THE EFFECTS OF ANTIOXIDANTS ON SOME RAT TISSUES AND MEMBRANES

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ABSTRACT

THE EFFECTS OF ANTIOXIDANTS ON DIABETIC RAT LIVER MICROSOMAL MEMBRANES AND ANTIOXIDANT ENZYME ACTIVITIES

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High blood glucose levels induce metabolic disorders that initiate a sequence of events including renal, arterial, cardiac and retinal disorders. Diabetes mellitus increases oxidative stress in tissues of animals including humans. The resulting oxidative stress might play role in the development of diabetic complications.

In the present study, 36 male Wistar rats (250-300g) were divided into 5 groups as Control (n=6), Diabetic (n=7), Diabetic + Vit C (n=7), Diabetic + α-Lipoic acid (n=6) and Diabetic + Combination of Vit C and α-Lipoic acid (n=10).

From the livers of all groups cytoplasmic and microsomal membrane fractions were prepared from liver and antioxidant enzymes namely, superoxide dismutase, glutathione peroxidase, catalase and glutathione S-transferase activities were measured. Microsomal lipid peroxidation, total lipid, total protein, reduced glutathione levels of each group was determined and compared. Microsomal fractions were also analyzed by FT-IR spectroscopy.

The total protein levels of diabetic rats were found to be decreased significantly ($p<0.05$) compared to controls and the α-lipoic acid and vitamin C supplemented groups tend to compensate the decreased levels of total proteins. Decreased catalase activity in diabetic group compared to control was restored by α-lipoic acid, vitamin C treatment and/or combination of both. Increased glutathione peroxidase activity was decreased to control levels by the treatement of both α-lipoic acid and vitamin C. Superoxide dismutase activities of diabetic rats were increased ($p<0.05$) compared to control group. Whereas glutathione S-transferase activities though showed some fluctuations, the results were not statistically significant. Total glutathione levels decreased in all groups significantly ($p<0.0.5$) compared to control group but any of the agent failed to compensate the reduced levels of glutathione. As an index of lipid peroxidation, TBA-reactivity (MDA) levels increased significantly in all diabetic groups and only combination group's TBARS levels decreased significantly compared to diabetic group.

FT-IR study of rat liver microsomal membranes was carried out in order to understand the effects of diabetes on membrane order, dynamics and lipid peroxidation status.

For this purpose CH_2 symmetric wavenumber, CH_2 antisymmetric bandwidth, =CH olefinic band area were compared. In temperature dependent FT-IR studies microsomal membrane phase behavior, order and dynamics were analyzed. Diabetic samples showed apparent decrease in both frequency and bandwidth. =CH olefinic band integrated area was increased for diabetic samples compared to controls. Alpha-lipoic acid and vitamin C supplemented groups showed similar effects. They tend to restore decreased levels of band frequency and bandwidth. Additive effect between α-lipoic acid and vitamin C was seen in some cases that only the combination group achieved to restore control values while α-lipoic acid and vitamin C were failed to restore alone.

In conclusion, STZ-induced diabetes mainly caused an increase in antioxidant enzyme activities. Also, increase in lipid peroxidation caused a decrease in the fluidity and order of the membrane resulting in more rigid membrane structures. The loss of cooperation between the antioxidant network may play a role in the secondary complications of diabetes.

Key words: antioxidant enzymes, FT-IR spectroscopy, diabetes, liver tissues, microsomal membranes, lipid peroxidation

ÖZ

ANTİOKSİDANLARIN BAZI SIÇAN DOKU VE ZARLARI ÜZERİNE ETKİLERİ

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Yüksek kan glukoz düzeyleri renal, arterial, kardiyak ve retinal bozukluklara yol açan bir dizi hastalığı tetikler. Diyabetes mellitus hem insan hem de hayvan dokularında oksidatif stresi artırır. Artmış oksidatif stres diyabetik komplikasyonların oluşumunda etkili olabilir.

Bu çalışmada, 36 erkek Wistar cinsi sıçan (250-300 g): Kontrol (n=6), Diyabetik (n=7), Diyabetik + C vitamini (n=7), Diyabetik + α-lipoik asit (n=6) ve Diabetik + α-lipoik asit ve C vitamini bileşimi (n=10) olmak üzere 5 gruba ayrıldı. Karaciğerler homojenize edildi. Sitoplazmik ve mikrozomal fraksiyonlar

ayrıldı. Antioksidan enzimler, süperoksit dismutaz, glutatyon peroksidaz, katalaz ve glutatyon S-transferaz aktiviteleri ölçüldü. Grupların mikrozomal lipid peroksidasyonu, total lipid, total protein, indirgenmiş glutatyon düzeyleri karşılaştırıldı. Mikrozomal fraksiyonlar aynı zamanda FT-IR çalışması için kullanıldı. Diyabetik sıçanların total protein düzeyleri kontrollere göre anlamlı düzeyde ($p<0.05$) artış gösterdi. Diyabetik grubun SOD aktiviteleri kontrole göre anlamlı düzeyde ($p<0.05$) yükseldi. Antioksidan uygulanmış gruplar düşmüş total protein düzeyini artırma eğilimi göstermiştir.Diyabetik grubun azalmış CAT aktivitesi α-lipoik asit ve C vitamini ve/veya her ikisinin kombinasyonunun verilmesiyle telafi edildi. Artmış GSHPx aktivitesi kombinasyon grubuna antioksidan kombinasyonu uygulanmasıyla normal düzeylere çekildi. GST aktivitesi anlamlı olmayan değişimler gösterdi. Tüm diyabetik gruplarda glutatyon düzeyleri kontrol grubuna kıyasla anlamlı azalma gösterdi, ve bu azalmayı hiçbir antioksidan telafi edemedi. Lipid peroksidasyonunun bir göstergesi olarak TBA-reaktivitesi (MDA) düzeyleri tüm diyabetik gruplarda anlamlı artış gösterdi. Bununla birlikte sadece kombinasyon grubunun MDA düzeyleri diyabetik gruba göre anlamlı düzeyde düştü. Sıçan karaciğer mikrozomal zarlarının FT-IR çalışmaları zar dinamiği, düzeni ve lipid peroksidasyonu gerçekleşme düzeyini anlamak için yapıldı. Bu amaçla CH_2 simetrik freakans CH_2 antisimetrik bant genişliği ve =CH olefinik bant alanı karşılaştırıldı. Sıcaklık bağımlı FT-IR çalışmalarında mikrozomal zar faz davranışı düzen ve dinamik analiz edildi. Diyabetik sıçanlar hem frekans hem de bant genişliğinde açık bir azalma sergiledi. Diyabetik grubun =CH olefinik bant integral alanı kontrole kıyasla artış gösterdi.

α-lipoik asit ve vitaminC verilmiş gruplar benzer etkiler gösterdi. Bu gruplar düşmüş bant frekansı ve bant genişliğini telafi etme eğilimindedir. α-lipoik asit ve C vitamini tek tek verildiğinde bant genişliğindeki azalmayı telafi edemezken birlikte verildiklerinde sinerjik etkileri sayesinde telafi edici özellik gösterdiler.

Sonuç olarak, STZ-diyabet, zar düzeni ve akışkanlığı üzerine azaltıcı etki göstermiş ve daha rijit yapılar oluşmasına yol açmıştır. Antioksidan ağındaki sinerjinin kaybolması diyabetin ikincil komplikasyonlarının oluşmasında rol oynamıştır.

Anahtar kelimeler: Antioksidan enzimler, FT-IR spektroskopi, diyabet, mikrozomal zar, lipid peroksidasyonu

To my daughter,

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CHAPTER I

INTRODUCTION

1.1 Free Radicals

Life arose approximately 3.5 billion years ago from basic chemicals by free radical reactions, initiated by solar irradiation. About 2.6 billion years ago, oxygen gradually appeared in the atmosphere, as a result of production of O_2 by blue-green algea. The early occurance of free radical reactions in the evolution of life helps to explain the apparent ubiquitous involvement of free-radicals in physiological processes (Harman, 1987).

A free radical can be defined as any species that has one or more unpaired electrons (Halliwell and Gutteridge, 1985). They can be neutral, or are positively or negatively charged. An unpaired electron can occur in many atoms with biological significance, like sulfur, carbon, hydrogen or nitrogen centered radicals. Since the discovery in 1968 of the enzyme superoxide dismutase, which catalyses the transformation of an oxygen radical to H_2O_2, the interest in the involvement of oxygen centered radicals in both pathological and physiological processes, has been renewed.

Free radicals are generally very reactive molecules possessing an unpaired electron. They are produced continuously in cells either as by products of metabolism, or for example, by leakage from mitochondrial respiration. The most important reactions of free radicals in aerobic cells involve molecular oxygen and its derivatives, peroxides and transition metals.

Diatomic oxygen itself is already a radical, because it contains two unpaired electrons. Hence one-electron reduction of oxygen is possible and gives the so-called superoxide anion radical ($\mathbf{O_2^{-\cdot}}$), with one unpaired electron. The $\mathbf{O_2^{-\cdot}}$ might undergo a spontaneous dismutation reaction in which one $\mathbf{O_2^{-\cdot}}$ reacts with another to produce hydrogen peroxide with a rate constant of $1.10^5 M^{-1}s^{-1}$. The reaction is catalyzed by Superoxide Dismutase (SOD).

$$2O_2^{-\cdot} + 2H^+ \longrightarrow H_2O_2 + O_2$$

Not only dismutation of $\mathbf{O_2^{-\cdot}}$, but also the addition of two electrons to O_2 with subsequent protonation produces hydrogen peroxide.

$$O_2 + 2e^- + 2H^+ \longrightarrow H_2O_2$$

In the reaction of superoxide with perhydroxyl radical yields oxygen and hydrogen peroxide. This is also a dismutation reaction.

$$HO_2^{\cdot} + O_2^{-\cdot} + H^+ \longrightarrow O_2 + H_2O_2$$

Superoxide radical may also be generated by the autooxidation of metal ions and these reactions are reversible. Therefore, they are called reversible

redox reactions (McCord and Fridovich, 1969). Various compounds eg. nitrofurantoin produce superoxide radical as their reduction products (İşcan *et al*., 1995).

$$Fe^{+2} + O_2 \rightleftharpoons Fe^{+3} + O_2^{-\cdot}$$

$$Cu^{+2} + O_2 \rightleftharpoons Cu^{+3} + O_2^{-\cdot}$$

Although hydrogen peroxide does not qualify a radical itself, it is involved in the formation of reactive oxygen species and plays an important role in the free radical biochemistry. The importance is due to its ability to produce the most reactive oxygen specie; hydroxyl radical (˙OH) through a metal-ion-dependent reaction. Iron and copper catalyze the formation of hydroxyl radical. Iron seems to be safely sequestered in the organism. However, transferrin (at lower pH) and ferritin (in existence of reducing agents) may release iron. The reaction is called Fenton Reaction with either ferrous (the so-called Fenton reaction) or cupric ions (Figure 1.1) (Bast and Goris, 1989).

