to a viscous and dense paste (probably due to the presence of sugars and fats); secondly, after addition of petroleum ether to marc extracts a solid precipitate was not separated.

On the basis of these initial results, the extraction procedure was tried to be improved focusing on employment of grape marc. The step of precipitation by petroleum ether was substituted by freeze-drying. Analysis of extracts before and after freeze-drying assured that the operation did not reduce antioxidant activity of extracts.

The particle size of 2mm and the solvent/sample ratio were not varied on the basis of the literature data already commented.

The next experiments were then aimed to verify whether an intermediate temperature between 28°C and 60°C could give the same recovery yield as 60°C (or even higher in case a certain degree of thermal degradation occurred at 60°C) in order to reduce the energy cost of the process; and to demonstrate if the equivalence of yield at 5 and 24 h was due either to the achieving of a plateau at 5 h, or to any antioxidants degradation taking place after an intermediate maximum.

Statistical analysis of results (Figure 5) indicated that both time and temperature highly influenced antioxidants yields with higher yields at 60°C. Tukey's post-hoc test confirmed that yield increased with length of maceration but at 60°C there seemed to be a reduction beyond 20 h due to thermal degradation, or phenols polymerization which may influence analytical quantification [63]. Effect of temperature cannot be generalised since it strongly depends on typology of compounds. For example, it has been reported [15] a maximum of 30-35°C for extraction of anthocyanins from ribes with ethanol 85%; while it has been indicated 20°C and 0°C for the highest yields of ethanolic extraction of carnosic acid, and of both ursolic acid and oleanolic acid, respectively, from Balm leaves [32]. On the other hand,

it was shown [45] that drying red grape pomace peels at 60°C did not significantly affect the stability of polyphenols and antioxidant activity, and, indeed, it was reported an increase in the antioxidant capacity of grape extract by means of a simple thermal treatment at 60°C, due to phenols polymerization [39, 65].

Time was a significant variable, particularly at 45°C, while at 60°C yields after 5 and 24 h were different but means fell into two adjacent homogenous groups (mean discrimination through Tukey's test), confirming the previous results. Intermediate and higher values were reached in agreement with other literature works [60] and many time-temperature combinations gave actually the same results. From a recovery point of view it would be more convenient to work at lower temperature for longer time. However, considering extraction rates of Figure 5 and bearing in mind an industrial application of the process, it could be more convenient and energetically less expensive to work at higher temperature for shorter times (possibly < 8 h). That's why extraction at 60°C for 5h was selected, even though leading to lower yields. An accurate economical evaluation of the incidence of energy cost of the extraction stage on the overall production cost per unit mass of final extract is required to confirm this choice.
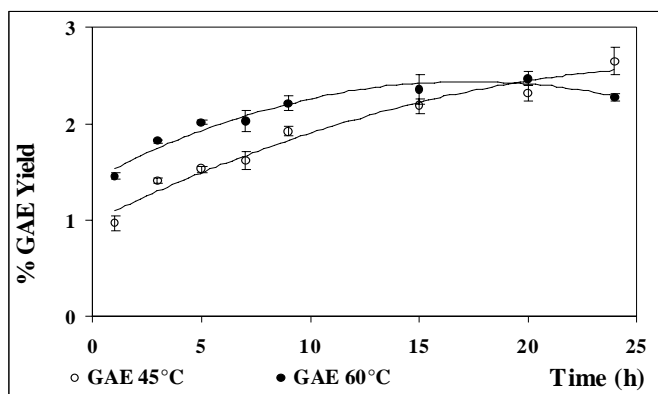
Figure 5. Phenols yield for trials at different temperatures and times (error bars indicate ± s.d.).

In order to further increase the recovery, we moved onto investigating the influence of addition of water to ethanol, since mixtures of alcohols and water have revealed to be more efficient in extracting phenolic constituents than the corresponding mono-component solvent system [61, 106]. Regarding grape and wine-making wastes, influence of water content of alcohols has been studied only for extraction from seeds [3, 106]. In fact, ethanol, a polar solvent, effectively extracts flavonoids and their glycosides, catecols and tannins from raw plant materials [12], but solubility of these compounds can be enhanced using a mixed solvent over a limited compositional range [15].

Phenols yields obtained at increasing water content of ethanol are shown in Figure 6a. In these trials the phenolic composition of the extracts were better characterised evaluating also the content of tannins, cinnamic acids, flavonols, and anthocyanins. Trend for the latter compounds was not shown because yields were always lower than 0.012%, due to the levels of anthocyanins in grapes and to the fact that for red wines, they are mainly extracted during fermentation and vinification processes. Total phenols were evaluated by both the Folin-Ciocalteu method and direct absorbance reading at 280nm. Figure 6b shows clearly that

yields of total phenols based on GAE-280 were lower but highly correlated to those based on GAE (Pearson's correlation coefficient +0.967, Sig. 0.000). The different results are due to the different principle of the two analytical methods: reaction with an oxidising reagent in the Folin-Ciocalteu analysis, absorption of the aromatic ring in direct reading at 280nm. This explains also why yields obtained in the second set of experiment (Figure 5) were higher than those obtained in the first one (Figure 4).
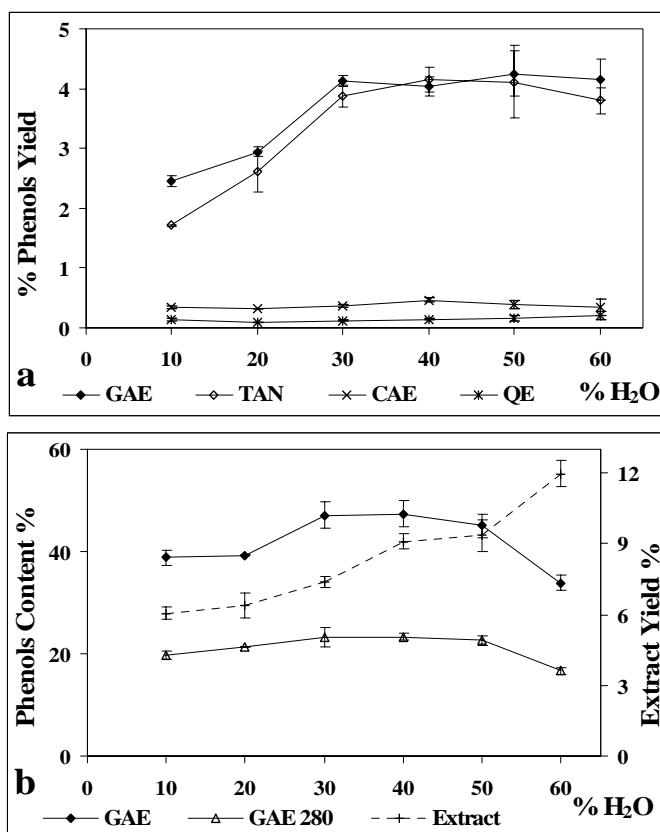
Figure 6. Influence of water content of ethanol on phenols yield (a), freeze-dried extract yield and phenols content (b) (error bars indicate ± s.d.).

Increase of water content of ethanol was statistically influent in improving extraction yield for GAE, GAE-280, tannins, and total extract, but not for cinnamic acids and flavonols. Tukey's post-hoc test confirmed that phenols yield was improved increasing the water percentage of ethanol from 10 to 30%, and, then, it did not significantly change for water content between 30 and 60%, while total extract yield kept on increasing with water content. Similar trends were reported by other authors. It was reported [106] that phenol content (as GAE) of ethanol extracts from grape seed powder increased increasing water in the mixture from 0 to 30%, kept constant for 30-40-50%, and decreased for higher percentage. In another study [15] it was obtained that extraction of anthocyanins from black currants using aqueous ethanol increased with ethanol concentration up to a maximum at about 60% and then

decreased with further increase in solvent concentration. In the same work it was suggested a different optimum ethanol content for the extraction of each group of phenols.

On the other hand, according to the fact the total extract yield kept on increasing with water content, concentration of phenolic constituents in the extracts increased for water content from 10 to 30%, and decreased for water content above 50% (Figure 6b), so a value of 40% was retained as the optimal one.

It must be pointed out that freeze-dried extracts had to be dissolved in the same mixture ethanol/water used for their extraction, in order to get a complete solubilization without heating assistance. Solubilization problems should be taken in consideration for final employment of the extracts in food systems. For example, flavonoids modification by lipase-catalysed esterification has been reported to improve their solubility in lipid-base media [28, 40], and in natural antioxidants commercially exploited a proper medium, such as maltodextrine and propylene glycol, is incorporated to the same purpose. Statistical analysis (Students' t-test for paired samples; $\alpha = 0.01$) confirmed freeze-drying did not lead to reduction in phenols content, except for the cinnamic acids which seemed to get lost through freeze-drying. Actually, they are very sensitive to oxidation processes due both to enzymes (such as tyrosinase which is easily co-extracted from marc) and oxygen. Liquid extracts were stored in closed flasks but not under nitrogen, under refrigeration (oxygen solubility in water is increased at low temperature) and for a variable period of time before freeze-drying, and this may have brought to their rapid degradation.

Antioxidant power of extracts was this time evaluated by the ABST test due to the limits encountered with the β-carotene assay, and since the ABTS assay is reported as one of the best method for antioxidant activity measurement [67]. Results (Figure 7a) confirmed the correlation between antioxidant power and phenols concentration, and showed also that there was no difference between the various extracts, water content seemed only to influence the amount of phenols recovered but not their composition. To compare our results with other similar literature works, % inhibition was transformed into TEAC values (Figure 7b). TEAC value was concentration-dependent as observed in other works [16, 26, 79, 101], because in the considered range antioxidant activity linearly increased with concentration up to a certain value, above which the increase was lower or absent, while a linear Trolox® calibration curve was used for TEAC calculation. Values of TEAC were comparable to those of many other phenolic compounds and vegetable extracts [6, 70, 101, 107]. Only a paper reported very high TEAC data (from 10 to 140) for grape marc extracts [29].

Recovery trials at the selected conditions of 60°C, 5h, ethanol with 40% water, were performed on different types of grape marc. For the same grape variety, a different vintage can greatly change the phenols yield without influencing the phenols content of the extract, due to the influence of weather and season on grape ripening and composition: this is the case of data indicated as 2004 and 2005 in Figure 8. Employment of different grape varieties inevitably leads to both different yield and phenols content: undistilled samples of Figure 8 were obtained from marc coming from a mixture of white and black grape. Finally, distillation seemed not to reduce the phenols recovery and extract purity. According to other authors [17, 60, 61] results confirmed the possibility of extracting antioxidants also from distilled grape marc, that should be even a cheaper by-products than marc collected after devatting.
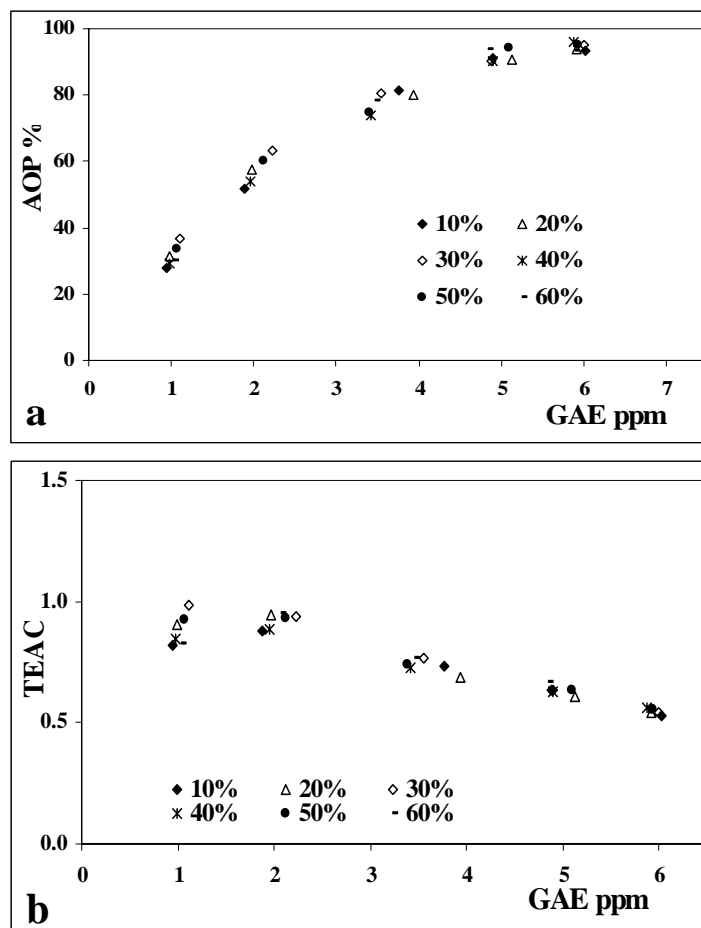
Figure 7. Antioxidant power (a) and TEAC values of extracts (b) as a function of phenols content.
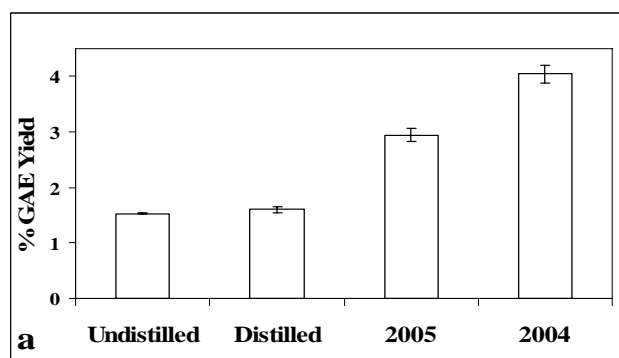
Figure 8. (Continued on next page.)
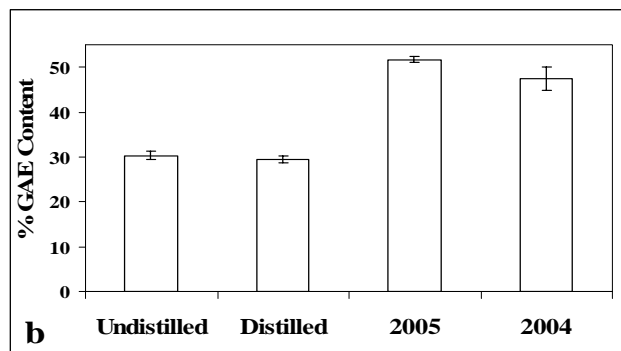
Figure 8. Phenols yield (a) and phenols content (b) of extracts obtained from Barbera grape marc of different vintages (2004 and 2005), and from grape marc other than Barbera before and after distillation (undistilled and distilled) (error bars indicate ± s.d.).

Knowledge of phenols extraction kinetics should provide a useful tool for scaling-up and process design. Data of Figure 5 were elaborated according to the quite simple model reported by [32]. The authors explained the kinetic plots of antioxidants extraction from Balm leaves by the presence of two extraction stages: an initial fast step corresponding to recovery of solutes from the superficial sites of the raw material, and a second lower step corresponding to molecular diffusion of solutes from the internal sites through the porous medium. For both the steps, application of the steady-state model leads to the first-order rate equation:

$$\ln\left(\frac{c_\infty}{c_\infty - c}\right) = k_{obs} \cdot t$$

where c is the concentration of the extracted constituent in the solution at time t, c_∞ is its concentration at equilibrium t = ∞), and k_{obs} is the overall rate constant (s^{-1}). The three rate governing steps of the process are: surface-controlled infusion, diffusion of the soluble constituents through the solid with a diffusion coefficient, and diffusion of the constituents through the Nerst layer with another diffusion coefficient. Considering the second step alone is rate determining, the k_{obs} takes into account the diffusion coefficient, the partition coefficient of the extracted constituents between the solvent and the solid, the total surface area, the volume of solvent and the size and geometry of solid particles. Considering the equilibrium concentration as that at 24 h for GAE extraction at 45°C, and that at 20 h before the apparent reduction for GAE extraction at 60°C of Figure 5, the second low step previously described (the fitting equations do not origin from zero) was able to describe our experimental results (Figure 9a), with an higher accuracy (smaller standard deviations) for the short extraction time range previously selected. Extraction rate for time <5h was then investigated, varying also the stirring rate (by using the rotary shaker or a magnetic stirrer). Experimental data obtained by both the stirring modalities could be described accurately by the use of the characteristic function in the general case of a polydispersed anisotropic solid [84]:

$$C_l = A - B\exp(-Ht)$$

where C_l is the liquid phase concentration. The equation is expression of a first order kinetics model. Values of equation parameters reported in Figure 9b were obtained by non linear regression (SPSS software). The increase in stirring rate almost duplicated the phenol concentration. Extract quality was monitored through estimation of the tannins percentage by the PVPP method. Percentage of tannins of the extracted phenols did not significantly change with extraction time (Figure 9b), such as the AOP which for all the samples showed the same trend as a function of GAE concentration.
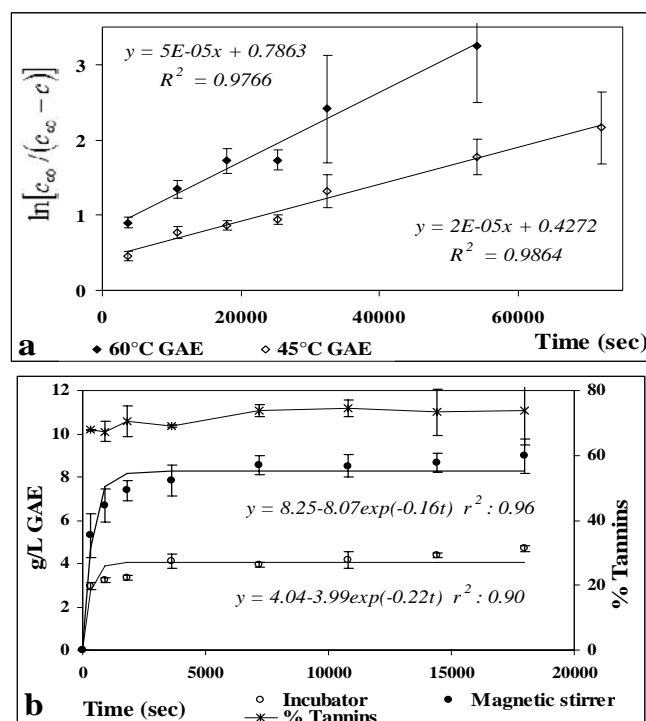
Figure 9. First order plot for the slow stage of phenols extraction at 45°C and 60°C (a), and extraction kinetics curves at different stirring modalities (b), with composition of phenols in terms of tannins percentage over time(error bars indicate ± s.d.).

From comparison of literature works on phenols recovery from grape, phenols content of our extracts were very high and comparable to those obtained from only grape seeds, the part of the fruit richest in phenols (average tannin content of 3-6% on fresh weight and 4-11% on d.w.) (Table 1). It should also be pointed out that many of the cited references in Table 1 reported the application of more complicated recovery procedures (multiple-solvent-multiple step extractions). Phenols concentration is always around 50%, this is because alcoholic and aqueous extracts (solvents not selective for phenols) from fruits processing by-products contain inevitably sugars and polysaccharides. From a commercial point of view, a higher purity would give a higher value to the extract. However, purification by C_{18} resins or other columns to eliminate sugars, non volatile acids and amino acids [85] is an expensive step,

Table 1. Comparison of different literature works on phenols extraction from grape and grape by-products.

Raw Material	Analysed compounds	Phenols Yield (%)	Phenols content of Extract (%)	References
Red Grape Marc	GAE	0.94-2.65	33.8-47.4	[93]
Red Grape Stalks	GAE	0.33	22.34	[90]
Red Grape Seeds	Catechins	0.86-1.56	43-54	[37]
Red Grape Seeds	Catechins	2.5	46	[36]
White Grape Seeds	Catechins	0.5	47	[60]
Fresh Grape Seeds	GAE	10.8	66.8	[11]
Fresh Grape Seeds	Catechins	0.078		[56]
Grape Seeds	Tannins		0.53	[1]
Fresh Red Grape Skins	GAE	0.25		[73]
Fresh Grapes		0.43-0.51		
Red Grape Marc	HPLC	0.11	0.011	[14]
Red Grape Marc	GAE	0.59	0.14	[53]
Seeds From Marc		2.4	0.28	
Skins From Marc		0.37	0.11	
Grape Marc	GAE	0.035-1.36		[44]
Grape Marc	Flavan-3-ols	0.06		[29]
Distilled Marc		0.08-0.14		
Grape Seeds		0.15		
Distilled Seeds		0.15-0.18		
Distilled Grape Marc	GAE	0.22	18.3	[17]
Grape Seeds From Marc	Epicatechin	0.08		[3]
Distilled Red Grape Marc	GAE	0.16	1.07	[62]
Distilled Red Grape Marc	GAE	0.25		[61]
Grape Marc Seeds	GAE	2.8-4		[106]

inevitably separates free from bound polyphenolics, or different phenols classes (mixing of which may bring to antioxidant synergistic effect) [79], while, depending on the extracts application (dietary supplies, antioxidants into food systems) sugars removal might be not necessary. Employment of colloidal gas aphrons as an alternative simpler and cheaper purification system has been investigated [91]. Furthermore, there are still many uncertainties about polyphenols effective bioavailability and metabolism, i.e. *in vitro* positive effects have been shown for concentrations that are not reached *in vivo*, and even though much has been learned about possible mechanisms of actions, it is largely unknown whether they can reach their multiple intended sites of action and whether they are better absorbed as aglycones or glycosides [103].

Food Applications of the Extracts

Addition of marc extracts to vegetable oil showed a prooxidant effect, since the peroxide value was higher than in the control (Figure 9a). It was visually observed that extracts did not well solubilize into the oil but formed a reddish layer on the glass at the interface oil/air. This is not in agreement with other works which found a certain (not always high) protection of oil oxidation by different natural extracts [9, 13, 96]. However these works did not report a detailed description of the solubilization procedure, and it has been reported about the problems of solubility of green tea extracts into edible oils [54].

Plant phenolics have been shown to be active against fungi. These compounds could then be exploited for developing improved and potentially safer technologies for postharvest disease control of fruits and vegetables [21, 46, 50, 71]. The shelf-life of strawberry is very short because of its perishability and susceptibility to rot-causing pathogens. During storage and shipping of strawberries, decay losses are mainly caused by *Botrytis cinerea* and *Rhizopus stolonifer*. Incorporation of different concentrations of our extracts into agar plates slowed down the growth of *B. cinerea* (Figure 10b). Complete inhibition, however, was not achieved at the concentrations tested, indicating that the phenols extracts are fungistatic rather than fungicidal. Their employment in combination with adequate packaging and refrigeration could greatly extend strawberry shelf-life.
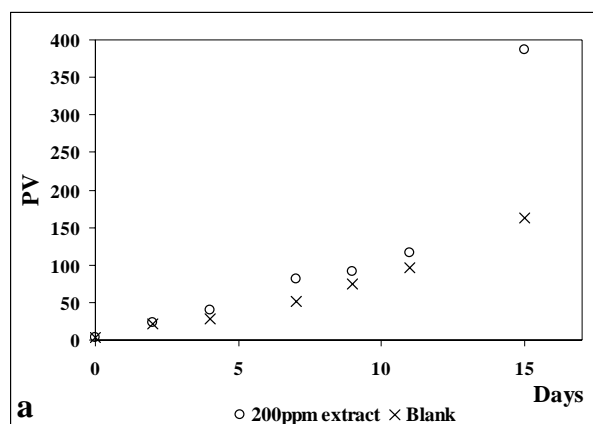
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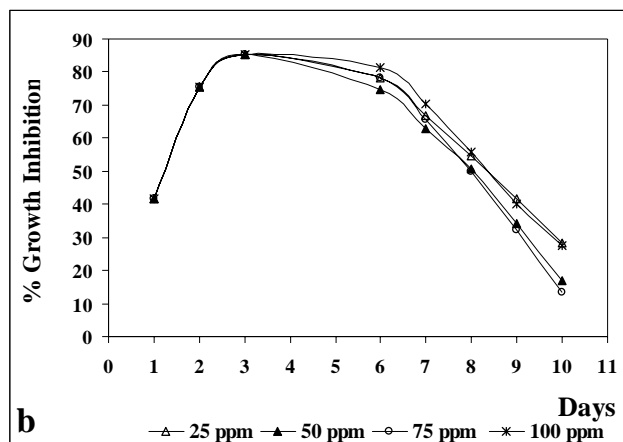
Figure 10. Application of marc extracts to inhibit oil oxidation (a) or to inhibit growth of moulds isolated from diseased strawberries (b).

CONCLUSION

The interest for production of natural antioxidants to be used instead of synthetic ones in food and pharmaceutical sectors has been greatly increasing in these last years.

Phenolic compounds represent the majority of natural antioxidants, and they can be potentially recovered from many sources since they are ubiquitous in plants. Their extraction from agricultural and food by-products is a great opportunity which would allow at the same time valorisation of wastes and reduction of production costs.

Recovery of phenols from natural sources involves many problematic aspects: choice of an adequate source (in terms of availability, cost, difference in phenolic content with variety and season); selection of the optimal recovery procedure (in terms of yield, simplicity, industrial application, cost); chemical analysis of extracts (for optimisation purposes a fast colorimetric method is preferable than a chromatographic one); evaluation of the antioxidant power (preferably by different assay methods).

Experimental results from a research carried out for recovery of phenolic compounds from wine-making by-products (grape marc and stalks) showed how it was possible to get a better insight into optimisation of the process, investigating some variables which were selected on the basis of the available literature about the same subject. The simple procedure chosen (one solvent-one step extraction) gave results comparable to other literature results obtained with similar, longer or more complicated systems, even though it could be further improved by means of ultrasound or microwave assistance.

Knowledge of extraction kinetics is necessary in order to minimise production costs and to provide a useful tool for scaling-up and process design.

Application of the extracts into real food systems should always be verified since antioxidant mechanism can be quite different than in the model systems used in the screening fast methods commonly employed for evaluation of antioxidant power.

The optimal storage conditions and shelf-life of extracts as a function of antioxidant activity has to be investigated and established.

ACKNOWLEDGMENTS

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Chapter VI

HISTIDINE-CONTAINING DIPEPTIDES AS HYDROPHILIC TISSUE ANTIOXIDANTS

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1. PRE-REQUISITES FOR THE ANTI-RADICAL ACTIVITY OF CARNOSINE

For the first time, the antioxidant activity of carnosine has been discovered in the pioneering experiments of Severin and co-workers (Северин и др., 1984; Дупин и др., 1984). The results of these experiments are taking new twists in the context of current concept of mechanisms of free radical processes in excitable tissues.

Among numerous hypotheses of biological activity of carnosine, the hypothesis of the pH-buffering capacity of this dipeptide has been the most popular over a long period of time. As a result, much effort has been directed toward selecting appropriate experimental conditions, in which the dipeptide-induced effects would be detected against the background of equal buffering capacities of control and experimental samples (Severin, 1964).

The history of the problem dates back to experiments of Meshkova and Karyavkina (Мешкова, Карявкина, 1965). It was shown in these experiments that carnosine and anserine are able to maintain the coupling between oxidation and phosphorylation in isolated mitochondria at a high level for a long time (see Part II). This suggested that the dipeptide-induced effect is directed toward conservation of low permeability of mitochondrial membrane for protons, which is a necessary prerequisite for ATP synthesis in mitochondria. It was shown later that carnosine increases efficiency of ion transporting ATPases, thereby supporting the ion asymmetry in the muscle cell (Болдырев, Северин, 1966; Северин, 1992).

In their experiments, Meshkova and Karyavkina compared the effects of carnosine to the effects induced by artificial pH buffers of the same pK_a value (see Table II-1). In addition to proton-binding capacity, carnosine and related compounds were shown to conserve the native

properties of membranes under un-favorable conditions. It was shown that carnosine prevents passive leakage of ions from the sarcoplasmic membrane vesicles. In the absence of carnosine, there was a rapid increase in the membrane permeability caused by lipid peroxidation (LPO).

A comparative survey of the efficiency of accumulation of peroxidation products in homogenates of hepatic and muscular tissues has been published in 1966 (Нейфах, 1966). E. A. Neifakh found that although muscles contain less α-tocopherol than liver, the rate of accumulation of peroxidation products in homogenates of muscular tissue is lower than in homogenates of hepatic tissue. It was concluded that the LPO inhibition in homogenates of muscular tissue is due to the presence of the histidine-containing dipeptides carnosine and anserine. Indeed, an addition of carnosine or anserine to the homogenates resulted in an increase in the tissue resistance to LPO. This was the first experimental evidence of the ability of histidine-containing dipeptides to protect cell membrane lipids from ROS. Neifakh suggested that the observed effects were due to the carnosine involvement in regeneration of α-tocopherol and other hydrophobic antioxidants in muscular tissue. This allows the stability of the tissue to be maintained at a high level even at relatively low intrinsic concentrations of α-tocopherol and ascorbate (Table IV-1).

Table lV-1. Comparison of LPO induction period with concentrations of endogenous unsaturated fatty acids (Нейфах, 1966).

Tissue	Concentration.of conjugated dienes, 10^{-4} mol/g			Total	Concentration of α-tocopherol, 10^{-6} mol/g	Induction period, min
	18:1	18:2	20:4			
Rat skeletal muscle	1.13	5.96	33.72	40.81	0.766	125.0
Rat liver	8.45	11.20	102.00	121.65	0.458	8.5
Mice liver	7.60	31.60	107.20	146.40	1.128	16.5

However, actual facts are often more surprising than the most daring hypotheses. Indeed, histidine-containing dipeptides themselves were found to prevent accumulation of peroxides during degeneration of membranes induced by the presence of Fe^{2+} and ascorbate. In addition to inhibition of accumulation of MDA, a terminal product of LPO, this was accompanied by protection of activity of Ca-ATPase and Ca-accumulating function of the sarcoplasmic reticulum membranes studied in these experiments (Figure IV-1).

It was interesting to assess the pH dependence of the protective effect of carnosine. As the pH of reaction medium goes below 6.8, the state of the ionogenic groups of imidazole ring is changed (pK_a of imidazole group is 6.8) giving rise to an increase in the lag-time of the protective effect of carnosine. This increase is observed even against the background of strong inhibition of LPO. Therefore, within the physiological range of pH (7.2-7.8), imidazole ring demonstrates definite nucleophilic properties, and carnosine actively prevents initiation of lipid peroxidation, although only insignificantly decreases the steady-state level of accumulation of LPO products. On the other hand, although the carnosine-induced effect on the lag-time at pH < 6.8 is significantly less pronounced, the steady-state level of accumulation of LPO products is inhibited much stronger (Figure IV-2).
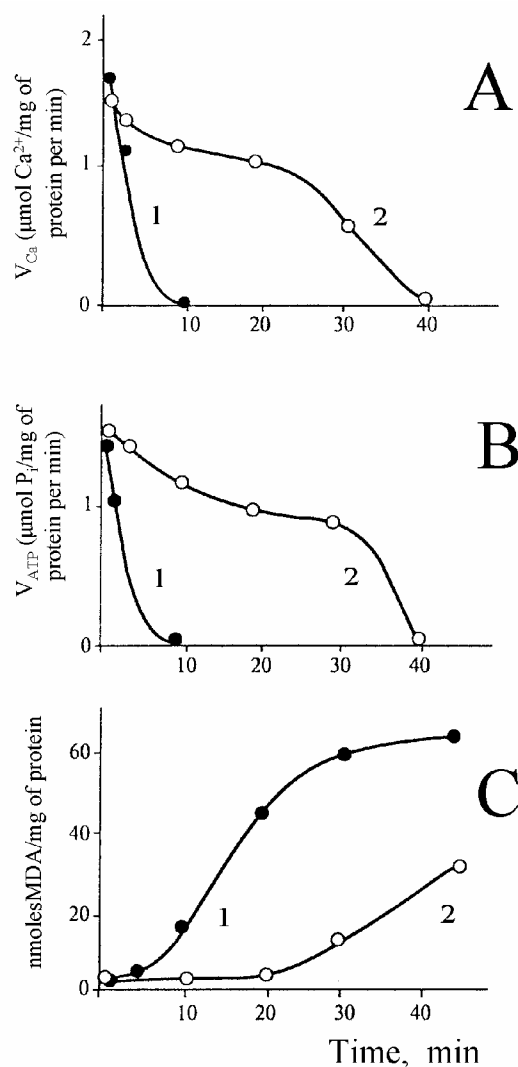
Figure IV-1. Ca-accumulation (A), Ca-ATPase activity (B) and lipid peroxidation (C) after addition of ferrous ions and ascorbic acid to frog muscle sarcoplasmic reticulum in the absence (1) or presence (2) of 25 mM carnosine.

It is not inconceivable that these properties of carnosine are employed by working muscle, in which intensive muscular work is accompanied by accumulation of lactate and more or less pronounced acidification. Moreover, in the presence of carnosine, the pH value of the medium is maintained at the level required to prevent development of peroxidation reactions. Thus, the pH-buffering properties of carnosine should be directly attributed to its antioxidant activity. Probably, the protective effect of this compound under these conditions is associated with scavenging of radicals and inhibition of LPO. The exhaustion of buffer capacity and acidification of medium bring the carnosine molecule into charged state. The anti-radical capacity of carnosine in the charged state is decreased, but the ability to neutralize toxic products of LPO is increased.
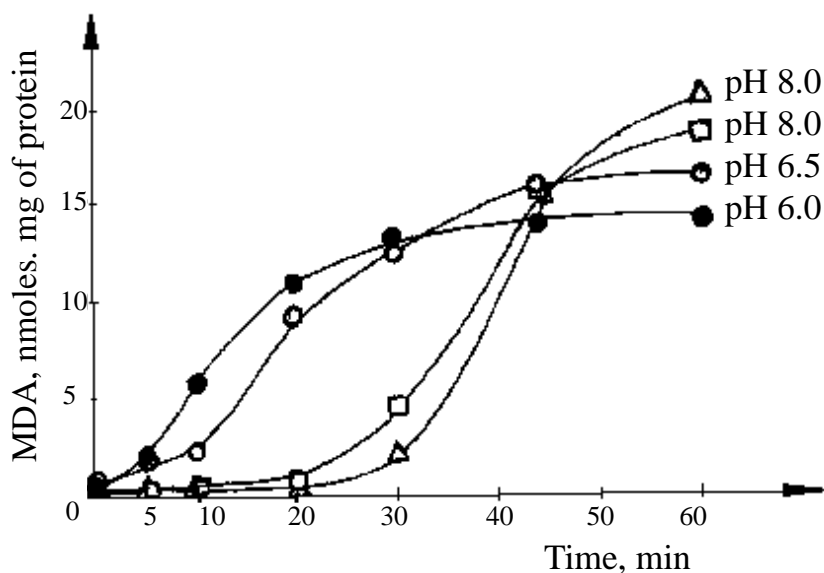
Figure IV-2. Effect of pH on the accumulation of MDA during (ferrous ions+ ascorbate) induced LPO in sarcoplasmic reticulum membranes in the presence of 20 mM carnosine.

Thus, our analysis of the pH dependence of carnosine-induced effects suggested that there are two components of the antioxidant activity of carnosine, which have different pH-dependence. In addition to inhibition of accumulation of the terminal product of LPO (MDA), carnosine was found to be able to interact with the intermediate products of this process (conjugate dienes and oxodienes). In the presence of carnosine, the rate of accumulation of these products was decreased, and their spectral characteristics were changed (Figure IV-3). We concluded that conjugate dienes (absorption maximum at 265 nm) and oxodienes (absorption maximum at 292 nm) - typical intermediate products of LPO under standard conditions - in the presence of carnosine form molecular complexes with this dipeptide.

Probably, the complexes formed in the presence of carnosine are less detrimental for membranes, because it is seen from Figure IV-1 that Ca-pump continues to function in the presence of carnosine and is completely inhibited in the absence of this dipeptide against the background of the same level of LPO products. These experiments can be regarded as the first experimental evidence of direct antioxidant activity of carnosine. Later, the conclusion of the direct antioxidant activity of carnosine was confirmed by Ames (Kohen et al., 1988), Hartman et al. (1988), Halliwell (Aruoma et al., 1989), Decker and Faraji (1990), Chan and Decker (1993), and other researchers.

Comparative study of carnosine-related compounds revealed that their antioxidant efficiency is determined by specific features of molecular structure. Histidine was found to be absolutely incapable of preventing MDA accumulation, whereas homocarnosine caused a significant increase in the lag-time of accumulation of the terminal products of LPO, although had virtually no effect on their steady-state level (Figure IV-4). The activity of anserine and D-carnosine, an optical isomer of L-carnosine, was similar to that for carnosine. Histidine, β-

alanine, and imidazole alone were inactive in this model of LPO initiation (Дупин и др., 1987; Boldyrev et al., 1988a).
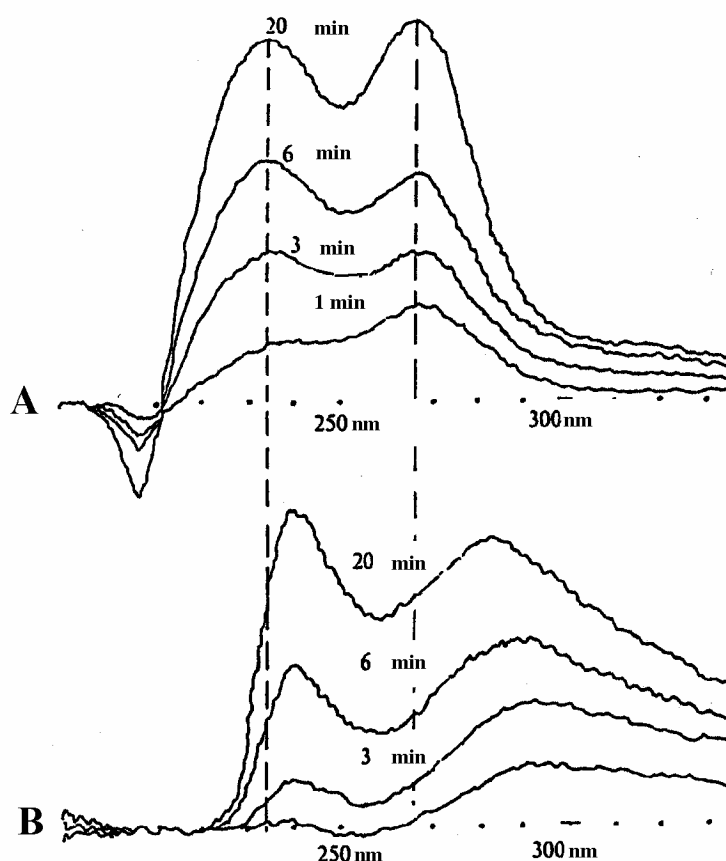
Figure IV-3. UV spectra of the primary LPO products, formed in sarcoplasmic reticulum membranes at different times of incubation. Conditions as in Figure IV-2. A- control, B - in the presence of 25 mM carnosine. First pick corresponds to conjugated dienes, second one - to oxodienes.