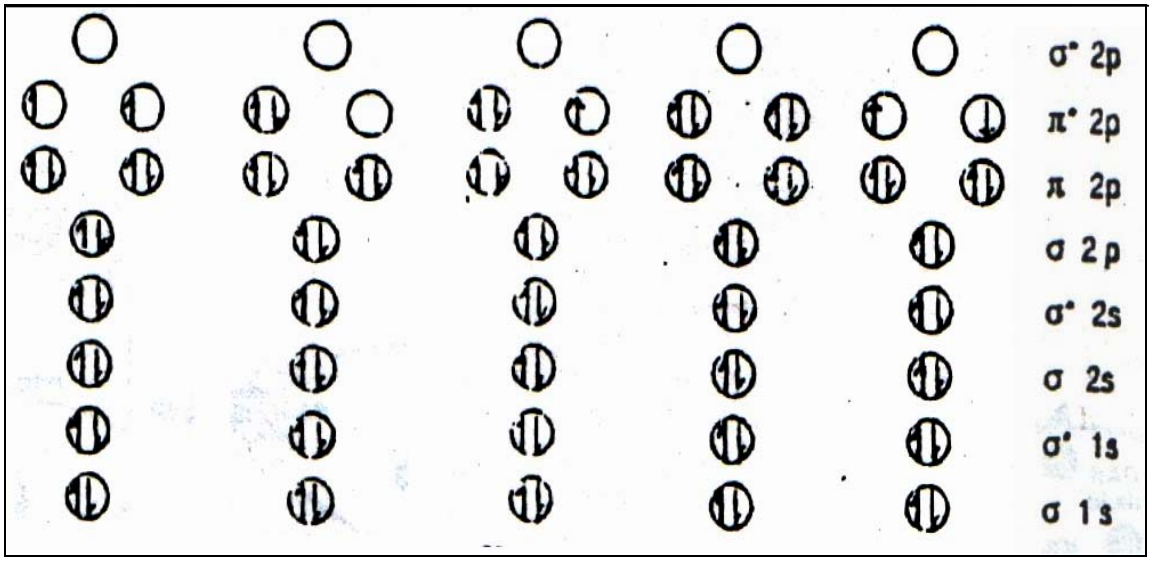
$$H_2O_2 + \underset{(Fe^{2+})}{Cu^{+}} \rightarrow OH^{\cdot} + OH^{-} + \underset{(Fe^{3+})}{Cu^{2+}}$$

$$\underset{(Fe^{3+})}{Cu^{2+}} + O_2^{\cdot -} \rightarrow \underset{(Fe^{2+})}{Cu^{+}} + O_2$$

$$H_2O_2 + O_2^{\cdot -} \xrightarrow[\text{catalysis}]{Fe,\ Cu} OH^{\cdot} + OH^{-} + O_2$$

Figure 1.1. Catalysis of hydroxyl radical formation from superoxide and hydrogen peroxide by iron and copper ions.

Singlet oxygen ($^1O_2$) is a reactive oxygen molecule that does not have any unpaired electron. Singlet oxygen is formed by the transfer of one of the electrons in the outermost shell to another orbital which has opposite spin direction. Energy gain induces this transfer. There are two types of singlet oxygen; sigma (Σ) and delta (Δ) (Figure 1.2).



Oxygen | Singlet oxygen $^1\Delta g\ O_2$ (Delta) | Superoxide ($O_2^{\cdot-}$) | Peroxide ion O_2^{-2} | Singlet oxygen $^1\Sigma g\ ^+O_2$ (Sigma)

Figure 1.2. Electron arrangements of native oxygen and other oxygen derived species

A further electron (e^-) carried with an H^+ ion converts $^{\cdot}OH$ into H_2O (Figure 1.3).

There are other types of radicals such as carbon-centered radicals ($R^{\cdot}$), peroxyl radicals ($ROO^{\cdot}$), alkoxyl radicals ($RO^{\cdot}$), and thyl radicals ($RS^{\cdot}$). Among them peroxyl radical which is derived from polyunsaturated fatty acids (PUFA) gets special attention because of its longer half life (Sies, 1991).

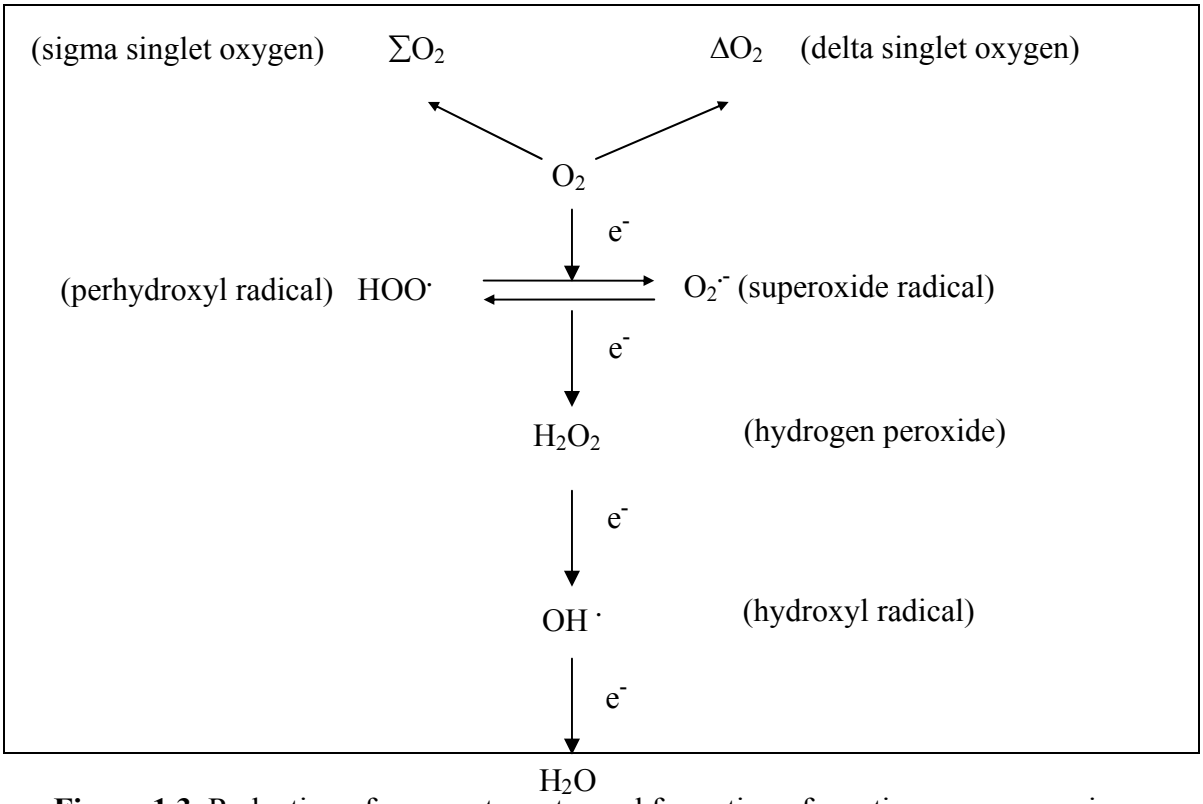


Figure 1.3. Reduction of oxygen to water and formation of reactive oxygen species

1.2 Oxidation of Macromolecules

Oxidative stress can be considered to occur when the flux of partially reduced forms of oxygen is greater than the ability of the biological system to cope with their production. Under stressed conditions, biological molecules are exposed to pro-oxidant (potantially oxidant) species resulting in irreversible oxidation reactions. Such reactions result in chemical modification of biological molecules that can lead to cellular dysfunction.

1.2.1 Lipid Peroxidation

Biological membranes define the extent of cells and organelles. The permeability characteristics of the bilayer allow gradients of metabolite and electrolyte concentrations to exist between the intra and extracellular spaces. Any loss in membrane integrity due to lipid damage rapidly dissipate these gradients and compromise cellular function. For example, the loss of the inner mitochondrial membrane permeability barrier preclude the establishment of a proton gradient and prevent ATP synthesis.

Under normal conditions, lipids are also subject to turnover and damaged lipids can be reprocessed and repaired. However, many of the products of lipid oxidation (Figure 1.4), such as hydroperoxides, alcohols, aldehydes and F2-isoprostanes, have biological activities (Esterbauer *et al*., 1991; Moore *et al*., 1995).

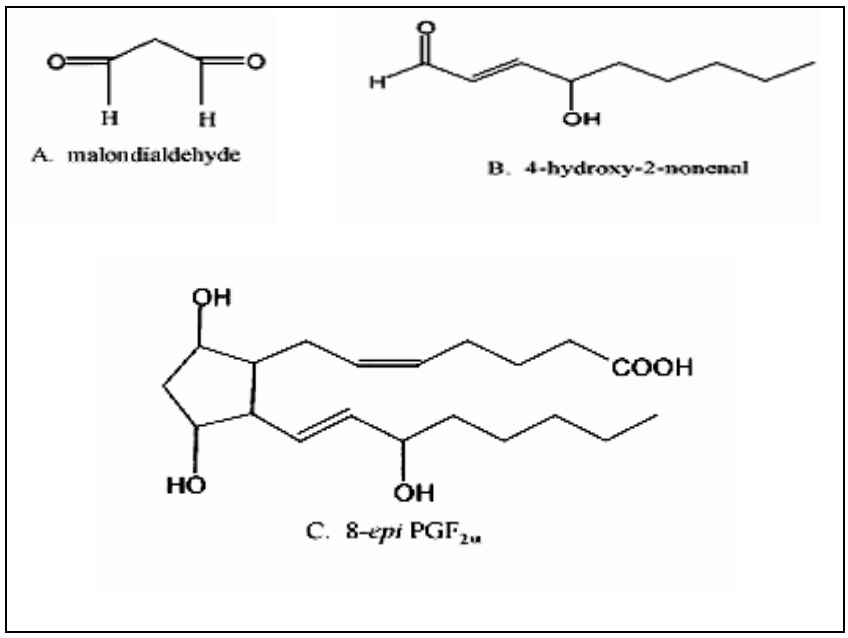


Figure 1.4. Chemical structures of selected lipid peroxidation products.