What is the mechanism of the antioxidant activity of carnosine? It was suggested that carnosine is able to regenerate oxidized forms of vitamin E (Нейфах, 1966). However, this suggestion was not confirmed in direct experiments, in which the efficiencies of interaction of oxidized form of α-tocopherol (semiquinone radical) with carnosine and ascorbate were compared (Горбунов, Ерин, 1992). Although 100 μM of ascorbate were able to quench the ESR signal of tocopheryl radical in the presence of 2,2'-diphenyl-1-picryl hydrazine, even a 50-fold excess of carnosine did not cause similar effect. Two important conclusions can be made from these experiments: 1) carnosine is unable to regenerate oxidized form of α-tocopherol and to implement this mechanism of antioxidant protection of tissues; 2) mechanisms of antioxidant protection of ascorbate and carnosine differ from one another.
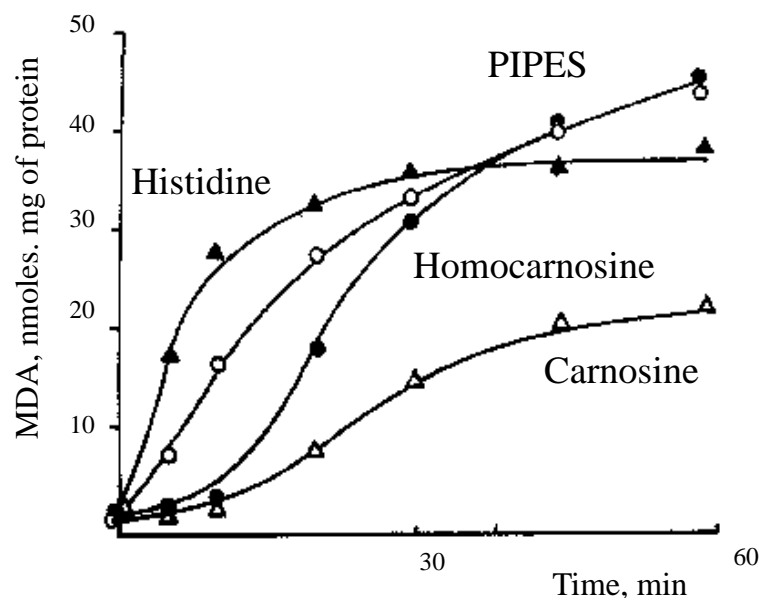
Figure IV-4. Accumulation of MDA in sarcoplasmic reticulum membranes isolated from rabbit skeletal muscle after addition of ascorbate+ferrous ions. The data obtained in the presence of PIPES, histidine, homocarnosine, and anserine, as indicated. It is seen that histidine demonstrate prooxidant effect, and homocarnosine and carnosine act as antioxidants. The former increases the lag-period of the process measured while the latter affects also the level of end products accumulated.

The antioxidant effect of carnosine could be attributed to its ability to form complexes with Fe^{2+} (Brown et al., 1979). This suggestion was supported by the results obtained in some experimental models (Ли Хо Ик и др., 1990). However, it is well known that Fe^{2+} remains catalytically active after incorporation in some complexes (Green, Hill, 1984). It was shown that the LPO dependence on concentration of $FeSO_4$ is invariant regardless of the presence or absence of carnosine in the reaction medium (Дупин, 1987). Later, A.M. Rubtsov in our laboratory showed that the antioxidant activity of carnosine can be observed in the absence of iron ions, when ROS generation is induced by the UV radiation (Болдырев и др., 1992). Therefore, the antioxidant activity of carnosine is not restricted only to its ability to chelate ferrous ions.

Direct interaction with ROS (LPO activators and/or products) can be suggested as the simplest explanation of the antioxidant activity of carnosine. For the first time, such an interaction has been demonstrated in 1984-1988 (Северин и др., 1984; Дупин и др., 1984; Boldyrev et al., 1988). Later, it was supported in a large number of independent studies (Kohen et al., 1988; Aruoma et al., 1989; Hartman et al., 1990; Chan, Decker, 1994).

Indeed, measurements of the content of 2'-thiobarbituric acid-reactive substances (TBARS) in the membrane preparation of frog sarcoplasmic reticulum oxidized in the presence of Fe^{2+} and ascorbate revealed that carnosine is capable of decreasing the initial level of oxidized products in the lipid phase, the initial rate of their accumulation during LPO induction, and their steady-state level. The extent of inhibition was proportional to carnosine concentration (Дупин, 1987).

Thus, these experiments revealed the previously unknown property of carnosine to prevent directly the accumulation of peroxide compounds. These compounds interfere with the physiological functions of membranes and modify various enzymatic processes in cells. This brings up the question if this property of carnosine is responsible for the Severin's phenomenon? In our laboratory, A.M. Dupin was concerned with by this problem. In collaboration with V.E. Kagan and Yu.V. Arkhipenko he assessed the antioxidant activity of carnosine. The experiments with the carnosine-induced effects on the contracting ability of the m. sartorius of frog have been revised (Дупин и др., 1987).

It is seen from comparison of Figures II-3 and II-12, that the resulting pattern was identical to that, observed in 1953. In further experiments, the main attention was focused on the carnosine content in muscle and in surrounding solution, because it was suggested that this content should be reduced as a result of interaction with peroxides produced during muscular work. Indeed, there was a significant decrease in the carnosine content in muscle, the extent of decline being proportional to the amount of work performed by the muscle.

Thus, one of the carnosine functions in muscles is to neutralize peroxides, which otherwise are able to interfere with the physiological functions of membranes and modify enzymatic processes (Северин и др., 1984).

2. HISTIDINE-CONTAINING DIPEPTIDES AS NATURAL ANTIOXIDANTS

The positive effects of carnosine on oxidative phosphorylation, sarcoplasmic reticulum Ca-pump, and Na/K-ATPase are, probably, the aftereffects of neutralization of peroxides. This neutralization allows the physiological processes in excitable tissues to be maintained at a normal level. Thus, the role of dipeptides in skeletal muscles consists in their ability to maintain the physiological functions at the level required to implement voluntary contraction. These findings provide a new insight into the importance of the histidine-containing dipeptides discovered by V.S. Gulevich. Indeed, many diseases associated with muscular pathology (pareses, palsy, Duchenne's disease, poliomyelitis, etc.) are accompanied by a decrease in the carnosine content in muscles (Степанова, Гринио, 1968).

Studies of the antioxidant properties of carnosine showed that this dipeptide affects various stages of LPO: from neutralization of ROS to interaction with molecular products of free radical oxidation (Boldyrev et al., 1988; Dahl et al., 1988; Kohen et al., 1988; Швачко и др., 1990). Carnosine and related compounds may serve as a trap of peroxyradicals (Kohen et al., 1988), hydroxide radicals (Aruoma et al., 1989; Rubtsov et al., 1991), singlet oxygen (Dahl et al., 1988; Егоров и др., 1992), as well as superoxide anion radical of oxygen (Гуляева,1987; Pavlov et al., 1993). They can also neutralize hypochlorite giving rise to stable chloramine complexes (Формазюк и др., 1992). Probably, the antioxidant properties of histidine-containing dipeptides are decisive component in the spectrum of their biological activity.

The ability of carnosine to interact with hydrogen peroxide has been studied quite long ago, the intensity of the interaction being insignificant (Shubert, 1968). The carnosine-induced quenching of hydroxide radical was demonstrated in both direct (Rubtsov et al., 1991) and indirect (Aruoma et al., 1989) experiments. Direct measurements were based on

the spin trap method and showed that carnosine was an equally effective quencher of the OH-radicals generated in the Fenton reaction or during exposure of aqueous solutions of hydrogen peroxide to the UV radiation. Such a coincidence suggested that the carnosine-induced effects were due to direct interaction with hydroxide radical.

The chemiluminescence assay of the ROS generation by activated neutrophils revealed that carnosine is capable of more than 50% quenching of the chemiluminescence signal. This effect was interpreted in terms of the carnosine-induced neutralization of hypochlorite anion (Sharonov et al., 1990), and this conclusion was later confirmed in the experimental studies of the effects of carnosine on generation of OCl⁻ by purified myeloperoxidase (see below).

Direct interaction between carnosine and hypochlorite in aqueous solutions was shown to produce stable chloramine complexes (Формазюк и др., 1992; Boldyrev, 1993). Hypochlorite anion is an extraordinary strong oxidizer. It is generated in the reaction between H_2O_2 and Cl^- catalyzed by myeloperoxidase. Macrophages contain significant amounts of this enzyme. The reaction is very effective, and virtually all hypochlorite anions interacting with carnosine become bound as stable chloramine complexes within 1 min after the initiation of the reaction. No more than 15% of these complexes degrade during further 2 h incubation. Anserine exerts similar quenching effect on OCl⁻. Although histidine, β-alanine, and glutathione also react with hypochlorite anion, they do not produce stable chloramine complexes. Probably, this ability can explain, to a certain extent, the wound-healing effect of carnosine in experimental animals (see Part II).

It is presently beyond doubt that carnosine is able to interact with singlet oxygen, hydroxide radical, and hypochlorite anion. As far as the interaction with superoxide anion radical is concerned, the data available from the literature are rather conflicting. For example, it was shown in two independent trials in different laboratories that carnosine itself has no effect on the content of superoxide anion radical in reaction medium, whereas carnosine complexes with copper and zinc demonstrate definite superoxide dismutase activity (Гуляева, 1987; Yoshikawa et al., 1991a). On the other hand, it was shown in an experimental model of generation of superoxide anion radical by the water pulse radiolysis that carnosine and superoxide anion radical form charge-transfer complexes. The formation of such complexes causes a significant decrease in the superoxide dismutation time (Павлов и др., 1990). The rate constant of disproportionation of superoxide anion radical in complexes with carnosine was found to be one fifth of that in solution. Therefore, carnosine makes this species of reactive oxygen more stable in aqueous solution rather than quenches it or causes its dismutation.

Similar interpretation can be suggested for the results of the carnosine-induced effects on the leukocyte chemiluminescence associated with generation of superoxide anion. The maximum yield of cell luminescence in the presence of *lucigenin*, a chemiluminescent probe specifically sensitive to superoxide anion radical of oxygen, was found to be increased by carnosine. On the other hand, although in the presence of *luminol*, a luminescent agent which has no selective sensitivity to individual species of active oxygen, the addition of carnosine caused an insignificant decrease in the luminescence intensity, the maximum level is attained significantly later (Тюлина и др., 1994, 1995). The discrepancy between the results of these studies can be attributed to different sensitivity of carnosine to different ROS.

The carnosine-induced effects are implemented not only as a result of binding to products of oxidation but also by neutralization or interaction with stimulators of ROS generation. The first indirect evidence of this suggestion was obtained in experiments with LPO initiation by

various species of active oxygen. For example, carnosine protected the genetic apparatus of bacteria exposed to singlet oxygen (Dahl et al., 1988). Carnosine also reduced the rate of oxidation of linoleic acid by peroxyl radicals in a model system (Kohen et al., 1988). The possibility of carnosine interaction with major species of active oxygen was later confirmed in direct experiments.

Carnosine interaction with the superoxide anion radical of oxygen is of particular interest because it is the first ROS in the chain of their mutual transformations. The superoxide anion radical concentrations typical of normal physiological state of biological tissue are insufficient to provide noticeable interaction with carnosine (Aruoma et al., 1989; Tanigawa et al., 1990; Yoshikawa et al., 1993). However, this interaction is more probable under conditions of oxidative stress, because the content of superoxide anion radical in tissues under these conditions is increased (Pavlov et al., 1993; Klebanov et al., 1997). Comparison between the efficiency of carnosine and a variety of other quenchers of superoxide anion revealed that carnosine is less effective than SOD but is on a par with vitamins E and C (Table IV-2).

Table IV-2. Ability of carnosine and other compounds to quench superoxide anion of oxygen (Klebanov et al., 1997).

Compound	I_{50}, M	K (M^{-1}. s^{-1})
Carnosine	$(7.1 \pm 0.2) \cdot 10^{-5}$	$(0.83 \pm 005) \cdot 10^{5}$
SOD	$(1.1 \pm 0.1) \cdot 10^{-9}$	$(5.35 \pm 0.07) \cdot 10^{9}$
Ascorbic acid	$4 \cdot 10^{-5}$	$2.7. \, 10^{5}$
α-Tocopherol	$5 \cdot 10^{-5}$	$2.0. \cdot 10^{5}$
N-Acetylcysteine	Does not interact with superoxide anion radical	

Thus, carnosine can provide real antioxidant protection against superoxide anion radical to those tissues that are deficient in SOD, ascorbate, and α-tocopherol but subjected to continuous threat of oxidative stress (excitable cells). Moreover, in contrast to SOD, the carnosine-mediated protection of cells against superoxide anion-radical is not accompanied by production of hydrogen peroxide.

In addition to interaction with various species of active oxygen, carnosine was shown to regulate activity of the enzymes involved somehow in free radical reactions. For example, carnosine and related compounds were found to inhibit tyrosine hydroxylase and myeloperoxidase (see below). Regulation of myeloperoxidase activity can explain the wound-healing effect of carnosine, because this enzyme contributes to formation of inflammation focus and determines its life-time in damaged tissues.

Thus, the discovery of the antioxidant activity of carnosine provided a new insight into the biological role of this compound and explained the diversity of its effects. In recent years it has been shown (mainly by Russian biochemists) that carnosine is a competent representative of the class of low-molecular hydrophilic antioxidants of direct action (Boldyrev, 1990) (for the list of the antioxidants, see Table III-3).

Carnosine derivatives were also tested for the antioxidant activity. Anserine was found to be equally active. Homocarnosine is less active than carnosine, whereas β-alanine is virtually

inactive. Histidine and imidazole interact only with one species of active oxygen (singlet oxygen, see below).

High hydrophily of histidine-containing dipeptides is of particular interest in the context of their antioxidant properties, because the LPO reactions occur mainly in the hydrophobic regions of membrane structures. Therefore, it can be expected that histidine-containing dipeptides interfere with the primary stages of LPO initiation.

Carnosine prevented membrane peroxidation not only in the sarcoplasmic reticulum of frog, rabbit, and fish (grayling) but also in the endoplasmic reticulum of rat liver and in the yolk lecithin liposomes (Болдырев, 1986; Boldyrev et al., 1988). Some other histidine-containing dipeptides demonstrated similar protective activity. In all cases, the specificity of the protective effect was determined by the method of ROS generation and damaging mechanism rather than by specific properties of tissue. Thus, all the data discussed above allows to conclude that inhibition of LPO by histidine-containing dipeptides is represented by a universal non-enzymatic mechanism of LPO regulation in biological membranes.

The effects of carnosine and related compounds on the level of singlet oxygen were studied at length at the laboratory of Hartman (Dahl et al., 1988; Z. Hartman and Ph. Hartman, 1992, 1993). It was shown that carnosine prevents oxidation of 2,5-*bis*-(hydroxymethyl)furan by singlet oxygen more effectively that histidine or homocarnosine, whereas imidazole was inactive in this model. Because this model proved to be rather difficult to interpret the results unambiguously, the effects of histidine-containing dipeptides were tested in direct measurements and the rate constants of interaction between superoxide anion radical of oxygen and carnosine-related compounds (anserine, histidine, imidazole) were calculated (Егоров и др., 1992). Fast spectrophotometric measurements allowed the kinetics of the singlet oxygen decay to be measured with high time resolution, and rate constants of its interaction with histidine-containing dipeptides to be calculated with sufficient confidence. All compounds containing imidazole ring were found to have equal and high affinity for singlet oxygen (rate constant, $(2-4) \cdot 10^9 \ M^{-1} \cdot sec^{-1}$). These experiments showed that imidazole ring is responsible for singlet oxygen quenching, whereas the results of measurement of chemical modification of furan derivative by singlet oxygen do not represent the actual processes of interaction between ROS and histidine-containing dipeptides.

Different hierarchy of antioxidant efficiency was observed in the comparative study of interaction between histidine-containing dipeptides and hydroxide radical (Figure IV-5). The ESR spectrum of the adduct produced as a result of interaction between OH$^\cdot$ and the ESR probe TEMPO showed that carnosine is the most effective quencher. Anserine and homocarnosine were less effective, whereas acetylcarnosine was the least active in this system (Figure IV-6).

The fact that carnosine exerts oppositely directed effects on two radical systems: ROS (generation and transformation of superoxide anion) and active chlorine (regulation of myeloperoxidase reaction and content of hypochlorite anion) has not been discussed yet. Carnosine stabilizes the first system and in some cases may prolong the ROS life-time. The second system is effectively inhibited by carnosine. Moreover, methylation of carnosine at different atoms of the imidazole ring modulates the effect of carnosine on superoxide anion radical: methylation to anserine attenuates, but methylation to ophidine significantly stimulates protecting effect of the molecule against superoxide anion radical.
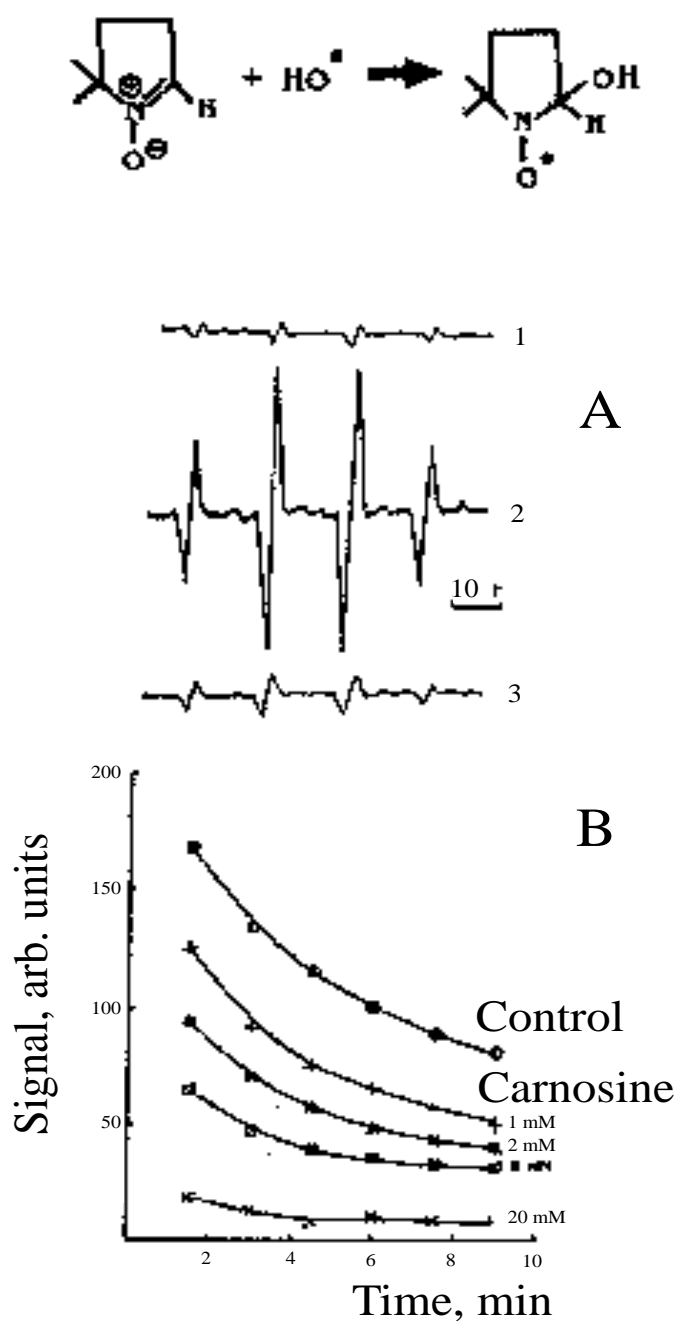
Figure IV-5. Spectrophotometric registration of adduct formed from EPR label TEMPO and hydroxide radical under different conditions. A: control (1), induction of OH-radical by ferrous ions in the presence of 10 mM PIPES (2), or carnosine (3). B - time dependence of signal quenching in the presence of different concentrations of carnosine.
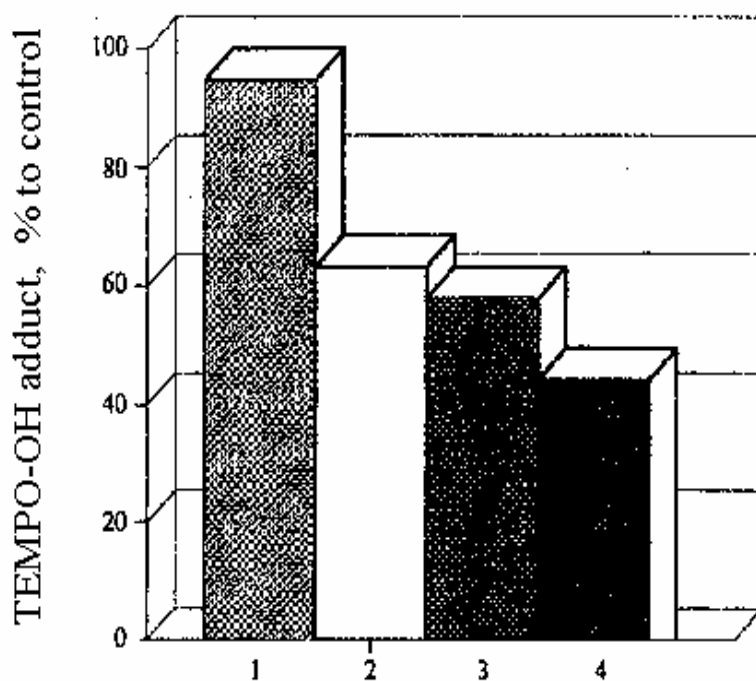
Figure IV-6. Suppression of TEMPO-OH adduct formed during Fenton reaction in the presence of 10 мМ acetylcarnosine (1), homocarnosine (2), anserine (3), or carnosine (4) in % to control (PIPES in the same concentration).

It was shown in some experiments with activation of cells in the presence of luminol that transformation of carnosine into ophidine causes an increase in the cell response, whereas anserine inhibits this response. The structure dependence of the protective efficiency of histidine-containing dipeptides is also observed in the reaction of oxidation of blood plasma lipoproteins (Bogardus et al., 1992). It was shown recently that within the concentration range from 0.1 to 2.5 mM carnosine inhibits the iron-dependent oxidation of low-density lipoproteins, neither duration of lag-period nor the maximum level of oxidation being affected (Ли Хо Ик и др., 1990). In a model of monolayer membrane, carnosine also decreased the latent period of the chemiluminescence burst associated with the iron-induced LPO (Klebanov et al., 1997).

Using the chemiluminescence assay, we also conducted a comparative trial of the protective effects of different histidine-containing dipeptides on oxidation of blood plasma lipoproteins. Detection of chemiluminescence of samples containing lipoproteins in the presence of Fe^{2+} allows both the content of preliminarily produced molecular peroxides (mainly, hydroperoxides) and kinetics of their production (inducibility of LPO) to be measured (Федорова и др., 1991; Vladimirov, 1996). All histidine-containing dipeptides tested in these experiments reduced the contents of preliminarily produced molecular peroxides and brought them to the same (low) level (Figure IV-7). On the other hand, different dipeptides exerted differing effects on the inducibility of lipoprotein oxidation: carnosine and anserine were the most active, whereas their acetylated derivatives were the less active. This indicated that free β-amino group is responsible for the protective effect of the dipeptides (Реброва, Болдырев, 1995).
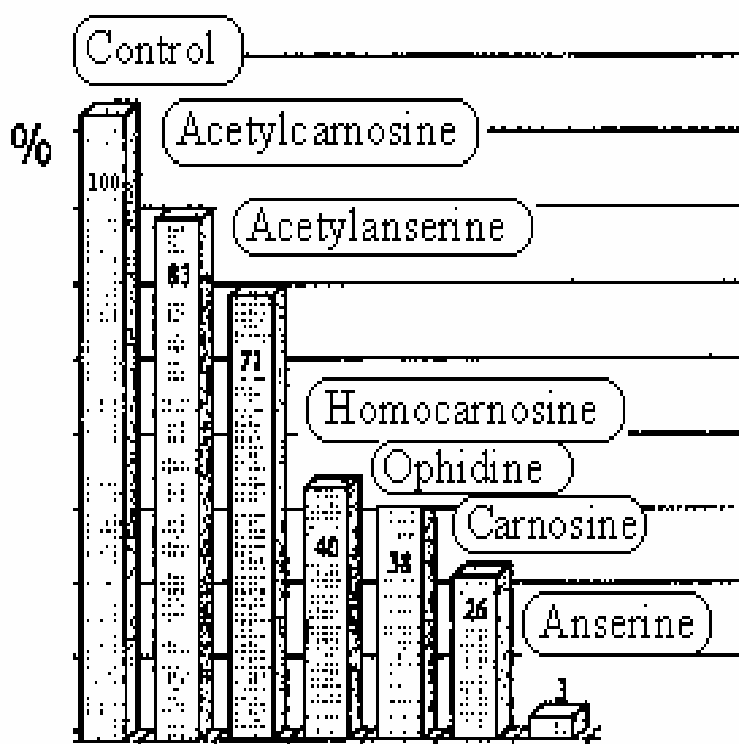
Figure IV-7. Effect of CRC (5 mM each) on the efficiency of LPO induced by 5 мM FeS0$_4$. Control corresponds to the reaction in the presence of 5 mM PIPES with the same pK a value (6.8). The figures within the bars correspond to per cent of LPO inhibition in each case.

   The antioxidant activity of the compounds tested in this model increases in the following ascending series: β-alanine < imidazole < acetylcarnosine < carnosine, homocarnosine < anserine. This series is consistent with the relative nucleophilic activity (proton donor capacity) of the compounds. Therefore, the concept of the biological role of histidine-containing dipeptides may combine the pH-buffering and antioxidant activities.

   The target-oriented modification of molecular structure can provide additional information on the contribution of individual molecular groups of carnosine to its antioxidant activity. The histidine-containing dipeptides artificially synthesized at the laboratory of Yu.P. Shvachkin are of particular interest in this context, because they are not met in natural objects (Figure IV-8). The comparative study of these synthetic analogs of carnosine in the efficiency of inhibition of Fe^{2+}-induced LPO in sarcoplasmic reticulum showed that although some of these dipeptides were more effective in increaseing the LPO lag-period than carnosine, only carnosine reduced the content of preliminarily produced molecular peroxides (Boldyrev et al., 1989). None of structural groups of synthetic dipeptides exerted specific antioxidant effect, and it was suggested that enhanced protection of membrane lipids was due to increased general hydrophobicity of artificial molecules. These compounds are of significant interest in terms of development of the carnosine-based pharmaceutical preparations of prolonged activity.
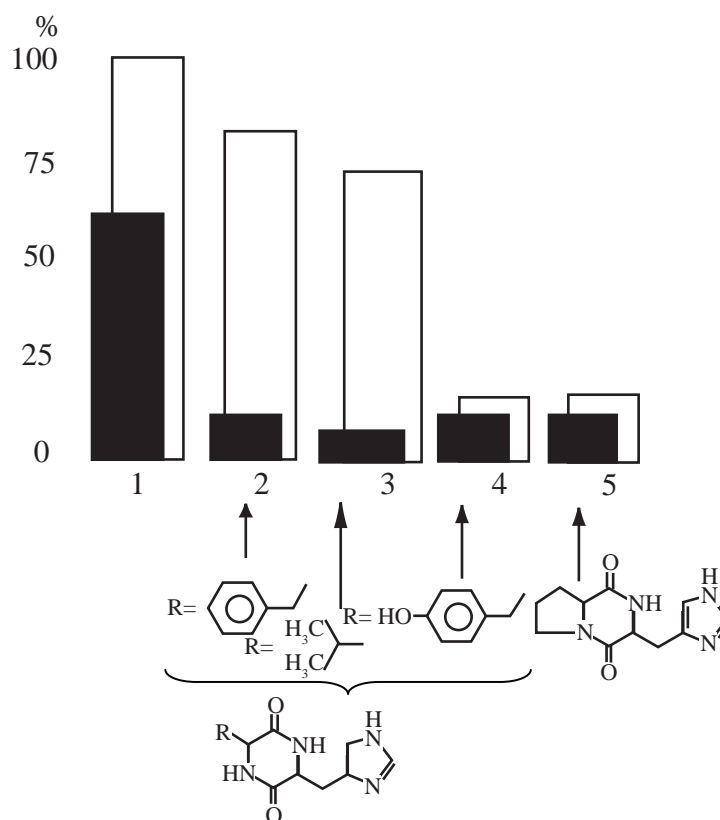
Figure IV-8. Effect of synthetic CRC on the MDA formation after 30 min (gray bars) and 3 h (wait bars) of LPO induced by ferrous ions+ascorbate additiopn to rabbit skeletal muscle sarcoplasmic reticulum. 1 - control (PIPES), cyclo-L-histidyl-phenylanaline, 3 - cyclo-L-histidyl-valine, 4 - cyclo-L-histidyl-tyrosine, 5 - cyclo-L-histidyl-proline, all in 50 mM concentration. The data are presented in % to the highest MDA level measured to 3 hr after LPO induction. Below the bars structures of the compounds tested are present.

Aruoma et al. (1989) suggested that individual histidine-containing dipeptides are unable to inactivate suiperoxide anion-radical or hydrogen peroxide at the rate appropriate to implement the antioxidant protection *in vivo*. Because mammal tissues often contain several species of histidine-containing dipeptides simultaneously, MacFarlane et al. (1991) tested the possibility of synergic interactions between individual histidine-containing dipeptides in the antioxidant activity. It was shown that such synergism indeed occurs at physiological concentrations of histidine-containing dipeptides. The ability of histidine-containing dipeptides to quench free radicals was determined by the method suggested by Fridovich (see: Fridovich, 1997). According to this method, free radicals are generated as a result of photoinduced oxidation of dianisidine sensitized by riboflavine. This process includes a series of free radical reactions starting from generation of suiperoxide anion radical of oxygen. Quenchers remove superoxide radical from the reaction medium and cause a decrease in the dianisidine consumption. For example, ascorbate, uric acid, glutathione, and ergothioneine at a concentration of 4 mM cause 23%, 31%, 74%, and 52% inhibition of this reaction, respectively.

It was found (McFarlane et al., 1991) that carnosine, histidine, N^1-methylhistidine, and anserine are capable of inhibiting this reaction within a broad range of concentrations. Probably, these compounds are non-specific rather than superoxide-specific radical quenchers. However, it is unclear if such efficiency of quenching (about 40%) is of substantial importance *in vivo*. Homocarnosine is an exception to this rule, because it inhibited or activated this reaction at low or high concentrations, respectively. For example, individual application of 10 mM homocarnosine, carnosine, anserine, and N^1-methtylhistidine induced 5%, 15%, 40%, and 50% decrease in the efficiency of oxidation of dianizidine, respectively. A combination of these compounds at the same concentration (10 mM) demonstrated pronounced potentiation of superoxide quenching. This effect may represent the synergism between carnosine and related compounds. It should be noted that the effect of synergism is the most pronounced at a concentration ratio of 8:1:1:2, which corresponds to the ratio of these compounds in the cardiac muscle.

3. Pro-Oxidant Component in the Spectrum of Biological Activity of Carnosine

It should be noted that in addition to obvious antioxidant properties, carnosine, under specific conditions, is capable of provoking the ROS generation (Ph. Hartman and Z. Hartman, 1993; Shi et al., 1993). The first (mostly, emotional) response to these findings was an attempt to explain them by a methodological discrepancy. However, in our opinion, there are objective grounds for the pro-oxidant activity of histidine-containing dipeptides. Indeed, as noted above, carnosine exerts oppositely directed effects on two radical systems: ROS (generation and transformation of superoxide anion) and active chlorine (myeloperoxidase reaction). In living cells there is a complex system of ROS generation, in which a variety of different radicals are produced simultaneously. Therefore, carnosine can shift the balance between the radical systems to either side. Moreover, carnosine undergoes metabolic transformations in cells, and the resulting histidine-containing dipeptides exert differing antioxidant effects. Therefore, being true natural antioxidants, histidine-containing dipeptides may have both antioxidant and pro-oxidant properties.

Modulation of immunocompetent cells is an important component of the biological activity of histidine-containing dipeptides. Carnosine modifies the differential blood count in animals (Nagai, Suda, 1988) and changes the proliferation activity of hemopoietic stem cells (Курелла и др., 1991; Мальцева и др., 1992). However, the carnosine-induced effects on the leukocyte system is not, probably, restricted to hemopoiesis alone, but includes the functional regulation of the immunocompetent cells and the whole immunic system.

Activated phagocytes are capable of simultaneous production of a number of free radical forms of oxygen: superoxide anion radical, hydroxide radical, peroxyl radical, hypochlorite anion, and, probably, singlet oxygen. This process proceeds against the background of active uptake of molecular oxygen. In the literature, this phenomenon is named the respiratory burst (Campbell, 1993). Because the active up-take of molecular oxygen is insensitive to inhibitors of the mitochondrial respiratory chain, it was concluded to be irrelevant to cell respiration.

The activity of the immunocompetent cells should be regulated to provide effective tissue regeneration at the inflammation focus. It seems probable that antioxidant drugs are able to

intervente in this stage of the inflammation process. We suggested that because carnosine exerts pronounced anti-imflammating, wound-healing, and immunomodulating effects, it could be a natural regulator of the leukocyte function.

To find the correlation between the leukocyte function and carnosine capacity to modulate the ROS generation, we tested the effects of this dipeptide and its derivatives on the leukocyte function *in vitro* and *in vivo*. This capacity of carnosine is of substantial importance for the immunocompetent system. The scavenger cells capable of the phagocytosis (mainly, neutrophils) attack, ingest, and destruct foreign particles using the mechanism of ROS generation. During this process, the species of active oxygen are released both in the phagocytic cavity and in the surrounding medium. The ROS release in the surrounding medium is typical of large-size foreign particles, which cannot be swallowed by the phagocyte. The generation of species of active oxygen and chlorine facilitates destruction of cell walls and membranes of microorganisms, thereby their digestion.

There are two systems of generation of free radicals in the immunocompetent cells: the NADPH-oxidase complex of the external membrane and myeloperoxidase. The NADPH-oxidase complex consists of a FAD-containing flavoprotein, a cytochrome *b* variety, and, probably, ubiquinols. Activation of immunocompetent cells by soluble (Ca-ionophors, complement components, phorbol ethers, etc.) or insoluble (bacteria, immune complexes, latex particles, suspensions of poorly soluble compounds, etc.) agents triggers a complicated cascade of enzymatic reactions including activation of adenylate cyclase and protein kinases (Владимиров, Шерстнев, 1989). On the one hand, the triggering of the cascade activates the glucose oxidation by the hexosomonophosphate pathway, which increases the intracellular content of NADPH. On the other hand, this activates the NADPH-oxidase complex, which catalyzes the following reaction:

$$2O_2 + NADPH ---\rightarrow 2O_2^{\cdot-} + NADP^+ + H^+.$$
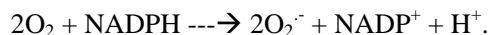
Dismutation of superoxide anion radical to hydrogen peroxide as well as other reaction of radical interconversion occurring in the presence of iron ions give rise to a broad spectrum of cellular ROS. Accumulation of intracellular hydrogen peroxide activates myeloperoxidase, which catalyzes generation of hypochlorite anion. Myeloperoxidase is accumulated in inactive phagocytes and is activated as a result of fusion of the phagocytes with phagosomes (or release of their content into the intercellular space). According to the type of stimulation and the cell fraction attacked by the phagocytes, either one of these reactions or several reactions simultaneously take place in the system.

Activation of leukocytes by $BaSO_4$ or phorbol myristate acetate (PMA) induces an abrupt increase in the chemiluminescence intensity, the shape of the burst being dependent on the inducer type. In case of PMA, the luminescence response has a lag-phase. The rate of increase of the luminescence response induced by PMA is higher than that induced by $BaSO_4$. Phorbol myristate acetate ether permeates into cells and activates protein kinase C. This enzyme activates the NADPH-oxidase complex, which provides production of superoxide anion. The insoluble inducer $BaSO_4$ mainly stimulates generation of hypochlorite anion, because ingestion of insoluble particles of $BaSO_4$ causes predominant activation of myeloperoxidase. In the presence of lucigenin, a chemiluminescent probe specifically sensitive to superoxide anion radical of oxygen, or luminol, a luminescent agent which has no selective sensitivity to

individual species of active oxygen, generation of different species of active oxygen can be detected.

The effect of carnosine and related compounds on the respiratory burst of activated leukocytes (ROS generation) was studied in our laboratory by O.V. Tyulina (Тюлина и др., 1994, 1995). The $BaSO_4$-induced activation of leukocytes gives rise to a luminescence burst, the shape of the luminescence peak detected in the presence of lucigenin being virtually indistinguishable from that detected in the presence of luminol. However, it should be noted that the luminescence response in the presence of luminol is much more intense than in the presence of lucigenin, thus to obtain the luminescence response of about the same amplitude, the amount of cells in the latter case should be ten times higher than the former system. In contrast to lucigenin, which is able to interact mainly with superoxide anion radical, luminol in this system can interact with other radicals, including the most abundant hypochlorite anion. Therefore, luminol is much more effective stimulator of luminescence in this system than lucigenin.

The addition of SOD caused complete or 70% inhibition of cell response detected in the presence of lucigenin or luminol, respectively. In the presence of luminol, imidazole (10 mM) had virtually no effect on the cell response, whereas the carnosine-induced effect depended on the state of the cells. It ranged from 10-15% activation in freshly isolated cells to 20-30% inhibition in cells stored for longer than 3 h after isolation. In the presence of lucigenin, imidazole (10 mM) was also virtually inactive, whereas carnosine prevented inhibition of chemiluminescnce by SOD addition (Тюлина и др., 1994).

These experiments showed that carnosine exerts a dual effect. In addition to neutralization of singlet oxygen and hypochlorite anion, which was previously reported by Dahl et al. (1988) and Formazyuk et al. (Формазюк и др., 1992), carnosine was shown to activate the system of superoxide anion radical generation. The variability of the carnosine-induced influence on the cells activation by $BaSO_4$ in the presence of luminol can be attributed to the fact that ageing of cells is accompanied by a trend toward an increase in the relative contribution of hypochlorite anion to the total pool of radicals generated by leukocytes.

The chemiluminescence assay also revealed that carnosine derivatives differ significantly from each other in the efficiency of inhibition of cell response (Тюлина и др., 1994). Anserine and histidine are the most and the least effective inhibitors, respectively. The inhibition efficiencies of homocarnosine and taurine are comparable. Anserine exerts a dual effect: in addition to neutralization of reaction product, it inhibits the enzyme itself. Carnosine, homocarnosine, and histidine (in the presence or in the absence of β-alanine) inhibit this reaction by neutralizing the reaction products. These results show that replacement of the β-alanyl residue in carnosine molecule by the γ-aminobutyric acid residue causes a decrease in the efficiency of the function of neutralization of hypochlorite anion; acetylation inhibits this function completely; whereas methylation increases this function significantly, providing the dipeptide with additional functions (Тюлина и др., 1994).