Lipid oxidation is a chain reaction which may be initiated by the formation of a carbon-centered radical by hydrogen abstraction from the lipid Different initiation processes (e.g., photolysis, Q-radiation, singlet oxygen) produce different initial radicals. The initiating event has little effect on the subsequent chemistry, though the rate of initiation controls the extent of the chain reaction (Wheatley, 2000).

A single oxidative event can oxidize many lipid molecules. In the presence of iron or copper ions (Fenton Reaction), the chain reaction may become a cascade and the oxidation process can rapidly become unstoppable (Halliwell and Gutteridge, 1984).

Biological strategies against lipid peroxidation involve both preventing the initial oxidation and breaking the chain before much damage is done.

1.2.1.1 Mechanism of Lipid Oxidation

Polyunsaturated fatty acids (PUFA) have a propensity to oxidize, resulting in the formation of alkanes, aldehydes, alcohols, and hydroperoxides among other products. This propensity arises from the fact that bis-allylic (double bonded) methylene ($=CH_2$) hydrogens are more susceptible to hydrogen abstraction by oxidizing radicals than are the methylene hydrogens from fully saturated lipids. This is partially due to the fact that the resulting free radical has multiple resonance structures that increase its stability. In the more heavily populated of these structures the double bonds are conjugated and the radical resides on an adjacent methylene carbon. The rapid reaction of these resonance forms with oxygen fixes the double bonds in a conjugated arrangement to form peroxyl radicals at positions +2 and -2 with respect to the carbon atom from which the original abstraction occurred

(Fig. 1.5). For example, oxidation of linolenic acid by hydrogen abstraction at carbon-11 can result in peroxyl radicals at both the 9- and 13-position.

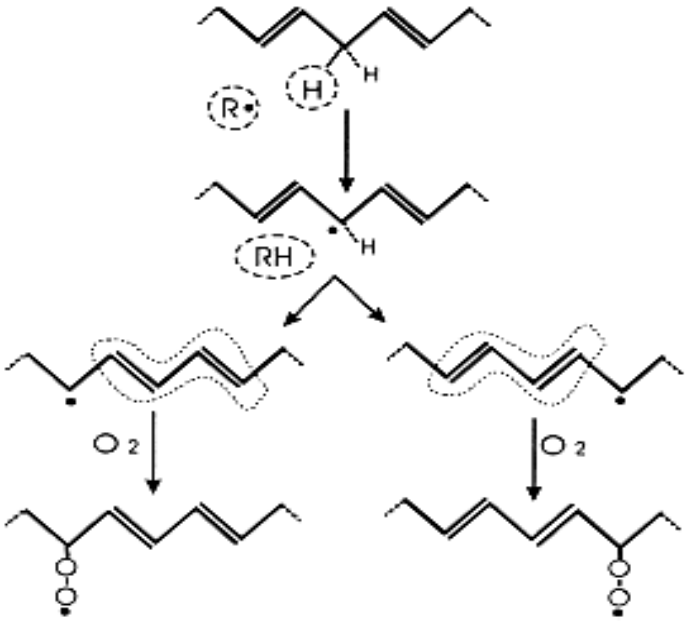


Figure 1.5. Lipid peroxidation. Abstraction of an allylic hydrogen and conjugated diene formation.

The lipid peroxyl radical is the central species of the lipid peroxidation chain reaction. This radical can abstract a bis-allylic hydrogen from an adjacent fatty acid to form a lipid hydroperoxide and a second lipid radical [eq. 1], which subsequently reacts with oxygen to regenerate a peroxyl radical [eq. 2].

$$LOO^{\cdot} + LH \longrightarrow LOOH + L^{\cdot} \quad \text{[eq. 1]}$$

$$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot} \quad \text{[eq. 2]}$$

These reactions are the chain-propagation steps of lipid peroxidation. Eq. [1] is rate limiting and so the rate of the propagation reaction is proportional to the concentration of lipid peroxyl radicals. Consequently any reaction that alters the concentration of peroxyl radicals will affect the rate of lipid peroxidation.

Figure 1.6 shows the overall mechanism of a polyunsaturated fatty acid (linoleic acid) oxidation. The initiating radical, $ROO^{\cdot}$, is usually of unknown origin or composition. Step 1 generates the first polyunsaturated fatty acid radical then, after electron redistribution, adds dioxygen in step 2. The hydroperoxyl radical from step 2 can attack linoleic acid, step 3, to generate the first linoleyl radical and a linoleyl hydroperoxide. This is a free radical chain reaction. Two linoleyl hydroperoxyl radicals can react to give non-radical products, step 4, terminating free radical chain oxidation. Antioxidation by vitamin E takes place at step 5, giving linoleyl hydroperoxide and α-tocopheroxyl radical. A second linoleyl hydroperoxyl radical can react with α-tocopheroxyl radical to give a non-radical product, step 6. If the concentration of linoleyl hydroperoxyl radical is too low, the α-tocopheroxyl radical will attack linoleic acid giving the linoleic acid radical and regenerating vitamin E, step 7. In vivo, the oxidation of ascorbic acid may regenerate vitamin E without oxidizing linoleic acid, step 8 (Figure 1.6).

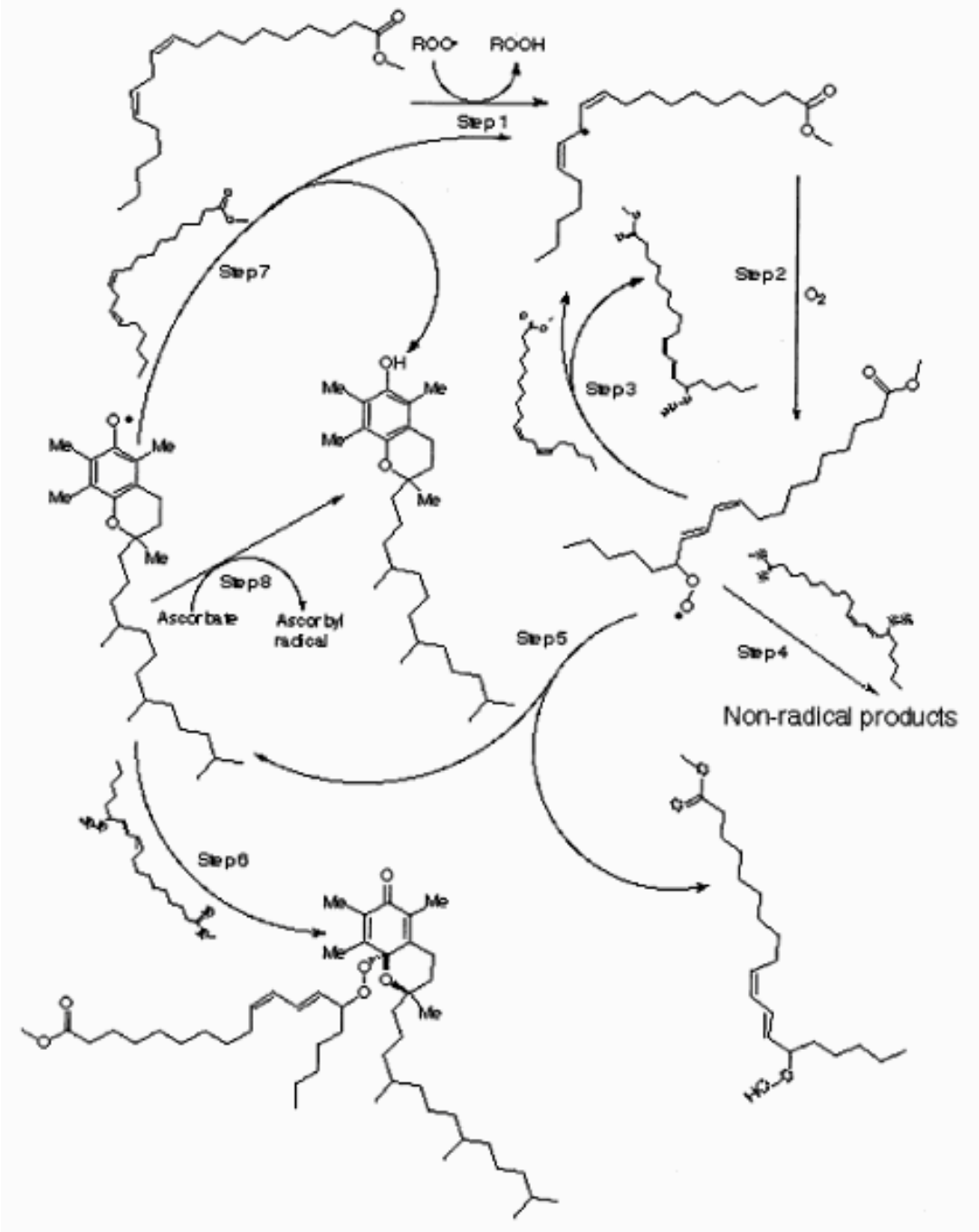


Figure 1.6. The mechanism of polyunsaturated fatty acid oxidation as exemplified by the autooxidation of lineloic acid.

1.2.1.2 Transition metal ions

A few ions of transition metal elements have redox transitions with potentials of a magnitude that allows the catalytic decomposition of hydroperoxides (Figure 1.7). The redox couples of most importance to biological systems are Cu^{+} / Cu^{2+} and Fe^{2+} / Fe^{3+}.

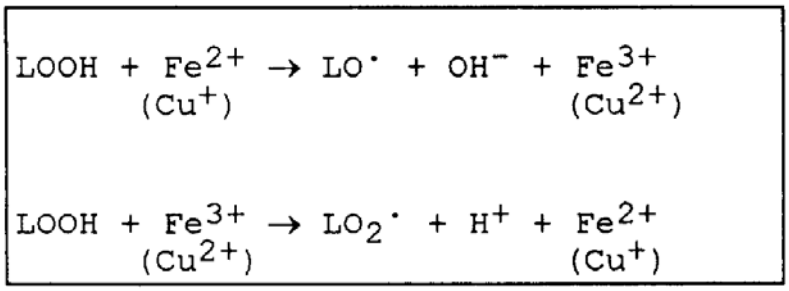


Figure 1.7. Decomposition of lipid peroxides (LOOH) by transition metal ions: $LO^{\cdot}$ =lipid alkoxyl radical; $LO_2^{\cdot}$ =lipid peroxyl radical (oversimplified)

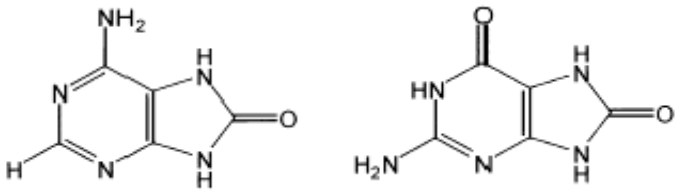
The one electron redox cycle results in the formation of peroxyl and alkoxyl radicals (Girotti, 1985) the latter of which rearrange and react with oxygen to form peroxyl radicals (Wilcox *et al.*, 1993). By this mechanism, transition metal ions increase the concentration of peroxyl radicals and accelerate lipid peroxidation. As lipid peroxidation generates lipid hydroperoxides, the effect of transition metal ions is autocatalytic. This behavior is observed during copper catalyzed oxidation of low-density lipoprotein (Esterbauer *et al.*, 1992; O'Leary *et al.*, 1993).