When carnosine had been infused to experimental animals, the activation component of its effect disappeared synchronously with the degradation of the dipeptide in blood, whereas inhibition of respiratory burst of leukocytes persisted much longer than the period of clearance of carnosine from the blood stream (24 h) (Гуляева и др., 1989a). These results represent an important direction in the modulation activity of carnosine in the system of

cellular immunity. Elucidation of specific mechanisms of its activity is of particular importance in the context of progressively increasing pharmacological applications of carnosine.

The effects of carnosine-induced modulation on the ROS production by leukocytes and stabilization of cell membranes can underlie the modulation effect of carnosine in the system of cellular immunity (Boldyrev et al., 1995). The wound-healing activity of carnosine can also be explained by the modification of activity of leukocytes and macrophages.

The pro-oxidant activity of carnosine was also observed by Onufriev et al. (1992). Single intraperitoneal injection of 0.45 mM carnosine activated macrophages and increased their cytostatic and phagocytic functions in mice. In the cytostatic test *in vitro*, incubation of macrophages in the presence of carnosine caused a 67% inhibition in the growth of ascitic tumoral cells against the background of a 45% inhibition by control macrophages. The stimulation activity of carnosine was higher than that of 4-methyluracil. It is well known, that magrophages implement these functions by rapid generation of superoxide anion-radical and hydrogen peroxide.

Other possible mechanisms of connection between the carnosine-induced effects on LPO and system of cellular immunity are also discussed in the literature. The effect of carnosine on the lipoxygenase pathway of oxidation of arachidonic acid is a typical example (Figure IV-9). Taking into consideration that the cascade of reactions of oxidation of arachidonic acid is an important source of biologically active lipoperoxy radicals and assuming that carnosine can interact with lipoxygenase during some pathological processes in human body, it should be concluded that these reactions can provide important information about the mechanisms of carnosine activity *in vivo*.

Oxidation of arachidonic acid is an important source of such biologically active regulators of metabolism as *prostaglandins* and *thromboxanes* (cyclooxygenase pathway) or *leukotrienes* and *lipoxins* (lipoxygenase pathway). For the first time, 15'-lipoxygenase of reticulocytes has been described in 1979. In addition to oxidation of arachidonic acid to leukotrienes, this enzyme catalyzes effective production of lipoxin B_4 without involvement of other lipoxygenases. Activation by lipoperoxides is a typical feature of the lipoxygenase reaction. None of other natural substances is presently known to activate this reaction, whereas it is inhibited by a variety of different compounds, including antioxidants. Antioxidants are effective inhibitors of this reaction, because oxidation of unsaturated fatty acids is a free radical process.

Within the concentration range from 0.25 to 0.65 mM, carnosine activates 15'-lipoxygenase, but at concentrations above 1 mM, activation is replaced by inhibition. Although histidine does not activate lipoxygenase, its inhibition effect in a concentration of 8 mM is comparable to the inhibition effect of the same concentration of carnosine. However, 20 mM carnosine inhibits the lipoxygenase reaction completely, whereas 20 mM histidine causes only a 60% inhibition of this reaction.

It should be noted that the direct antioxidant effect of carnosine in the nonenzymatic systems of peroxidation *in vitro* is also observed at concentrations above 10 mM. The antioxidant effects of carnosine *in vivo* described in the literature were observed at one to two orders of magnitude lower concentrations of carnosine (Гуляева и др.,1989a). For example, intraperitoneal injection of 0.2 mg/kg carnosine to experimental animals exposed to stress

A

B
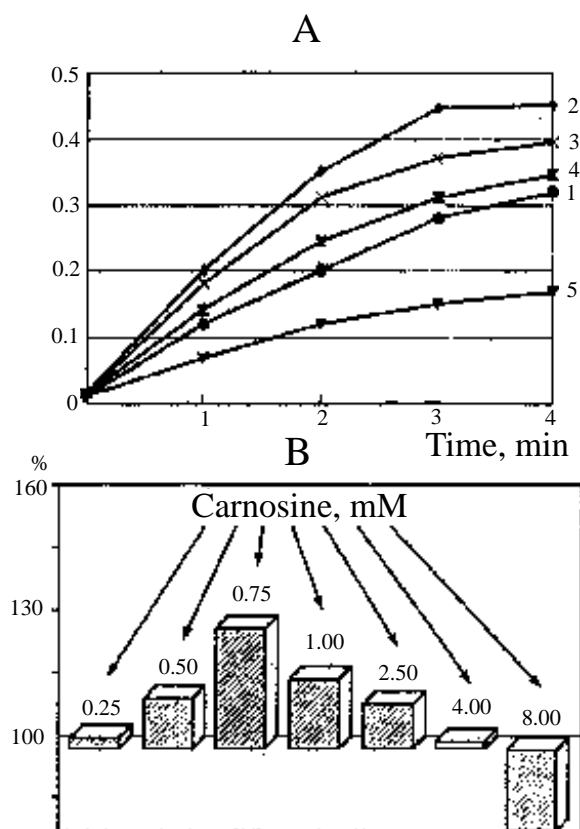
Figure IV-9. Modulation of rabbit platelet 15'-lipoxygenase by carnosine. A - time dependence (OD_{235}) in control (1), or in the presence of 1, 2, 4, and 8 мM carnosine (2, 3, 4, and 5 respectively), B - relative rates of the activity measured in the stationary period of the reaction (% to control rate).

conditions caused a statistically significant decrease in the rate of accumulation of LPO products in blood and brain tissues. In LPO models *in vitro*, this dose of carnosine exerted virtually no inhibition effect.

The concentrations of carnosine that activated lipoxygenase in the experiments described above are comparable to the carnosine concentration in blood serum but they are 10-20 times less than the carnosine concentration providing direct antioxidant effect *in vitro*. Therefore, it can be suggested that carnosine is a natural regulator of lipoxygenase, and its administration *in vivo* would provide a two-phase response. The first stage is inhibition of blood cell lipoxygenase. As carnosine undergoes hydrolysis catalyzed by carnosinase, its content goes down, and inhibition at this stage is replaced by activation. Thus, the carnosine-induced effects *in vivo* are not restricted to changes in the content of peroxides in tissues alone.

Possible interaction between carnosine and eicosanoids in regulation of biological processes is of substantial interest. It should be noted in this context that lipoxin B_4, a product of the lipoxygenase reaction of oxidation of arachidonic acid, demonstrates biological activity at very low concentrations. Indeed, the addition of 10^{-9} M lipoxin B_4 affects proliferation and differentiation of granulocyte-monocytic colony-forming units. Therefore, this substance is a

strong biological effector, which regulates maturation and abundance of leukocytes (Попов и др., 1989).

It was shown that carnosine increases activity of bone marrow stem cells in rats, thereby increasing the radiation resistance. However, until recent time, the mechanism of this process remained obscure. The results obtained in our laboratory suggest that appearance of low concentrations of carnosine in blood against the background of induction of the lipoxygenase-catalyzed cascade of reactions of oxidation of arachidonic acid, which is typical of many pathological states, provides appropriate conditions for mobilizing immature white blood cells and multilateral protection against adverse factors (Стволинский и др., 1996).

Thus, the conditions in which carnosine modulates the systems of ROS generation were determined. In addition to a comprehensive description of the biological effects of carnosine, these findings allowed apparent contradictions between immunomodulation and antioxidant effects to be explained. The difference between the carnosine-induced effects in young and old animals can also be understood (Мальцева и др. 1992). The results discussed in this chapter also suggest that the evolution of carnosine transformation in tissues is consistent with the evolution of oxidative metabolism and specific features of generation and conversion of different species of active oxygen.

4. PROTECTION OF BRAIN NEURONS AGAINST OXIDATIVE STRESS

The ability of carnosine to protect the ovary cells of Chinese hamster from the damage inflicted on their chromosomes induced by hyperoxia (80-90% O_2) has been discovered by Gille et al. (1991). Of a large variety of antioxidants tested, only carnosine was able to reduce significantly the number of chromosomal breaks. It should be noted that thus far carnosine is the first agent capable of providing protection against normobaric hyperoxia. This ability of carnosine is of considerable interest for studies of the mechanisms of protecting phenomenon.

It was shown in recent works (McFarland, Holliday, 1994; Hipkins et al., 1995; Kanta et al., 1996; Holliday, McFarland, 1996; Hipkiss, 1998) that carnosine inhibits non-enzymatic glycosylation of proteins (glycation). The terminal amino group of the side chain of lysine in protein molecule (especially in a combination Lys-Pro) can interact with the aldehyde of linear saccharide molecules giving rise to a Schiff's base, which is then transformed into carbonyl bond as a result of the Amadory's rearrangement (see below). Free amino group of carnosine (β-Ala-His, which is so close to Lys-Pro) reacts with aldehydes much faster than that of lysine. Therefore, carnosine prevents lysine from aldehyde attack. There is a large body of evidence that aging cells accumulate progressively increasing amounts of abnormal proteins. Probably, the products of advanced glycation of these proteins may contribute to the aging phenotype (Holliday, 1996).

Experiments with an HFF-1 human cell culture and an MRC-5 culture of hamster embryonic cells showed that the addition of high concentrations of carnosine (10-50 mM) causes an aging delay and makes the aged culture "younger" (McFarland, Holliday, 1994). In the presence of carnosine, the morphology of cultures with a large number of passages is maintained at a less aged level, as compared to the morphology of control cultures subjected to normal (physiological) aging (for the description of normal aging of cell cultures see:

Hayflick, Moorhead, 1988). The morphology typical of aging phenotype was rapidly restored in cells with large number of passages if these cells were transferred from the carnosine-containing culture medium into medium without carnosine.

Thus, carnosine causes a delay in the onset of morphological changes inherent in aging cells and even makes the aged cells "younger". Neither D-carnosine nor homocarnosine, anserine, or β-alanine demonstrate similar activity. Carnosine conserves native cells by affecting protein metabolism. Probably, carnosine is able to reduce the content of abnormal proteins in cells. Preliminary experiments revealed that protein metabolism in cell cultures grown in the presence of carnosine are significantly modified (McFarland, Holliday, 1994; Kanta et al., 1996). Whatever are the initial causes of aging, the final result of this process is the loss of cell homeostasis. Cell homeostasis exerts a multiple impact on phenotype, and protective effect of carnosine may indicate that this dipeptide is capable of supporting cell homeostasis.

Changes in the oxygen supply of tissues (hypoxia or hyperoxia) are the main physiological mechanism of peroxidation initiation (Владимиров, Шерстнев, 1986). Individual tissues differ from each other in the LPO initiation reactions. Besides, changes in normal physiological states or development of pathology may affect the LPO initiation reactions.

For example, the rate of initiation of free radical reactions is noticeably increased at the early stages of malignant degeneration, whereas the late stages of the degeneration are characterized by inhibition of free radical reactions. Lipid peroxidation is a probable mechanism of the damage induced by UV or ionizing radiation (Владимиров, Арчаков, 1972). The damaging effect of mutagenic agents on genes and cell cycle is usually mediated by generation of hydroxide radical (Gille et al., 1991).

Acute stress causes considerable changes in the level of free radical oxidation, the first and the second phases of the stress being accompanied by inhibition and activation of oxidation, respectively (Гуляева и др., 1989а). The partial pressure of oxygen in intensely working muscles is subject of considerable variation. The extent of the oxidative damage of biomacromolecules in such muscles is directly proportional to the intensity of muscular work.

As for brain, the content of presently known natural antioxidants in skeletal muscles is rather low, and enzymes of antioxidant protection are relatively inactive in this tissue (Kagan, 1988). This brings up the problem of the mechanism of the antioxidant protection of muscular tissue *in vivo* and possible contribution of carnosine to this protection.

Carnosine protects membrane lipids in high concentrations, but the carnosine concentration in living objects is high indeed. The first experiments showed that the biological significance of carnosine as an antioxidant is beyond doubt. Indeed, the $K_{0.5}$ value for the carnosine-induced inhibition of LPO is 2-5 mM. In skeletal muscles carnosine often coexists with anserine, and these dipeptides together make up to 5-20 mM (Crush, 1970) or 7-25 mM (Северин и др., 1984; Miller et al., 1992) The appearance of carnosine in embryonic muscles strictly correlates with appearance of contractility, i.e., it appears at the late stages of embryonic development coinciding with the reflex arch formation (Вульфсон, 1962). It is intense contraction activity that presents the hazard of exposure of muscular fibers to free radical products. Histidine-containing dipeptides could be regarded as elements of the antioxidant protection.

The dynamics of the survival rate of brain neurons in patients with progressive parkinsonism can be considered as an illustration of possible protection of carnosine-related

dipeptides. A non-compensated increase in the ROS content is a major cause of cell death in this disease. Individual brain structures differ from each other in their sensitivity to ROS (Figure IV-10). The neurons enriched with neuromelanin are more sensitive than neurons depleted with this substance (Hirsh et al., 1988). The brain structures enriched with histidine-containing dipeptides contain neurons with the highest resistance to ROS (see Part I for the data on carnosine and homocarnosine distribution in brain structures).
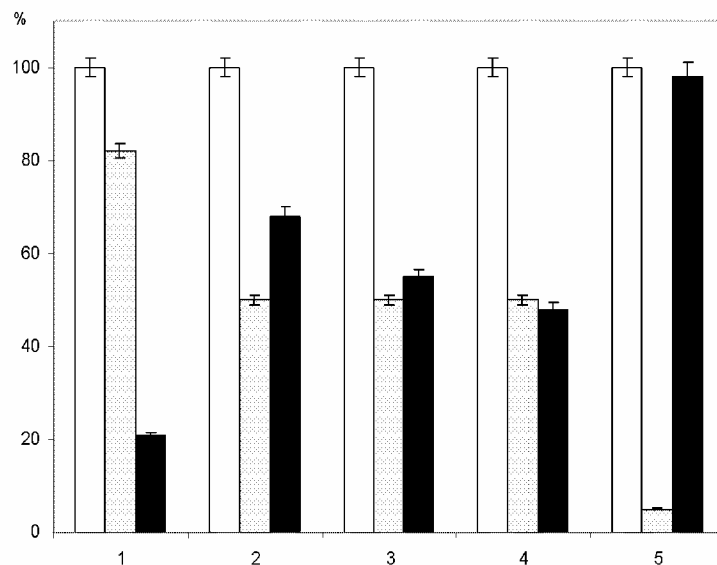
Figure IV-10. Dopaminergic neurons in parkinsonian brain (Hirsch et al., 1988; Javoy-Agid, 1992). 1 - compact part of substancia nigra, 2 - lateral part of substancia nigra, 3 - peri- and retrorubral areas, 4 - ventral area, 5 - central part of gray matter. Bars correspond to the amount of normal (dark) and melanized (wait) neurons in % to total amount of neurons.

Disorders of cell homeostasis are accompanied by a rapid increase in the rate of ROS generation and cell death of oxidative stress. Depending on a variety of factors (including the type of ROS generated by the system), the cell death may proceed as necrosis or apoptosis (Figure III-2). It is evident that the outcome of these events is quite adverse, and they may aggravate the neurodenerative disease (parkinsonism, Alzheimer's disease), aging processes, and ischemia. As noted above, excitable cells are extremely vulnerable to oxidative stress. However, lack of adequate models makes it difficult to study the molecular mechanisms of these processes.

Methodological approaches to studies of individual ischemic neurons were suggested recently (Hori, Carpenter, 1994; Oyama et al., 1995). In these models, the ischemic damage to individual neurons is induced by activation of receptors of excitotoxic mediators. Although cerebellum of young (10- to 12-day-old) rats contains sufficiently mature cells, the connections between the cells are no stable enough. Enzymatic treatment of slices of this tissue gives a suspension of individual neurons, which remain alive for at least several hours after isolation (Boldyrev et al., 1997c).

These neurons can be activated by NMDA, kainate, or other ligands of glutamate receptors. If these neurons are preliminarily loaded with ROS-sensitive fluorescence probes,

like DCF the content of generated free radicals can be measured (Table III-4). It was shown in our experiments that carnosine or its derivatives (homocarnosine, anserine, and acetylcarnosine, 10 mM each) reduce the sensitivity of the cells to the activation effects of glutamate analogs (ligands of glutamate receptors). The amplitude of the NMDA- or kainate-induced increase in the fluorescence yield of the probe in control neurons was significantly higher than in cells pre-incubated for 1 h in the presence of carnosine or other histidine-containing dipeptide (Figure IV-11).
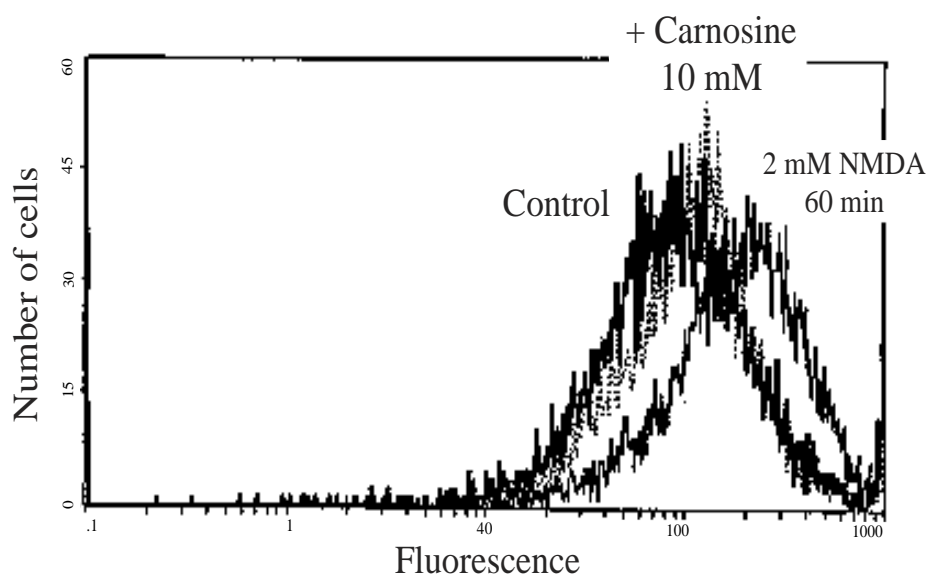
Figure IV-11. DCF fluorescence of cerebellum neurons under control conditions or after 60 min exposure to 2 mM NMDA with or without pre-loading of the cells with 10 mM carnosine (as indicated).

It is interesting to note that the population of live neurons, which were not stained with *propidium iodide* (PI-negative neurons) contained a sub-population of cells with enhanced content of ROS, as measured by the DCF fluorescence (Figure IV-12). According to their size, these cells were basically shrinked rather than swollen. It seemed that they were on the verge of apoptosis. Indeed, incubation with NMDA caused an increase in this fraction. If the neurons were pre-loaded with carnosine, the fraction of apoptotic cells was not observed at all (Figure IV-12). These results showed that carnosine reduces the content of ROS in cells, thereby preventing development of apoptosis. This effect definitely deserves additional analysis.
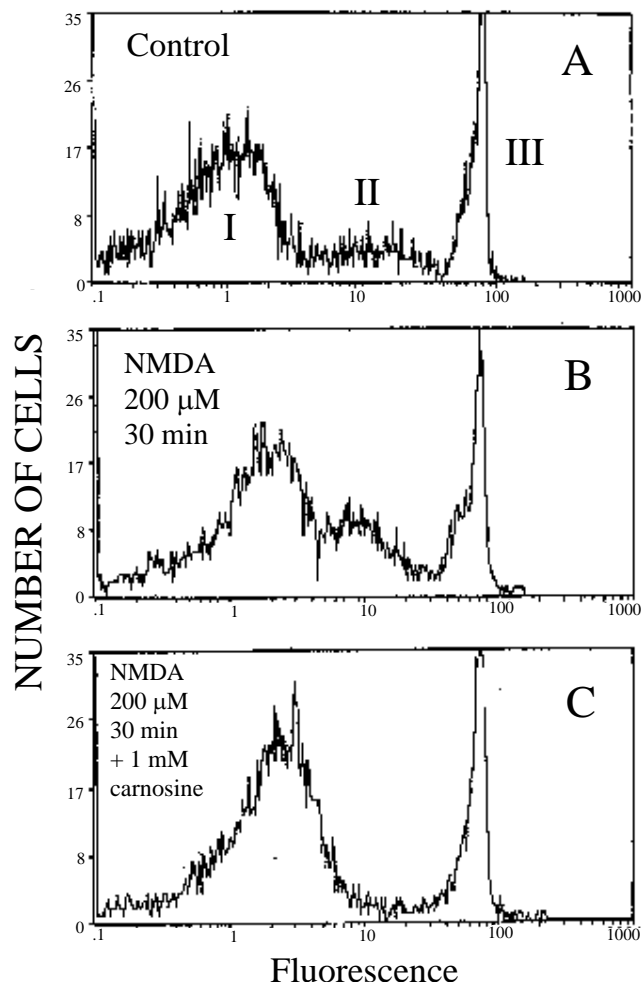
Figure IV-12. Distribution of the cells between different sub-populations in control (A) and after their incubation with NMDA (B and C). In C neurons preincubated with 10 mM carnosine were used. I and II - viable sub-populations non-sensitive to PI, having low (I) and high (II) level of intrinsic DCF fluorescence, III - sub-population of non-viable (necrotic) PI-sensitive cells.

In addition to the ROS content in each individual cell of given population, the method of flow cytometry allows the type of the cell death induced by various factors to be identified. This can be achieved by using different fluorescent dyes (Oyama et al., 1995). Necrotic and apoptotic cells were identified by staining with propidium iodide and *annexin V*, respectively.

A typical pattern of distribution of neurons is shown in Figure IV-13 (Boldyrev et al., 1997c). The whole population of neurons falls into four sub-populations: intact cells (left bottom quadrant), necrotic cells (left top quadrant), cells at early stages of apoptosis (pre-apoptotic, right bottom quadrant), and virtually dead cells (right top quadrant).
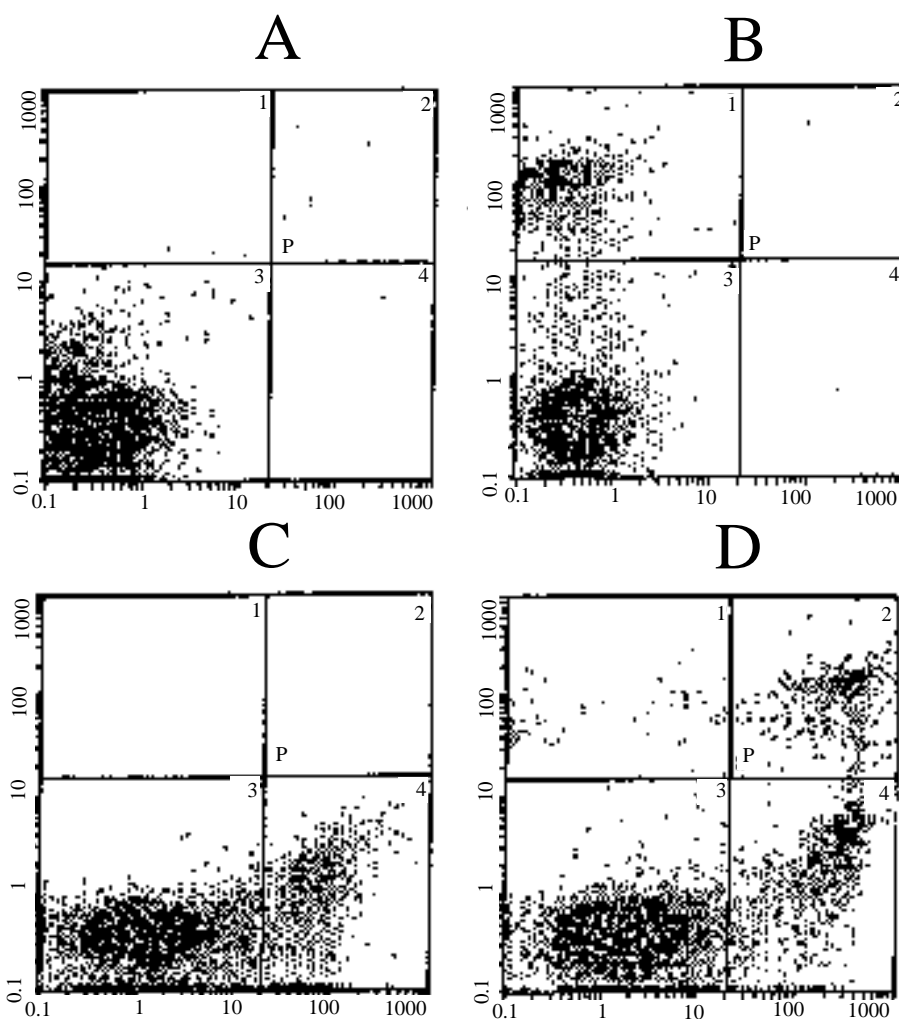
Figure IV-13. Distribution of the neurons among different sub-populations using different dyes in flow cytometric study. A - no dye added, B - PI is added, C - the same with annexin V, D - in the presence of both dyes. Ordinate - intensity of PI fluorescence, abscissa - intensity of the fluorescence of annexine V labeled with FITS.

Induction of free radical reactions increased the death rate of cells incubated with NMDA or kainate. Kainate was less toxic for neurons than NMDA, but in both cases the death rate was determined by the concentration of the excitotoxic agent and duration of its action. The fractions of neurons died of necrosis and apoptosis before and after their incubation with NMDA are shown in Figure IV-14. It is well established that activation of glutamate receptors of brain neurons is a mechanism for triggering cell death under unfavorable conditions. Because carnosine and other histidine-containing dipeptides inhibit the NMDA-induced fluorescence of neurons, it was interesting to assess the effects of these dipeptides on the resistance of neurons to necrosis and apoptosis.
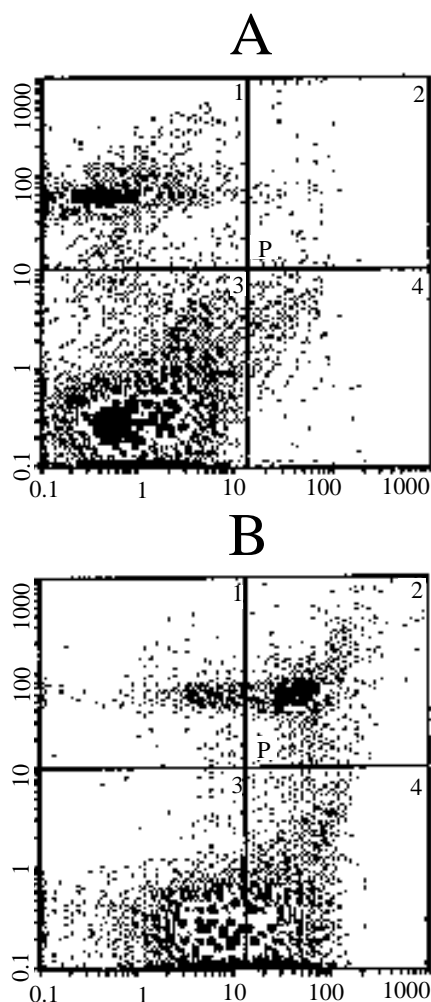
Figure IV-14. Distribution of the cells between of necrotic (1+2), viable (3) and apoptotic (4) sub-populations before (A) and after (B) 3 hr incubation with 1 мM NMDA. Other descriptions as in Figure IV-13.

It was found that addition of carnosine prevents the NMDA-induced apoptosis, the carnosine-induced inhibition of ROS generation and prevention of apoptosis being observed within the same range of concentrations of carnosine (Table IV-3). Carnosine also caused a decrease in the rate of the kainate-induced apoptosis of neurons (Figure IV-15). The carnosine-induced effect on the necrotic death of neurons was slightly less pronounced, but it can also be explained by the antioxidant activity of the dipeptide. However, although homocarnosine and acetylcarnosine reduced the ROS-induced fluorescence, neither of these histidine-containing dipeptides prevented apoptosis. Moreover, under certain conditions they even increased it (Figure IV-16). Thus, it became evident that in addition to ROS, histidine-containing dipeptides may affect other signal mechanisms in cells.

Table lV-3. Distribution of cerebellar granular cells (%) among different sub-populations under exposure to NMDA or kainite.

Conditions	DCF-positive cells I	PI-positive neurons II	PI-positive neurons III
Control	66.7 ± 2.1	16.7 ± 1.5	16.7 ± 1.2
NMDA, 2 mM, 60 min	47.0 ± 12.3	28.0 ± 9.6	24.0 ± 2.6
+ carnosine, 1 mM	76.2 ± 12.4	4.3 ± 1.4	19.3 ± 4.9
Kainate, 2 mM, 60 min	43.0 ± 14.1	34.8 ± 10.8	22.0 ± 7.6
+ carnosine, 1 mM	79.6 ± 9.8	3.0 ± 0.6	17.8 ± 5.8
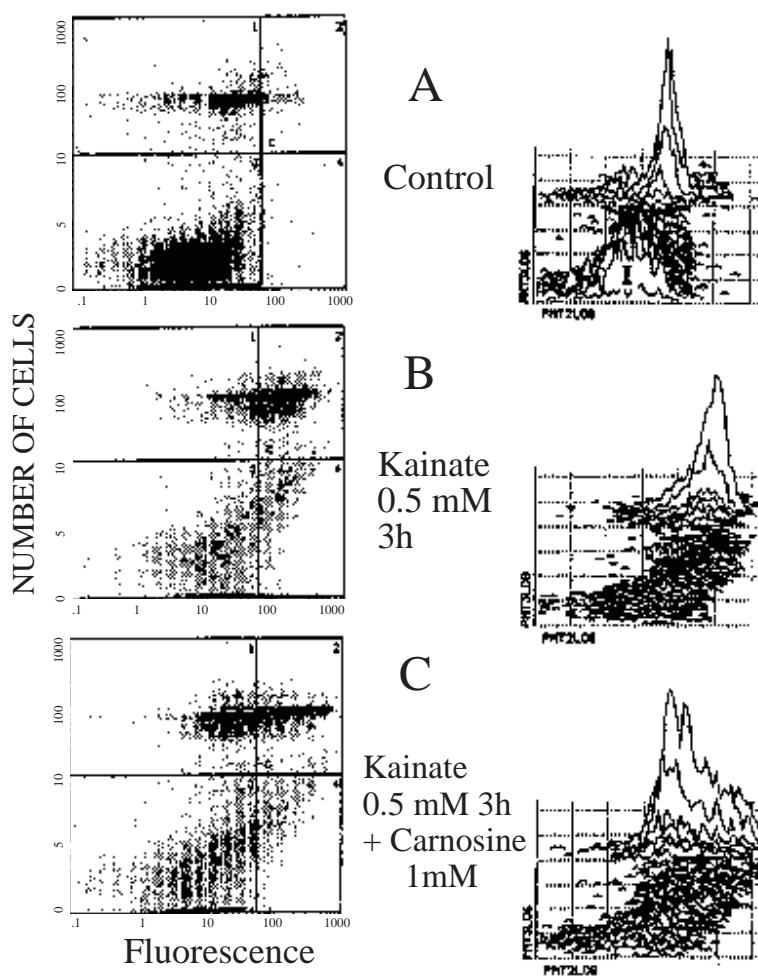
Figure IV-15. Effect of carnosine on excitotoxic action of kainate on cerebellum granule cells pre-loaded with DCF. A - control (distribution of the cells onto two sub-population is seen - viable, PI insensitive, and necrotic, PI-stained); B - the same after 30 min exposure to 0.5 mM kainate (the percentage of viable cells is decreased while their fluorescence rises up); C - the same with the cells pre-loaded 1 hr with 10 mM carnosine (carnosine prevents both increase the number of dead cells and decrease the level of DCF signal). Left part - dot plot, right part - tri-dimentional picture.
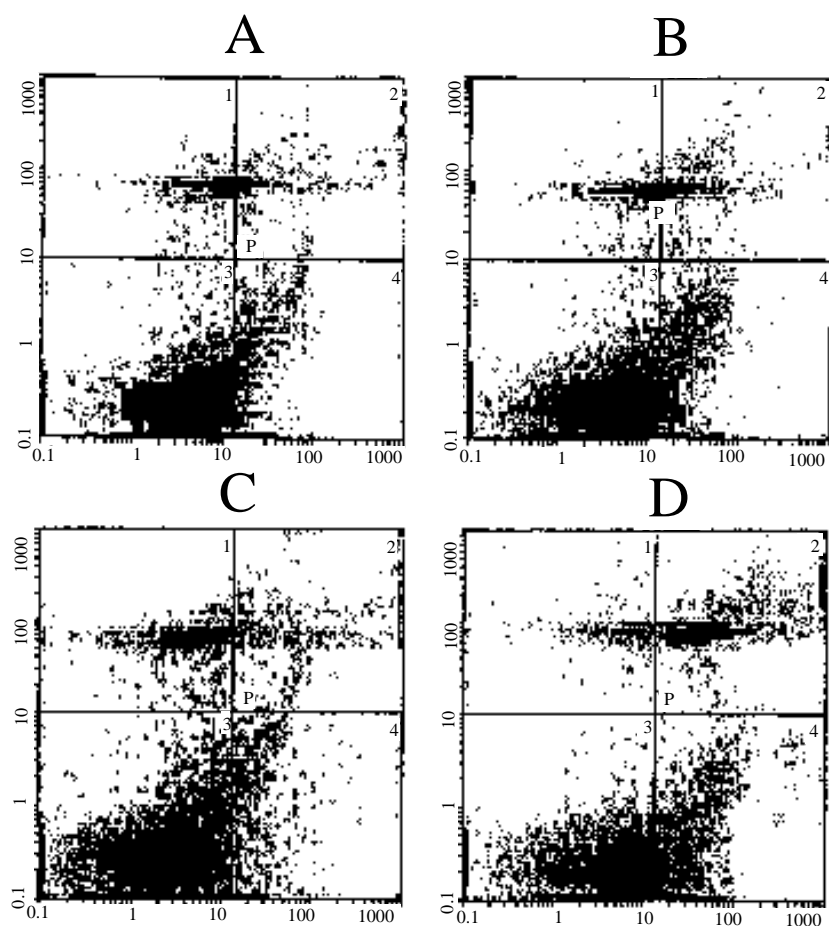
Figure IV-16. Distribution of rat cerebellar granule cells between different sub-populations in the absence (A) or in the presence (B-D) of NMDA (100 μM, 3 hr) with no protection (B) or in the presence of carnosine (C) or acetylcarnosine (D). Descriptions as in Figure IV-13.

The correlation between the efficacy of protection of neurons from apoptosis and antiradical properties of biologically active substances is ambiguous. We compared efficacy of carnosine, *taurine*, and *melatonin* in the neuron protection against oxidative stress induced by various factors. According to the literature, the three biologically active compounds are hydrophilic antioxidants specific for nerve tissue. It is seen from Table IV-4 that only carnosine exerts the two effects simultaneously: it reduces the ROS content and protects cells from death. Taurine also protects cells but has virtually no effect on the ROS level, whereas melatonin may even increase ROS production. The concentrations of the protectors used in these experiments fell within normal physiological ranges. In all cases, the carnosine-induced protection of neurons was the most pronounced.

Table lV-4. Antioxidant defense of cerebellum neurons against oxidative stress.

Conditions	ROS, arb. units	Viability, %
Control	86 ± 7	75 ± 0.6
Kainate (0.5 mM)	134 ± 0.4	71 ± 1
+ Carnosine 2.5 mM	102 ± 4	87 ± 2
+ Taurine 1 mM	139 ± 9	83 ± 3
+ Melatonin 0.1 mM	156 ± 6	83 ± 1
SIN-1 (100 μM)	225 ± 5	62 ± 2
+ Carnosine 2.5 mM	162 ± 15	76 ± 2
+ Taurine 1 mM	243 ± 19	65 ± 2
+ Melatonin 0.1 mM	422 ± 44	65 ± 1
NOC-7 (100 μM)	470 ± 3	44 ± 7
+ Carnosine 2.5 mM	374 ± 3	69 ± 2
+ Taurine 1 mM	461 ± 8	58 ± 1
+ Melatonin 0.1 mM	433 ± 31	56 ± 1

Experimental models of cell death induced by *amyloid-β*, a factor responsible for the neurotoxic effect in patients with Alzheimer's disease, revealed different mechanisms, which are insensitive to carnosine. Amyloid-β induces cell necrosis, which develops without increase in the level of free radicals. These effects of amyloid-β were observed in our experiments with thymocytes (Figure IV-17) and neurons (Figure IV-18). Although carnosine inhibits the ROS level increase in the presence of amyloid-β, this does not protect neurons from the toxic effect and death.
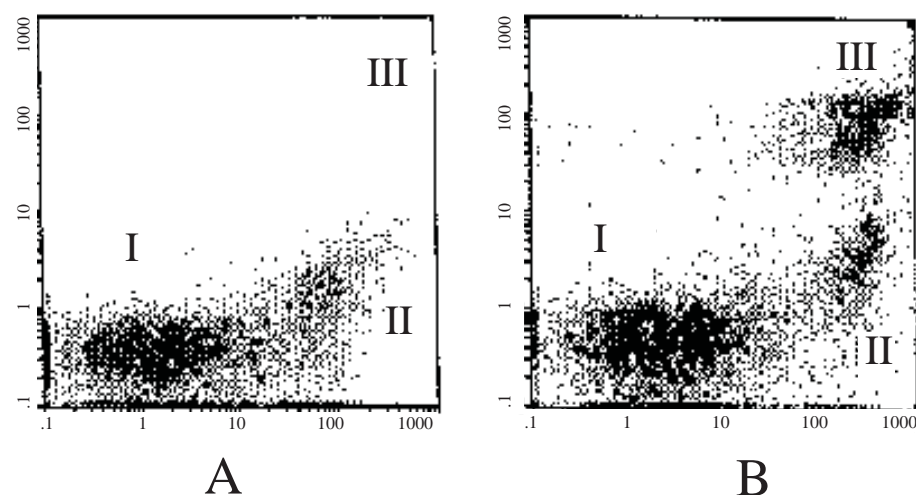
Figure IV-17. Effect of amyloid-β on distribution of thymocytes between viable (I), apoptotic (II), and necrotic (III) sub-populations. A - control, B - after 1 hr exposure to amyloid-β (1 μg/ml medium). Vertical axis - PI fluorescence, horizontal axis - FITS-annexin V fluorescence.
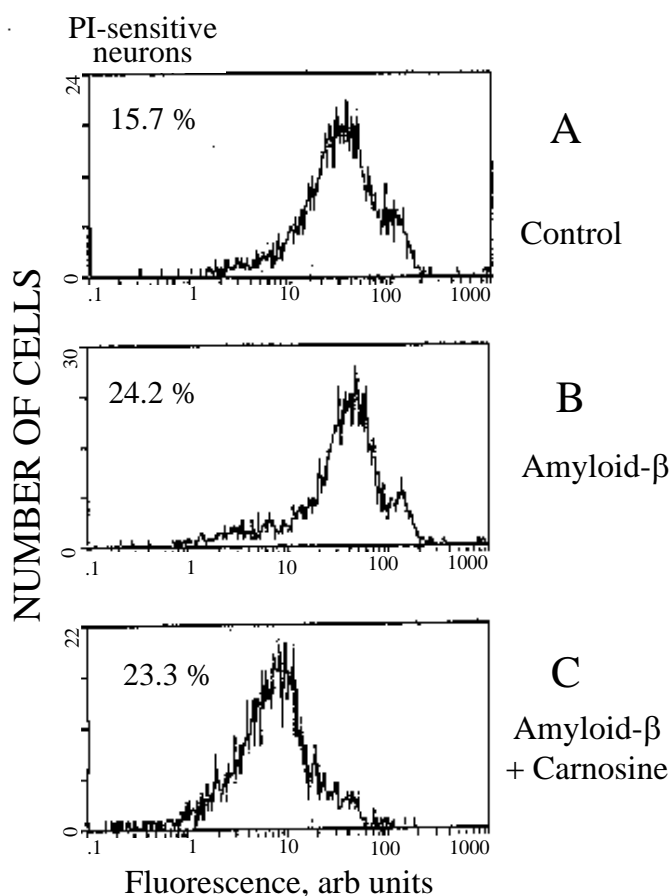
Figure IV-18. Effect of amyloid-β (1 μg/ml) on the fluorescence and viability of rat cerebellum neurons. A - control, B - 60 min after incubation with amyloid-β, C - the same with neurons pre-loaded 1hr with 10 mM carnosine. Position of maximum relative to horizontal axis reflects the mean fluorescence value, % of necrotic cells is noted at the upper left of each picture.

Two main types of natural death of cells - apoptosis and necrosis - differ significantly from one another in mechanisms and aftereffects on the surrounding tissues. Apoptosis is a programmed cell death (Скулачев, 1996). In contrast to necrosis, apoptosis is a normal physiological process that offsets cell proliferation. Apoptosis is initiated for various reasons, such as when a cell is no longer needed within the body or when it becomes a threat to the health of the organism. The apoptotic cell macromolecules can be consumed by the neighboring cells as a construction material. Apoptosis is a safe death of cells, which is not accompanied by the inflammation reaction. During maturation of the mammalian brain, many of embryonic cells (about 40% of the initial neurons that were not incorporated into the integral network of brain) are sacrificed to create the final form. Apoptosis allows a great amount of biological construction material to be saved during the life cycle metamorphosis of insets.

The importance of regulation of apoptosis is beyond doubt. In brain it is as important as in other tissues. Therefore, the ability of histidine-containing dipeptides to control the

processes of apoptosis and necrosis is of significant importance for regulation of vital activities of neurons. Specific features of carnosine and homocarnosine activity can modify neuronal resistance to different types of cell death. The neurons containing carnosine or homocarnosine are resistant or vulnerable to apoptosis, respectively.

This hypothesis is indirectly supported by the fact that during ontogeny histidine-containing dipeptides emerge when the brain formation has been completed, and the number of neurons in brain attains the level typical of adult organism. However, the agents regulating the content of free radicals are always in urgent need in the neuronal tissue. That is why many age-related and neurodenerative processes in brain (e.g., Alzheimer's disease) develop against the background of inhibited anti-radical protection and enhanced content of ROS. These processes are also characterized by an increased rate of apoptosis causing too many neurons to die.

5. BIOLOGICAL SIGNIFICANCE OF CARNOSINE METABOLISM IN EXCITABLE TISSUES

Exhausting muscular work is accompanied by accumulation of LPO products and activation of myeloperoxidase in muscular tissue (Морозов и др., 1991; Novelli et al., 1990). Therefore, possible radical-quenching activity of carnosine is of particular importance (Болдырев, 1986; Boldyrev, 1993).

The biological significance of interconversion between different histidine-containing dipeptides during ontogeny and their predominant localization in excitable tissues (nerves and muscles) against the background of their absence in liver, kidney, blood, etc. (i.e., the tissues enriched with carnosinase, which prevents accumulation of these dipeptides) are the main points that should be explained by this hypothesis.

In our experiments conducted in 1986-1990, we compared intensity of LPO reactions and activity of Ca-pump in membrane preparations of sarcoplasmic reticulum. It was found that the same concentration of LPO products accumulated in cells could be either toxic or nontoxic for protein functions in the absence or in the presence of carnosine, respectively (Дупин и др., 1984; Boldyrev, Severin, 1990, 1991; Boldyrev, 1993). Therefore, in addition to antioxidant activity, carnosine is able to stabilize macromolecules exposed to unfavorable environment. Later, this conclusion was supported in other models, including oxidation of fatty acids (Kohen et al., 1988), nucleotides (Hartman et al., 1990), and cytochrome P_{450} (Антипов и др., 1989); non-enzymatic glycosylation of proteins (McFarland, Holliday, 1994); and oxidative modification of nucleic acids (Gille et al., 1991). It was also found that carnosine prevents macromolecules from damaging effects of copper ions (Chikira, Mizukami, 1991).

Thus, there is no unambiguous correlation between various useful properties of carnosine and specific localization of histidine-containing dipeptides in body tissues of various levels of structural organization and functional intensity. By analogy with the pH-buffering capacity of histidine-containing dipeptides, which varies significantly in accordance with tissue functions and pK_a value of dipeptides (see Table III-1), it can be suggested that individual histidine-containing dipeptides differ from each other in their ability to serve as ROS buffers during induction of free radical reactions.

Carnosine and related compounds differ from each other in biological activity and distribution in tissues and animal species. Individual histidine-containing dipeptides exert oppositely directed effects on certain biochemical reactions. Some biological processes are affected only by individual dipeptides.

As noted above, L-carnosine causes a delay in the senescence of a culture of diploid human fibroblasts, whereas neither D-carnosine nor homocarnosine, anserine, or β-alanine demonstrate similar activity (McFarland, Holliday, 1994). According to the chemical structure, D-carnosine, homocarnosine, and anserine should be as active as carnosine itself, provided that they are taken up by the cells. However, this particular effect is inherent only in carnosine. Probably, this indicates that cell functions are regulated by products of carnosine metabolism rather than the dipeptide itself.

Johnson and Hammer (1989) showed that carnosine and anserine modulate the inhibitory effect of *calpastatin* on *calpain II*. Calpains (Ca-dependent serine proteinases) play an important role in degradation of skeletal muscle proteins. Both carnosine and anserine activate muscular calpain II (Ward, Preedy, 1992), but the former activates calpastatin, whereas the latter inhibits this enzyme. Therefore, the replacement of carnosine by anserine during ontogeny may accelerate the protein degradation induced by calpain II and adapt the "protein portrait" of the cell to the development demands.

Although the structures of carnosine and anserine are very much similar, these dipeptides exert remarkably opposite effects on Ca^{2+} accumulation by isolated mitochondria. These effects are observed in the presence of physiological concentrations of dipeptides inherent in mammal tissues. Anserine activates and carnosine inhibits accumulation of Ca^{2+} by mammalian mitochondria (Figure II-11). Perhaps, these dipeptides regulate the affinity of a mitochondrial calcium transporter for Ca^{2+} (Daniel et al., 1992).

The functional difference between the molecules containing free or methylated imidazole ring was also demonstrated in some histidine-containing protein hormones (Giralt et al., 1986). Methylation of the histidine imidazole ring at N^1 position (as in anserine) or at position N^3 (as in ophidine) in thyrotropin releasing hormone (TRH) caused activation or almost complete inhibition of the hormonal activity, respectively (Table IV-5).

Table lV-5. Interrelation between tautomerism and biological activity of native thyroliberine (A), [5-nitro-*L*-histidine]^2-thyroliberine (B) and its N$_1$- (C) and N$_3$- (D) methylaed derivatives (Giralt et al., 1986).

Compounds	Substituting group	% of the molecules with the substituting group in the N_1 position	% of the molecules with the substituting group in the N_3 position	Biological activity
A	H	82%	18%	100%
B	H	0%	100%	0.01%
C	N_1-CH$_3$	100%	-	800%
D	N_3^-CH$_3$	-	100%	0.04%

Analysis of the antioxidant function of carnosine and its metabolic derivatives (methylated, decarboxylated, acetylated, etc., see Figure I-9) revealed that in addition to pH-buffering capacity, they may serve as buffers of free radical species of oxygen in human or animal body. Most actively carnosine interacts with hydroxyl radical. However, its

methylation to anserine causes an increase in the ability to interact with superoxide anion-radical and to neutralize hypochlorite anion, a product of the myeloperoxidase reaction. Homocarnosine exerts a pronounced stabilization effect on cell structures and biomacromolecules, including DNA.

Comparative evaluation of activities of histidine-containing dipeptides in various models of induction of free radical reactions provides a deeper insight into the biological significance of carnosine metabolism in tissues. Indeed, direct measurements of singlet oxygen quenching by histidine-containing dipeptides revealed a considerable decrease in the lifetime of the excited state of O_2. The quenching rate constant values for all compounds tested (imidazole, histidine, carnosine, anserine, and ergothioneine) was found to be virtually equal to each other. Therefore, if the Nature were interested to select the most effective quencher of singlet oxygen, it would fail to solve this problem by modifying the imidazole ring with various additional groups.

A different situation arises in studies with the ability of histidine-containing dipeptides to neutralize hydroxyl radical. The ability of carnosine to neutralize OH· has been predicted by Pavlov et al. in 1990. Later, this reaction was observed experimentally using the spin trap method. The adduct produced as a result of interaction between the spin trap and the hydroxyl radical generated in Fenton reaction is characterized by a specific ESR spectrum (Болдырев и др., 1992a). It was found in these experiments that carnosine is an effective quencher of OH·; histidine and imidazole are completely inactive; whereas homocarnosine, acetylcarnosine, and anserine are arranged as a descending series of activity (Figure IV-6). Thus, the "improvement" of the molecular structure of histidine-containing dipeptides during the evolution of metabolism made the tissue protection against hydroxyl radical less rather than more effective.

Although in the presence of zinc or copper ions, carnosine is an effective scavenger of superoxide anion-radical (Гуляева, 1987), this dipeptide has virtually no direct dismutase activity (Yoshikawa et al., 1991a). Therefore, the concept of superoxide-binding activity of carnosine (Павлов и др., 1990; Sharonov et al., 1990) should be revised in the context of recent findings. A decrease in the lifetime of the superoxide anion-radical generated by water radiolysis is, most likely, due to carnosine interaction with other species of active oxygen rather than with the superoxide anion-radical itself. The carnosine-induced inhibition of the chemiluminescence burst of leukocytes (Тюлина и др., 1995) can mainly be attributed to inhibition of myeloperoxidase reaction and interaction with hypochlorite anion, a product of this reaction (Болдырев и др., 1992b).

In our laboratory, O.V. Tyulina conducted a comparative trial of the efficiency of histidine-containing dipeptides in inhibition of chemiluminescence of rabbit leukocytes. Carnosine was found to be rather ineffective in this reaction. Homocarnosine was slightly more active, whereas activity of acetylcarnosine and anserine was sharply pronounced. Histidine was inactive, and activity of imidazole was intermediate between carnosine and homocarnosine. It should be noted that efficiency of histidine-containing dipeptides in inhibition of myeloperoxidase reaction in this model differed from that in the model described above. For example, N-acetylcarnosine, imidazole, and β-alanine were inactive, whereas histidine, homocarnosine, carnosine, and anserine were arranged as a ascending series of inhibition activity. The effective concentrations providing half-maximum inhibition were: 0.2 mM (anserine), 0.5 mM (carnosine), 1 mM (homocarnosine and histidine) (Тюлина и др., 1995). Therefore, modification of structure of histidine-containing dipeptides may improve

their antioxidant function. The protective effect against superoxide anion-radical increases in the following series: histidine (inactive) < carnosine < homocarnosine < anserine = acetylcarnosine. The protective effect against the product of the myeloperoxidase reaction (hypochlorite anion) increases in the following series: imidazole = acetylcarnosine (inactive) < histidine < homocarnosine < carnosine < anserine.

D.Yu. Yakovlev et al. (Яковлев и др., 1991) described the reaction of photoinduced reduction of thymine to uracil, in which carnosine exerts a pronounced protective effect preventing the UV-induced damage to nucleotides. Taking into account the facts of specific binding of carnosine to nucleic acids *in vitro* (Chikira, Mizukami, 1991) and pronounced stabilization activity of carnosine *in vivo* (prevention of DNA breakdown) (Gille et al., 1991), we used the Yakovlev-Khomutov's reaction as a test for carnosine ability to protect *thymine* from the UV-induced degradation. Comparative efficiency of histidine-containing dipeptides in this test was studied in our laboratory by E. Krinitsyna in collaboration with D.Yu. Yakovlev (Engelgardt Institute of Molecular Biology, Russian Academy of Sciences and A.R. Khomutov (Bach Institute of Biochemistry, Russian Academy of Sciences).

One of the stages of the reaction described by Yakovlev et al. is formation of a charge-transfer complex. It is quite probable that carnosine is involved in this stage, because it is able to bind to hydroxyl radical. Indeed, it was shown that in the presence of carnosine there is a decrease in the rate of the photoinduced modification of thymine. Moreover, homocarnosine is even a more effective protector of the thymine modification in this reaction than carnosine. In a concentration of 10 mM the two dipeptides inhibit the reaction of photoinduced reduction of thymine almost completely. The other histidine-containing dipeptides are significantly less active even at much higher concentrations. The inhibition efficiency series is: anserine = imidazole (inactive) < carcinine < histamine < histidine = N^1-methylhistidine << carnosine < homocarnosine. Thus, this model demonstrated a particularly important role of homocarnosine molecule in the nucleotide structure protection.

Analysis of all antioxidant effects of histidine-containing dipeptides suggest that individual dipeptides differ from each other in spectra of antioxidant activity. For example, carnosine and homocarnosine are effective against hydroxyl radical. Acetylcarnosine and anserine are effective quenchers of superoxide anion-radical. Myeloperoxidase reaction is the most effectively inhibited by carnosine and anserine.

These spectra of the antioxidant activity of different histidine-containing dipeptides provide a new insight into their distribution over biological tissues. It is well known that exhausting muscular work is accompanied by activation of myeloperoxidase in muscular tissue (Морозов и др., 1991). Cell protection against the product of the myeloperoxidase reaction (hypochlorite anion) is an important prerequisite of working ability of muscles. This conclusion is consistent with the data of high content of anserine in intensely working muscles. Moreover, it was shown in experiments *in vivo* that intense muscular work induce carnosine conversion into anserine (Стволинский и др., 1992).

In contrast to carnosine, N-acetylcarnosine more effectively protects against superoxide anion-radical. This can, probably, explain the fact that cardiac muscle is enriched with acetylated derivatives of carnosine.

On the other hand, cardiac muscle contains neither anserine nor carnosine. These dipeptides are particularly effective in the inhibition of myeloperoxidase reaction. This could imply an important role of myeloperoxidase in cardiac muscle, which should not be inhibited. Only inhibition of singlet oxygen was not selective. All dipeptides tested, including

imidazole, were effective in this reaction. In the other cases, imidazole was remarkably inactive. It can be suggested that imidazole transformation into histidine, carnosine, and other histidine-containing dipeptides was motivated by the necessity to provide tissue protection against different species of active oxygen.

Carnosine is the most versatile protective agent, and its versatility is consistent with the widest abundance of this dipeptide. On the other hand, enzymatic transformations adapted it to specific features of oxidative metabolism in different tissues.

Predominant localization of homocarnosine in brain can be attributed to the requirements of effective protection of neurons from the hydroxyl radical attack (it is shown in Figure IV-4 that homocarnosine inhibits the primary stage of peroxidation induction). Histidine conversion into carnosine and then into anserine during ontogeny of muscular tissue can be associated with a progressively increasing resistance of muscular tissue to products of myeloperoxidase reaction. This is of particular importance for protecting actively working muscles. Contrastingly, detoxication of xenobiotics in liver involves free radical intermediates. This process would be less effective, if any of ROS quenchers were present in the system. That is probably why liver does not contain carnosine synthetase, but carnosinase activity is high, and dipeptides are accumulated in liver only for a short time after injection to blood (Figure V-7).

Although the hypothesis of general biological feasibility of distribution of histidine-containing dipeptides in tissues has not been proved in full measure yet, further experimental study in this direction could provide new approaches to a variety of interesting questions, which remain to be answered. Why acetylated derivatives of histidine-containing dipeptides are specific for cardiac muscle? Why muscles of whales and dolphins, who live under conditions of prolonged hypoxia, are enriched with ophidine rather than anserine? What is the biological implication of carnosine decarboxylation resulting in accumulation of carcinine? In our opinion, the answers to these questions would provide a deeper insight into the problem of oxidative metabolism in biological tissues.

However, the attempts to reduce the biological significance of histidine-containing dipeptides to the antioxidant activity alone would oversimplify the multidimensional pattern of metabolic pathways. Perhaps, the true function of histidine-containing dipeptides in biological tissues is such a fine regulation of the level of free radical compounds that allows the regulatory functions of ROS without oxidative damage to cells.

Thus, it can be suggested that the biological significance of metabolic transformations of histidine-containing dipeptides and resulting formation of a variety of metabolic derivatives is to provide the distribution of the dipeptides in tissues accordance with specific features of the ROS metabolism (Boldyrev et al., 1994a). This provided a basis for the hypothesis that the metabolic transformations of carnosine in tissues could be driven by the evolution of oxidative metabolism.

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Chapter VII

N-H OR C-H BONDS CLEAVAGE IN *N*-PHENYL-*N'*-ALKYL-P-PHENYLENEDIAMINE ANTIOXIDANTS ?

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ABSTRACT

In analogy with p-phenylenediamine and N,N'-diphenyl-p-phenylenediamine it has been supposed that the degradation of all *N*-phenyl-*N'*-alkyl-p-phenylenediamine (PPD) antioxidants begins with amine N-H bonds cleavage and benzoquinonediimine intermediates are formed. Nevertheless, the results of non-isothermal DSC measurements of a series of PPD antioxidants indicate that the N-bonded alkyl carbon atom is crucial for their antioxidant effectiveness and the existence of aromatic ketimine structures may be supposed. It has been shown that IR spectra of commercial PPD antioxidant samples heated on air correspond to the double dehydrogenated ketimine structures with Phenyl-N=C double bond and not to their N,N´-dehydrogenated benzoquinonediimine counterparts. DFT quantum-chemical calculations of the optimized structures of a series of PPD antioxidants and of their possible dehydrogenation products have been used for IR spectra interpretation as well as for energy and electronic structure data evaluation. These results support the idea of preferential ketimine Ph-N=C structures formation (they are more stable than the corresponding quinonediimine structures) despite the tertiary carbon centered radicals formation in the first dehydrogenation step need not be energetically preferred over the nitrogen centered ones. The results of recent PPD degradation studies using DFT and semiempirical methods of quantum chemistry are compared.

Keywords: DSC measurements; quantum-chemical calculations; IR spectroscopy; antioxidant effectiveness; dehydrogenation

ABBREVIATIONS

6PPD	N-phenyl-N'-(1,3-dimethyl-butyl)-p-phenylenediamine
6QDI	N-phenyl-N'-(1,3-dimethyl-butyl)-p-quinonediimine
AEM	Antioxidant Effectiveness related to 1 mole of antioxidant (1 mol/kg concentration)
AEX	Antioxidant Effectiveness related to mass fraction of antioxidant (pure antioxidant)
AM1 method	Austin Model 1 semiempirical method of quantum chemistry
AOx	AntiOxidant
B3LYP/6-31G*	Becke 3-parametric – Lee-Yang-Parr functional using 6-31G basis set with polarization functions for non-hydrogen atoms
CPPD	N-(1-methyl-1-phenylethyl)-N'-phenyl- p-phenylenediamine
DFT	Density Functional Theory
DPPD	N,N'-diphenyl-p-phenylenediamine
DQDI	N,N'-diphenyl-p-quinonediimine
DSC	Differential Scanning Calorimetry
FTIR spectroscopy	Fourier Transform InfraRed spectroscopy
H_f	Heat of Formation
IP	Induction Period
IPPD	N-phenyl-N´-isopropyl-p-phenylenediamine
IR spectroscopy	InfraRed spectroscopy
MBPPD	N-(2-methoxybenzyl)-N'-phenyl- p-phenylenediamine
MNDO method	Modified Neglect of Diatomic Overlap semiempirical method of quantum chemistry
M_s	spin multiplicity
oC-6PPD	N-o-cumyl-phenyl-N'-(1,3-dimethyl-butyl)-p-phenylenediamine
pC-6PPD	N-p-cumyl-phenyl-N'-(1,3-dimethyl-butyl)-p-phenylenediamine
PED	Potential Energy Distribution
PF	Protection Factor
PIR	PolyIsoprene Rubber
PM3 method	Parametric Method number 3 semiempirical method of quantum chemistry
PPD	p-phenylenediamine
SPPD	N-phenyl-N´-(1'-methylbenzyl)-p-phenylenediamine
ZPE	Zero Point Energy
$\Delta_r H$	reaction enthalpy

INTRODUCTION

The reactions of organic compounds with oxygen from the air are the basis of energy production of life, but they are also the major cause of irreversible deterioration and ultimate death. All living biological systems can, however, produce in vivo certain antioxidants to prevent or retard this oxidation, which is also known to be the main cause of foodstuff

deterioration, biological ageing and synthetic polymer degradation. These degradative processes are significantly accelerated by sunlight irradiation, heat and dynamic stress. Addition of small amounts of antioxidants into most synthetic polymer products retards such degradative reactions and increases the service lifetimes during processing and end-uses. The most important reactive radical intermediates formed during degradative reactions are hydroxyl (HO•), alkoxyl (RO•) and peroxyl (ROO•) radicals. These radicals are so reactive that they can readily abstract hydrogen atoms from polymer molecular backbones, ultimately breaking down the polymer molecules. Degradation results in the change of the molecular weight distribution together with changes in chemical properties and mechanical behavior [1].

Thermal oxidation of rubber is an autocatalytic, free radical chain reaction where the oxidation products are carboxylic acids, ketones, aldehydes, epoxides, etc. The oxidation process has been classified into four stages using FTIR spectra as a function of time [2,3], viz. the induction period (IP), rapid oxidation period, slow oxidation period and the diffusion-limited period. The degradation process starts with the formation of free radicals during the induction period. The concentration of free radicals determines the rate of the oxidation reaction and can be reduced using antioxidants. Chain-breaking antioxidants (such as sterically hindered phenolic compounds, aromatic amines and thiophenols) donate labile hydrogen to a peroxy radical so interrupting the propagation step [4].

The most widely used antioxidants in the rubber industry are N,N‘-substituted p-phenylenediamines (PPDs) [5,6], being effective also as antiozonants with anti-fatigue effects. The supposed mechanism of inhibition by phenylenediamines (I) is given in Scheme 1 [7]. Primarily formed amine radical (II) is very reactive and may react in two ways. Its reaction products (III) and (IV) have antioxidative effects as well. The benzoquinonediimine-type products (III) may undergo hydrolytic or condensation reactions by forming benzoquinonemonoimine-type structure or N-heterocyclic compounds as well. Benzoquinonediimines (III) are able to trap R• radicals. This contributes to the antioxidant efficiency of the originally added phenylenediamine derivative due to the regeneration of the RO• trapping species (V)[4]. Another group of products may contain a nitroxyl radical (IV) with antioxidative effect.
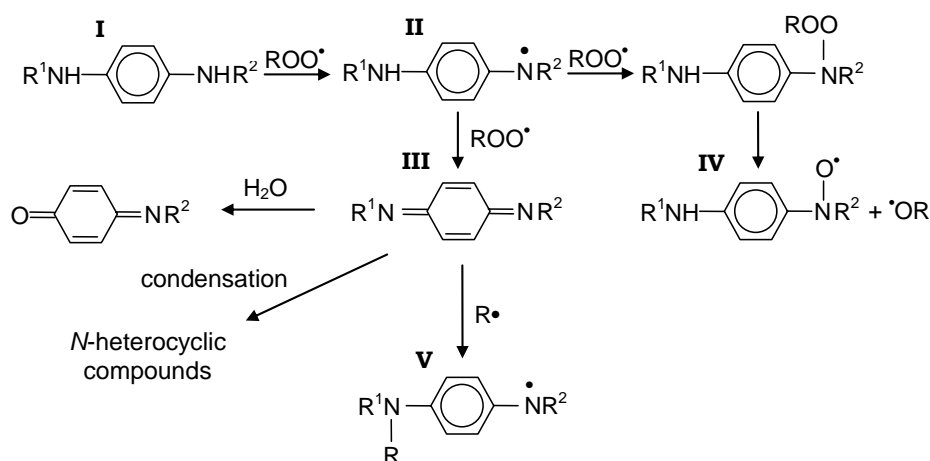
Scheme 1. Classical reaction scheme of PPD degradation [7].
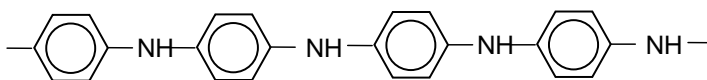
a)
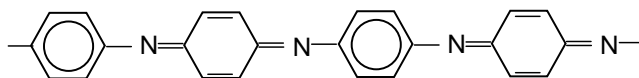
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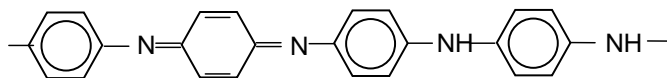
c)

Scheme 2. Different oxidation forms of polyaniline: a) leucoemeraldine (reduced form), b) pernigraniline (oxidized form) and c) emeraldine (partially oxidized form).

It is well known that N,N'-diphenyl-p-phenylenediamine (DPPD) reacts with peroxy radicals to give N,N'-diphenyl-p-quinonediimine (DQDI) [8,9]. Analogous products are formed by oligoanilines and polyaniline (leucoemeraldine) oxidation [10- 22] (see Scheme 2). On the other hand, the formation of the nitroxide radicals originating from the -NH- bridge oxidation by ROO$^\bullet$ radicals has been confirmed by EPR spectroscopy [23].

It is evident that the relative stability of the above reaction intermediates depends on reaction conditions. Based on these results it has been concluded that nitrogen atoms are the reaction sites for the dehydrogenation (oxidation) reactions of all PPD antioxidants. IR spectral measurements of 6PPD oxidation at higher temperatures [3] have been interpreted in this sense. The new band at $1670 \, \text{cm}^{-1}$ in the IR spectrum of oxidized 6PPD products has been ascribed to its N,N´-dehydrogenated quinonediimine-type analogue.

However, the most recent results of our group indicate that the problem is much more complicated and that this assignment is wrong [24 - 26]. This peak really corresponds to the C=N stretching of a little bit different structure of ketimine type. This implies that under specific reaction conditions the nitrogen atoms need not be the preferred dehydrogenation sites of PPD antioxidants.

DSC MEASUREMENTS

Thermal analytical methods represent an established way of investigating the antioxidant activity. Thermogravimetry and differential scanning calorimetry (DSC) were used to study the oxidation of ethylene/propylene rubber stabilized with several antioxidants, where the onset temperature of the oxidation peak was considered as a measure of the antioxidant activity [27,28]. Synergistic effects of antioxidants in natural rubber, including PPDs, were studied recently by isothermal DSC [29].

In the study [30] a method for the evaluation of kinetic parameters of induction periods for non-isothermal processes is suggested. The method has been employed for the kinetic description of the lengths of induction periods of rubber compounds vulcanisation [31], oxidation of edible oils [32], oxidation of polyolefins [30], oxidation of pharmaceuticals [33] and antioxidants (AOx) effectiveness [34, 35] under non-isothermal conditions. The advantage of the method is that it provides kinetic parameters describing the length of induction period under any temperature regime. It has been verified that the induction periods determined by the method [30] are free of systematic errors and, in many cases, the calculated isothermal induction periods coincide with the measured isothermal values.

i) Kinetic Analysis of the Reactions with Induction Period

For any mechanism, the rate of reaction can be described by the general rate equation [30]

$$\frac{d\alpha}{dt} = kf(\alpha)$$
(1)

where α is the conversion, $f(\alpha)$ is the conversion function and $k(T)$ is the rate constant. The temperature dependence of the rate constant is usually expressed by the Arrhenius equation

$$k = A_k \exp\left[-\frac{E}{RT}\right]$$
(2)

where A_k is the pre-exponential factor, E is the activation energy, T is absolute temperature and R stands for the gas constant. Combining Eqs. (1) and (2), after separation of variables and integration under the assumption of the same conversion for any temperature, gives the result

$$1 = \frac{1}{A} \int_0^{t_\alpha} \exp\left[-\frac{B}{T}\right] dt$$
(3)

where t_α is the length of induction period and the constants A and B are given as

$$A = \frac{F(\alpha) - F(0)}{A_k} \tag{4}$$

$$B = E/R \tag{5}$$

where $F(\alpha)$ is the primitive function of $1/f$ and α is the conversion of the reactions occurring during IP and corresponding to the end of IP. The physical meaning of the denominator in Eq. (3) can be simply demonstrated for a special case of isothermal processes where the denominator is a constant equal to the length of the induction period at given temperature. Thus the temperature dependence of the induction period can be expressed by an Arrhenius-like relationship

$$t_i = A \exp\left[B/T\right] \tag{6}$$

In the case of linear increase of temperature ($\beta = \Delta T/\Delta t$), the parameters A and B in Eq. (3) can be obtained as

$$\beta = \int_0^{T_i} \frac{\mathrm{d}T}{A \exp\left[B/T\right]} \tag{7}$$

From the nonisothermal measurements, the kinetic parameters A and B have been obtained by minimizing the sum of squares between experimental and theoretical values of heating rates by the simplex method [30-35].

ii) Protection Factor [30-35]

The temperature range of accelerated DSC stability tests differs from the temperature range where the stability of polyisoprene rubber (PIR) should be predicted. The change of the temperature can lead to a change in the reaction mechanism, i.e. to a change of the conversion function. Thus, the extrapolation of absolute values of the lengths of induction periods can lead to non-realistic estimations.

A better estimation can be obtained using the ratio of the lengths of the induction periods t_i of stabilized (PIR + AOx) and unstabilized (PIR) polymer since the stability or non-stability of the polymer is brought about the same structural units both in stabilized and unstabilized polymer. This ratio is called the protection factor (*PF*)

$$PF = \frac{t_i\left(\text{PIR+AOx}\right)}{t_i\left(\text{PIR}\right)} \qquad (8)$$

If the value of *PF* is greater than one, the additive has stabilizing effect on polyisoprene. Otherwise, the additive exhibits destabilizing effect. The greater is the value of *PF*, the higher is the antioxidant effectiveness of the additive. The values of protection factors decrease with increasing temperature and decreasing concentration of the antioxidant. The dependence of protection factor on the relative mass fraction of antioxidant is almost linear. This fact offers an opportunity to define a criterion characterizing the antioxidant effectiveness, AEX, as a slope of the dependence PF = f(X):

$$AEX = \frac{PF-1}{X} \qquad (9)$$

where X is a relative mass ratio of the antioxidant.
In a molar scale, the effectiveness *AEM* can be defined as follows

$$AEM = \frac{PF-1}{m} \qquad (10)$$

where *m* is the concentration of antioxidant in polymer matrix expressed in mol kg^{-1}.
Both criteria bring about a normalization of the protection factor so that the values of PF for various stabilizer content can be mutually compared. It is obvious that a relationship exists between AEX and AEM:

$$AEM = 10M.AEX \qquad (11)$$

where M is the molar mass of the antioxidant expressed in mol kg^{-1}. Since PF depends on temperature, also both AEX and AEM are functions of temperature.

iii) PPD Antioxidant Effectiveness [34, 35]

The antioxidant activity of several N,N'-substituted p-phenylenediamines (see Table 1 and Figures 1-6) in PIR matrix has been studied by DSC under non-isothermal conditions with the aim to explore the influence of antioxidant concentration on its activity and the dependence between the structure of the substituent in PPD molecule and the antioxidant effectiveness (for details see original papers [34, 35]). The thickness of the samples used in measurements is about 0.1 mm. Hence, the sample is very thin and the diffusion of oxygen plays a negligible role in the determination of antioxidant effectiveness. The experimental results thus reflect pure effects of the structure and concentration of antioxidant on its effectiveness. The antioxidant effectivenesses of individual stabilizers for the temperature of 180 ^{o}C are listed in Table 1.

Table 1. The antioxidants under study (see Figs. 1-6) and their antioxidant effectiveness (AEX and AEM) at 180 ^{o}C [35, 36]

Acronym	Compound name	AEX	AEM [kg/mol]
DPPD	N,N'-diphenyl-p-phenylenediamine	283	738
SPPD	N-phenyl-N´-(1'-methylbenzyl)-p-phenylenediamine	122	351
6PPD	N-phenyl-N'-(1,3-dimethyl-butyl)-p-phenylenediamine	103	277
pC-6PPD	N-p-cumyl-phenyl-N'-(1,3-dimethyl-butyl)-p-phenylenediamine	97	375
oC-6PPD	N-o-cumyl-phenyl-N'-(1,3-dimethyl-butyl)-p-phenylenediamine	68	263
IPPD	N-phenyl-N´-isopropyl-p-phenylenediamine	78	177
MBPPD	N-(2-methoxybenzyl)-N'-phenyl- p-phenylenediamine	50	152
CPPD	N-(1-methyl-1-phenylethyl)-N'-phenyl- p-phenylenediamine	0	0
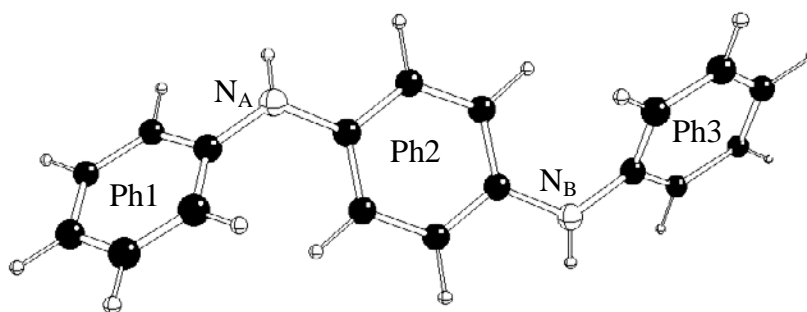
Figure 1. Optimal geometry and active sites notation of DPPD.

Lower antioxidant effectiveness of oC-6PPD in comparison with its pC-6PPD and 6PPD counterparts may be explained by a shielding effect of the cumyl group in ortho-position of phenyl ring. Nevertheless, the relative effectivenes of pC-6PPD and 6PPD using AEX and AEM criteria is different.

If omitting both cumyl derivatives of 6PPD, the order of antioxidant effectivenesses, using both criteria, is

$$DPPD > SPPD > 6PPD > IPPD > MBPPD > CPPD \qquad (12)$$

The highest ranking of DPPD is not surprising since its high antioxidant efficiency is well known and may be explained by its high mobility in rubber. However, a weak spot of DPPD is its low solubility in rubber [36]. The rest of the order is very surprising. This order is inversely proportional to the dissociation energy of the C-H bond at the carbon atom in the neighbourhood of the nitrogen atom. Substitution of all hydrogen atoms at the carbon atom leads to a loss of antioxidant properties and, consequently, to zero value of antioxidant effectiveness in the case of CPPD.

This fact leads to a justifiable assumption [34] that, in the mechanism of antioxidation effect of PPDs, instead of the classical benzoquinonediimine-type structure
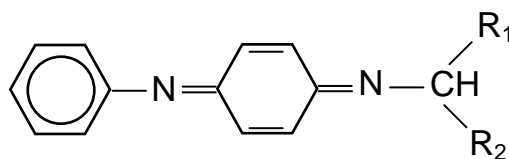
the ketimine molecule should be included:
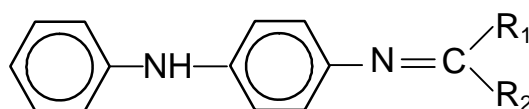
where R_1 and R_2 are substituents. In our futher studies this hypothesis has been tested using various treatments.

QUANTUM-CHEMICAL STUDIES

Molecular geometry and electronic structure of PPD antioxidants have been studied by semiempirical [37, 38] and ab initio [22, 24 - 26, 39 – 40] methods of quantum chemistry. Their experimental antioxidation effectiveness has been correlated with some properties of the optimized structure [37-39].

Semiempirical theoretical methods (PM3, AM1, MNDO) are specially designed to obtain the enthalpy of formation of chemical systems. Dewar's MNDO [41] and AM1 [42] methods have been widely employed in enthalpies of formation calculations and represent a standard tool for both theoretical and experimental organic chemists. Later, Stewart proposed a mathematical reparametrization of MNDO method known as PM3 method [43, 44]. Recently, DFT (especially B3LYP functional [45]) method is suitable for the study of various organic compounds. However, the applicability of this approach is restricted in case of great number of conformations of large molecules. Semiempirical calculations in all our studies have been performed using Hyperchem software [46] whereas Gaussian [47] program package has been used for DFT calculations.

The suitability of PM3 method for PPD antioxidants has been tested [48, 49] using theoretical (DFT/B3LYP) and experimental data available for various aniline derivatives in the work of Li et al. [50]. PM3 calculations provided bond dissociation energies in very good agreement with experimental values. From the accuracy viewpoint PM3 method gives comparable results with B3LYP method using 6-311+G(2d,2p) basis set in significantly shorter time.

The ground-state geometry of PPD antioxidants was optimised at the Hartree-Fock level using standard semiempirical PM3 method [37]. In the first step, the conformational analysis was carried out for the studied compounds and their radicals centered at N_A, N_B or C_X atoms (see Figures 2 - 6) after the abstraction of corresponding hydrogen atom. In the case of studied antioxidants the presence of many dihedral angles indicates the existence of a large number of conformations. Conformational analysis by varying the angles between the

benzene rings and nitrogen (and carbon) atoms has been performed. All obtained structures are non-planar and twisted, the benzene ring with the two nitrogen atoms remains planar in all molecules.

Table 2 compares the data of the the Boltzmann's weighted PM3 mean reaction enthalpies of formation (up to 1.5 kcal/mol over the optimal conformations) [37] of the radicals obtained after hydrogen abstraction from the N_A, N_B or C_X (C_Y) atoms at 180 ^oC (the temperature correction is under 1 kcal/mol) with our data based on the most stable conformations obtained at AM1 [38] and B3LYP/6-31G* levels of theory [39]. DFT enthalpies are approximated by zero-point-energy (ZPE) corrected total energies.