1.2.1.3 Termination reactions

In the absence of any additional reactions, the lipid peroxidation chain reaction will terminate when two lipid radicals react to form non-radical products. These reactions decrease the level of peroxyl radicals and slow the rate of lipid oxidation. The mechanism and products of such reactions are complex and only partially understood. It has been reported that endogenous chemiluminescence is associated with such reactions, perhaps indicating the formation of singlet state oxygen (Sugioka *et al*., 1976).

1.2.2 DNA Oxidation

Free radical attack upon DNA generates a whole series of DNA damage, including modified DNA bases. Hydroxyl radical causes the formation of a large number of pyrimidine- and purine-derived lesions in DNA (Dizdaroglu, 1992, Breen and Murphy, 1995). Some of these modified DNA bases have considerable potential to damage the integrity of the genome [Wang 1998, Wallace 1998]. 8-Hydroxyguanine (8-OH-Gua) is one of the most widely studied lesions. The presence of 8-OH-Gua residues in DNA leads to GC to TA transversions unless repaired prior to DNA replication (Grollman, 1993). Therefore, the presence of 8-OH-Gua in cells may lead to mutagenesis. Several other modified bases (Figure 1.8) such as 2-hydroxyadenine (2-OH-Ade), 8-hydroxyadenine (8-OHAde), 5-hydroxycytosine (5-OH-Cyt), and 5-hydroxyuracil (5-OH-Ura) have also been shown to possess miscoding potentials. It is conceivable that these lesions may be premutagenic as well (Wallace, 1998).



8-oxoadenine **8-oxoguanine**

Figure 1.8. Chemical structures of selected oxidized purine bases.

In view of the importance of DNA damage in carcinogenesis, it is obvious that any agent capable of reacting with DNA and chemically modifying it could be carcinogenic; and the reactive oxygen species (ROS) belong to this group. There is evidence that ROS play a role in the etiology of atherosclerosis (Halliwell and Gutteridge, 1999) and it has been suggested that alterations of cellular DNA may contribute to atherosclerotic plaque formation (Penn *et al*., 1986). Moreover, an increase in the level of oxidative damage to DNA bases in patients infected by human immunodeficiency virus (HIV) might influence the progression of the infection into acquired immunodeficiency syndrome (AIDS) (Jaruga et al., 1999).

1.2.3 Protein Oxidation

Radical mediated damage to protein may be initiated by electron leakage, metal-ion-dependent reactions and autoxidation of lipids and sugars. Oxidized proteins are functionally inactive and their unfolding is associated with enhanced susceptibility to proteinases. Cells can generally remove oxidized proteins by proteolysis. However, certain oxidized proteins are poorly handled by cells, and

together with possible alterations in the rate of production of oxidized proteins, this may contribute to the observed accumulation and damage, like in the case diabetes and atherosclerosis (Reznick *et al*., 1994).

Two major molecular mechanisms leading to structural changes in plasma proteins are metal-catalyzed protein oxidation, which is characterized by carbonyl (PCO) formation, (Cakatay *et al*., 2000) and loss of thiol (SH) groups (Hu *et al*., 1994). Attack by reactive oxygen species upon proteins can damage several amino acid residues, including histidine, proline, arginine and lysine. Oxidative damage to several of these amino acid residues and/or to the peptide backbone of proteins can generate PCO products. Indeed, measurement of PCO has been used as a sensitive assay for oxidative damage to proteins Free radicals also cause oxidation of protein SH groups in plasma (Hu *et al*., 1994). Essentially all of the plasma groups are protein associated. Albumin is the most abundant plasma protein and is a powerful extracellular antioxidant. The measurement of plasma thiol (T-SH) is a good reflection of excess free radical generation as the conformation of albumin is altered, allowing SH groups to be oxidized. Nitrotyrosine (NT) formation is another molecular mechanism causing oxidative protein damage. Peroxynitrite ($ONOO^-$) is a cytotoxic species generated when superoxide radicals ($O_2^{\cdot -}$) combine with nitric oxide (NO), and its formation has been implicated in both the origin and in the progression of diabetes. Nitration of the ortho position of tyrosine is a major product of $ONOO^-$ attack on proteins, leading to NT (nitrotyrosine), and it has been considered a specific marker for the detection of $ONOO^-$ in vivo. Peroxynitrite is highly toxic due to its ability to oxidize thiol groups, lipids, proteins, enzymes and DNA. Since NT is a stable end product of $ONOO^-$ oxidation, assessment of its plasma concentration may be useful as a marker of $NO^{\cdot}$ dependent damage in

vivo. On the other hand, $ONOO^-$ induces the formation of PCO, the oxidation of tryptophan, tyrosine and cysteine residues and causes protein fragmentation (Ischiropoulos *et al*., 1995).

1.2.4 Carbohydrate Oxidation

Autooxidation of monosaccharides forms hydrogen peroxide, peroxides and oxoaldehydes. These end products play important role in various pathological processes like diabetes and smoking related chronical diseases (Lunec and Blake, 1990).

Oxoaldehydes possesses anti-mitotic effect due to their DNA, RNA and protein binding and croos-link forming properties. Therefore, they play important roles in cancer and aging processes.

Hyaluronic acid, an important mucopolysaccharide of the connective tissue, is found abundantly in synovial fluid. In inflammatory joint diseases a number of polymorph cells pass through the synovial fluid, then probably due to the activation by immune complex polymorph cells release H_2O_2 and $O_2^{-}\cdot$ to the extracellular fluid and the released H_2O_2 and $O_2^{-}\cdot$ are able to breakdown hyaluronic acid. Hyaluronic acid is also found in vitreous humor of the eye. Oxidative damage resulting the breakdown of hyaluronic acid would also accelerate the formation of cataract (Delibas *et al*, 1995).

1.3 Diabetes Mellitus

Diabetes affects a large number of people of all social conditions throughout the world. In untreated state diabetes mellitus is recognized by chronic elevation of glucose level in blood. This is sometimes accompanied by symptoms of severe

thirst, profuse urination, weight loss, etc. The high concentration of blood glucose and other biochemical abnormalities results from a deficiency in β-cells of endocrine pancreas and/or from a sub sensitivity to insulin in target cells. Exact etiology of diabetes mellitus has not been established yet. Although there are several diseases and syndromes associated with hyperglycemia, it has been traditional to classify all forms of diabetes as either insulin-dependent (IDDM) or noninsulin-dependent (NIDDM) on functional grounds (Öztürk *et al*, 1996).

1.3.1 Insulin-Dependent Diabetes Mellitus (IDDM)

Insulin dependent diabetes mellitus (IDDM) usually begins before the age 40, often in childhood and adolescence. The clinical picture is usually more severe in patients with IDDM than in patients with NIDDM. Characteristically, the plasma insulin is low or immeasurable, because a total deficiency in pancreatic β-cells has occurred in IDDM. Glucagon levels are elevated but suppressible with insulin. This type of diabetes is unresponsive to the treatment with oral antidiabetic drugs. Therefore, insulin therapy is always required for patients with IDDM (Pozzilli, 1998).

1.3.2 Noninsulin-Dependent Diabetes Mellitus (NIDDM)

This form of diabetes usually begins in middle life or after and the symptoms begin more gradually than in IDDM so the diagnosis is frequently made when an asymptomatic person is found to have elevated plasma glucose on routine laboratory examination. In contrast to IDDM, plasma insulin levels are normal to high in absolute term, although they are lower than predicted for the level of the plasma glucose, i.e., relative insulin deficiency is present. Although these types of patients

are responsive to the treatment with oral antidiabetic drugs, many of them are treatable with diet and exercise alone. However in some cases treatment with combination of oral antidiabetic drugs and insulin is required (Akerblom and Knip, 1998)

1.3.3 Complications of Diabetes Mellitus:

Various short-term and long-term complications may occur in diabetic patients. These complications fall into following categories:

Neurological Complications

Cardiovascular Complications

Gastrointestinal Complications

Urological Complications

Respiratory Complications

Ophthalmic Complications

Reproductive Complication

Haematological Complications

Biochemical Complications

Complications related to drug metabolism and pharmacokinetics

Certain diabetic complications such as gastroenteropathy or the development of urinary bladder dysfunction are not fatal in nature, although they may seriously affect the quality of life in diabetic patients. However some of the diabetic complications like neuropathy, nephropathy, angiopathy and cardiomyopathy may be deleterious for diabetic patients; development of these complications has been shown to confer an increased incidence of morbidity and mortality of diabetic patients (Öztürk *et al*., 1996).

1.3.4 Experimental Models of Diabetes Mellitus

There are many advantages to study diabetes mellitus in experimental animals. Almost every diabetic complication can be detected in experimental models of diabetes to investigate the molecular of the disease mechanism. Furthermore, models of experimental diabetes permit the study of the involvement of environmental factors, such as diet, exercise, drugs, toxins and infective agents. In addition they exhibit many features of clinical diabetes. However none of the models is exactly equivalent to clinical diabetes.

The following methodologies have been applied for experimental diabetes:

Surgical Diabetes: This method, which consists of total or subtotal pancreatomy in animals, is now rarely used for investigation of diabetes and its complications.

Spontaneous Diabetes: Diabetes mellitus may occur spontaneously in the population of various animal species like Chinese Hamster and BB Wistar Rat.

Viral Diabetes: Viral infection has been implicated as a cause of diabetes in both man and animals. Several viruses have been reported to induce experimental diabetes in certain animal species: Coxsackie viruses, foot and mouth disease virus, rubella virus, reoviruses etc.

Chemical Diabetes: Various drugs and chemicals have been reported to cause an experimental situation somewhat similar to diabetes. Among these diabetogenic substances alloxan and streptozotocin are the most specific and convenient ones (Lohmann, 1998).

1.3.4.1 STZ Induced Diabetes Mellitus

Streptozotocin is an antibiotic extracted from *Streptomyces achromogenes*. STZ causes diabetes by a direct action on the pancreatic β-cell. The exact site of STZ interaction with β-cell is somewhat speculative. In previous studies, 7 to 10 h after STZ treatment, massive β-cell degranulation and necrosis associated with an increase in serum insulin levels and hypoglycemia was observed. This was followed by prolonged hyperglycemia (1-28 days), which coincided with a reduction in pancreatic insulin levels t <5% of normal values. The diabetogenic effects of STZ were found to be dose dependent, ranging from mild diabetes following a dose 35 mg kg^{-1} to a severe ketotic state, leading to death within 2 to 3 days after a dose of 100 mg kg^{-1}. When treated with intermediate doses of STZ (55-65 mg kg^{-1}), weight loss and blood glucose level 3 to 4 times higher than normal one was seen (Tomlinson *et al*., 1992).

1.4 Biological Membranes

Every cell is enclosed in a complex structure called membrane, which serves as the interface between the machinery in the interior of the cell and the extracellular fluid that bathes all cells.

Since the structure-function paradigm is of central importance in modern molecular biology, studies on the structure and dynamics of natural membranes have become particularly important in recent years. An understanding of the physical principles that govern the molecular organization of membranes is essential for identification of their physiological roles since structure and function are strongly

interdependent in membranes (Sandorfy and Theophanides, 1984).

1.4.1 Membrane Lipids

Membranes contain several types of lipid, all of which are amphipathic; that is, they contain both hydrophilic and hydrophobic regions. Phospholipids are the primary lipids of biological molecules.

The amphipathic nature of phospholipids is essential to their biological function in cell membranes. The phospholipids of the cell membrane form into a sheet of two molecules thick with the fat-soluble portions inside shielded on both sides by the water-soluble portions. This stable structure provides the cell membrane with its integrity (Figure 1.9).

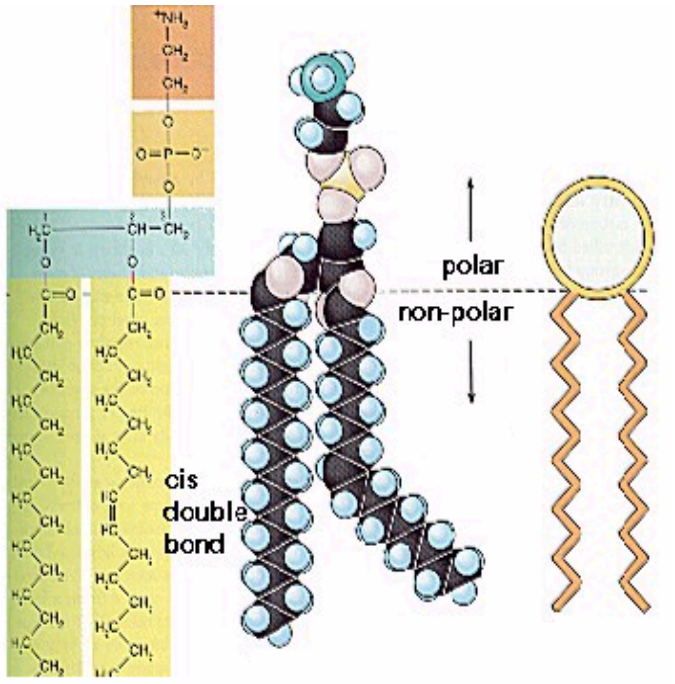


Figure 1.9. Phospholipid structure

1.4.1.1 Membrane Lipids and Membrane Fluidity

The physical state of the lipid of a membrane can be described by its fluidity or viscosity (fluidity and viscosity are inversely related; fluidity is a measure of flow, and viscosity is a measure of resistance to flow). Lipids can exist in a crystalline solid phase or a liquid phase of varying viscosity depending on the temperature. At sufficiently low temperatures, the lipid molecules are solid like. In many ways they resemble hydrocarbon crystals. When the temperature is raised, the properties of bilayers show abrupt changes at one or more temperatures. The abrupt small increase in the phase transition curve corresponds to the pretransition *(T_p)* associated with the tilting of the hydrocarbon chains and the abrupt larger increase corresponds to the main transition *(T_m)* of hydrated phospholipids from gel to the liquid crystalline phase which is associated with complete melting of the hydrocarbon chain (Severcan and Cannistraro, 1988).

The fluidity of membrane depends on three aspects in the structure of the lipid matrix namely: the length of the fatty acids, the degree of saturation, and the amount of cholesterol present.

Longer fatty acids contribute to a more stable membrane structure, at which the fluidity of the membrane decreases with increasing the number of carbon atoms in the fatty acid chains and thus the temperature at which fatty acids melt (the transition temperature) increases with increasing the number of carbon atoms in saturated fatty acids.

Membrane fluidity assumes a direct relation with the degree of unsaturation of fatty acids (the number of double bonds present). The transition

temperature is relatively high for fatty acids with no double bonds (saturated), and it is low for unsaturated ones with a low number of double bonds present. This direct relation between the fluidity and the degree of unsaturation is so apparent since linear saturated fatty acids form stable lipid structure by packing together tightly. While the presence of kinks, resulting from the double bonds, in the unsaturated fatty acids prevents the chains from flitting together tightly and thereby, minimizing their interactions.

Furthermore, as its position minimizes interaction between neighbouring fatty acids hydrocarbon chains in the membrane, cholesterol decreases membrane fluidity at higher temperatures, and increases it at low temperatures (Choi *et al*., 1991).

1.5 Non-Enzymatic Antioxidants

Biological antioxidants are natural molecules which can prevent the uncontrolled formation of free radicals and activated oxygen species, or inhibit their reactions with biological structures. The destruction of most free radicals and activated oxygen species relies on the oxidation of endogenous antioxidants, mainly scavenging and reducing molecules.

Antioxidant scavengers are either chemical traps of oxidizing free radicals and activated oxygen species, or physical quenchers of excited species such as singlet oxygen and triplet states of photosensitizers. The kinetics of their scavenging reactions are very fast, that is, much faster than those of most other biological targets. Free radical products of scavengers decay through dismutation, recombination or reduction by secondary scavengers, and therefore do not serve as initiators of uncontrolled chain reactions.

Examples of activated oxygen species which are continuously "scavenged" within our cells include peroxyl radicals $ROO^{\cdot}$, hypervalent iron species, singlet oxygen and halogenating oxidants such as hypochlorous acid HOCl.

Superoxide ($O_2^{\cdot -}$), hydroxyl ($OH^{\cdot}$) and alkoxyl ($RO^{\cdot}$) radicals are efficiently trapped by endogenous antioxidant scavengers: superoxide free radical anion is actually a one-electron reductant, and its biological degradation is under exclusive control of superoxide dismutases in most cells. Conversely, $OH^{\cdot}$ and $RO^{\cdot}$ radicals are extremely oxidizing species and the rate constants of their reactions with many biological molecules are close to the diffusion limit. This means that a very large concentration of scavenger would be necessary to prevent undiscriminate molecular damage from such radicals (J. Chaudiea Re, 1999).

1.5.1 α-Lipoic Acid

α-Lipoic acid, a disulphide derivative of octanoic acid (Figure 1.10), has been known for decades to be a crucial prosthetic group of various cellular enzymatic complexes. Recently, α-lipoic acid has been characterized as an efficient antioxidant (Bustamante *et al*., 1998). It has been proposed to be a potential therapeutic agent in the treatment or prevention of different pathologies that may be related to an imbalance of the oxido-reductive cellular status. This occurs in the case of neurodegeneration, ischemia–reperfusion, polyneuropathy, diabetes, AIDS, and hepatic disorder status (Packer *et al*., 1995).

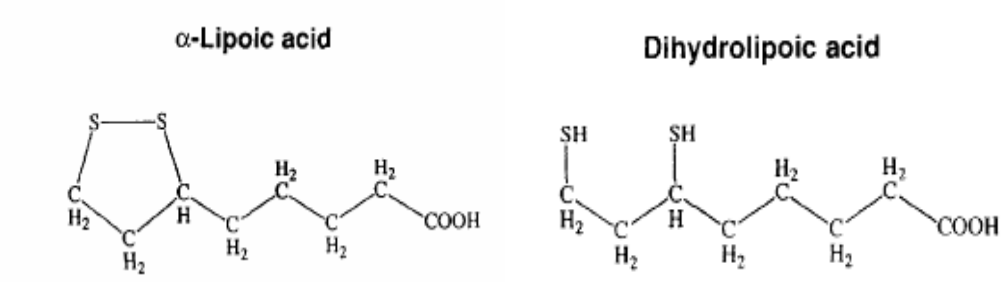


Figure 1.10. Chemical stuctures of α-lipoic acid and dihydrolipoic acid

1.5.1.1 Uptake, Metabolism, and Degradation of α-Lipoic Acid

Various studies on the distribution of radioactivity in rat tissues after intraperitoneal or oral administration of *d*l-[^{14}C]- or [^{35}S]-lipoic acid led to the observation that α-lipoic acid is rapidly absorbed in the gut, taken up into various tissues where it is metabolically altered and then excreted. After supplementation with α-lipoic acid for 5 weeks, free α-lipoic acid was found in various tissues, the highest being the heart. When given to cells in vitro, α-lipoic acid is rapidly taken up by the cells and reduced to dihydrolipoic acid, which is released by the cell. Urinary excretion is maximal 3–6 h after administration of α-lipoic acid, suggesting that the clearance of lipoate metabolites from the rat is even faster than indicated in many studies. After 4 h of intraperitoneal or oral administration of *d*l-[^{14}C]-lipoic acid, the highest amount of radioactivity was observed in the liver, but 24 h later, radioactivity was found in the skeletal muscle. Liver has a high capacity for uptake and accumulation of these compounds, in accordance with the *in vivo* studies. It has been demonstrated that catabolic pathways involved in lipoate metabolism are largely through β-oxidation of the valeric acid side chain, but the carbon skeleton of the dithioline ring portion is much more resistant to alteration (Spence *et al*., 1976).

1.5.1.2 Role of α-Lipoic Acid on Physiological Functions of Cells

The unique physico-chemical properties of α-lipoic acid make it a powerful and reactive biological molecule, carrying out biochemical reactions crucial for oxidative metabolism, and shown by researchers to modulate various cellular functions. The low negative redox potential (E_0= - 0.29 V) makes the lipoic acid/dihydrolipoic acid couple a strong reducing agent.