Unlike DFT data, semiempirical methods predict preferential dehydrogenation at C_X sites (Tab. 2). Klein et al. [37] suppose that overall antioxidant action expressed in terms of AEM can be ascribed to all three reaction centres (N_A, N_B and C_X) in the PPD antioxidant molecules. The main aim of their work is to find relation between AEM and dehydrogenation reaction enthalpies at various rection sites, which can be useful for other PPDs AEM prediction. If restricting to SPPD, 6PPD, pC-6PPD, IPPD, and MBPPD antioxidants, the resulting linear dependence may be found [37] in the form

$$AEM = 39876.0 - 330.1 \, \Delta_rH(N_A) - 44.5 \, \Delta_rH(N_B) - 93.6 \, \Delta_rH(C_X) \tag{13}$$
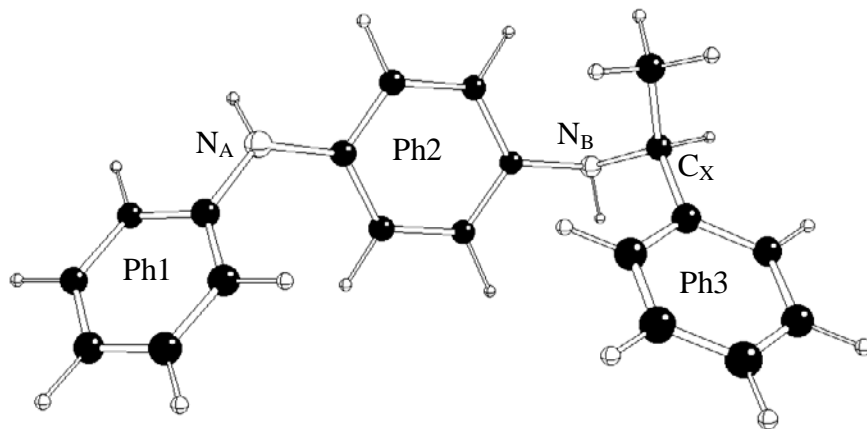
Figure 2. Optimal geometry and active sites notation of SPPD.
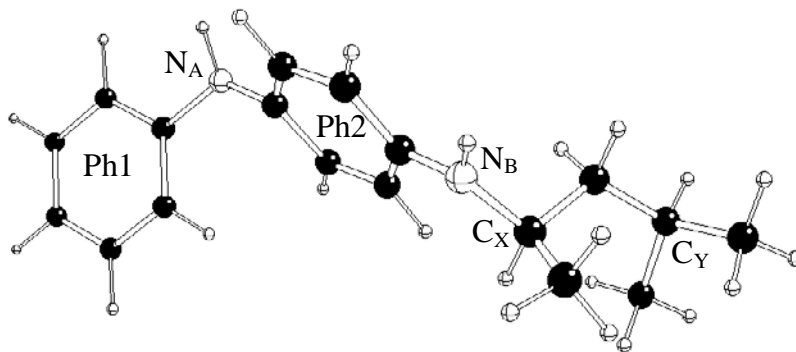
Figure 3. Optimal geometry and active sites notation of 6PPD.
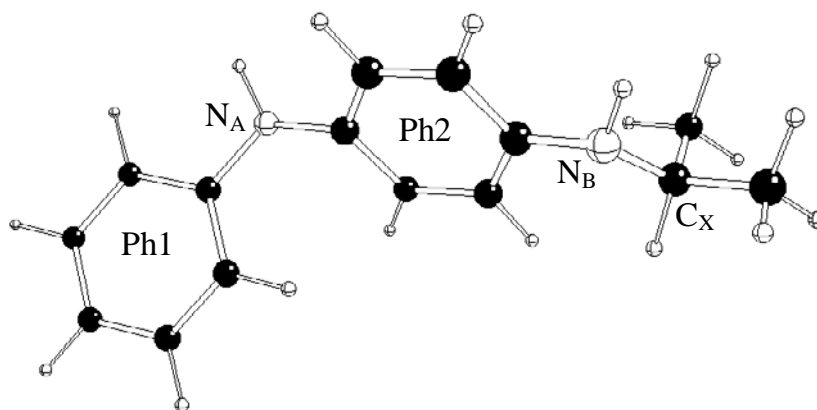
Figure 4. Optimal geometry and active sites notation of IPPD.
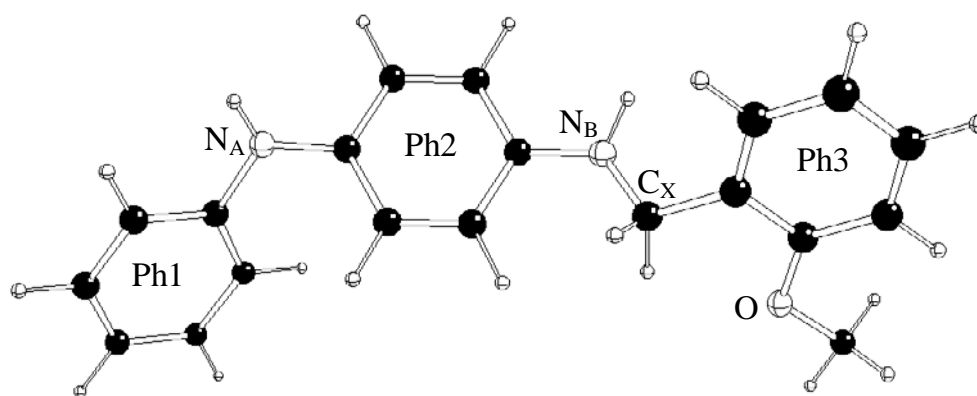
Figure 5. Optimal geometry and active sites notation of MBPPD.
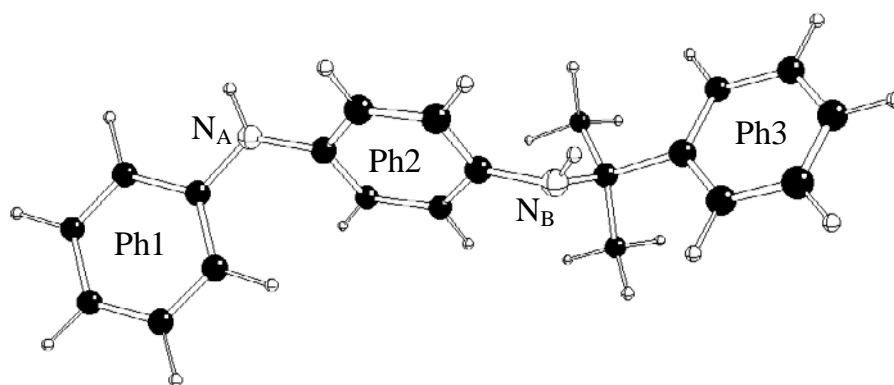
Figure 6. Optimal geometry and active sites notation of CPPD.

Table 2. The first dehydrogenation enthalpies, $\Delta_r H(X)$ [kcal/mol], of PPD antioxidants for various reaction sites X calculated by various methods (see Table 1 and Figures 1-6)

Compound	X	Method		
		PM3[a]	AM1[b]	B3LYP[c]
DPPD	N_A or N_B	-	71.0	74.7
SPPD	N_A	85.9	71.8	75.3
	N_B	88.6	75.8	77.6
	C_X	77.4	60.8	70.8
6PPD	N_A	85.8	66.1	73.4
	N_B	87.9	70.7	78.0
	C_X	78.6	62.8	86.1
	C_Y	-	66.3	86.9
pC-6PPD	N_A	85.7	-	-
	N_B	87.6	-	-
	C_X	78.2	-	-
oC-6PPD	N_A	88.2	-	-
	N_B	88.4	-	-
	C_X	77.5	-	-
IPPD	N_A	85.9	71.2	73.6
	N_B	87.8	75.4	77.6
	C_X	79.4	61.9	86.4
MBPPD	N_A	85.6	70.4	-
	N_B	87.9	74.2	-
	C_X	80.7	60.7	-
CPPD	N_A	86.2	71.3	74.3
	N_B	88.7	76.7	78.6

[a] weighted mean reaction enthalpies at 180 ^{o}C [38].
[b] based on heat of formation data in ref. [39].
[c] based on B3LYP/6-31G** total energies with ZPE correction in ref. [40].

Correlation coefficient value is 0.9996. The differences between calculated and experimental AEM values are in 0.3 - 1.7 percent range. $\Delta_r H(X)$ values (in kcal/mol) correspond to the dehydrogenation at X reaction sites (X = N_A, N_B or C_X). All three active centres have to be without any steric hindrance. It would be appropriate to use larger number of the antioxidants in the linear regression, but more antioxidants have not yet been synthesized.

If restricting to N_A and C_X reaction sites only, the following equation has been obtained

$$AEM = 37614.1 - 353.8\, \Delta_r H(N_A) - 88.7\, \Delta_r H(C_X) \qquad (14)$$

In this case, the relative deviations between experimental and calculated AEM values do not exceed 6%.

However, in both cases the absolute value of the coefficients corresponding to the N_A-H bond dissociation is significantly higher than the C_X-H one what indicates the highest

importance of N_A sites for the antioxidant activity. Moreover, our DFT data (Table 2) have confirmed the preferential dehydrogenation at C_X sites in the case of SPPD only. The high reliability of PM3 tested on aniline derivatives [50] cannot be confirmed for PPD antioxidants with two or more phenyl rings. Our results show that the above treatment has not confirmed this conclusion based on the DSC data in the previous chapter.

Table 3. The AM1 heat of formation (H_f) [39] and B3LYP/6-31G total energy data without (E_{tot}) and with ZPE correction [40] ($E_{tot}+ZPE$) of the double dehydrogenated antioxidants at various reaction sites in triplet ($M_s = 3$) and singlet ($M_s = 1$) ground spin states (see Table 1 and Figures 1-6)**

Antioxidant	Dehydrogenated sites	M_S	H_f [kcal/mol]	E_{tot} [a.u.]	$E_{tot} + ZPE$ [a.u.]
DPPD	$N_A N_B$	3	136.3	-803.77676	-803.50736
		1	136.8	-803.81072	-803.53879
SPPD	$N_A N_B$	3	104.9	-882.37965	-882.05334
		1	96.2	-882.42116	-882.09207
	$N_A C_X$	3	86.1	-882.40737	-882.08098
		1	102.9	-882.42184	-882.09243
	$N_B C_X$	3	89.4	-882.40588	-882.07964
		1	68.6	-882.46869	-882.13950
6PPD	$N_A N_B$	3	83.5	-808.59400	-808.23524
		1	80.3	-808.63213	-808.27044
	$N_A C_X$	3	71.2	-808.59004	-808.23142
		1	90.3	-808.61139	-808.24962
	$N_A C_Y$	3	73.1	-808.59201	-808.23463
		1	134.0	-808.56830	-808.21023
	$N_B C_X$	3	75.3	-808.59074	-808.23202
		1	53.7	-808.66452	-808.30305
	$N_B C_Y$	3	78.6	-808.58448	-808.22695
		1	73.2	-808.64248	-808.28576
	$C_X C_Y$	3	72.5	-808.56962	-808.21333
		1	72.9	-808.58436	-808.22783
IPPD	$N_A N_B$	3	104.9	-690.66017	-690.38715
		1	96.2	-690.69781	-690.42200
	$N_A C_X$	3	86.1	-690.65518	-690.38274
		1	102.9	-690.67709	-690.40131
	$N_B C_X$	3	89.4	-690.65354	-690.38058
		1	68.6	-690.72954	-690.45408
MBPPD	$N_A N_B$	3	98.5	-	-
		1	96.0	-	-
	$N_A C_X$	3	80.3	-	-
		1	101.5	-	-
	$N_B C_X$	3	85.8	-	-
		1	70.4	-	-
CPPD	$N_A N_B$	3	136.4	-922.69382	-921.33980
		1	133.0	-921.73596	-921.37929

Here it must be mentioned that such a treatment, in principle, cannot be succesful because the thermodynamic quantities (bond dissociation energies) are correlated with the kinetic ones (AEM is the relative reaction time) that depend on the reaction barriers. The relations between reaction barrier height and the reaction enthalpy of the hydrogen atom abstraction in C-H and N-H bonds are different as shown in the DFT study of hydrogen abstraction from phenol and toluene by the hydroperoxyl radical with similar reaction enthalpies but significantly different reaction barrier heights [51].

An alternative treatment is based on quantum-chemical studies of relative stability of various double dehydrogenated products of the antioxidants under study [38, 39]. In this way, the optimal geometries of analogous structures obtained by removing two hydrogens (in singlet or triplet spin state, $M_S = 1$ or 3) from the starting antioxidant molecule have been obtained in our recent studies. The B3LYP/6-31G* data of total energy without (E_{tot}) as well as with zero-point energy corrections ($E_{tot} + ZPE$) for our model systems are summarized in Table 3 and compared with heat of formation data (H_f) obtained by semeimpirical AM1 method. For the sake of simplicity, our AM1 and DFT studies are restricted to a single conformation for each of the molecules.

Among the second dehydrogenation step products of SPPD, 6PPD and IPPD, both AM1 and DFT data indicate the highest stability of the ketimine structures with double $N_B=C_X$ bonds in singlet spin state what is in agreement with the supposition based on DSC data [34, 35] (with the $N_BC_X > N_AC_X > N_AN_B$ sequence of stability for SPPD and $N_BC_X > N_AN_B > N_AC_X$ for 6PPD and IPPD, respectively). It must be mentioned that the relative stability of biradicals is substantially different and AM1 data are not reliable for such systems. Except N_AC_Y double dehydrogenated 6PPD biradical ($M_s = 3$), singlet states are more stable than the corresponding triplets. The singlet-triplet energy separation of ketimines (37-45 kcal/mol) is higher than this one of quinonediimines (20 – 25 kcal/mol) and of the remaining isomers (< 12 kcal/mol).

As expected, we have not found any correlation between the relative energies of individual isomers of double dehydrogenated antioxidants (thermodynamic parameters) and the antioxidant effectiveness (kinetic parameters). Extra high DPPD antioxidant effectiveness is usually ascribed to its high mobility (and thus low solubility) in rubber [36]. Relatively high AEM value of SPPD might be explained by energetically advantageous formation of C_X radical in the first dehydrogenation step (unlike the remaining antioxidants under study) that supports the $N_A=C_X$ ketimine structure formation in the second reaction step. The decreasing number of the active sites for dehydrogenation in 6PPD, IPPD and CPPD might explain their relative antioxidant effectiveness as well.

IR SPECTRA MEASUREMENT AND INTERPRETATION

Several studies have been focused to vibrational spectroscopy of DPPD, oligo- and polyanilines (leucoemeraldine) as well as to their dehydrogenation products [15 - 22, 52, 53]. In all cases, N-H bonds have been released and benzoqiunonediimine type compounds (such as DQDI, emeraldine and pernigraniline) have been identified (see Scheme 2).

In analogy with these studies it has been [3] supposed that the thermal aging of polyisoprene rubber with the 6PPD commercial antioxidant in hot air (140 ^{o}C) leads to the

formation of N-(1,3-dimethyl-butyl)-N´-phenyl-quinonediimine (6QDI) and its FTIR spectra have been interpreted in this sense as well. The new broad band of oxidized 6PPD at 1670 cm^{-1} has been ascribed to the C=N stretching of a quinonediimine-type structure. Nevertheless, this product has not been isolated till now and this assignment is not based on any theoretical model.

In the light of the above mentioned DSC studies [34, 35] we have investigated the thermal ageing of some PPD antioxidants. Powder samples of DPPD, SPPD and IPPD (ca 2g) on a glass dish were heated in an oven at 140 °C on air until the evident formation of dark coloured oxidation products occurred (ca 1 - 5 days). Transmission mid-infrared FTIR spectra of such treated samples were taken by Nicolet model NEXUS 470 FTIR spectrometer using standard KBr technique for various thermal degradation times (for more details see original literature [24 - 26]). Standard B3LYP/6-31G* geometry optimizations of the most stable conformations of DPPD, SPPD and IPPD antioxidants and of their doubly dehydrogenated products at N_A, N_B, C_X and/or C_Y sites in singlet (spin multiplicity $M_S = 1$) and triplet spin ground states ($M_S = 3$) have been performed using Gaussian03 program package [47]. In the next step, the vibrational frequencies of the above mentioned singlet state molecules have been computed and compared with the experimental ones. Calculated DFT vibrational frequencies have been scaled by factor 0.9614 [54]. IR spectra analysis is restricted to singlet ground states because their energy is significantly lower than that of triplet states in all the systems under study (see Table 3).

Survey IR spectra of unheated DPPD, SPPD and IPPD samples in KBr pellet together with IR spectra of their oxidized forms after 1 - 5 days thermal treatment in the hot air at 140 ºC are shown in Figures 7 - 10 and compared with the DFT calculated IR spectra in Tables 4 - 6. The assignment of individual vibrations will be discussed in more detail.
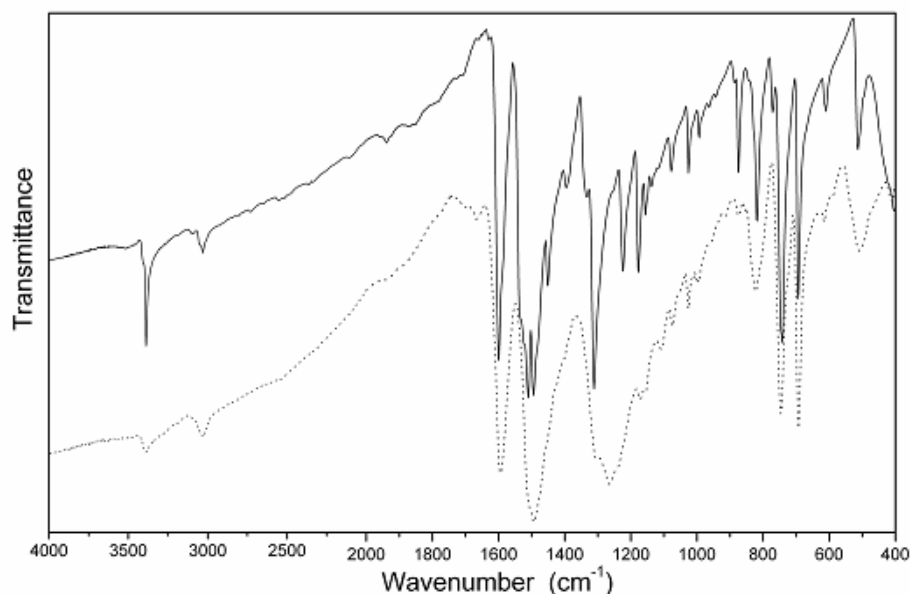
Figure 7. IR spectra of DPPD in KBr pellet before (full line) and after heating at 140 °C on air for 5 days (dotted line).

Table 4. Experimental vibrational wavenumbers of the heated DPPD sample (ν_{exp}, [cm^{-1}], see Figures 7 and 10) and DFT calculated vibrational wavenumbers scaled by the scale factor 0.9614 (ν_{scaled}, [cm^{-1}]), IR intensities (I, [km/mol]) and bands assignment for DPPD and DQDI

Heated DPPD	DPPD			DQDI		
ν_{exp} [a]	ν_{scaled}	I	Assignment[b]	ν_{scaled}	I	Assignment[b]
				1632	0.0	C=C ss (Ph2)
	1615	0.07	CC s (8a, Ph2)			
1600 vs	1601	23.6	CC s (8a, Ph1, Ph3 s)	1598	17.9	CC s (8a, Ph1, Ph3 a)
1590 s,sh	1600	329.7	CC s (8a, Ph1, Ph3 a)	1587	0.1	CC s (8a, Ph1, Ph3 s)
	1584	1.7	CC s (8b, Ph1, Ph3 s)			
1575 w,sh	1582	44.6	CC s (8b, Ph1, Ph3 a)			
	1571	3.6	CC s (8b, Ph2)	1576	99.0	C=N as
				1567	18.4	C=C as (Ph2)
				1562	2.9	CC s (8b, Ph1, Ph3 a)
				1562	4.0	CC s (8b, Ph1, Ph3 s)
				1528	0.1	C=N ss
1533 m	1516	848.5	NH aδ			
1522 m						
1510 vs	1501	78.7	CC s (19a, Ph2)			
1494 s	1498	32.4	NH sδ			
1484 s,sh	1485	393.1	CC s (19a, Ph1, Ph3 a)	1472	50.0	CC s (19a, Ph1, Ph3 a)
	1483	0.03	CC s (19a, Ph1, Ph3 s)	1470	1.0	CC s (19a, Ph1, Ph3 s)
1451 m	1446	6.3	CC s (19b, Ph1, Ph3 a)	1437	2.0	CC s (19b, Ph1, Ph3 a)
	1421	2.5	CC s (19b, Ph1, Ph3 s)	1436	2.2	CC s (19b, Ph1, Ph3 s)
1395 w	1390	33.7	CC s (19b, Ph2)	1393	0.0	C-C s (Ph2)
				1374	1.2	CCH δ (Ph2)
	1327	0.7	CC s (14, Ph1, Ph3, s)			
1333 m	1321	100.5	CC s (14, Ph1, Ph3, a)	1310	0.8	CC s (14, Ph1, Ph3 a)
	1317	2.0	CCH δ (3, Ph1, Ph3)	1310	0.0	CC s (14, Ph1, Ph3 s)
				1296	15.8	C-C s (Ph2)
1311 s	1304	122.9	CCH δ (3, Ph1, Ph3)			
	1302	4.5	CCH δ (3, Ph2)			
1293 m,sh	1300	519.3	C-N$_A$-C as, CC s (14, Ph2)			

[a] s strong; m medium; w weak; v very; sh shoulder;

[b] s stretch; δ deformation; wa wagging; s symmetric; a asymmetric.

i) DPPD [26]

The IR spectrum of unheated DPPD as well as of pure DQDI compounds is well known and has been published elsewhere [11-14]. During hot air acting on DPPD many split bands

became broader and combined into one band with changed intensity. In general, both spectra (before and after thermal treatment) are similar and only few bands almost disappeared or decrease their intensity (for instance bands around 3388, 1533, 1522, 1333, 1311, 1224, 1177 and 875 cm^{-1}) and some new bands arose which can be assigned to the oxidized products (see for example bands at 1264 cm^{-1} and 1108 cm^{-1} in Figures 7 and 10). Nevertheless, any band formation around 1660 cm^{-1} was not observed.

The hydrogen splitting-off from the nitrogen atoms and creation of the DQDI during the thermal DPPD dehydrogenation can be supported by the strong decrease of the intensity of the N-H stretching vibration band at 3388 cm^{-1} in comparison with the C-H stretching bands and by the disappearing of the bands at 1533 and 1522 cm^{-1} which are assigned as NH deformation modes according to the potential energy distribution (PED) calculations.

The most significant changes between both spectra may be seen in the 1350-1200 cm^{-1} region. In the 1350-1250 cm^{-1} region the bands of CC ring stretch mode (denoted as mode 14 according to the Wilson's notation) and CCH ring deformation mode (mode 3) can be expected and in the 1200-1300 cm^{-1} region the C-N-C asymmetric and symmetric stretch vibrations [55]. For 1,4-disubstituted benzene ring these modes are at about 10-15 cm^{-1} lower frequencies than for the monosubstituted ones [55]. In ref. [12], one of the strongest bands in IR spectra of DPPD around 1300 cm^{-1} was not assigned to any vibrational mode because no intense band was predicted by the calculation in this area and it was interpreted as a coupling between mode 14 and C-N-C asymmetric stretching vibration. However, in our calculations this band was evaluated as the second most intense band at 1300 cm^{-1} and according PED really corresponds to the mode 14 of the 1,4-disubstituted Ph2 benzene ring and C-N-C asymmetric stretching vibration. Also the DPPD band at 1224 cm^{-1}, which changes its intensity dramatically, is (according to PED) connected with the C-N-C asymmetric stretching vibration. During the transformation of DPPD to DQDI the Ph2 benzene ring is converted to a benzoquinone ring and two C=N double bonds are formed. Therefore it is not surprising that after thermal treatment both mentioned bands decrease their intensity and appearing the new band at 1264 cm^{-1} can be ascribed to DQDI [12].

Also the decreasing intensity of the DPPD band at 1177 cm^{-1} is in agreement with the calculated about 15 cm^{-1} downshift of the CCH deformation modes (mode 9a) for all three benzene rings and calculated decreasing of its intensities.

ii) SPPD [25]

Similarly to DPPD, the IR spectrum of unheated SPPD has the features which are more typical for crystalline state whereas the spectra after hot air acting are more similar to the amorphous state with broad bands (Figures 8 and 10). This reflects that SPPD is molten at 140 ºC. During hot air acting many split bands became broader and combined into one band with changed intensity. Only several bands in IR spectra almost disappeared or some new bands arose which can be assigned to the oxidized products (see for example bands around 1660 cm^{-1}, 1340 cm^{-1} and 1140 cm^{-1} in Figures 8 and 10). Especially in the region of the double bonds stretching vibration the most dramatic changes can be observed where a new very strong band around 1660 cm^{-1} appeared. In general, also here it may be declared that many features of both spectra (before and after thermal treatment) are similar.
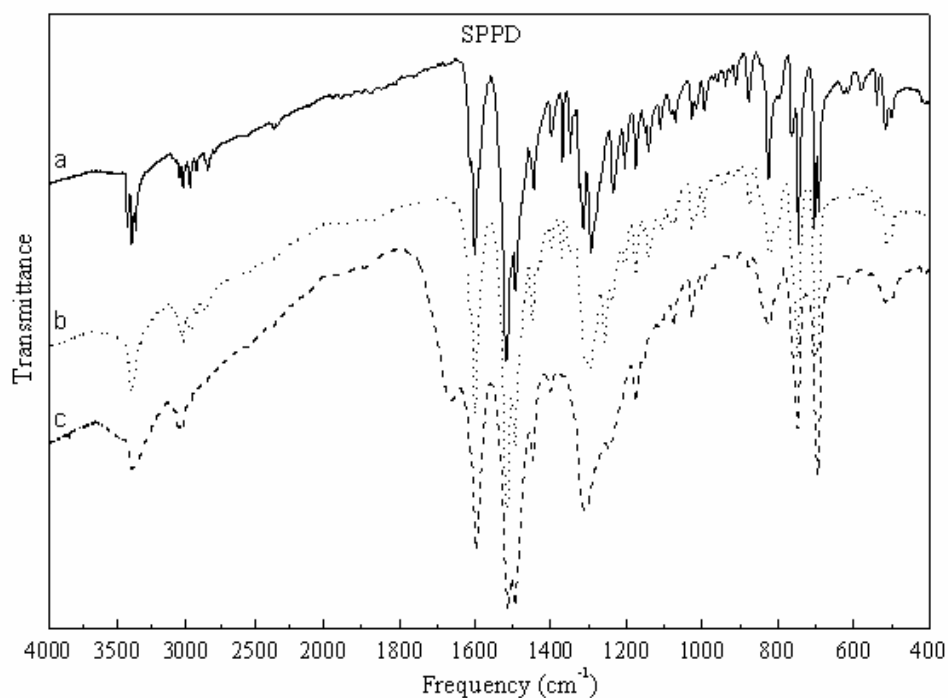
Figure 8. IR spectra of SPPD in KBr pellet before (full line – curve a) and after heating at 140 °C on air for 1 hour (dotted line - curve b) and 50.5 hours (dashed line – curve c).

Some vibrational modes of SPPD and its possible oxidation products (including their IR intensity) are collected in Table 5. SPPD has no band over 1620 cm^{-1}. We may see that the calculated scaled frequencies of SPPD fit well with the experimental ones. In the region of 1620-1420 cm^{-1} the substituted benzene ring has four characteristic modes occurring near 1600, 1580, 1490 and 1440 cm^{-1}. These bands are good group vibrations and are denoted as 8a, 8b, 19a and 19b according to the Wilson's notation. The first two of them correspond prevailingly to CC ring stretch whereas the remaining ones represent the mixture of the CC ring stretch and CCH in-plane deformation modes. Because our compounds under study have the last 19b mode in the region of the CH_3 group deformation modes we will focus our discussion on the first three modes only. For monosubstituted benzene rings, the modes 8a, 8b and 19a are expected in the regions 1605 ± 15 cm^{-1}, 1585 ± 15 cm^{-1} and 1485 ± 25 cm^{-1}, respectively, and for 1,4-disubstituted benzene in the regions 1610 ± 20 cm^{-1}, 1585 ± 30 cm^{-1} and 1500 ± 30 cm^{-1}, respectively [55]. Ph2-N_B symmetric stretching at 1216 cm^{-1} contains small N_B-C_X contribution. Besides the benzoquinonediimine-type compounds (N_A,N_B-double dehydrogenated SPPD and N_A,C_X-double dehydrogenated SPPD), the mentioned three modes have been calculated in the expected regions of all other studied compounds. The highest ring mode is shifted to the slightly higher frequency than 8a benzene ring mode for N_A,N_B-double dehydrogenated SPPD (1634 cm^{-1}). Because in the case of N_A,N_B-double dehydrogenated SPPD this mode is created by the symmetric stretch vibration of both C=C bonds its intensity is very low and probably not measurable in the IR spectra. Therefore if the quinonediimine structure is created during the SPPD oxidation we would not expect the new strong band at 1660 cm^{-1}. Despite the character of the Ph2 ring of N_A,C_X-double dehydrogenated SPPD is

more close to the benzoquinonediimine structure there is no calculated band over 8a benzene ring mode and therefore the new created band around 1660 cm^{-1} can be explained neither by this N_A,C_X-double dehydrogenated SPPD structure. Moreover, the calculated differences in the IR spectra for N_A,C_X-double dehydrogenated SPPD structure and unheated SPPD were higher than for next two N_A,N_B- and N_B,C_X-double dehydrogenated SPPD structures. For example, the modes 8b for all three rings are shifted markedly down and the two strongest bands calculated at 1568 cm^{-1} and 1420 cm^{-1} corresponding to the NH deformation and N_B-C stretching modes, respectively, are in the area of IR spectrum where no bands for unheated SPPD are present. Therefore if the N_A,C_X-double dehydrogenated SPPD structure is created during the SPPD oxidation process we should expect some new bands around 1560 cm^{-1} and 1420 cm^{-1}. Because such new bands are not present in the IR spectrum of heated SPPD the N_A,C_X-double dehydrogenated SPPD can be excluded from our discussion.

The next calculated band for N_A,N_B-double dehydrogenated SPPD at 1609 cm^{-1} corresponds to the C=N asymmetric vibration of both C=N bonds and belongs to the strongest bands in the IR spectrum (C belongs to Ph2 ring and N denotes N_A/N_B atoms, its symmetric counterpart is at 1550 cm^{-1}). This agrees well with the experimental spectrum of para-benzoquinonediimine where the band with the highest frequency in this region has been measured at 1594 cm^{-1} and assigned to C=N mode [56]. The Ph1 ring stretching 8a and 8b modes of N_A,N_B double dehydrogenated SPPD are calculated at lower frequencies than the corresponding unheated SPPD modes whereas the Ph3 ring ones are practically unchanged. These Ph2 ring modes are transformed into C=C symmetric and asymmetric stretching vibrations and their frequencies are outside the corresponding unheated SPPD region. In agreement with the SPPD calculations and the mentioned intervals for 8a mode the strong SPPD band at 1601 cm^{-1} can be assigned to the monosubstituted benzene ring and its high frequency shoulder at 1613 cm^{-1} to the disubstituted benzene ring. The same assignment of the band around 1600 cm^{-1} to the monosubstituted benzene ring was used also for 6PPD in [3] as well as for its 6QDI oxidation product and subsequently the intensity of this band was used in the evaluation process as the normalized scaler. If the N_A,N_B-double dehydrogenated SPPD structure is created during oxidation process (as supposed in [3]) the above mentioned procedure cannot be correct because (according to our calculations) the corresponding ring 8a CC stretching band has downshift about 13 cm^{-1} for Ph1 ring, the Ph2 ring modes are outside this region and moreover this band is mixed with the C=N asymmetric stretching mode and thus it is very improbable to suppose the unchanged 1600 cm^{-1} band. On the contrary the invariance of this band is explainable by N_A,C_X-double dehydrogenated SPPD for which the 8a modes are practically the same as for unheated SPPD. Also the 19a mode bands of all three SPPD benzene rings calculated at 1500, 1487 and 1485 cm^{-1} represent N_A,N_B-double dehydrogenated SPPD downshift at 1472 cm^{-1} (for the monosubstituted Ph1 ring) and at 1389 cm^{-1} (for the Ph2 ring) to the CC stretching mode. Moreover, there are no calculated bands for N_A,N_B-double dehydrogenated SPPD in the region 1550-1488 cm^{-1} and therefore again: if the N_A,N_B-double dehydrogenated SPPD structure is created then both bands of 19a mode should decrease their intensity. It is in contradiction with [3] where only the decrease of the 6PPD band at 1514 cm^{-1} assigned just to disubstituted benzene ring is considered. This band for unheated SPPD correspond to the very strong band at 1517 cm^{-1} and our calculations, however, reveal that this band is mixed also with the NH deformation modes and decreasing intensity of this band can be explained by changing this mode as well.

The appearing new band at 1660 cm^{-1} in the IR spectrum is explainable if the N_B,C_X-double dehydrogenated SPPD structure is created. The calculated frequency for N_B,C_X-double dehydrogenated SPPD at 1634 cm^{-1} corresponds to the symmetric stretching vibration of $N_B=C_X$ double bond (analogous symmetric stretching of N_B-C_X bond in N_A,N_B-double dehydrogenated SPPD is at 1100 cm^{-1}). Calculated intensity of this band is very high and such a band is visible in IR spectrum even at lower concentrations. Intensity changes of SPPD 1517 and 1503 cm^{-1} bands can be explained by the calculated IR spectrum downshift of the strongest band of NH deformation mode and 19a mode of Ph2 between SPPD and its N_B,C_X-double dehydrogenated structure. Additional argument for N_B,C_X-double dehydrogenated SPPD and splitting off the tertiary C_X hydrogen is in dramatic decrease of the intensity of SPPD 1349 cm^{-1} band. For unheated SPPD the band at 1349 cm^{-1} together with the 1330 cm^{-1} band have been calculated as tertiary C_XH deformation modes. This is in excellent agreement with the classical force field study of saturated hydrocarbons [57] where isobutane 1330 cm^{-1} and 2-methylbutane 1351 and 1337 cm^{-1} bands were assigned to this mode. In our calculations the N_B,C_X-double dehydrogenated SPPD bands in this area are missing unlike the N_A,N_B double dehydrogenated SPPD ones. Therefore the disappearance of the mentioned 1349 cm^{-1} band in the IR spectrum of the 50 hours heated SPPD in hot air can be explained by the splitting off the tertiary C_X hydrogen and thus ketimine structure formation.
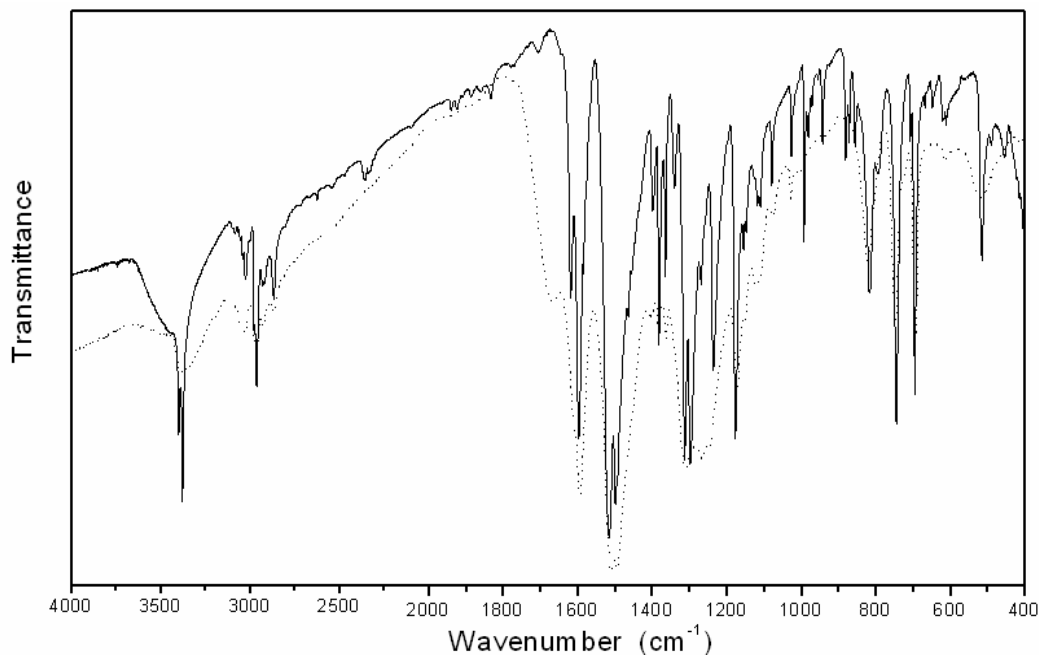
Figure 9. IR spectra of IPPD in KBr pellet before (full line) and after heating for 28.5 hours (dashed line).