Similar to other vicinal thiols, dihydrolipoic acid (which has a high pK value for the —SH, around 10.7) is more easily oxidized in comparison with monothiols, leading to high activity in —SH/S—S— interchange reactions. Furthermore, both α-lipoic acid and dihydrolipoic acid have high hydrophobicity, which allow them to permeate biological membranes at a high rate.

α-Lipoic acid is present as a cofactor in α-keto-acid dehydrogenases and in the glycine cleavage system. These enzyme complexes are involved in the metabolic pathways of pyruvate oxidation, the citric acid cycle, and amino acid degradation and biosynthesis. The α-keto acid dehydrogenase complexes, i.e., the pyruvate dehydrogenase complex, the α-ketoglutarate dehydrogenase complex, and the branched chain α-ketoacid dehydrogenase complex constitute an almost ubiquitous family of enzymes. The complexes share structural similarities being composed of multiple copies of three enzymes: the a-keto dehydrogenase component or E1 (pyruvate, 2-oxoglutarate or 2-oxo-isovalerate dehydrogenase), the dihydrolipoyl acyltransferase component or E2, and the dihydrolipoyl dehydrogenase component or E3, which is an enzyme common to all complexes.

α-Lipoic acid has been reported to be a substrate for a lipoamide

dehydrogenase detected in human serum (Pelley *et al*., 1976). In this case, the efficacy of α-lipoic acid as substrate was eightfold lower than that of lipoamide. The ability of the α-lipoic/dihydrolipoic couple to act as hydrogen donors in the reaction catalyzed by Se– peroxidase has also been studied (Maiorino *et al*., 1994). In the presence of H_2O_2 as a substrate, dihydrolipoic acid reduced the active site of glutathione peroxidase and of hydroperoxide glutathione peroxidase. However the efficacy of dihydrolipoic acid was far lower than that of the natural substrate GSH.

1.5.1.3 Activity of α-Lipoate as a Scavenger of Free Radical Species

The dithiol nature of lipoate renders this compound highly reactive against a number of reactive oxygen species, and it also has the ability to regenerate oxidized antioxidants. Interest in the antioxidant properties of lipoate originated from the studies of Rosenburg and Culik who noticed that the administration of α-lipoic acid prevented the symptoms of both vitamin C and E deficiency in guinea pigs and vitamin E deficiency in rats (Rosenberg and Culik., 1959).

As mentioned before, various biochemical reaction pathways result in the formation of free radicals. Further, the respiratory burst of neutrophils in response to inflammatory stimuli produces highly reactive oxygen species. Superoxide is formed, which in the presence of superoxide dismutase, forms H_2O_2. This can then be converted to hyperchlorous acid (HOCl) by the action of myeloperoxidase. Studies showed that α-lipoic acid and dihydrolipoic acid can scavenge both hydrogen peroxide and HOCl while dihydrolipoic acid can scavenge superoxide. Hydrogen peroxide may react with transition metals producing highly reactive hydroxyl radicals. Both α-lipoic acid and dihydrolipoic acid have been shown to scavenge hydroxyl radicals in a metal-catalysis system and in a metal-free reaction of

ultraviolet irradiation (UV) induced by decomposition of the aromatic hydroperoxide model compound NP-III (Matsugo *et al*., 1995). During the course of lipid peroxidation, peroxyl radicals are formed that propagate the reaction. Dihydrolipoic acid can scavenge these radicals, formed from both lipophilic and hydrophilic peroxyl radical generators. Therefore, all those results showed that the lipoate couple represents a potent radical scavenging unit.

When antioxidants react with reactive oxygen species, the antioxidant is converted to a form that is no longer able to function, and is said to be consumed. Therefore, this oxidized product needs to be recycled to its native form to function again. Vitamin E, being a potent peroxyl radical scavenger, is the major chain-breaking antioxidant protecting biological membranes from lipid peroxidation. This task appears difficult because there are approximately 1500 phospholipid molecules to 1 molecule of vitamin E; however, membrane oxidation does not naturally occur and vitamin E is not rapidly depleted. This apparent paradox can be explained by vitamin E recycling from circulating antioxidants. A number of antioxidants can recycle vitamin E including vitamin C, ubiquinols and glutathione. Dihydrolipoic acid has only a weak interaction with the tocopheroxyl radical, so the major recycling of vitamin E by dihydrolipoic acid occurs via the intermediary recycling of other antioxidants. Electronic spin resonance studies have demonstrated the recycling of the ascorbyl radical by dihydrolipoic acid, which in turn recycles the α-tocopheroxyl radical produced by oxidation. Dihydrolipoic acid may also recycle vitamin E by reducing oxidized glutathione, which then reduces the vitamin E radicals. The observed metabolic effects of α-lipoate/dihydrolipoate couple is summarized in Table 1.1.

Table 1.1. Summary of the antioxidant and metabolic effects of the α- lipoate /dihydrolipoate couple *in vitro*

Action	Effect
Involvement of lipoate in scavenging reactive oxygen species	• $OH^{\cdot}$ scavenging • $O_2^{\cdot -}$ scavenging • $^1O_2$ scavenging • $ROO^{\cdot}$ scavenging • HOCl scavenging • metal chelation
Interactions of lipoate with other antioxidants	• reduction of GSSG • vitamin C/E recycling • ubiquinol recycling • thioredoxin reduction • NADH/NADPH
Effects of supplemented lipoate on metabolic processes	• increased NAD^+/NADH ratio • increased intracellular GSH stabilization of oxidant-induced increase in intracellular Ca^{+2} • inhibition of NF-κB activation

1.5.1.4 Effect of α-Lipoic Acid as a Metal Chelator

In addition, α-lipoic acid and dihydrolipoic acid can chelate a number of metal ions including Cu^{2+}, Fe^{3+} (Scott *et al*., 1994), Mn^{2+}, Zn^{2+} and Cd^{2+}. Chelation of Cu^{2+} (Sigel *et al*., 1978) by α-lipoic acid was reported to inhibit the Cu^{2+} catalyzed oxidations in vitro, assist the partition of Cu^{2+} into n-octanol, and inhibit the flux of H_2O_2 induced within erythrocytes by exposure to ascorbic acid (Peimian *et al*., 1995). It has been shown in vitro that dihydrolipoic acid as well as dihydrolipoamide can remove iron stored inside ferritin by complexing it in the ferric form. The rate of the reaction increased as a function of the ratio of dihydrolipoic acid/ferritin,

was inaffected by the iron content of the ferritin itself, and was pH dependent (Bonomi *et al*., 1986).

1.5.1.5 Effect of α-Lipoic Acid on Glutathione Metabolism.

Modulation of cellular glutathione status has long been discussed as a potential therapeutic strategy, taking into account the role of reduced glutathione in a variety of detoxification reactions against oxidizing species, produced during the metabolism of xenobiotics, as well as its involvement in the formation of conjugates with electrophilic metabolites (Sigel *et al*., 1978).

The influence of α-lipoic acid on the cellular status of glutathione was investigated in various *in vitro* and *in vivo* systems. In accordance with the lower redox potential of the α-lipoic acid/dihydrolipoic acid couple, with respect to the GSH/GSSG couple, dihydrolipoic acid is a powerful reductant for GSSG (Jocelyn 1967).

Dihydrolipoic acid (DHLA) (formed via cellular α-lipoic acid reduction) reduced extracellular cystine to cysteine, which is transported to the cells more efficiently than cystine, and is promptly used as sources for glutathione synthesis. An increase in the tissue levels of glutathione in the lung, liver, and kidney, concomitantly with an improvement in the survival after whole body irradiation, was observed in rats after 11 days of an i.p. treatment of α-lipoic acid at doses of 4.8 –16 mg/kg/day (Busse *et al*., 1992).

An increase in total glutathione levels in the liver and blood, but not in the kidney, heart, and skeletal muscles of rats, occurred after intragastric supplementation with lipoate (150 mg/kg for 8 weeks). α-Lipoate-supplemented

rats also had lower levels of thiobarbituric reactive species (TBARS) in the liver, heart, and red gastronemius muscle (Khanna *et al*., 1997).

1.5.2 Vitamin C (Ascorbic Acid)

Ascorbic acid participates in a variety of enzymatic reactions like collagen and catecholamine synthesis as an electron donor and it is one of the most important water soluble antioxidants of mammalian tissues (Levine, 1986). Its role as a free radical scavanger and the redox interrelationships between ascorbate and other antioxidants are in the focus of continuous interest.

1.5.2.1 Ascorbate Synthesis

Ascorbate can be synthesized *de novo* in the hexuronic acid pathway of the liver or the kidney of species having gulonolactone oxidase activity or it can be regenerated from its oxidized form in any cells of any mammalian species. Hexuronic acid pathway can use glucose units theoretically originating from glycogenolysis, gluconeogenesis or glucose uptake, therefore, the hepatic (or renal) carbohydrate metabolism and the ascorbate synthesis could influence each other. The first phase of this pathway is cytosolic, while the last three enzymatic steps are performed at the endoplasmic reticulum (Figure 1.11).

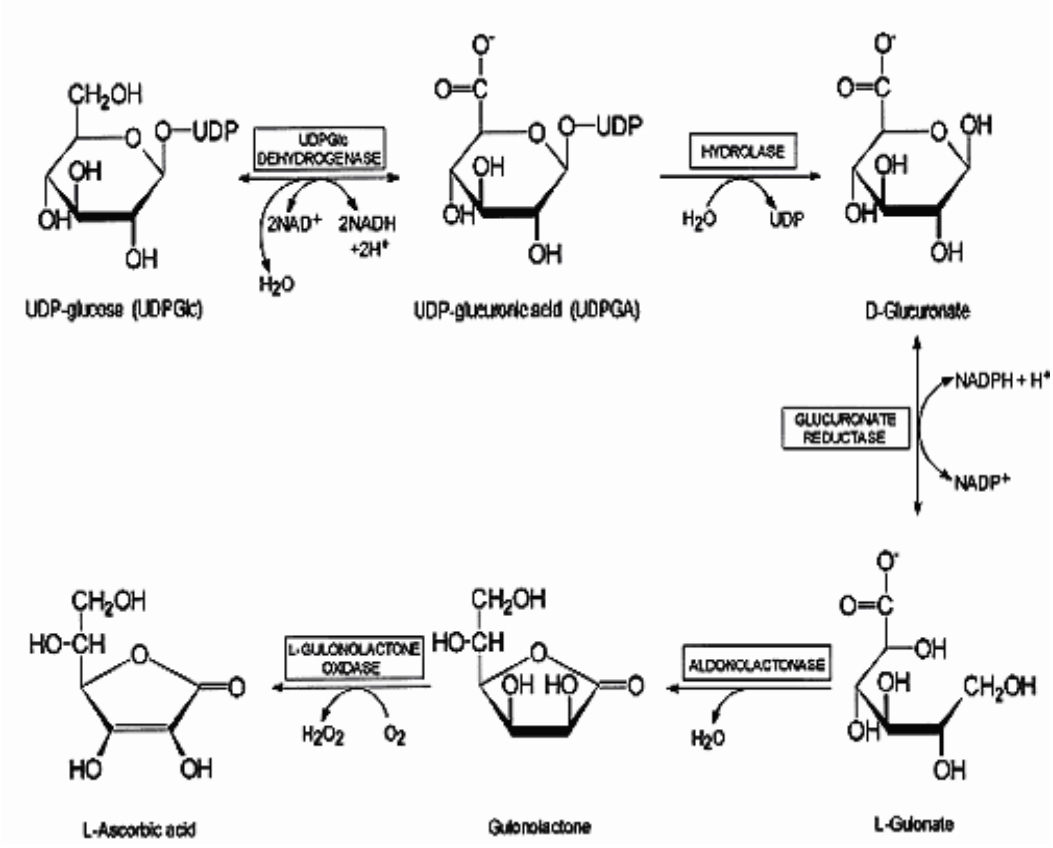


Figure 1.11. Biosynthesis of L-ascorbate from UDP-glucose (Banhegyi, 1997)

1.5.2.2 Ascorbate Recycling

All of the ascorbate utilizing reactions are based on its property of being easily oxidized (Figure 1.12). Ascorbic acid is oxidized in animal cells to dehydroascorbic acid. The reaction occurs by one-electron oxidation of ascorbate to semidehydroascorbate radical followed by further oxidation to dehydroascorbate or by very rapid disproportionation to dehydroascorbate and ascorbate. From an economical point of view, it is more advantageous for the cell to reduce back the oxidized forms of ascorbate, than to synthesize it *de novo.*

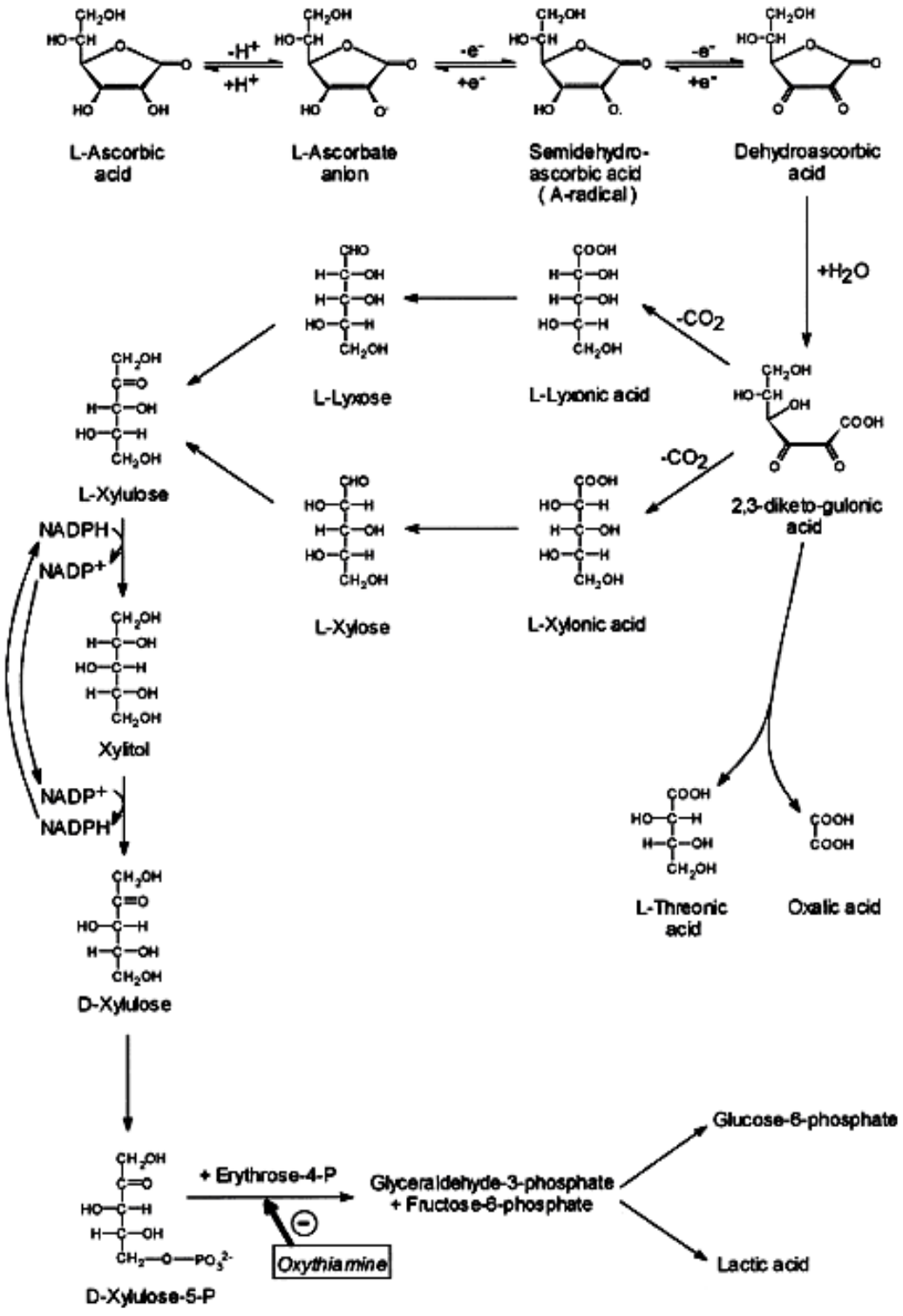


Figure 1.12. Ascorbate catabolizing pathways in animals (Tolbert *et al*., 1975)

In species unable to produce ascorbate, the recycling mechanisms are even more important. NADH-dependent semidehydroascorbate reductase activity has been described in cellular membranes. Dehydroascorbate can be reduced by non-enzymatic or enzymatic reactions. The non-enzymatic recycling at the expense of GSH has been known for decades, but the existence of an enzymatic reaction was doubtful till the last years. It has been reported recently that glutaredoxin and protein disulphide isomerase exhibit GSH-dependent dehydroascorbate reductase activity. Furthermore, a novel GSH-dependent enzyme has been purified from rat liver cytosol (Maellaro *et al*., 1994), where another NADPH-dependent enzyme, identical with 3α-hydroxysteroid dehydrogenase, has also been found. During normal conditions the redox state of the cells probably permits that the majority of the oxidized forms of ascorbate can be reduced by these reactions. In prolonged oxidative stress or pathophysiological states characterized by a dominancy of prooxidant effects (e.g., diabetes mellitus), ascorbate consumption and dehydroascorbate accumulation can be observed *in vivo*. In these conditions the ascorbate catabolising pathways come into prominence (Bode *et al*., 1993).

1.5.2.3 Ascorbate Breakdown

Dehydroascorbate, if not reduced back to ascorbate, decomposes with a half-life of a few minutes, since this compound is unstable at physiologic pH. The product of the hydrolysis is 2,3-diketo-L-gulonate, which does not possess antiscorbutic effects any more. 2,3-diketo-L-gulonate is decarboxylated to L-xylonate and L-lyxonate. These 5-carbon compounds can enter the pentose phosphate pathway and the L- to D-conversion is suggested to occur through xylitol. Another minor

pathway of ascorbate catabolism is a carbon chain cleavage yielding oxalate and 4-carbon intermediates (Fig. 1.12) (Tolbert *et al*., 1975).

In murine and human erythrocytes, which are unable to synthesize glucose (glucose-6-phosphatase is lacking), ascorbate or dehydroascorbate addition resulted in the increase of lactate, the end product of anaerobic glycolysis. Lactate production could be stimulated by the addition of menadione or inhibited by oxythiamine treatment of the cells indicating that the pentose phosphate pathway is involved in ascorbate catabolism both in hepatocytes and in erythrocytes. These results show that ascorbate does not get lost but is effectively reutilized even in case of diminished recycling and it should be taken into account not only as a vitamin, but also as a source of energy.

1.5.2.4 Ascorbate/Dehydroascorbate Transport, Ascorbate Cycles

Ascorbate synthesis and breakdown carried out in the same cell or separately in different tissues together with the transport mechanisms necessitate the existence of intra- and interorgan cycles. In species able to produce ascorbate two metabolic cycles can be operative. An intracellular cycle in the ascorbate-synthesizing cells may exist involving enzymes of the pentose phosphate cycle, gluconeogenesis and hexuronic acid pathway (Fig. 1.13a). This sequence may be particularly important during long-term starvation when the major glycogenolysis-dependent ascorbate synthesis and the exogenous supply are missing, or in oxidative stress which favours ascorbate catabolism directly and by GSH depletion. This cycle saves dehydroascorbate for both glucose production and ascorbate resynthesis. Another, interorgan ascorbate cycle with participation of hepatic production and

peripheral breakdown through the intermediacy of the bloodstream forms a metabolic route similar to the Cori cycle (Fig. 1.13b). In species unable to synthesize ascorbate the breakdown of the exogenous ascorbate can fuel the Cori cycle both in the liver and in the periphery (Fig. 1.13c) (Banhegyi, 1997).