Table 5. Experimental vibrational wavenumbers of the heated SPPD sample (ν_{exp}, [cm^{-1}], see Figures 8 and 10) and DFT calculated vibrational wavenumbers scaled by the scale factor 0.9614 (ν_{scaled}, [cm^{-1}]), IR intensities (I, [km/mol]) and bands assignment for SPPD and its double dehydrogenated structures

Heated SPPD	SPPD			N_B,C_X-dehydrogenated SPPD			N_A,N_B-dehydrogenated SPPD			N_A,C_X-dehydrogenated SPPD		
ν_{exp}[a]	ν_{scaled}	I	Assignment[b]	ν_{scaled}	I	Assignment[b]	ν_{scaled}	I	Assignment[b]	ν_{scaled}	I	Assignment[b]
				1634	143.9	N_B=C s	1634	1.6	C=C ss (Ph2)			
1613 m	1610	1.0	CC s (8a, Ph2)	1606	8.3	CC s (8a, Ph2)	1609	95.3	C=N as	1606	112.7	CC s (8a, Ph2)
1601 vs	1599	198.5	CC s (8a, Ph1)	1597	237.2	CC s (8a, Ph1)	1597	5.8	CC s (8a, Ph3)	1590	19.8	CC s (8a, Ph3)
	1597	14.9	CC s (8a, Ph3)	1591	68.4	CC s (8a, Ph3)	1586	18.6	CC s (8a, Ph1)	1582	70.9	CC s (8a, Ph1)
										1568	515.3	NH δ
1588 m	1584	21.2	CC s (8b, Ph1)	1583	26.2	CC s (8b, Ph1)	1581	4.4	CC s (8b, Ph3)	1557	1.8	CC s (8b, Ph3)
1577 w,sh	1579	0.6	CC s (8b, Ph3)	1573	14.4	CC s (8b, Ph3)	1573	1.1	C=C as (Ph2)			
	1572	5.5	CC s (8b, Ph2)	1559	29.5	CC s (8b, Ph2)	1563	3.8	CC s (8b, Ph1)	1557	4.7	CC s (8b, Ph1)
							1550	25.7	C=N ss	1521	299.4	N_A-Ph2 s
1517 vs	1511	376.3	NH δ	1504	553.5	NH δ				1502	59.8	CC s (8b, Ph2)
1503 s	1500	140.1	CC s (19a, Ph2)	1492	13.2	CC s (19a, Ph2)	1488	12.4	CC s (19a, Ph3)	1487	83.5	CC s (19a, Ph3)
1492 s	1487	133.2	CC s (19a, Ph1)	1484	71.0	CC s (19a, Ph1)	1483	5.3	CH$_3$ aδ	1476	413.5	CC s (19a, Ph2)
	1485	7.0	CC s (19a, Ph3)	1482	60.5	CC s (19a, Ph3)	1472	24.1	CC s (19a, Ph1)	1470	15.7	CC s (19a, Ph1)
	1476	4.6	CH$_3$ aδ	1460	7.7	CH$_3$ aδ				1455	9.1	CH$_3$ aδ
1475 m	1465	26.0	NH δ									
1453 w	1463	5.7	CH$_3$ aδ	1455	3.2	CH$_3$ aδ	1461	6.6	CH$_3$ aδ	1449	363.3	CH$_3$ aδ

Table 5. Continued

Heated SPPD ν_{exp}[a]	SPPD ν_{scaled}	I	Assignment[b]	ν_{scaled}	I	Assignment[b]	ν_{scaled}	I	Assignment[b]	ν_{scaled}	I	Assignment[b]
	SPPD			N_B,C_X-dehydrogenated SPPD			N_A,N_B-dehydrogenated SPPD			N_A,C_X-dehydrogenated SPPD		
1445 m	1444	5.4	CC s (19b, Ph3)	1437	19.1	CC s (19b, Ph1)	1442	11.5	CC s (19b, Ph3)	1441	21.0	CC s (19b, Ph3)
1431 vw,sh	1430	17.6	CC s (19b, Ph1)	1436	13.6	CC s (19b, Ph3)	1436	2.1	CC s (19b, Ph1)	1435	2.6	CC s (19b, Ph1)
										1420	557.4	N_B-C s
1399 m	1391	6.7	CC s (19b, Ph2)	1394	14.5	CC s (19b, Ph2)				1400	201.4	CC s (19b, Ph2)
1386 vvw							1389	6.3	CC s (Ph2)			
							1380	9.7	CC s (Ph2)			
1369 m	1379	8.6	CH$_3$ $s\delta$	1372	22.2	CH$_3$ $s\delta$	1374	9.3	CH$_3$ $s\delta$	1388	30.3	CH$_3$ $s\delta$
1349 m	1349	4.5	CH δ				1348	62.0	CH δ			
1337 vw	1330	0.2	CH δ				1326	1.3	CH δ			
1324 m	1324	102.0	CC s (14, Ph1)	1323	134.7	CC s (14, Ph1)	1312	2.7	CC s (14, Ph3)	1339	132.7	C-Ph3 s
	1313	1.3	CC s (14, Ph3)	1316	4.2	CC s (14, Ph3)	1310	0.3	CC s (14, Ph1)	1322	15.7	CC s (14, Ph3)
1315 m	1311	132.2	C-N_A-C as CCH δ (3, Ph1)	1311	205.0	C-N_A-C as CCH δ (3, Ph1)	1291	10.6	CCH δ (Ph2)	1310	1.6	CC s (14, Ph1)

[a] s strong; m medium; w weak; v very; sh shoulder.

[b] s stretch; δ deformation; a asymmetric; s symmetric; ro rocking; wa wagging.

Table 6. Experimental vibrational wavenumbers of the heated IPPD sample (ν_{exp}, [cm^{-1}], see Figures 9 and 10) and DFT calculated vibrational wavenumbers scaled by the scale factor 0.9614 (ν_{scaled}, [cm^{-1}]), IR intensities (I, [km/mol]) and bands assignment for IPPD and its double dehydrogenated structures

Heated IPPD	IPPD			N_B,C_X-dehydrogenated IPPD			N_A,N_B-dehydrogenated IPPD		
ν_{exp} [a]	ν_{scaled}	I	Assignment[b]	ν_{scaled}	I	Assignment[b]	ν_{scaled}	I	Assignment[b]
				1674	166.5	C=N s			
							1637	0.5	C=C ss (Ph2)
1619 m	1618	23.1	CC s (8a, Ph2)	1607	4.2	CC s (8a, Ph2)			
1598 s	1602	179.1	CC s (8a, Ph1)	1597	246.8	CC s (8a, Ph1)	1602	69.7	N=C as
							1586	39.6	CC s (8a, Ph1)
1586 m	1582	18.8	CC s (8b, Ph1)	1582	22.5	CC s (8b, Ph1)	1576	14.3	C=C as (Ph2)
1578 w,sh	1573	2.2	CC s (8b, Ph2)	1562	19.6	CC s (8b, Ph2)	1562	3.7	CC s (8b, Ph1)
							1544	16.2	N=C ss
1517 vs	1512	332.7	NH δ	1504	484.9	NH δ			
							1480	7.0	CH₃ δ
1500 vs	1506	279.6	CC s (19a, Ph2)	1491	13.3	CC s (19a, Ph2)			
1494 s,sh	1488	188.3	CC s (19a, Ph1)	1483	116.4	CC s (19a, Ph1)	1471	25.2	CC s (19a, Ph1)
1484 vw,sh	1479	1.4	CH₃ δ	1462	15.3	CH₃ δ			
1474 vw	1477	7.3	CH₃ δ	1453	6.7	CH₃ δ	1464	5.1	CH₃ δ
1465 w	1462	16.7	NH δ						
1456 vw	1459	1.7	CH₃ δ	1444	1.2	CH₃ δ	1459	0.3	CH₃ δ
1447 vw	1457	2.3	CH₃ δ	1442	6.4	CH₃ δ	1452	1.3	CH₃ δ
1437 vw	1429	51.3	CC s (19b, Ph1)	1437	14.8	CC s (19b, Ph1)	1436	1.7	CC s (19b, Ph1)
1400 w	1395	1.3	CC s (19b, Ph2)	1392	13.6	CC s (19b, Ph2)	1399	6.5	CCH δ (Ph2)
1385 m	1387	10.5	CH₃ s	1376	4.6	CH₃ s	1382	0.6	CH₃ s
							1374	0.2	CCH δ (Ph2)
1372 m	1369	8.6	CH₃ a	1370	25.8	CH₃ a	1363	4.1	CH₃ a
1341 w	1332	19.5	CH δ				1339	69.9	CH δ
1315 s	1326	50.0	CC s (14, Ph1)	1324	102.5	CC s (14, Ph1)			
	1320	6.3	CH δ				1311	7.0	CH δ

[a] s strong; m medium; w weak; v very; sh shoulder; [b] s stretch; δ deformation; *a* asymmetric; *s* symmetric; Wilson notation in parentheses.
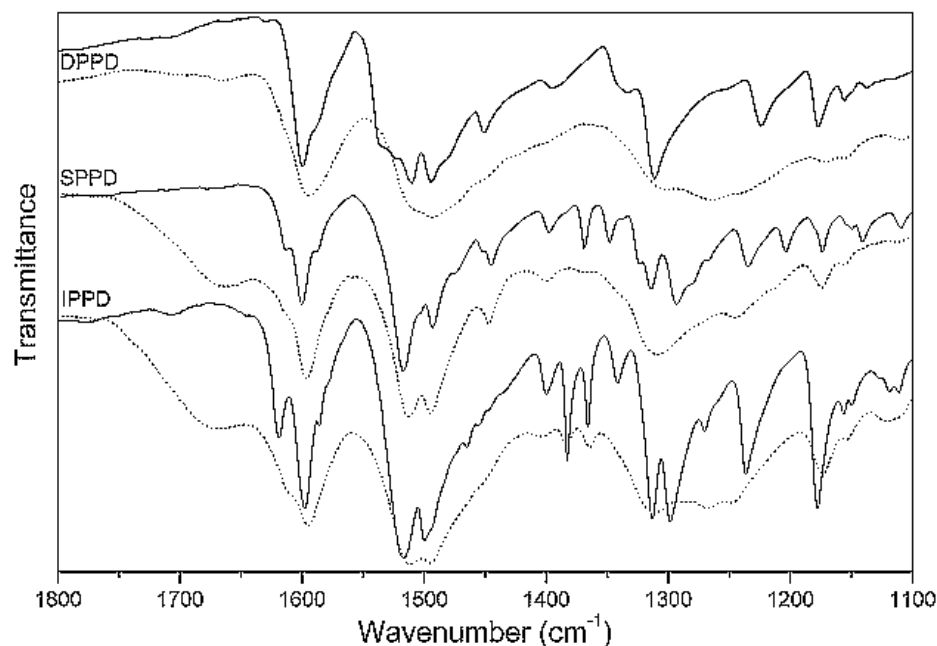
Figure 10. Comparison of relevant parts of DPPD, SPPD and IPPD IR spectra in KBr pellet before (full lines) and after heating (dotted lines).

iii) IPPD [24]

Also here the unheated IPPD spectrum has the features more typical for crystalline state, whereas the spectra after hot air acting are more similar to the amorphous state with broad bands. Similarly as above, heating causes the most dramatic IR bands changes in the region of the double bonds stretching vibrations where a new very strong band around $1670 \, cm^{-1}$ has appeared (Figures 9 - 10). Because the energy of N_A,C_X-double dehydrogenated IPPD molecule is significantly higher than its N_A,N_B- (quinonediimine) and N_B,C_X- (ketimine) counterparts (see Tab. 3) and its formation is thus highly improbable, it may be excluded from our further discussions.

The most important vibration modes calculated for unheated IPPD as well as its N_A,N_B- and N_B,C_X-double dehydrogenated forms are collected in Table 6. These results indicate that the calculated scaled frequencies of IPPD fit well with the experimental ones and that IPPD has no bands over $1620 \, cm^{-1}$. In the region of $1620 - 1420 \, cm^{-1}$, the substituted benzene ring has four characteristic modes occurring near 1600, 1580, 1490 and $1440 \, cm^{-1}$. These bands are good group vibrations and are marked as 8a, 8b, 19a and 19b according to the Wilson notation. The first two of them correspond mainly to CC ring stretch whereas the second two ones are the mixture of the CC ring stretch and CCH in-plane deformation modes. Because the last 19b mode of our compounds is in the region of the CH_3 group deformation modes, we will focus our discussion on the first three modes only. For monosubstituted benzene ring the modes 8a, 8b and 19a are expected in the regions of $1605\pm15 \, cm^{-1}$, $1585\pm15 \, cm^{-1}$ and

1485 ± 25 cm^{-1}, respectively, and for 1,4-disubstituted benzenes in the regions of 1610 ± 20 cm^{-1}, 1585 ± 30 cm^{-1} and 1500 ± 30 cm^{-1}, respectively [55]. For all the calculated compounds except the compound with the quinonediimine-type structure (N_A,N_B-double dehydrogenated IPPD), there are three modes in the expected region. For this compound the highest ring mode is shifted to the slightly higher frequency (1637 cm^{-1}). Because this mode corresponds to the symmetric stretch vibration of both C=C ring bonds, its intensity is very low and probably not measurable in the IR spectra. On the other hand, the next calculated band at 1602 cm^{-1} for the N_A,N_B-double dehydrogenated IPPD corresponds to the C=N asymmetric vibrations of both C=N bonds and it is one of the strongest bands in the IR spectrum. This agrees well with the experimental spectrum of the p-benzoquinonediimine where the highest frequency band in this region has been found at 1594 cm^{-1} and assigned to the C=N mode [56]. The Ph1 ring stretching modes of the N_A,N_B-double dehydrogenated IPPD are calculated at lower frequencies than the corresponding unheated IPPD modes. Therefore if the structure of N_A,N_B-double dehydrogenated IPPD is created during the IPPD oxidation process, the new band at 1670 cm^{-1} would not be expected according to our calculations. As mentioned before, the band around 1600 cm^{-1} is assigned in [3] to the monosubstituted benzene rings of 6PPD as well as of its oxidation quinonediimine-type product 6QDI. Our calculations show that using its intensity as normalized unit is not very correct because the corresponding ring CC stretching band is shifted down by ca 15 cm^{-1} and, moreover, this band is mixed with the C=N asymmetric stretching mode. Also the 19a mode bands of both benzene rings calculated for unheated IPPD at 1506 and 1488 cm^{-1} are in the N_A,N_B-double dehydrogenated IPPD shifted down to 1471 cm^{-1} for the monosubstituted Ph1 ring. There are no calculated bands between 1544 cm^{-1} and 1480 cm^{-1} for the N_A,N_B-double dehydrogenated IPPD. Thus if this quinonediimine structure is created, the both bands of 19a mode should decrease their intensity. It is in contradiction with [3] where only the band at 1514 cm^{-1} of disubstituted benzene ring decreases.

The appearing of new band at 1670 cm^{-1} in the IR spectrum may be explained by the structure of N_B,C_X-double dehydrogenated IPPD formation. The calculated frequency of 1674 cm^{-1} for this ketimine structure corresponds to the stretching vibration of the $N_B=C_X$ double bond. Calculated intensity of this band is very high and so it can be seen in IR spectrum even at lower concentrations. Such conclusion agrees with the calculated and experimental IR spectra of N-isopropylideneaniline for which N=C stretching vibration was calculated as a strong band at 1684 cm^{-1} and measured as a characteristic band at 1660 cm^{-1} [58]. Intensity changes of 1517 and 1500 cm^{-1} IPPD bands can be explained by the calculated downshift of the strongest IR band of NH deformation mode and 19a mode of Ph2 from unheated IPPD to N_B,C_X-double dehydrogenated IPPD.

Analogously as in the case of SPPD, an additional argument for N_B,C_X-double dehydrogenated IPPD and splitting off the tertiary C_X hydrogen is in the decrease of the intensity of IPPD 1341 cm^{-1} band. For unheated IPPD the band at 1332 cm^{-1} together with the 1320 cm^{-1} band have been calculated as tertiary C_XH deformation modes. In our calculations the N_B,C_X-double dehydrogenated IPPD bands in this area are missing unlike the N_A,N_B-double dehydrogenated IPPD with more intense 1339 cm^{-1} band. Therefore the disappearance of the mentioned 1341 cm^{-1} band in the IR spectrum of the 50 hours heated IPPD in hot air can be explained by the splitting off the tertiary C_X hydrogen.

Finally it may be concluded that in the IR studies of heated IPPD and SPPD [24, 25] (and the re-interpreted 6PPD one as well [3]), in the region of the double bonds stretching

vibration the most dramatic changes were observed and a new very strong band around 1660 cm^{-1} appeared. Such changes in the IR spectra of DPPD are not visible and the mentioned band was not observed (Figure 7). The appearing of the new band at 1660 cm^{-1} in the IR spectrum of SPPD and IPPD was explained by the formation of the Phenyl-N_B=C-ketimine structure in singlet spin state during their oxidation and not of its N,N´-dehydrogenated quinonediimine-type structure as supposed in the literature [3] for 6PPD. In 6PPD, SPPD and IPPD the formation of the ketimine structure is possible because amino-nitrogen is bonded with the tertiary carbon. On the other side the formation of the ketimine structure is not possible for DPPD and therefore appearing the new band at at 1660 cm^{-1} in its IR spectrum is impossible in agreement with the experimental results.

CONCLUSION

Our results show that oxidation of PPD antioxidants depends on reaction conditions. In addition to standard N-H bonds cleavage and quinonediimine-type products formation in solutions at room temperature, C-H bonds cleavage with subsequent ketimine-type products formation has been observed by heating on air at 140 ^{o}C in the case of *N*-phenyl-*N'*-alkyl-p-phenylenediamine antioxidants. This prediction based on DSC measurement has been confirmed by IR spectral data supported by quantum-chemical calculations at DFT level of theory. Nevertheless, our results indicate the reaction mechanisms of PPDs oxidation is very complex.

Unlike semiempirical PM3 and AM1 data, more reliable DFT data predict that from the thermodynamic point of view the formation of C centered radicals (i.e. C-H bonds cleavage in the first reaction step) is preferred for SPPD only. Nevertheless, the N_B=C_X ketimine structures are the most stable isomers formed in the second dehydrogenation step both for SPPD, 6PPD and IPPD molecules. Here it must be mentioned that the reaction rates are controlled by reaction barriers heights (i.e. kinetic factors). It is highly probable that the reaction barrier for N-H bond split is lower than the C-H one. Nevertheless, after long time periods the relative populations of individual isomers are re-distributed according to thermodynamics factors – the relative stabilities of individual isomers. The isomerisation rate increases with temperature and decreases with isomerisation barrier heights (for low temperatures and very high barriers the metastable state may be preserved for very long time). Our samples were heated ca 1- 5 days at 140 ^{o}C and the energy data in Table 3 predict only vanishing equilibrium concentrations of N_A,N_B-double dehydrogenated quinonediimine-type products that cannot be detected by IR spectroscopy.

Nevertheless, it must be mentioned that the possibility of different dominant reaction mechanisms of PPDs oxidation at lower and at higher temperatures cannot be excluded. Some deviations from linearity at DSC measurements of antioxidant effectivity might be interpreted in this sense as well [59]. Further experimental as well as theoretical studies in this field are desirable.

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Chapter VIII

A Novel 88 kDa Acute Phase Protein in Inflammations and its Antioxidant Function

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Abstract

Identification, purification and characterization of a new protein from the serum of patients with Eales' disease are presented. The protein was purified using preparative electrophoresis and HPLC. It resolved in α_2 globulin region. The purified protein had a retention time of 9.2 min in RP-HPLC. The molecular weight as determined by gel permeation chromatography was 88 kDa. Hence the protein is referred as 88 kDa protein. Periodic acid Schiff's staining revealed it as a glycoprotein. It was completely denatured above 70º C. Its isoelectric pH was 5. The protein was present in the vitreous and epiretinal membrane of Eales' patients and the blood of patients with systemic diseases like tuberculosis, leprosy and rheumatoid arthritis, but was absent in diabetic retinopathy and healed vasculitis of Eales' disease, as in the latter there is no inflammation. Hence 88 kDa protein is an acute phase protein produced in inflammatory conditions. The protein of different sources and diseases was immunologically the same.

The N terminal amino acid sequence by automated Edman's degradation chemistry is

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Ala-Asp-Asp-Pro-Asn-Ser-Leu-Ser-Pro-Ser-Ala-Phe-Ala-Glu-Ala-Leu-Ala-Leu-Leu-Arg-Asp-Ser-
 A   D   D   P   N   S   L   S   P   S   A   F   A   E   A   L   A   L   L   R   D   S

X-Leu-Ala-Arg-Phe-Val
 X   L   A   R   F   V

Keywords: 88 kDa protein, purification, physico chemical properties, sequence of N terminal 28 amino acids and 3 internal peptides, found in serum, vitreous and epiretinal membrane in Eales' disease with inflammation, serum of other diseases like leprosy and tuberculosis with inflammation, acute phase protein, immunological identify, quenches Reactive oxygen species and Reactive Nitrogen Species, anti TBARS activity, 8% thiols, ferro oxidase, arrests Fenton's reaction, preventive and protective endogenous antioxidant, novel protein.

THE SEQUENCE OF 3 INTERNAL PEPTIDES IS AS FOLLOWS:

SKVHDSYN	K = Lysine	G = Glycine
SSGTSYAALA	H = Histidine	T = Threonine
SPSYEFEEDE	Y = Tyrosine	

As the protein is expressed in inflammatory conditions with accumulation of Reactive Oxygen Species (ROS) $O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$, it was predicated that the 88 kDa protein expressed in inflammation might have antioxidant function. Protein purified from both serum and vitreous exhibited antilipid peroxidation effect on erythrocytes when added during *in vitro* assay of thiobarbituric acid reactive substances (TBARS). The antiTBARS activity was completely inhibited by 0.1 mM concentration of para chloro mercuric benzoate (PCMB) and 5,5' dithio bis (2/nitro) benzoic acid (DTNB). Inhibition by PCMB suggested the presence of thiol groups (-SH) which may be involved in antioxidant function. On analysis, the protein was found to contain 8% thiols by mass. It also oxidized ferrous iron to ferric iron and is a ferro oxidase. As such, by removing ferrous iron, the protein could arrest the Fenton's reaction and Haber-Weiss-Fentons' reaction which produce $OH^{\bullet}$ free radical. So, it is a preventive antioxidant like ceruloplasmin which is also a ferro oxidase and arrests the Fenton's reaction and the Haber-Weiss-Fentons' reaction. By virtue of its 8% thiol content, it can also quench the oxygen free radicals directly like metallothioneins. In this respect, it could function as a protective antioxidant by removing the oxygen free radicals after their formation. i.e. protecting from oxidation by ROS and even by Reactive Nitrogen Species (RNS).

The protein and DNA data base search revealed no match to 88 kDa protein.

The molecular weight, sequence of N-terminal amino acids, functions as a preventive and protective antioxidant certify that the 88 kDa protein is a novel protein different from other antioxidant or iron-sequestering proteins like transferrin, hemopexin, haptoglobins, ceruloplasmin metallothioneins and Retinal pigment epithelial protective protein (RPP). It is expressed to offer natural defense against oxidant – assault during inflammation.

A novel 88 kDa protein was discovered in the Biochemistry Research Department, Vision Research Foundation, Sankara Nethralaya, Chennai, India, in the course of Research work done to unearth the etiology of Eales' disease (ED) (Sulochana et al, 2001). The disease is named after the scientist Henry Eales who described the disease in 1882 in a patient. In spite of many years of active research by different groups, the etiology of Eales' disease is not known till this date. It is an idiopathic venous occlusion that primarily affects the peripheral retina of young adults (Eales, 1880, 1882). The clinical symptoms in most cases are retinal vasculitis with inflammation within the eye that involves the retinal vessels including arterioles, recurrent vitreous hemorrhage and retinal neovascularization (Spitzans et al., 1995). Bleeding from neovascularization is common, usually recurrent and is one of the major causes for visual loss in such cases (Das et al., 1994). Vitreous hemorrhage is a prominent manifestation of the disease and is the major cause of visual impairment. In addition, the formation of epiretinal membrane imposes a tractional pull on the retina causing retinal detachment.

Timely intervention with pars plana vitrectomy, laser photocoagulation and removal of epiretinal membrane helps in the partial restoration of the vision. The treatment for ED is based on the symptoms. It includes corticosteroids, photocoagulation with or without cryoablation and vitrectomy as the case may be, depending on the clinical appraisal of the disease process (Das et al., 1994 Biswas et al 2002).

Vitreous hemorrhage is the prominent manifestation of the disease and is the major cause of visual impairment. Retinal vasculitis can be associated with (a) systemic disease (b) infectious disease and (c) disease exclusively affecting the eye (primary ocular disorders. ED and uveitis fall under category c i.e. primary eye disease.

ED is rare in developed countries like USA but common in the Indian subcontinent with an incidence of one in 200-250 ophthalmic patients (Puttana, 1970). The disease predominantly affects males in the age groups of 20-40 years and is often bilateral. The peak age of onset of symptoms is 20-30 years.

There are 2 stages in ED, the active vasculitis stage and the healed stage. The active stage is characterized with inflammation. Rengarajan et al. (1987) have identified and purified a 23 kDa protein from the serum of patients with ED and speculate that this protein might be involved in the pathogenesis of ED. We have found for the first time, a protein of molecular weight 88 kDa (hereafter referred to as 88 kDa protein) to be present in the sera of almost all the patients of ED with active vasculitis and inflammation. The protein was absent in healed vasculitis in which the inflammation had been cured by medical treatment.

THE PROTEIN WAS IDENTIFIED BY THE FOLLOWING METHOD

The serum samples of patients (25 μg/50 μl) with active vasculitis were run on 7.5% native polyacrylamide gel electrophoresis. After electrophoresis, the gel was stained with Coomassive brilliant blue R 250. It resolved in α_2 globulin region (Figure 1). Samples showing the presence of the candidate protein were stored at – 20º C until they were used to get the purified protein. Serum samples from usually six to eight patients (1.5 gm protein / 25 ml serum) found to contain the protein were pooled and subjected to 70% ammonium

sulphate precipitation. The precipitate was dialyzed against 10 mM phosphate buffer pH 7.4 for 48 hrs with frequent changes of buffer at every 4 to 6 hours interval. After dialysis, the sample was centrifuged at 5000 rpm for 30 min. at 4° C, the supernatant was subjected to further purification by preparative gel electrophoresis. During each step of purification, protein content was determined by method of Lowry et al. (1951). Preparative electrophoresis (Biorad 491 Hercules, CA, USA) was done on a 5% Native PAGE (NPAGE) and the proteins were eluted using 0.05 mM Tris-glycine buffer pH 8.3. Fractions of 5.0 ml were collected at a flow rate of 1.0 ml/min using continuous buffer elution system (Bio Rad Echno PumpEP1). All the fractions were screened at 280 nm using spectrophotometer (Beckman DU 640, Fullerton, CA, USA) for protein content and to asses the elution profile. They were also subjected to NPAGE and silver staining (Morrissey, 1981). The new protein eluted between 150-175 fractions (Figure 2). The fractions positive for the protein were further subjected to anti TBARS activity. The active fractions were pooled and stored at -20°C until further purification. During every stage of purification, the presence of the protein was confirmed by performing 7.5 % NPAGE along with positive serum obtained from patients with Eales' disease.
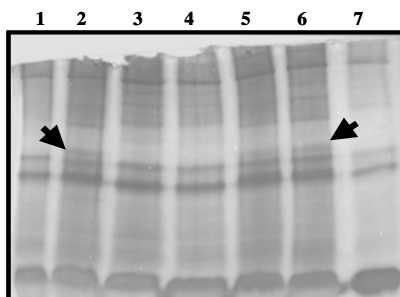
Figure 1: 7.5% native PAGE, stained by Commassie brilliant blue G250. Lane 1, and 7 serum from normal healthy volunteer, Lane 2,5 and 6 are serum from patients with Eales' disease, Lane 3 and 4 serum from patients with diabetic retinopathy. The presence of 88kDa protein in Lanes 2,5 and 6 is indicated by arrow in the photomicrograph. 20 mg of serum protein was uniformly used for protein profile.
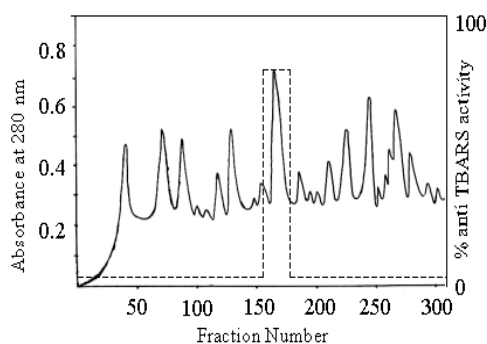
Figure 2: Elution profile of proteins from preparative cell electrophoresis system. 88 kDa protein gets eluted from 150 -175 fractions, which is represented by dashed line in the graph. Anti TBARS activity was maximum in these fractions.

PURIFICATION OF THE PROTEIN: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Active fractions from preparative electrophoresis were pooled and protein content was measured and then concentrated by lyophilization using Virtis freeze drier (NY, USA). The protein concentrate (50 mg) was further purified in batches using ODS reversed phase HPLC [LKB Bromma, Sweden].

Solvent A consisted of 0.05 % trifluroacetic acid (TFA) in Milli Q water (Millipore Co, MA, U.S.A). Solvent B consisted of 80 % acetonitrile and 0.05 % TFA in Milli Q water. The separation was performed using linear gradient 0.0 – 20.0 min 0.0 – 100 % B. The fractions were subjected to silver staining and anti TBARS activity. Active fractions were pooled and rechromatographed in the same conditions until the protein resolved as a homogeneous peak. In the RP-HPLC, the protein had a retention time of 9.2 min (Figure 3). The purity was further checked by sodium dodecyl sulphate (SDS) PAGE electrophoresis followed by silver staining. The purified protein was dialyzed against 10 mM. N- (2 hydroxyethyl) piperazine – N'- (2 ethanesulfonic acid) [HEPES] buffer pH 7.4, and used for further characterization studies.
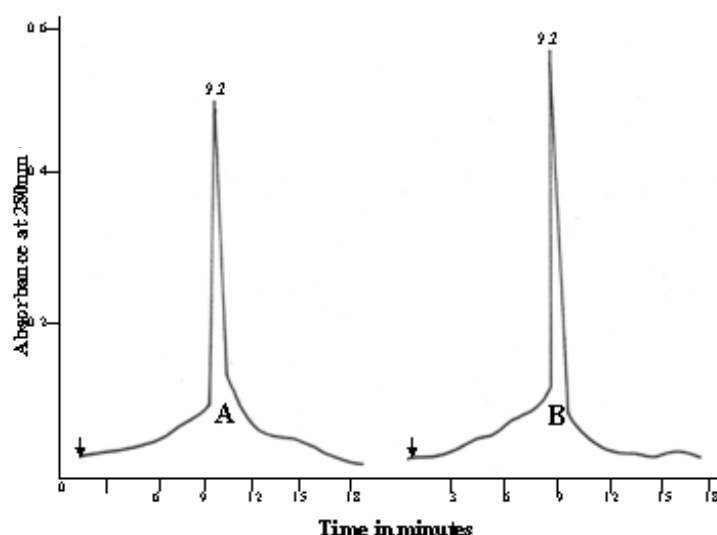
Figure 3: HPLC elution profile showing the homogeneity of the 88kDa protein from (A) serum and (B) vitreous (5 mg each) with retention time of 9.2 min. The separation was performed using RP C18 column 5 mm particle size with 300 A° pore size (Lichrosphere, Merck, Germany).

PHYSICO CHEMICAL PROPERTIES OF THE PROTEIN

Molecular Weight Determination

This was carried out using a gel permeation HPLC column (TSK 3000, Amersham Pharmacia, UK) and Sigma molecular weight markers. Along with these markers, 2 µg of

purified protein was loaded to gel filtration column. The proteins were eluted with 10 mM phosphate buffer pH 7.4 containing 20 % methanol in 50 mM sodium chloride, at a flow rate of 0.2ml/min. The elution was monitored at 280 nm (LKB Bromma UV detector). Molecular weight was calculated from the graph plotted with retention time against log molecular weight (Figure 4). In addition to the above, molecular weight of the purified protein was also determined by SDS PAGE (Figure 5). In both the methods, the molecular weight of the new protein was found to be ~ 88 kDa.
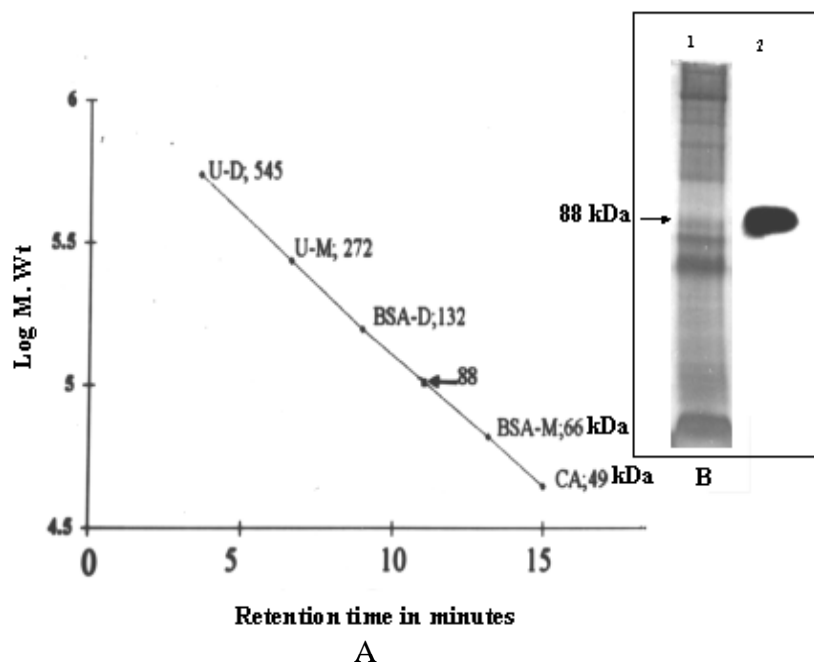
Figure 4: Molecular weight determination of the purified protein. This was done using gel filtration column (TSK 3000, Amersham Pharmacia, Sweden) using LKB Bromma HPLC system.. The gel permeation molecular weight markers used were urease dimer (545 kDa), urease monomer (272 kDa), BSA dimer (132 kDa), BSA monomer (66 kDa) and carbonic anhydrase (49 kDa). **B:** 7.5% native PAGE of purified protein stained by silver nitrate indicating its homogeneity. Lane 1, Serum (20 mg) from patient with Eales' disease, Lane 2, purified 88 kDa protein (30 mg).

Determination of Isoelectric Point (pI)

Determination of pI of 88kDa protein was performed using Mini-Portean II tube cell apparatus (Bio Rad, U.S.A). In brief, the capillary tubes were filled with monomer solution consisting of 9.2 M urea (5.5 g), 4 % acrylamide (total monomer 1.33 ml), 20 % triton X 100 (2 ml), kaleidoscope pI marker i.e 1.6 % Bio- Lyte 5/7 ampholyte (0.4 ml) and 0.4 % Bio-Lyte 3/10 ampholyte (0.1 ml) (Bio Rad) dH$_2$O, 0.01 % ammonium sulphate (10 μl) and 0.1 % TEMED (10 μL). This makes total volume of 10 ml sufficient to cast a set of 8 capillaries. The purified 88 kDa protein (5 μg/20μl) was mixed with 25 μl of sample buffer containing, 9.5 M urea, 2.0 % triton X 100, 5 % β mercaptoethanol and 1.6 % 5/7, 3/10 ampholyte

markers and incubated at room temperature for 15 min. During the incubation time, the gel was pre-electrophoresed (300 V / 15 min) with the upper chamber buffer containing 100 mM NaOH and lower chamber containing 10 mM H_3PO_4. After pre-run, the buffers were discarded and the sample was loaded on to the tubes and overlaid with sample buffer 20 µl , which contained 9 M urea along with 0.8 % 5/7 ampholyte and 0.2 % 3/10 ampholyte. Then the focussing was performed by running the gels for 500V for 15 min, increasing to 750 V and continuing the run for 3.5 hrs. After the completion of the run the pI of the 88 kDa protein was determined by comparing with samples that contained standard proteins with ampholytes and with that of 88 kDa protein.

The protein had a pI at pH 5.
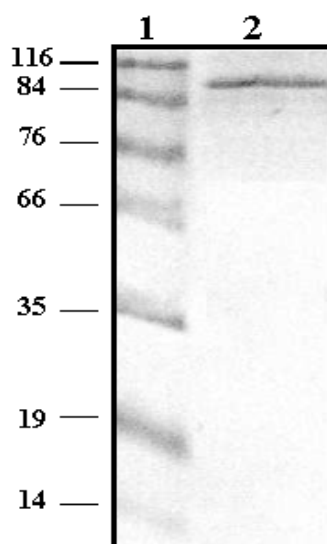
Figure 5: 12.5 % SDS PAGE gel profile for purified protein. Lane 1: molecular weight markers, Lane 2: purified protein from either serum or vitreous. The presence of homogeneous protein ~ 88 kDa was observed.

Thermal Stability of the 88 kDa Protein

0.5 µg 88 kDa protein /100 µl PBS pH 7.4 was incubated at various temperatures starting from 37°C - 70°C for varying time points (10 min – 60 min) and the anti TBARS activity was determined in these tubes. Results showed that 88 kDa protein was completely destroyed at 70°C when heated for more than 20 min.

88kDa protein - a Glycoprotein

Purified 88 kDa protein along with standard glycoprotein (ovalbumin) was resolved in SDS PAGE and PAS stain was performed. A positive pink staining indicated that 88 kDa is a glycoprotein (Figure 6).
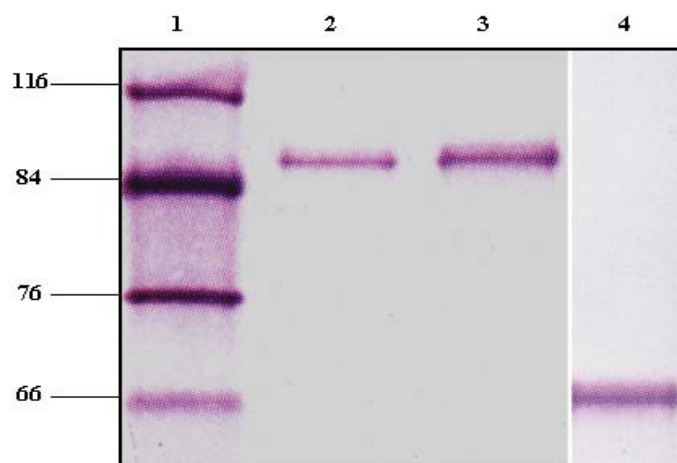
Figure 6: PAS stain for detecting glycoprotein. 12.5 % SDS PAGE gel profile, indicating the glycosylation of 88 kDa protein. Lane 1: Molecular weight markers, Lane 2: purified protein from vitreous of patients with ED, Lane 3: purified protein from serum of patients with ED. Lane 4: human albumin (monomer) was used as positive control.

Amino Terminal Sequencing of 88 kDa Protein (Matsudaria, 1987)

The purified protein was subjected to 12 % SDS PAGE electrophoresis and electrotransferred to polyvinylidine difluoride membrane (PVDF, Millipore Co., Bedford, MA, U.S.A) using Bio Rad Trans blot equipment. The electrotransfer was performed in buffer containing (3.3 g Tris base, 14.4 g glycine, 1.0 g SDS, 800 ml dH$_2$O and 200 ml CH$_3$OH) for 1hr at 100 V/400mA. The transferred protein was visualized by staining the membrane with 0.1 % ponceau S (dissolved in 1.0 % acetic acid in water). The protein band was then carefully excised, de-stained with several changes of Milli Q water. Then the PVDF membrane containing the protein was directly subjected to N terminal sequencing by using automated Edman degradation chemistry (Matsudaria, 1987) in Applied Biosystems 470A gas phase amino acid sequencer (Foster City, CA, USA). The derived sequence was analyzed by protein and DNA data base analysis using SWISS Prot, and NCBI programs.

Sequence of 28 amino acids from N-terminus of 88 kDa protein

1
Ala-Asp-Asp-Pro-Asn-Ser-Leu-Ser-Pro-Ser-Ala-Phe-Ala-Glu-Ala-Leu-Ala-Leu-Leu-Arg-Asp-Ser-
   A   D   D   P   N   S   L   S   P   S   A   F   A   E   A   L   A   L   L   R   D   S

X-Leu-Ala-Arg-Phe-Val ^{28}
X   L   A   R   F   V

Conventional one letter symbols are used for amino acids
X - not determined

DETERMINATION OF INTERNAL PEPTIDE SEQUENCE

250 µg of purified protein was precipitated with 300 µl ice cold 100 % TCA, left in ice for 45 min and centrifuged at 4000 rpm at 4°C for 15 min. The supernatant was removed carefully and discarded. The precipitate was washed twice with ice-cold acetone (200 µl) by centrifuging as described above. Finally the precipitate was dissolved in 300 µl of 8 M urea solution. The solubilized protein was reduced with 10 µl of 45 mM dithiothreitol (DTT). This procedure was done in sealed ampules containing sample under nitrogen gas at 52°C for 30 min. Subsequently 10 µl of 25 mM iodoacetamide was added, after cooling the tubes to room temperature. The reaction was allowed to take place at room temperature for 15 min. To this, 150 µl of milli Q water was added. The protein was then digested with trypsin at the concentration of 0.2 mg / ml in 20mM ammonium carbonate buffer pH 8.0 for 12 – 16 hrs at 37°C. The contents were frozen rapidly in liquid nitrogen to stop the reaction and were reduced to near dryness in rotary speed vacumn concentrator (Savant Instruments, U.S.A). The residue was finally dissolved in 100 µl of Milli Q water and injected into C_8 column (Phenomax, U.S.A) and the peptides were separated using RP HPLC (Shimadzu –SLC6A, Japan). The solvents used were as follows **A**: 0.1 % TFA in water. **B**: 80 % CH_3CN in 0.75 % TFA in water. The gradient program was: 0-5 min 0 % B, 5 – 65 min 100 % B, 65-70 min 100 % B, 80 min 0 % B. Elution was monitored at 214 nm. The major peaks were collected and used for further processing. Data collection and processing was done using C-R7A Plus software (Shimadzu Corp. Japan). The volume of the major fractions was reduced to 50 µl and then rechromatography was performed to obtain pure peptides. The chromatographic conditions were similar as described above. The fractions containing pure peptides were reduced in volume and loaded on to automated amino acid sequencer (PSQ –1, Shimadzu, Japan) to obtain the internal peptide sequence. The derived sequence was analyzed by protein and DNA data base analysis using SWISS Prot, and NCBI programs.

Internal peptide sequence derived from tryptic digestion of purified 88 kDa protein

Peak	Retention time	Sequence
12	18	S K VHDSYN
14	21	SSGTSYAALA
17	23	SPSYEFEEDE

K = Lysine G = Glycine H = Histidine
T = Threonine Y = Tyrosine

Occurrence of protein in the vitreous: The protein was found to be present in the vitreous of patients with active vasculitis. (Sulochana et al. 2001).

PURIFICATION OF THE PROTEIN FROM THE VITREOUS

Purification of 88 kDa protein from vitreous was done by adopting the same procedure used for serum, but for the omission of ammonium sulfate precipitation. The undiluted vitreous samples (4.0 ml) from six patients were pooled, homogenized with phosphate buffered saline pH 7.4 and centrifuged at 5000 rpm for 30 minutes at 4ºC. The protein content in the supernatant was measured by the method of Lowry et al. (1951). The supernatant (3ml) was directly subjected to 5% preparative polyacrylamide gel electrophoresis. The fractions were analyzed as for serum. The active fractions were pooled and purified further by RP-HPLC. The purified protein was dialyzed against 10 mM HEPES buffer pH 7.4 and then used for characterization. All these procedures were done in a cold room maintained at 4ºC.

PROTEIN IN EPIRETINAL MEMBRANE

Immuno histochemistry was performed on epiretinal membranes (ERM) obtained from ED patients. 88 kDa protein was localized in inflammatory cells and in nonvascular endothelium in ERM obtained from patients with ED (Swami et al, 2002). (Figure 7)
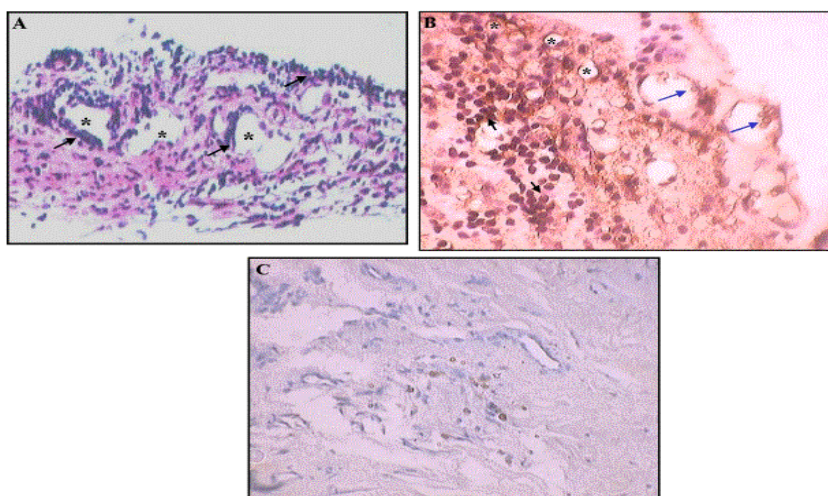
Figure 7: Immunohistochemical localization of 88 kDa protein in ERM of patients with Eales disease. (A) Haematoxylin and eosin staining of ERM obtained from patients with Eales disease, showing neovascular channels with dense leukocyte infiltration. Neovascular channels are indicated by black asterisks and the infiltrating leukocytes in the neovascular endothelium are represented by black arrows, X 200 magnification. (B) Immunolocalization for 88 kDa protein in ERM in Eales' disease. The expression of 88 kDa protein in infiltrating leukocytes are indicated by solid black arrow, while in the neovascular channels endothelium it is denoted by solid blue arrow, while the immunoreactivity of vascular endothelium for 88kDa protein is represented by black asterisks, X 400 magnification. (C) ERM obtained from patients with diabetic retinopathy did not reveal 88 kDa protein expression. X 200 magnification.

PRESENCE OF THE PROTEIN IN THE SERUM OF UVEITIS AND SYSTEMIC INFLAMMATORY AND OTHER DISEASES

Serum samples from healthy volunteers and from patients with ED, uveitis, pars planitis, ocular sarcoidosis, toxoplasmosis, leprosy, diabetic retinopathy, viral hepatitis, and rheumatoid arthritis were analyzed for 88 kDa protein by polyacralymide gel electrophoresis. It was detected in the serum samples obtained from patients with posterior uveitis, tuberculosis, leprosy and rheumatoid arthritis, all of which have inflammatory reaction. It was not detectable in diabetic retinopathy and proliferative diabetic retinopathy which do not have inflammatory process (Rajesh et al., 2003; Umamaheswari et al., 1999).

WESTERN BLOT

The purified 88kDa protein (20μg) from serum and vitreous was subjected to 12% SDS PAGE. Then the proteins were electrotransfered to nitrocellulose membrane (Millipore Co., Bedford, MA, USA). The membrane was blocked with 5% skimmed milk in PBS pH7.4 for 1hr at room temperature in a rocking platform. The membrane was then probed with anti 88kDa antibody (goat polyclonal 1:500dilution). Mouse anti goat IgG antibody was used as secondary antibody (Sigma, St Louis, CA, USA. 1:1000 dilution) and the protein was visualized using NBT/BCIP as substrate (Brunette, 1981). (Figure 8)
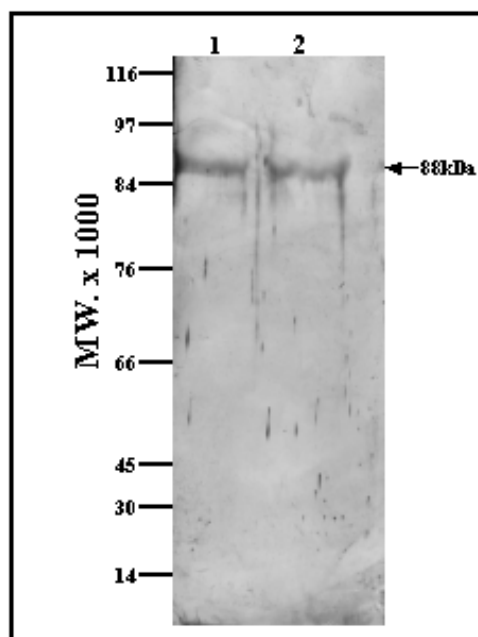
Figure 8: Western blot analysis of purified 88kDa protein. Lane 1, 30 mg purified 88kDa from serum. Lane 2, 30 mg purified 88kda from vitreous.

IMMUNOLOGICAL STUDIES ON 88 KDA PROTEIN

Partial Purification of IgG From Anti Serum

One mg of purified 88kDa protein was used for raising polyclonal antibody in goat as described earlier (Chase,1967). The antibody was custom made by Chemicon Inc., (Temecula, CA, USA). Purification of IgG from polyclonal antibody was done by adopting the method described by Talwar (1967). This essentially involved salt fractionation using sodium sulphate (18%) followed by ion exchange chromatography on DEAE column. The immunoglobulins were eluted using 100mM phosphate buffer pH 8.0. This fraction was used for further analysis.

IMMUNOELECTROPHORESIS

Immunoelectrophoresis was performed using the method described by Culliford (1964) and Lowrell (1972). Briefly, 1% agarose was dissolved in 0.04M Veronal buffer pH 8.6. The same buffer was used as tank buffer. Electrophoresis was carried out for 2 hrs with 10mA/slide. Following electrophoresis, another horizontal trough was made in between the two antigen wells and filled with 0.1ml of partially purified antibody (1:500). The slides were then kept in a humid chamber at room temperature for 24 to 36 hrs to allow the passive diffusion of antigen and antibody. Precipitin arcs were viewed directly under dark back ground, the slides were then dried completely (60°C for 2 hrs) and subsequently stained with Coomassie brilliant blue (R250) for permanent documentation (Figure 9).
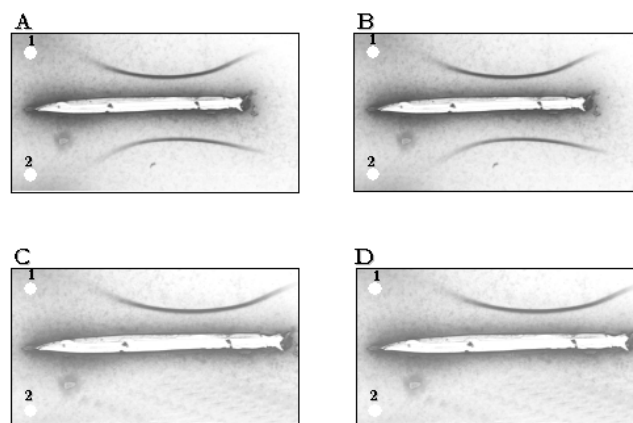
Figure 9: Immunoelectrophoresis of serum 88kDa protein with Eales' disease. (A) Well 1 serum (60 mg) from patient with Eales' disease, well 2 vitreous (10 mg) from patient with Eales' disease. (B) Purified 88kDa protein from vitreous (2 mg, well 1) and serum (2 mg, well 2) from patients with Eales' disease.(C) Well 1 vitreous (60 mg of protein) from a patient with Eales' disease, well 2 vitreous (10 mg of protein) from patient with diabetic retinopathy. (D) Well 1 serum (60 mg of protein) form patient with Eales' disease, well 2 serum (10 mg of protein) obtained from normal healthy volunteer. In all the above experiments (Fig 4A-D), the central well was probed with anti 88kDa antibody (goat polyclonal 1:500 dilution.

The immunological identify of the 88 kDa protein found in ED and in other diseases was investigated by Western blot. The 88 kDa protein found in serum from patients with ED was immunologically identical to that found in the serum of patients with uveitis, tuberculosis, leprosy, rheumatoid arthritis and the vitreous of patients with Eales' disease, all of which have inflammation. The protein was not present in diabetic retinopathy and proliferative diabetic retinopathy in which there is no inflammation (Figure 7).

BIOLOGICAL EFFECTS

The research work done has convincingly and conclusively shown that the novel protein is produced and found in inflammatory conditions. As such, its biological effect might have something to do with inflammation. This idea gave the clue for future work.

The polymorphonuclear neutrophils and the mononuclear phagocytes are the two types of cells very much involved in inflammation due to phagocytosis. During this process, the oxygen consumption of the macrophages is increased. The increased oxygen consumption is not utilized for energy purposes; in stead, oxygen is reduced to a series of highly reactive and toxic metabolites which include free radicals (with unpaired electrons) and ultimately water. The pathway of oxygen utilization is called "respiratory burst" (Ramakrishnan and Rajiswami 1995) and the metabolites including free radicals are collectively known as reactive oxygen species (ROS). They are (i) superoxide anion with an unpaired electron and negative charge $O_2^{\bullet-}$, (ii) hydrogen peroxide H_2O_2 (iii) hydroxyl free radical $OH^{\bullet}$, and through the latter lipid free radicals $L^{\bullet}$, $LO^{\bullet}$, $LO_2^{\bullet}$ and lipid peroxide LO_2H (Ramakrishnan 1992). The lipid free radicals and peroxide are converted to melondialdehyde which reacts with thiobarbituric acid giving a chromogen. There are a few other end products also which react with thiobarbituric acid. As such, all of them are collectively called thiobarbituric acid reactive substances (TBARS). The quantity of TBARS is decided by the extent of lipid oxidation which, in turn is decided by the quantity of ROS produced. So, the ROS formed due to oxygen thrust (or respiratory burst) is measured as TBARS.

In view of the relationship of inflammatory condition with production TBARS due to respiratory burst of macrophages, TBARS were estimated in the erythrocytes and vitreous in Eales' patients.

Inflammation $\longrightarrow$ Increased oxygen uptake by macrophages $\longrightarrow$ Respiratory burst

ROS $\longrightarrow$ $OH^{\bullet}\uparrow$ $\longrightarrow$ $L^{\bullet}$ TBA $\longrightarrow$ TBARS $\uparrow$
$LO^{\bullet}$
$LO_2^{\bullet}$
LOOH
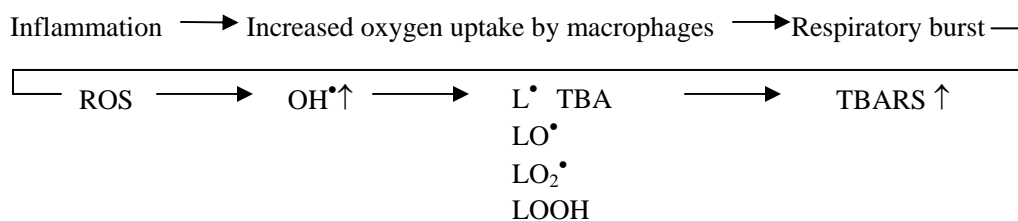
DETERMINATION OF THIOBARBITURIC ACID REACTIVE SUBSTANCES (TBARS) IN ERYTHROCYTES

(Devasagayam & Tarachand, 1987)

Malondialdehyde (MDA) produced during peroxidation of lipids, served as an index of lipid peroxidation. MDA reacts with Thiobarbituric acid (TBA) to generate a coloured product, which absorbs at 532 nm.

The erythrocytes were washed thrice with saline after removal of plasma. To the packed cells 1.5 ml of 10 % TCA was added and was allowed to stand for 15 minutes at room temperature. The tubes were centrifuged and to the supernatant 1.5 ml of TBA solution was added and heated in boiling water bath for 15 min. After cooling the tubes, the absorbance was measured at 532 nm.

TBARS were found to be increased in both the erythrocytes and the vitreous confirming the increased production of ROS (Bhooma et al., 1997), (Sulochana et al 1999) and in platelets (Srivastava et al, 2000).

In a living system which utilizes oxygen for respiration, there will always be the formation of ROS, but it is quenched by antioxidants which are also present in the living beings (just as pathogens are handled by immunoglobulins and interferons). Some antioxidants are endogenously produced while a few are exogenous – taken in the diet.

The endogenously produced antioxidants are glutathione, ceruloplasmin, metallothioneins, hemopexin and the enzymes superoxide dismutase, glutathione peroxidase, glutathione reductase, catalase, glutathione S-transferase and glucose 6 phosphate dehydrogenase. Those taken in the diet to build up a good antioxidant defence are vitamins E, C, A, carotenoids, lycopene, polyphenols, lipoic acid etc.

The antioxidants are also classified as preventive antioxidants and protective antioxidants that is, if the formation of ROS itself is blocked, the antioxidant is called preventive antioxidant (eg ceruloplasmin). If the antioxidant reacts and neutralises the oxidant after the formation of the latter, it is called protective antioxidant (eg. Vitamins E, C).

CHEMICAL BASIS FOR THE FORMATION OF ROS

How are the Reactive oxygen species (ROS) formed in living systems even under normal circumstances?

To keep life giving, one has to respire air for his oxygen requirement. The oxygen is utlizaed by each cell at the level of the inner membrane of the mitochondria for biological oxidation and oxidative phosphorylation. The hydrogens and electrons from the metabolites are routed through red-ox systems like $NAD^+/NADH$ to react with molecular oxygen catalyzed by the vital enzyme cytochrome a_3 (cytochrome oxidase). In this process a little oxygen undergoes univalent reduction as it is para magnetic and produces superoxide anion. (*Ramakrishnan, 2001*)

$$O_2 \longrightarrow O_2^{\bullet-}$$

(of air)   superoxide anion
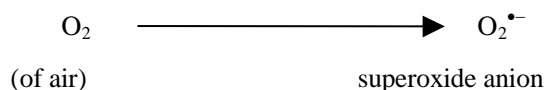
This superoxide anion, in its turn, triggers a chain of events and causes the production of H_2O_2 and $OH^\bullet$ if it is not removed immediately by the enzyme superoxide dismutase. The $OH^\bullet$ (free radical) reacts with long chain unsaturated fatty acids like arachidonic acid (Johnson et al, 1986) giving Lipid free radical ($L^\bullet$), lipoxide free radical ($LO^\bullet$), and lipid peroxide free radical ($LO_2^\bullet$) and ultimately lipid peroxide LOOH (Ramakrishnan, 1992). All those, which have odd electrons, are free radicals and being highly reactive, oxidize membrane proteins and lipids; the membrane integrity is lost.

It was thought that as 88 kDa protein is formed during inflammation with formation of ROS, it could have a role in quenching some oxidants and function as an antioxidant. To prove this prediction, protein purified from both serum and vitreous of patients with Eales disease was tested for anti TBARS activity on erythrocytes as detailed below.

The anti TBARS activity was measured using the method of *Ledwozywa et al* (1986). The extent of lipid peroxidation in fresh erythrocytes was measured as TBARS. The results were expressed as nmoles of malondialdehyde (MDA)dL^{-1} RBC. The quantity of the product, MDA was decreased when the assay was performed in the presence of 100ng of the purified 88 kDa protein to 1.0ml of erythrolysate. This property was used to identify the presence of 88 kDa protein during its purification in fractions collected from preparative electrophoresis and HPLC. The same assay was carried out to assess the effect of various thiol inhibitors such as para chloromercury benzoate (PCMB) and 5, 5^1, dithiobis 2 Nitro benzoic acid (DTNB).

The conventional thiol group inhibitor namely DTNB and PCMB inhibited this activity of the protein, indicating the importance of thiol residues in its function. This was further confirmed by the fact that cysteine residues contributed 8% of total mass of the protein. The *in vitro* demonstration of anti TBARS activity in neutral pH is suggestive of the fact that the contribution by carbohydrate moiety is negligible as enolization of sugar (in alkaline pH) is needed for antioxidant function. The above experiments proved that 88 kDa protein exhibits antioxidation property.

88 kDa Protein as a Preventive Antioxidant

In addition, the thiol containing protein oxidized Ferrous iron to Ferric iron and behaved as ferrooxidase like ceruloplasmin (an endogenous antioxidant)

Ferrous iron reacts with H_2O_2 and generates hydroxyl free radical in Fenton's reaction

$$H_2O_2 + Fe^{2+} \longrightarrow OH^- + OH^\bullet + Fe^{3+}$$
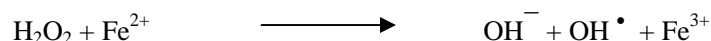
An extension of Fenton's reaction is Haber, Weiss Fentons' dismutation reaction with $O_2^{\bullet-}$ catalysed by Ferrous iron.

$$H_2O_2 + O_2\, Fe^{2+} \longrightarrow {}^\bullet OH^- + OH^\bullet + O_2$$
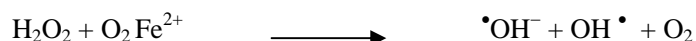
These reactions are arrested by ferro oxidases like ceruloplasmin and the newly found 88 kDa protein. The latter is thus functioning as a preventive antioxidant i.e. it prevents the very formation of $OH^\bullet$ and thereby lipid free radicals, through chain reactions as given below.

$$L^\bullet + O_2 \quad LO_2^\bullet \qquad\qquad LH + OH^\bullet \longrightarrow L^\bullet + H_2O$$

$$LO_2^\bullet + LH \longrightarrow LOOH + L^\bullet \qquad LH + LO_2^\bullet \longrightarrow L^\bullet + LOOH$$

$$2LOOH \xrightarrow{\text{Fe, Cu}} LO^\bullet + LO_2^\bullet + H_2O \qquad 2LOOH \xrightarrow{\text{Fe, Cu}} LO^\bullet + LO_2^\bullet + H_2O$$
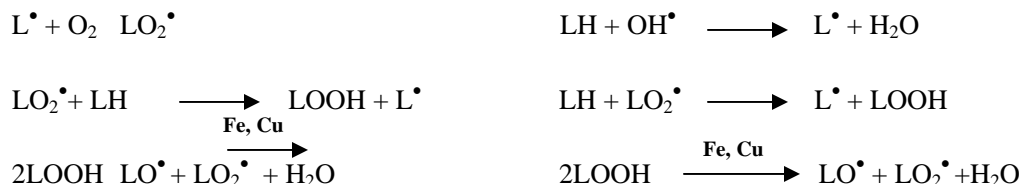
LOOH can be scavenged by glutathione peroxidase and glutathione (Ramakrishnan,1992) 88 kDa protein can sequester Ferrous iron and prevent the very formation of OH$^\bullet$ (preventive antioxidant).

88 KDA PROTEIN AS A PROTECTIVE ANTIOXIDANT

At the same time, it could also have a role as a protective endogenous antioxidant. This is by virtue of its thiol content. In this, its behaviour is similar to that of metallothioneins. The latter contain SH groups in cysteine residues and sequester metal ions including Fe, Cu etc. So the –SH groups in 88 kDa protein too can form complexes with divalent ions through their cysteinyl SH. (sequestering of iron, copper etc). In addition, metallothioneins can quench $O_2^-$$^\bullet$ and OH$^\bullet$ through their thiol groups. The same property can be expected of 88kDa protein which has cysteinyl SH and which, like metallothioneins, could quench $O_2^-$$^\bullet$ and OH$^\bullet$. In this, 88 kDa protein could function as a protective (endogenous) antioxidant like GSH i.e. these protective antioxidants protect the membrane from oxidation by destroying ROS after it (ROS) has formed.

88 KDA PROTEIN AND REACTIVE NITROGEN SPECIES (RNS)

Superoxide anion reacts with Nitric oxide (NO) to produce peroxynitrite (ONOO)$^-$ (Rajesh et al. 2003). The reaction of NO and O_2^- $^\bullet$ is facilitated during inflammatory conditions since phagocytes have been shown to generate simultaneously NO and $O_2^-$$^\bullet$ and at a similar rate (Rodenas et al, 1995) (ONOO)$^-$ is considered a more powerful oxidant than O_2^- $^\bullet$ because of its higher diffusion coefficient and half life. ONOO$^-$ on entering the cell, rapidly nitrates a variety of macro molecules chiefly at the aromatic rings (say tyrosine). Nitration affects the structure and functions of many proteins including. SOD.

In addition, peroxynitrite radical gets protonated and the product formed decomposes to give OH$^\bullet$ free radical. This hydroxyl free radical initiates a chain of reactions on poly unsaturated fatty acids of lipids giving lipid free radicals and ultimately TBARS. Just as, vitamin C and metallothioneins quench oxygen free radicals. 88 kDa protein also with –SH groups can quench oxygen free radicals and protect the membranes. So 88 kDa protein with its – SH groups can be considered to have the dual function of preventive antioxidant and protective antioxidant from both (i) ROS and (ii) ROS derived from RNS.

COMPARISON OF THE STRUCTURE AND FUNCTIONS OF 88 KDA PROTEIN WITH OTHER ANTIOXIDANT/ACUTE PHASE PROTEINS

Though 88 kDa protein is an antioxidant like ceruloplasmin, transferrin, haptoglobins, hemopexin and metallothioneins it stands alone as a different acute phase protein with antioxidant function. The functions of the various antioxidant proteins and their differences from 88 kDa protein are given below.

Transferrin, a glycoprotein is an iron sequestering protein binds Fe^{3+} and has a mol. wt of 77 kDa. There is no homology in the sequence of N-terminal amino acids of the two proteins.

Haptoglobins (glycoproteins) bind hemoglobin and have a molecular wt of about 90 kDa. They are raised in inflammation (Murrey et al., 1988). One of the members of ROS i.e. H_2O_2 is decomposed by haptoglobin-hemoglobin complex. The sequence of N terminal amino acids is different from that of 88 kDa protein..

Hemopexin, also a glycoprotein, is an iron-sequestering protein. It keeps heme in a soluble form. N terminal homology in different .

Ceruloplasmin incorporates Cu. as a structural component. It is a ferroxidase and quite different from -88 kDa protein in sequence and constitution. But both are ferrooxidases and arrest Fenton's reaction.

METALLOTHIONEINS

Metallothioneins are low molecular weight proteins (6.5 to 7.5 kDa) and sequester iron, copper and other divalent ions through their cysteinyl SH groups. Their expression is triggered in heavy metal toxicity and inflammatory conditions. In addition to removing iron and arresting Fenton's reaction, their high cysteine content makes them function as a scavenger of $O_2^{-\bullet}$ and $OH^{\bullet}$ radicals. So the metallothioneins are antioxidants – both preventive and protective. The new protein also like metallothioneins can function as a preventive (i.e. before production of ROS) and protective (i.e. destroying ROS after formation) antioxidant. But, in mol. wt. and N-terminal sequence the two are entirely different.

RETINAL PIGMENT EPITHELIAL PROTECTIVE PROTEIN (RPP)

A novel protein secreted by retinal pigment epithelial cells has been found by *Wu et al* (1996). It inhibits generation of superoxide ($O_2^{-\bullet}$) by activated neutrophils *in vitro*. It is well known that $O_2^{-\bullet}$ is one of the most injurious species through its secondary conversion to H_2O_2, $OH^{\bullet}$ (free radical) and peroxy nitrite (ONOO⁻) (Rao et al 1985, 1995). Most

antioxidants are intracellular – unable to move to extra cellular compartment. In the event of inflammation, RPE releases a protein to extra cellular space. The RPP protein is synthesized endogenously by RPE cells. The mechanism of its antioxidant effect is not by quenching the released superoxide (i.e. protective antioxidant); rather the protein intervenes in the activation cascade of the neutrophils and thereby arrests the consequential release of $O_2^{-\bullet}$. (i.e. preventive antioxidant)

Sequence studies have shown that in three of the amino acid sequences, there is some degree of homology with transferrin family protein while 5 other sequences are totally different.

88 kDa protein is different from RPP in sequence homology.

Comparative table of 88 kDa protein with other similar proteins.

Protein	Special property	Different from 88 kDa protein
Transferrin	Binding with iron	Molecular weight (77 kDa), No homology in N terminus
Haptoglobins	Hemoglobin binding, Agglutination, Electrophoretic movement (α_2 region) raised in inflammation, mol.wt 90 kDa.	Seen in all individuals and irrespective of disease.No sequence homology with 88 kDa protein in N terminus.
Ceruloplasmin	Contains copper, ferooxidase activity, endogenous antioxidant.	88 kDa protein not having ceruloplasmin activity with paraphenylene diamine, No sequence homology in N terminus
Metallothioneins	Low molecular wt,both preventive and protective antioxidant, contain – SH	High mol. wt. Though a preventive and protective antioxidant, 88 kDa protein is different in mol wt and / N-terminus.
Retinal pigment epithelial protective protein (RPP)	Has sequence homology in 3 domains with transferrin, but different in 5 domains; arrests the release of $O_2^{-\bullet}$; preventive antioxidant	Mol. wt and N terminus homology are different, also ferro oxidase.
Superoxide Dismutase	Endogenous antioxidant	88 kDa protein has no SOD activity; does not inhibit epinephrine auto oxidation.
Glutathione Peroxidase (GP_x)	Removes H_2O_2	88 kDa does not require GSH for activity, while GP_x needs GSH for its activity.

In summary, the 88 kDa protein is a novel protein, an endogenous preventive and protective antioxidant, different from other antioxidant or iron-sequestering proteins like transferrin, hemopexin, haptoglobins, ceruloplasmin, metallothioneins and Retinal pigment epithelial protective protein (RPP). It is expressed to offer natural defence against oxidant – assault during inflammation and is an acute phase protein.

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Chapter IX

Influences of Alpha-Ketoglutarate and Ornithine Alpha-Ketoglutarate on Lipid Peroxidation and Antioxidants and on Their Temporal Patterns

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Abstract

α-Ketoglutarate (α-KG) is a Krebs cycle intermediate. Ornithine α-ketoglutarate (OKG) is a salt formed from one molecule of α-ketoglutarate and two molecules of ornithine, which is a urea cycle intermediate. We investigated the effects of α-KG as well as OKG against ammonia induced oxidative stress in rats. Administration of ammonium acetate significantly elevated the levels of lipid peroxides and depleted the antioxidants (superoxide dismutase, catalase, glutathione peroxidase and reduced glutathione). α-KG as well as OKG positively modulated these changes. Thus it can be concluded that α-KG as well as OKG would exert their antihyperammonemic effects by positively modulating the lipid peroxides and antioxidants.

In the other study, we investigated the effects of α-KG on biochemical circadian rhythms during N-nitrosodiethylamine (NDEA) induced hepatocarcinogenesis in rats. NDEA caused a delay in acrophase of thiobarbituric acid reactive substances (TBARS) and antioxidants and an increase in mesor values of TBARS and decrease in mesor values of antioxidants. Administration of α-ketoglutarate reversed the circadian rhythm alterations caused by NDEA treatment. It can be suggested that α-KG would exert its

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chemopreventive effect by positively modulating the circadian rhythms of lipid peroxides and antioxidants.

INTRODUCTION

α-KG is a Krebs cycle intermediate. It is a carbon skeleton of glutamate and glutamine. It functions as an energy donor and ammonium ion scavenger as well as provides a source of glutamine that stimulates protein synthesis, inhibits protein degradation in muscle and constitutes an important metabolic fuel for cells of the gastrointestinal tract (Jones *et al.,* 1999). Enteral feeding of α-KG significantly increases circulating plasma levels of hormones such as insulin, growth hormone and growth factor IGF-1 (Jeevanandam et al., 1999).

Glutamine and glutamate with proline, histidine, arginine and ornithine, comprise 25% of the dietary amino acid intake and constitute the "glutamate family" of amino acids, which are disposed off through conversion to glutamate (Tapiero *et al.,* 2002). In major trauma, major surgery sepsis, bone marrow transplantation, intense chemotherapy and radiotherapy, when glutamine consumption exceeds its synthesis, it becomes a conditionally essential amino acid (Tapiero et al., 2002). In cells, glutamine is a key link between carbon metabolism of carbohydrates and proteins and plays a vital role in the growth of fibroblasts, lymphocytes and enterocytes.

Glutamine supplementation could be considered as metabolic support in patients undergoing treatment for cancer. It attenuates glutathione depletion in plasma, liver and gut after chemotherapy and upregulates systemic and tissue immune function during catabolic stress (Darmaun, 2000 and Ziegler et al*.,* 2000). Deamidation of glutamine via glutaminase produces glutamate a precursor of γ-amino butyric acid, a neurotransmission inhibitor. Glutamate plays an important role in neuronal differentiation, migration and survival in the developing brain via facilitated Ca^{++} transport (Yano et al., 1998). It also plays a critical role in synaptic maintenance and plasticity (McDonald and Johnston, 1990). It contributes to learning and memory through use dependent changes in synaptic efficacy and plays a role in the formation and function of cytoskeleton. Glutamine via glutamate is converted to α-KG. It is a component of the antioxidant glutathione and of the polyglutamated folic acid. Foly-polyglutamate synthesis is required for normal folate retention by cells (Shane, 1989).

Glutamine requirements are increased during injury, in particular to sustain the needs of rapidly growing cells like fibroblasts involved in wound healing. During injury, cell membrane depolarization and dissipation of Na^+ gradient may limit cellular glutamine uptake. Aussel et al. (1996) have reported that (1) the uptake of α-KG was independent of the presence of Na^+ in the medium. (2) 4-hydroxy α-cyanocinnamate, a known inhibitor of anion transport was ineffective on α-KG uptake and concluded that α-KG uptake occurs by an unmediated diffusion process and this uncontrolled character of α-KG uptake could be an advantage in extreme situations (Aussel et al.*,* 1996).

It has been reported that supplementing the diet with α-KG would spare dispensable amino acids as well as provide other benefits (Kristensen et al., 2002). α-KG is an important source of glutamate and glutamine, functioning as an energy donor and ammonium ion scavenger via its transformation into glutamate in perivein hepatocytes (Wiren et al., 2002). Glutamine is a precursor of proline which in turn plays a key role in collagen fiber formation

through its hydroxylation to hydroxyproline. In pigs, α-KG administration increases the level of proline in the portal and arterial blood by 45% and 20% respectively (Kristensen, 2002). Due to increased proline synthesis and its further hydroxylation to hydroxyproline, the major constituent of collagen, enteral α-KG administration is believed to enhance bone tissue formation (Harrison et al., 2004). It is evident that α-KG or its metabolites positively influence bone metabolism, making it an important factor to be considered in the treatment of osteoporosis (Harrison et al., 2004).

Yamamoto and Mohanan (2003) investigated the effects of α-KG on kainic acid induced brain mitochondrial DNA damage and seizures in mice. The increased lipid peroxidation *in vivo* and *in vitro* due to kainic acid exposure was completely inhibited by cotreatment of α-KG. They concluded that α-KG would inhibit reactive oxygen species-dependent oxidative damage to macromolecules in brain cells.

α-KG has a very substantial anticataractogenic effect which is related to its action as a ROS scavenger (Varma and Hegde, 2004). Kjellmann et al. (1997) studied the cardioprotective effect of α-KG in 24 patients undergoing heart operations and in control subjects; α-KG treated patients exhibited less severe signs of tissue injury and had a higher myocardial oxygen extraction and a lower lactate release 30 min after declamping. In addition, glutamine arterial concentrations were higher in the α-KG treated group but arteriovenous differences were similar in the two groups. This cardioprotective effect may be mediated through the generation of succinate (Laplante et al., 1997).

α-Ketoglutarate is involved in acid-base homeostasis through its hepatic and renal metabolism. In the liver, α-ketoglutarate uptake is mainly perivenous. During acidosis, the inhibition of periportal glutaminase and ureagenesis will decrease the bicarbonate utilization and increase the ammonia reaching the perivenous cells (Stoll and Haussinger, 1989; De Bandt et al*.,* 1994). In this situation α-KG is extensively used for glutamine synthesis. Furthermore, Welbourne (1983) indicated that in the presence of glutamine, α-KG induces a 80% decrease in ammonia production with a five-fold increase in bicarbonate release; α-ketoglutarate also raised glomerular filtration and bicarbonate reabsorption (Welbourne, 1983).

α-KG and to a lesser extent pyridoxal phosphate were involved in the proper activation of transaminases (Garabedian and Vermeersch, 1989). White (1989) evaluated that α-KG acts as a precursor for many biologically important medium chain ketodicarboxylic acids in methanogenic archaebacteria.

In cyanide toxicity, α-KG acts as an effective antidote. It binds to the cyanide molecule and prevents the circulation of free cyanide (Dunaley et al., 1991). Toxicity of cyanide is related to its inhibitory action of cytochrome C oxidase. The results suggest that oxygen displaces cyanide from the enzyme and the poison is then trapped by the ketoacids to form the respective non-toxic cyanohydrin (Niknahad et al., 1994). The combination of both high oxygen concentration and the presence of either α-KG (or) pyruvate is necessary to effectively protect cytochrome C oxidase against cyanide poisoning (Delhumeau et al., 1994).

Zimmermann et al. (1996) reported that long term administration of calcium alpha-ketoglutarate normalized secondary hyperparathyroidism by correcting calcium/ phosphate ratio in serum. Administration of α-KG in combination with calcium carbonate effectively

improved amino acid metabolism and decreased hyperphosphatemia in hemodialysis patients (Riedel et al., 1996).

Antioxidative properties of various concentrations of different alpha-ketoacids, including α-KG and their effects on the hemolysis of human erythrocytes induced by hydrogen peroxide were studied by Sokolowska et al. (2000). They concluded that α-KG offers protection against oxidative stress by participating in hydrogen peroxide decomposition process (Sokolowska et al., 2000).

ORNITHINE α-KETOGLUTARATE (OKG)

Ornithine α-ketoglutarate (OKG) is a salt formed from two molecules of ornithine (ORN) which is a urea-cycle amino acid and one molecule of α-ketoglutarate (α-KG).
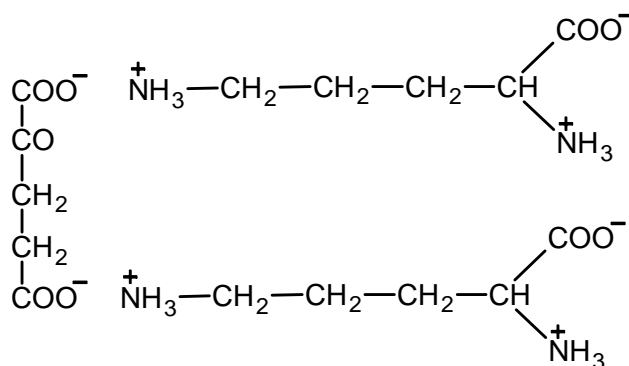
Figure 1. Ornithine α-ketoglutarate

It has been reported that in humans, the 2:1 molar combination of ORN and α-KG could modify the amino acid metabolism and hormonal patterns in a way that is not observed when they are administered separately (Vauborudolle et al., 1989; Cynober et al., 1990). This suggests that simultaneous administration of ORN and α-KG is necessary for expected anabolic effects.

METABOLIC PROPERTIES OF OKG

The metabolic properties of OKG are multiple (Cynober, 1995). Since OKG is an ionic salt, it dissociates into α-KG and ORN after administration (Cynober, 1991). The metabolic effects of OKG are related to α-ketoglutarate and ornithine by themselves and specific interactions between these two compounds (De Bandt and Cynober, 1998). Ornithine is processed via several metabolic pathways. This amino acid is a central part of urea cycle. In hepatocyte mitochondria, in the presence of carbamoyl phosphate, ornithine produces citrulline and this nonreversible reaction is catalyzed by ornithine carbamoyltransferase (Jones, 1985). At the "end" of the cycle, ornithine is regenerated from arginine by an

arginase-dependent reaction with the release of a urea molecule. Ornithine can also be decarboxylated via an ornithine decarboxylase into aliphatic polyamines, putrescine, spermine and spermidine (Grillo, 1985). Finally, ornithine undergoes a transamination reaction forming glutamate-semialdehyde, the precursor of glutamate and proline, via the formation of Δ'-pyrroline-5-caboxylate (Ohura et al., 1984; Jones, 1985).

Experiments performed on mice and with enzyme inhibitors suggest strongly that the latter pathway plays a major role in the metabolism of ornithine in vivo (Alonso and Rubio, 1989; Seiler et al., 1989). Furthermore, after (^{14}C)-Orn administration to rats, Glu and Pro appear to be the main metabolites (Vaubourdolle et al., 1989). Orn and α-KG follow several metabolic pathways but one is common to both molecules giving rise to glutamate in both cases (Cynober, 1991).

α-Ketoglutarate is synthesized from oxalosuccinate and gives rise to succinyl CoA. α-KG can also be aminated by glutamate dehydrogenase or transaminated into glutamate. In the latter case, the nitrogen donor is an amino acid thus transformed into a keto acid. Glutamate itself can be aminated into glutamine via a reaction catalyzed by glutamine synthetase (Meister, 1984).

1. Ornithine aminotransferase
2. Ornithine carbamoyltransferase
3. Arginase
4. Ornithine decarboxylase
5. Transaminase

6. Glutamate dehydrogenase
7. Glutamine synthetase
8. Glutaminase
P5C, pyrroline 5-carboxylate
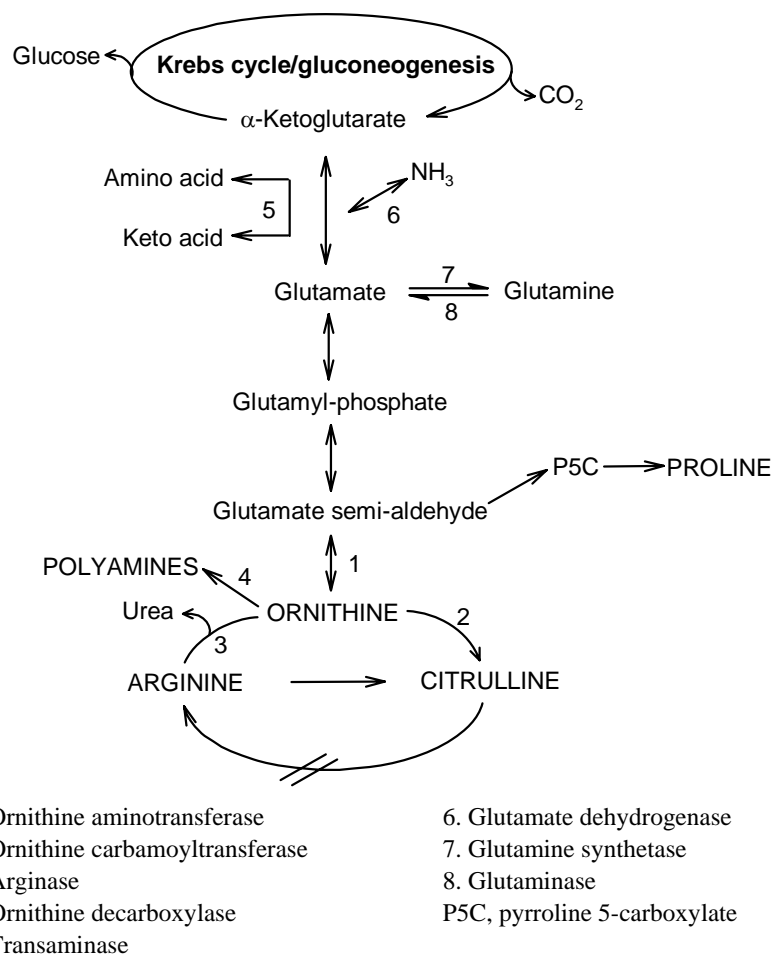
Figure 2. Metabolic pathways of ornithine and α-ketoglutarate

MECHANISM OF OKG ACTION

The mechanism of action of OKG is probably multifactorial, linked to the stimulation of anabolic hormone secretion and the production of Orn and/or α-KG metabolites (Figure 3) (Cynober, 1991).
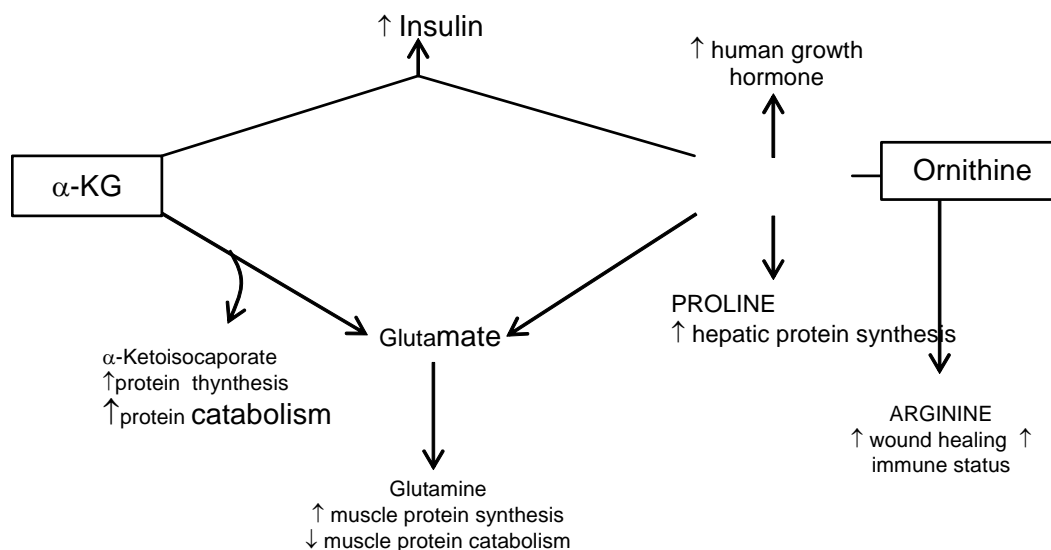
Figure 3. Possible mechanisms of OKG action

The initial purpose of OKG use was to improve the neurologic status of patients with hepatic encephalopathy at a time when ammonia was thought to play a major role as a causal agent of coma (Le Boucher et al., 1998).

OKG is a potent antihyperammonemic agent (Molimard et al., 1982) and is now being used for treatment. The rationale of treatment is as follows: α-ketoglutarate traps ammonia to form glutamate, which in turn is degraded in the pathway of ureagenesis, which may be activated by ornithine (Le Boucher et al., 1998).

HYPERAMMONEMIA, α-KG AND OKG

Ammonia intoxication reduces α-KG levels in the Krebs cycle and impairs the mitochondrial function (Kosenko et al., 1997a) which could lead to decreased ATP synthesis and also to an increased formation of free radicals (Kosenko et al., 1997b). It has been reported that sustained hyperammonemia in mice leads to increased lipid peroxidation in liver and brain, reflecting an oxidative stress condition (O'Connor et al., 1990).

We investigated the effects of α-KG as well as OKG on hyperammonemic rats and concluded that both these compounds are effective against ammonia induced toxicity which can be attributed to their participation in (1) the detoxification of excess ammonia, (2) non-

enzymatic oxidative decarboxylation during hydrogen peroxide decomposition process and (3) enhancement of the proper metabolism of fats that could suppress oxygen radical generation and thus prevent lipid peroxidative damage in rats (Dakshayani et al., 2002; Velvizhi et al., 2002a).

ALCOHOL INDUCED TOXICITY AND α-KG

Chronic alcohol intake is known to cause direct and indirect toxic effects in mammals and humans and is known to cause heptocellular damage due to the effects of byproducts such as acetaldehyde and acetate (Lieber, 1997). Chronic ethanol ingestion is known to increase hepatotoxicity and produce fatty liver with striking ultrastructural lesions, hepatitis and cirrhosis (Enomoto et al., 2000) and also to promote oxidative stress in mammals and humans (Morell et al., 1998). An increase in the free radical production is likely to play a role in the induction of severe cellular damage. The hydroxyl radical ($OH^\bullet$) formed by the action of hydrogen peroxide (H_2O_2) with Fe^{2+} in Fenton reaction would also cause cell membrane damage (Nordmann and Rouach, 1995). Formation of protein adducts with reactive aldehydes resulting from ethanol metabolism and lipid peroxidation has also been suggested to play a role in the pathogenesis of alcohol-evoked liver injury (Niemela et al., 1998).

We analysed the effects of α-KG on alcohol induced toxicity in rats and concluded that α-KG would positively modulate alcohol induced alterations in transminase activities, lipid peroxidation and antioxidants and offer protection against alcohol induced toxicity (Velvizhi et al., 2002b).

HEPATOCELLULAR CARCINOMA, DAILY RHYTHMS AND α-KG

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world (Okuda and Kojiro, 1993; Ferlay et al., 2001). Chronic infections with hepatitis viruses B and C, contamination of food stuffs with chemicals such as aflatoxin B_1 and nitrosocompounds and chronic abuse of alcoholic beverages are the major risk factors for the development of HCC (Tiribelli et al., 1989; Hecht, 1997). Tumors and tumor bearing hosts exhibit markedly altered circadian rhythms (Mormont and Levi, 1997). Amplitude damping, phase shifts and period change become more prominent at later stages of cancer (Mormont and Levi, 1997). These alterations would serve as markers in early diagnosis and prognosis of cancer.

It is a well established fact that oxidative stress plays a significant role in the pathogenesis of cancer and antioxidants exert chemopreventive effect against cancer (Karbownik et al., 2001). The effect of α-KG on circadian patterns of lipid peroxides during N-nitrosodiethylamine (NDEA) induced hepatocarcinogensis in rats has been studied. The circadian rhythm characteristics (acrophase, amplitude and mesor) of thiobarbituric acid reactive substances, antioxidants; superoxide dismutase, catalase, glutathione peroxidase and reduced glutathione were markedly altered in NDEA treated rats. The delays in acrophase observed in NDEA treated rats were brought back to near normal range by the administration

of α-KG. Increase in mesor values of TBARS and a decrease in mesor values of antioxidants in NDEA administered rats were reversed by α-KG administration (Figs. 4a-4e). We concluded that α-KG would exert chemopreventive effect by restoring antioxidants and their circadian rhythms (Dakshayani and Subramanian, 2004).

Fig. 4a. Thiobarbituric acid reactive substances (TBARS)
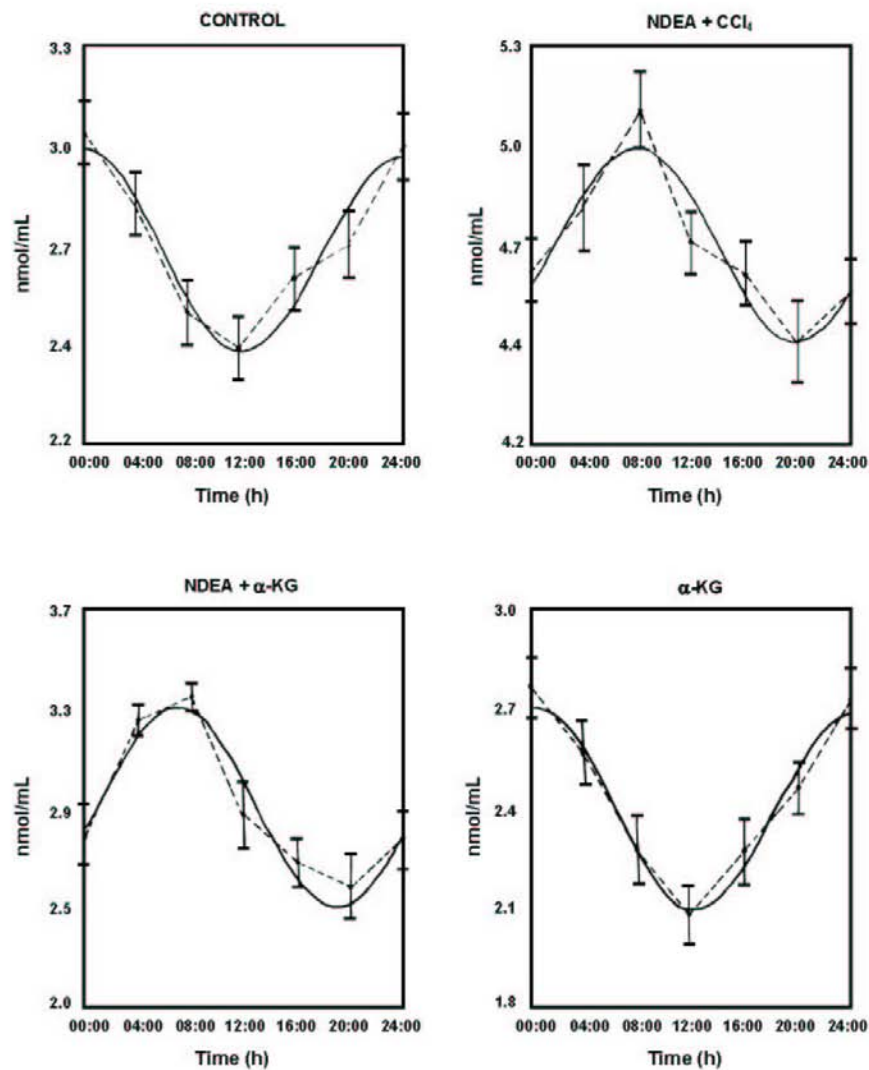
Figure 4a. Temporal characteristics of thiobarbituric acid reactive substances at 4 h intervals in control and experimental groups. Dotted line represents the raw data and solid line represents the best fitting cosinor curve obtained using consinorwin computer software programme.

Fig. 4b. Superoxide dismutase (SOD)
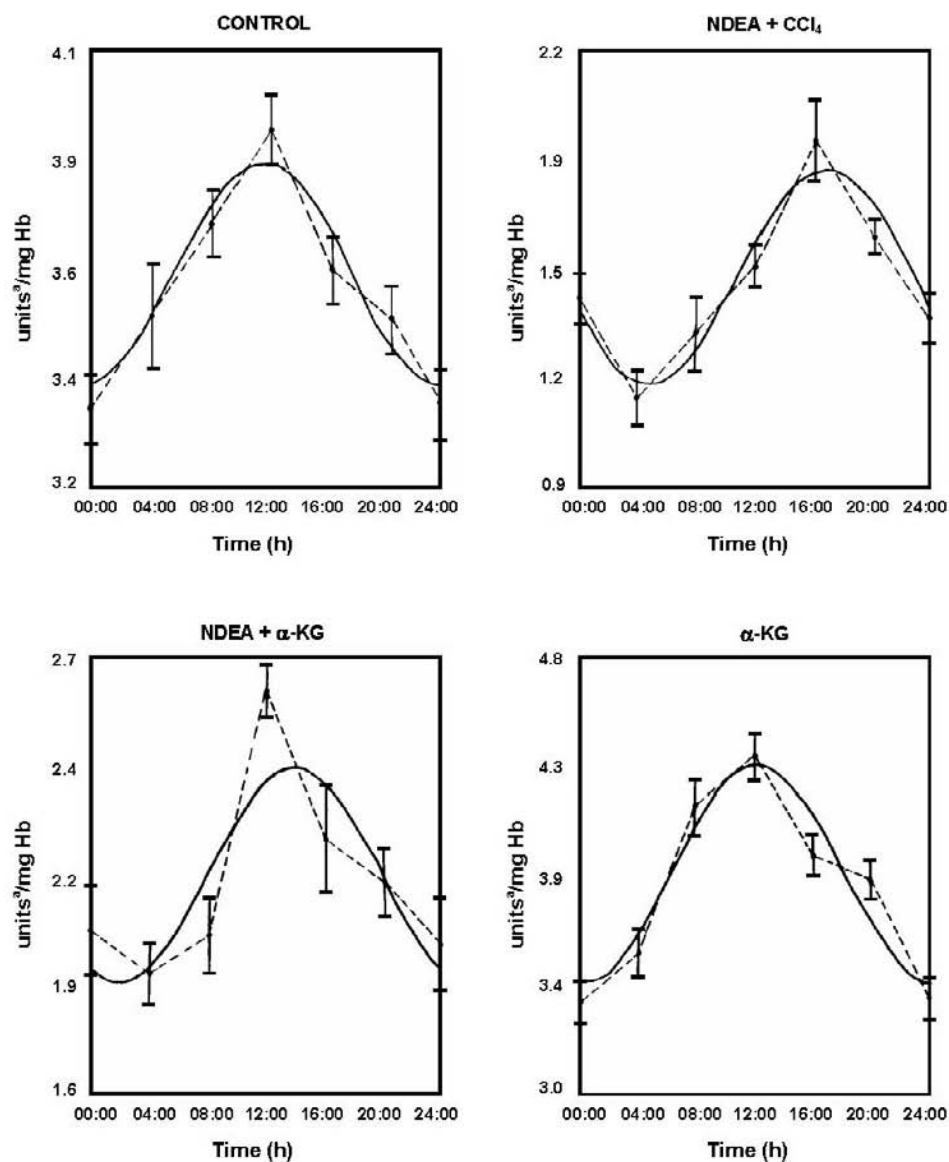
Figure 4b. Temporal characteristics of superoxide dimutase at 4 h intervals in control and experimental groups. Dotted line represents the raw data and solid line represents the best fitting cosinor curve obtained using consinorwin computer software programme.

Fig. 4c. Catalase
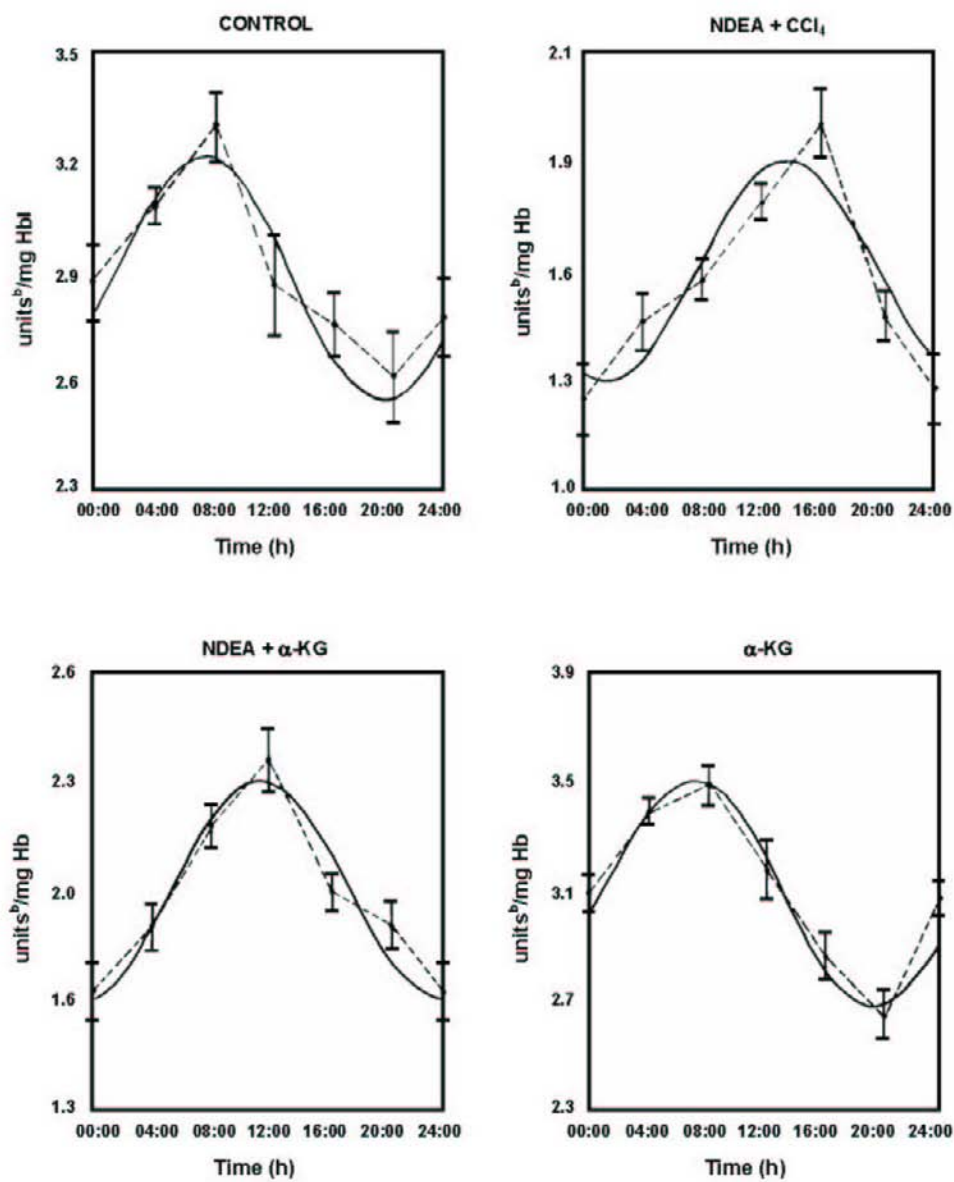
Figure 4c. Temporal characteristics of catalase at 4 h intervals in control and experimental groups. Dotted line represents the raw data and solid line represents the best fitting cosinor curve obtained using consinorwin computer software programme.

Fig. 4d. Glutathione peroxidase
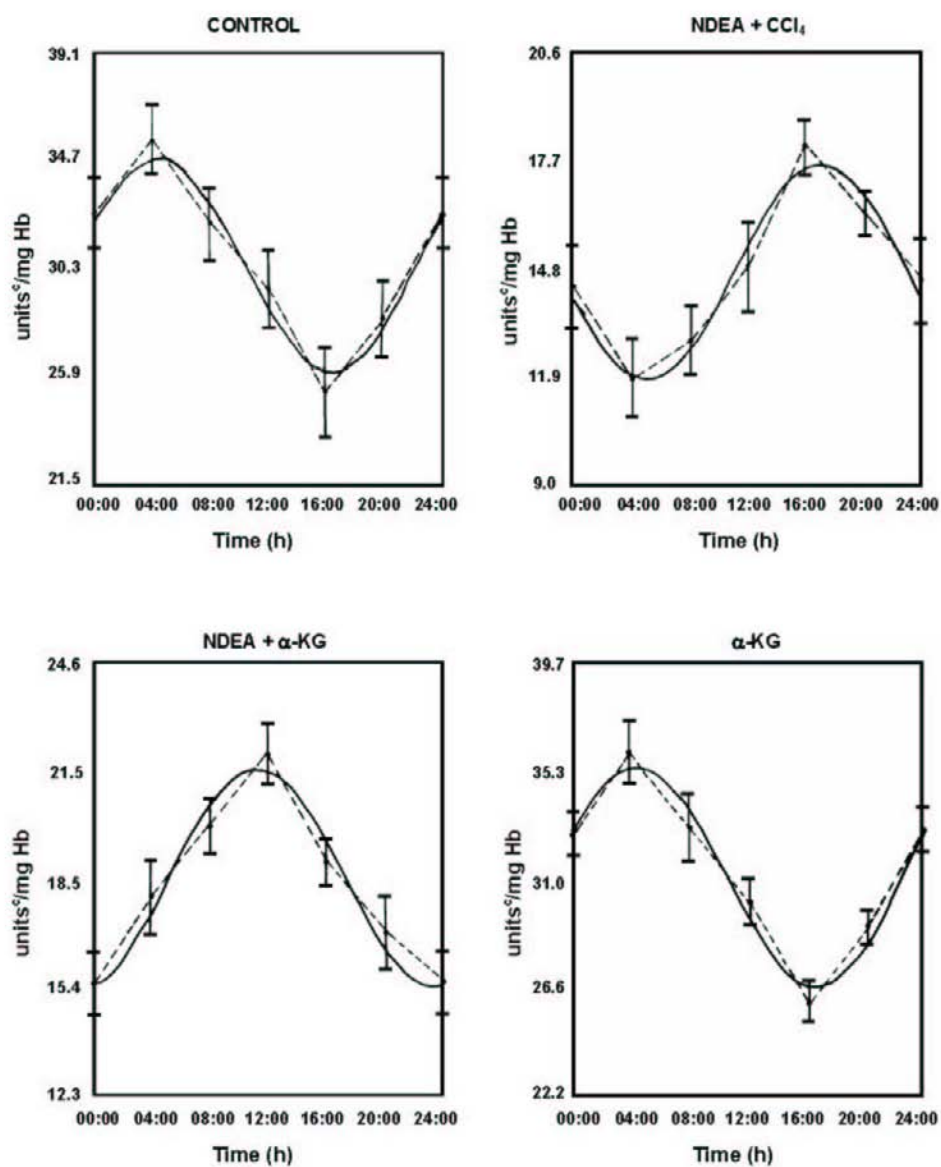
Figure 4d. Temporal characteristics of glutathione peroxidase at 4 h intervals in control and experimental groups. Dotted line represents the raw data and solid line represents the best fitting cosinor curve obtained using consinorwin computer software programme.

Fig. 4e. Reduced glutathione
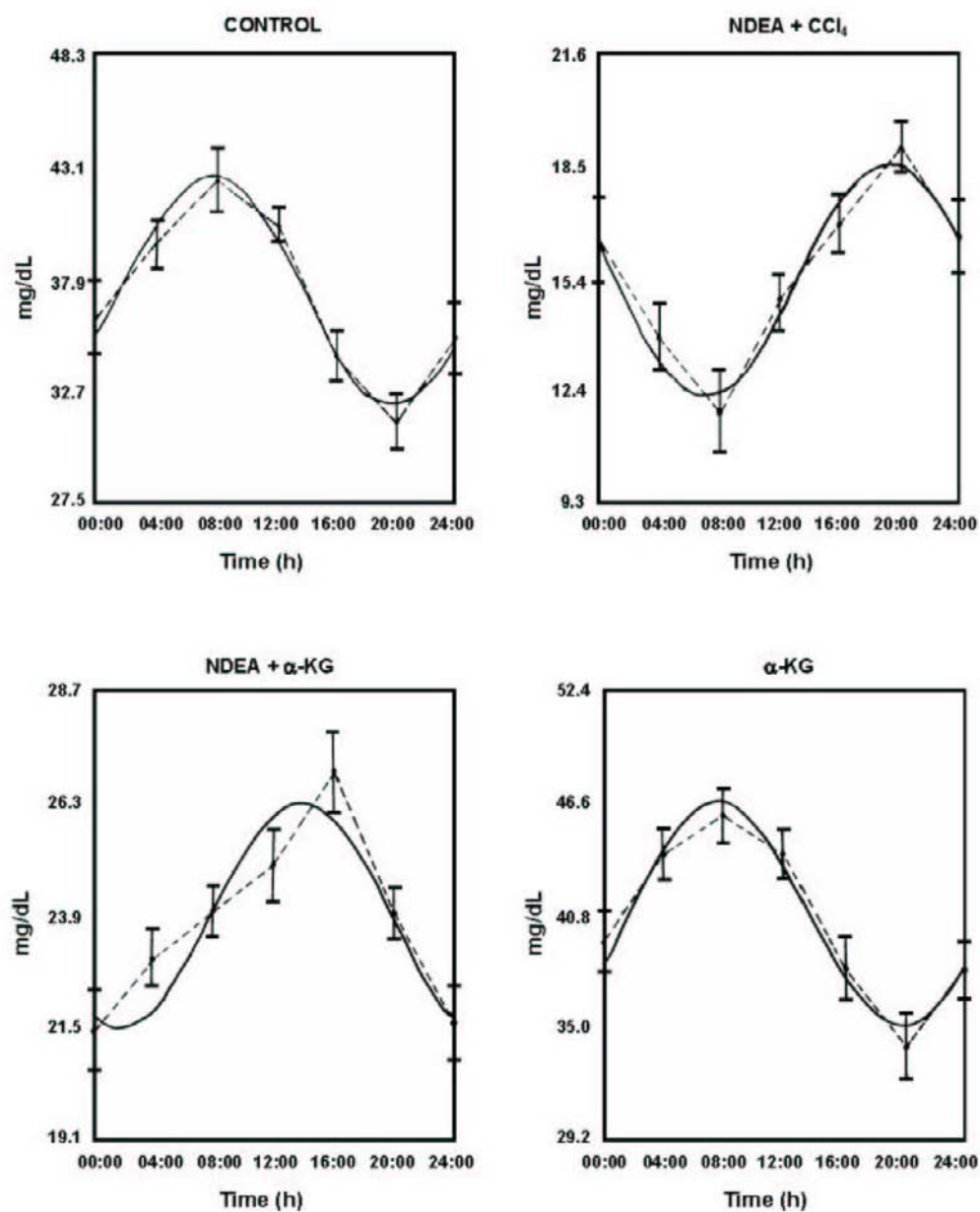
Figure 4e. Temporal characteristics of reduced glutathione at 4 h intervals in control and experimental groups. Dotted line represents the raw data and solid line represents the best fitting cosinor curve obtained using consinorwin computer software programme.

CONCLUSION

α-KG and OKG would have wide spectrum of applications in treating several pathological conditions like trauma, sepsis, osteoporosis, cataractogenesis, acid-base imbalance, cyanide poisoning, hyperparathyroidism, hyperammonemia, alcohol induced toxicity and cancer. These two compounds would hold a unique position in the field of pharmacy.

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Chapter X

DETERMINATION OF THE ANTIOXIDANT CONTENT OF CERTAIN PROPHYLACTIC AND TREATMENT PREPARATIONS

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ABSTRACT

The antioxidant content of a number of prophylactic and treatment preparations of the "Vision" company was determined. These were found to lie in the range $(7.5 – 1.7) \cdot 10^{-3}$ mole/kg, which is close to the antioxidant content of dry medicinal plants.

Key words: antioxidants, prophylactic and treatment, inhibitor, oxidation.

INTRODUCTION

Antioxidants play an important role in many processes of transforming organic matter, including biochemical ones. They provide a regulating function in these processes and often determine their direction.

All over the world today, biologically active food preparations containing antioxidants as matter securing normal functioning of the organism, i.e., normal interaction of all complex biological processes occurring in it, are widely used.

A deficiency of such matter in the organism leads to various disturbances and development of all kinds of pathology. Antioxidants enter a living organism with food, mainly vegetables. Their deficiency is the result of deteriorating ecology and large time

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interval between production of food and its consumption. Consuming food additives can compensate for their deficiency.

It should be noted that antioxidants of food additives by normalizing the functioning of the organism stave off the development of illnesses, while medicaments, as a rule, fight their consequences.

In the present work, the antioxidant content of a series of prophylactic and treatment preparations of the "Vision" company, obtained from plant extracts, was investigated. Their action covers a broad spectrum: from elimination of toxins from the organism to a prophylactic for cardiac-vascular and oncological illnesses. They are also used in case of abnormal metabolism, arthritis, liver illnesses, stomach ulcers and for strengthening the immunity system.

For antioxidant analysis an express method developed in the Institute of Chemical Physics of the Russian Academy of Sciences was used in this work [1-3]. It is based on the reaction of initiated oxidation of cumene (isopropyl benzene) by molecular oxygen. The method permits to determine the concentration of antioxidant in a complex composition and its antiradical activity. The oxidation reaction is conducted under conditions in which the oxidation process occurs at a constant rate over a long period of time. With the introduction in the reaction of antioxidant, an inhibitor of the oxidation process, the reaction is significantly slowed down, and as the inhibitor is expended increases to the value of an uninhibited reaction.

The inhibitor (antioxidant) expenditure time τ (period of induction) and the quantity of oxygen consumed ΔO_2 are determined from the following formulas [2-4].

$$\tau = \frac{2[\ln H]_0}{W_i} \tag{1}$$

$$\frac{\Delta O_2}{[RH]} = -\frac{k_3}{k_7}\ln(1 - t/\tau) \tag{2}$$

W_i - the initiating rate, is calculated from the formula

$$W_i = 6,8 \cdot 10^{-8}[\text{AIBN}] \text{ mole}/1 \cdot s , \tag{3}$$

where [AIBN] (AZO-bis-ISOBUTYRONITRILE) is the initiator concentration in mg per ml of hydrocarbon; RH - hydrocarbon and InH - antioxidant.

The concentration of antioxidant being analyzed [InH] is calculated from expression (1) using experimentally determined induction period τ and known initiating rate W_i. The inhibiting rate constant k_7, determining the antiradical activity of the antioxidant and being its quality indicator, is found from relation (2) using the chain continuation rate constant k_3, hydrocarbon concentration [RH], experimentally determined induction period τ and quantity of absorbed oxygen ΔO_2.

The method is functional, i.e., braking of the oxidation reaction is determined only in the presence of antioxidants in the system being analyzed. Other possible components of the system (not antioxidants) do not significantly affect the oxidation process, which enables one

to analyze antioxidants in complex systems without going through a process of their isolation. A description of the method and its application for concrete purposes can be found in [1-9].

RESULTS AND DISCUSSION

The following prophylactic and treatment preparations of the "Vision" company were taken for investigation: Antiox, Lifepack Probiotic, Lifepack Antiox, Sveltform, Pax, Chromvital, Nutrimax, and Detox. The antioxidant content of these products was determined.

As an example, the figure shows the kinetic dependences of oxygen absorption in a model reaction of initiated cumene oxidation in the absence of antioxidant (straight line 1) and in the presence of Nutrimax (curve 2).

ΔO_2, ml
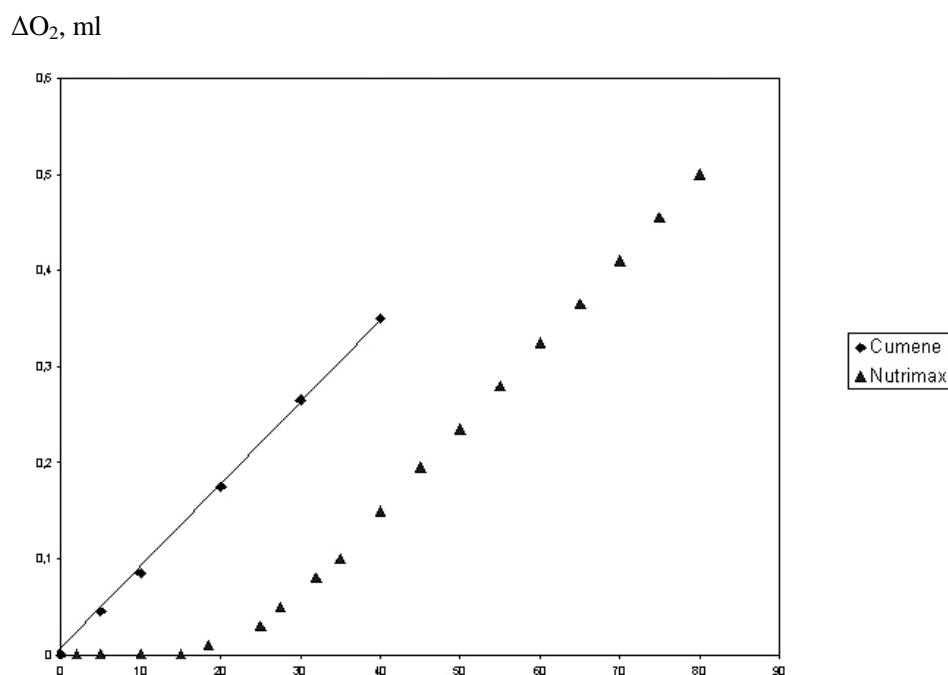
Figure. Kinetic dependences of oxygen absorption
    1 – hydrocarbon (cumene) alone,
    2 – with Nutrimax added (33.2 mg)
    1 ml of hydrocarbon, 1 mg of initiator, t = 60^0 C.

It can be seen from the figure that in the absence of the additive hydrocarbon oxidation proceeds at constant rate (straight line 1). When the preparation is added, the oxidation rate at the beginning is strongly retarded but begins to increase after a certain period of time. This is indicative of the presence of antioxidant in the additive.

The rise in reaction rate is due to the expenditure of antioxidant. When it is used up, the reaction proceeds at the constant rate of an uninhibited reaction. The time of antioxidant expenditure (τ) is determined graphically by the intersection of two straight lines on the kinetic curve. One of these is the straight-line portion of the kinetic curve after antioxidant

has been used up. The other is the tangent to the kinetic curve whose inclination angle is one-half the tangent angle of the first [2, 9].

Data on the antioxidant content of investigated preparations are presented in the table. These data are illustrated by the diagram.

Table. Concentration of Antioxidants in Studied Preparations of "Vision" Company

№	Preparations	Antioxidant Concentration	
		AO	(mole/kg)
1	Antiox	7.5	10^{-3}
2	Lifepack Probiotic	3.1	10^{-3}
3	Lifepack Antiox	3.0	10^{-3}
4	Sveltform	2.9	10^{-3}
5	Pax	2.3	10^{-3}
6	Chromvital	1.9	10^{-3}
7	Nutrimax	1.8	10^{-3}
8	Detox	1.7	10^{-3}
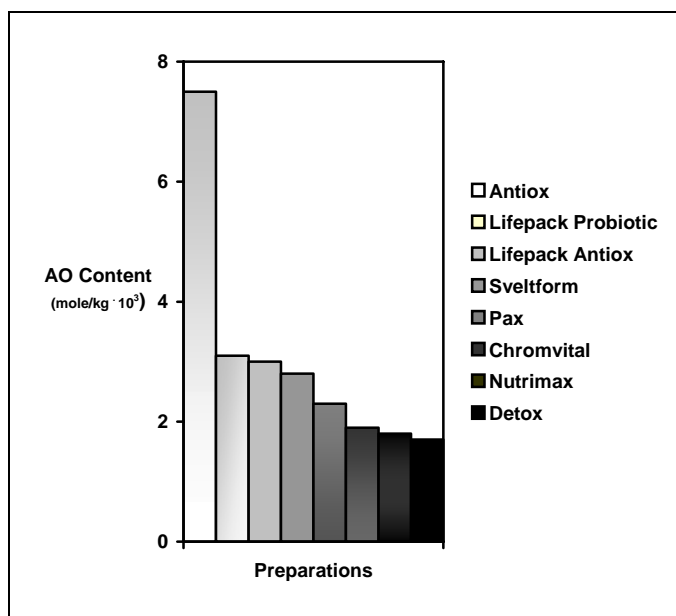
Diagram

It can be seen that the concentration of antioxidant for investigated preparations is in the range $(1.7\text{-}7.5) \cdot 10^{-3}$ mole/kg.

We have found earlier such a quantity of antioxidant, on the average, in dry medicinal plants: Hyssopus officinalis, Ocimum basilicum, Coriandrum sativum, Origanum vul gare, Padus racemosa and Melissa officinalis [10]. The closeness of values of antioxidant concentrations in the studied preparations and medicinal herbs gives reason to suppose that the preparations of "Vision" may be effective in acting on the organism.

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