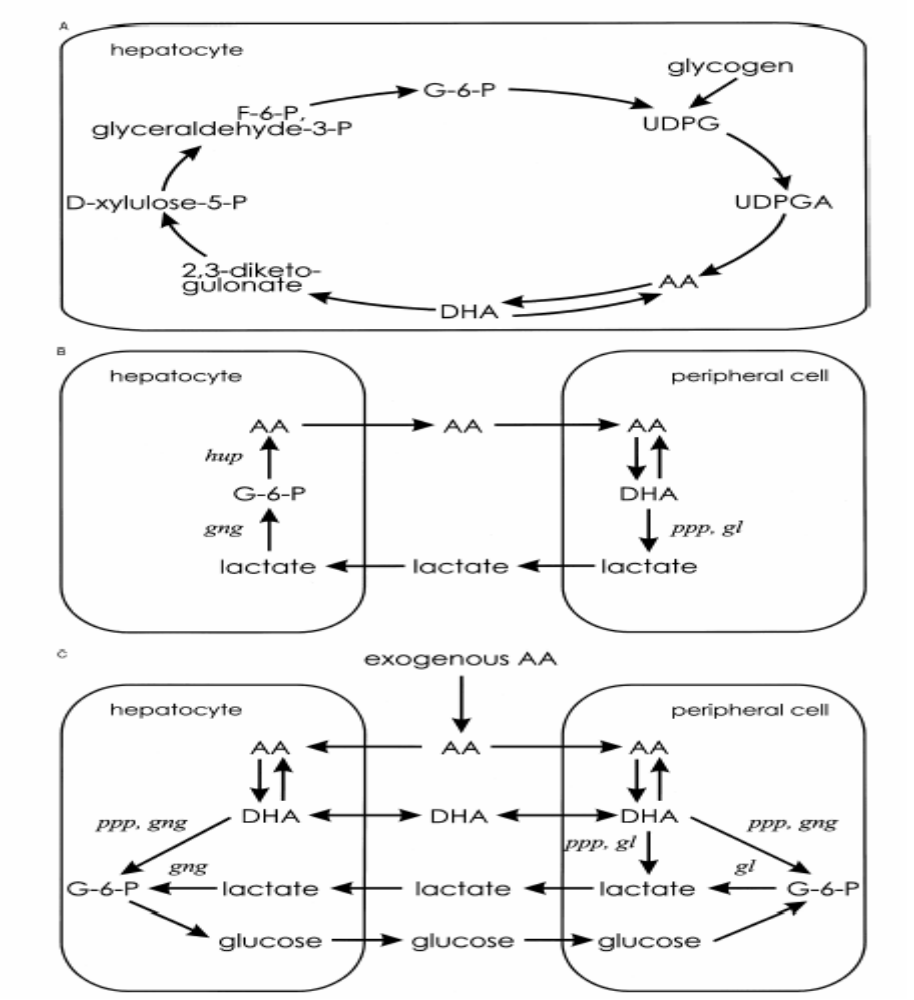


Figure 1.13. Ascorbate cycles. A: Ascorbate catabolism and resynthesis in isolated murine hepatocytes. B: Interorgan cycle with the participation of hepatocytes and peripheral cells (e.g., erythrocytes) in ascorbate-synthesizing species. C: Ascorbate catabolism in species unable to produce ascorbate. Abbreviations used: AA, ascorbic acid; DHA, dehydroascorbic acid; G-6-P, glucose-6-phosphate; UDPG, UDP-glucose; UDPGA, UDP-glucuronic acid; F-6-P, fructose-6-phosphate; *gl,* glycolysis; *ppp,* pentose phosphate pathway; *gng,* gluconeogenesis; hup, hexuronic acid pathway (Banhegyi,1997).

1.6 Enzymatic Antioxidants

Non-enzymatic scavengers are essential in the protection of cellular components from undiscriminate damage by the most reactive oxidizing species, but this protection does not cope with reducing radicals such as superoxide, or with metastable hydroperoxides. Such primary peroxides are continuously produced by aerobic cells (Chaudiére, 1994) and they provide a major source of much more oxidizing species such as hypervalent iron or copper complexes, peroxynitrite, and hydroxyl or alkoxyl radicals, which all behave as initiators of chain reactions. Specific antioxidant enzymes have been designed by nature to destroy superoxide and hydroperoxides. The advantage of using enzymes is that the steady-state concentration of peroxides can be adapted to cellular requirements: several of the antioxidant enzymes can be induced, inhibited or activated by endogenous factors (Harris, 1992), and they play an important role in the regulation of metabolic pathways and specific functions of aerobic cells. Enzymatic degradation of superoxide is ensured by superoxide dismutases (SOD), while that of hydroperoxides is ensured by catalase, glutathione peroxidases (GSHPx) or ascorbate peroxidases. SOD and catalase are dismutases, and therefore they do not consume cofactors. Thus, the primary reactions catalysed by such enzymes are not associated with any energetic cost. Conversely, glutathione peroxidases and ascorbate peroxidases are reductases whose reducing coenzymes are regenerated by NAD(P)H equivalents produced in metabolic pathways.

1.6.1 Superoxide Dismutase (SOD) (EC 1.15.1.1)

SODs catalyse the one-electron dismutation of superoxide into hydrogen peroxide and oxygen (Fridovich, 1997, McCord and Fridovich, 1969)

$$2O_2^{-\cdot} + 2H^+ \longrightarrow H_2O_2 + O_2$$

At physiological pH, the rate of the non-enzymatic dismutation of superoxide is significant, but it is considerably increased in the presence of SOD. The turnover numbers of SODs are indeed very high over a wide range of pH. For example, the bimolecular rate constant associated with the reaction of superoxide with Cu/Zn-SOD is higher than $2x10^9$ $M^{-1}.sec^{-1}$ (Fielden et al., 1974).

In animal cells, Cu/Zn-SOD is present in the cytosol and mitochondria, while Mn-SOD, a remnant of its bacterial ancestor, is only present in the mitochondrial matrix (Steinman, 1982). The fact that intracellular SOD concentrations range from 10^{-6} to 10^{-5} M supports the concept that superoxide is strongly toxic. At least three types of reactions could be responsible for this cytotoxicity:

1. Formation of unstable complexes of dioxygen with transition metals of enzyme prosthetic groups, followed by oxidative self-destruction of the active site and some times release of inorganic iron (Keyer and Imlay, 1996).

2. Fast production of peroxynitrite $O=N-OO^-$ on reaction of superoxide with nitric oxide (Koppenol *et al*., 1992). In equilibrium with its conjugate acid (pKa = 6.8 M^{-1}), peroxynitrite behaves as a powerful oxidant and protein nitrating species:

$$O_2^{-\cdot} + NO^{-} \longrightarrow N\text{-}OO^{-2} / O{=}N\text{-}OO\text{-}H \qquad k\sim 6.7 \times 10^{9}\ M^{-1}.sec^{-1}$$

3. Reduction of ferric complexes to their ferrous homologs (Halliwell, 1978), which in the presence of hydroperoxides will catalyse the production of hydroxyl or alkoxyl radicals through Fenton reactions

$$O_2^{-\cdot} + Fe^{+3} \longrightarrow O2 + Fe^{+2}$$
$$H_2O_2 + Fe^{+2} \longrightarrow OH^{\cdot} + OH^{F-} + Fe^{+3}$$
$$ROOH + Fe^{+2} \longrightarrow RO^{\cdot} + OH^{F-} + Fe^{+3}$$

Another property of SODs is that they may act as "free radical sinks" in cell compartments where GSH concentration is high, although this is probably restricted to situations where ascorbate concentration is very low. If a hydrophilic X^{-} radical is scavenged by GS(H) in the presence of physiological concentrations of oxygen, that is, 20±50 mM, SOD activity will drive the following reactional sequence to the right:

$$GSH + X^{\cdot} \longrightarrow GS^{\cdot} + X(H)$$
$$GS^{\cdot} + GS^{-} \longrightarrow GSSG^{\cdot -}$$
$$GSSG^{\cdot -} + O_2 \longrightarrow GSSG + O_2^{\cdot -}$$
$$2\ O_2^{\cdot -} + 2H^{+} \xrightarrow{(SOD)} H_2O_2 + O_2$$

This reactional pathway bypasses the direct recombination of $GS^{\cdot}$ thiyl radicals:

$$GS^{\cdot} \longrightarrow GSSG$$

This may have the advantage to prevent undesirable side reactions of $GS^{\cdot}$, but then scavenging of oxidizing free radicals by GSH will require further degradation of hydrogen peroxide by other enzymes to translate into efficient protection.

1.6.2 Catalase (CAT) (EC 1.11.1.6)

Catalase is a porphyrin-containing enzyme of high molecular weight which destroys hydrogen peroxide in high concentration by catalysing its two electron dismutation into oxygen and water (Schonbaum and Chance, 1976):

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Alternatively, it can act as a peroxidase at low hydrogen peroxide concentration, using reducing co-substrates such as alcohols, including ascorbate and phenols:

$$RHXH + H_2O_2 \longrightarrow RX + 2H_2O$$

In mammalian cells, NADPH is bound to catalase and may protect the enzyme from inactivation by H_2O_2. Because in many animal cells, most of the enzyme is located in peroxisomes where the steady state concentration of hydrogen peroxide is very high, that is, greater than 10^{-4} M. Although erythrocytes a high content of non-peroxisomal catalase, hydrogen peroxide is essentially degraded by glutathione peroxidases (Nicholls, 1972). This is consistent with a very high Km of catalase for H_2O_2, that is, close to 1 M. However, cytosolic catalase freely diffuses through membranes and it is conceivable that non-cytosolic catalase could protect cells during short time

exposure of the cytosolic compartment to high concentrations of H_2O_2. In this connexion, it is noteworthy that in guinea pigs, the lack of glutathione peroxidase activity in many tissues appears to be compensated by increased levels of cytosolic catalase. Interestingly, catalase is also present in small amounts in the mitochondrial matrix, where its function remains controversial. There are cells such as lymphocytes, in which the catalase/GSHPx ratio is high, and the autocrine production of extracellular catalase was shown to prevent apoptosis in a T-cell line (Sandstrom and Buttke, 1993). As a rule, however, further investigations are required to assess the antioxidant function of catalase in vivo.

1.6.3 Glutathione Peroxidase (GSHPx) (EC 1.11.1.9)

In animal cells, glutathione peroxidases (GSHPx) are selenoenzymes which catalyse the reduction of hydroperoxides at the expense of GSH (Flohé, 1989; Ursini et al., 1995). In this process, hydrogen peroxide is reduced to water whereas organic hydroperoxides are reduced to alcohols:

$$ROOH + 2GSH \longrightarrow ROH + H_2O + GSSG$$

or,

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

As in other selenoenzymes of animal cells, such as deiodinases and thioredoxin reductase, GSHPx active sites contain selenium in the form of a selenocysteine residue which is incorporated into the polypeptide backbone. Hydrogen peroxide and other hydrophilic hydroperoxides are reduced by water-soluble Se-GSHPx, a

homotetrameric enzyme which is generally abundant in cytosolic and mitochondrial compartments. The advantage of using selenium rather than sulfur to reduce hydroperoxides at the active site of GSHPx has been demonstrated by site directed mutagenesis by Rocher *et al*., (1992): the sulfur Se-GSHPx mutant of murine Se-GSHPx exhibits extremely weak GSHPx activity, and it is rapidly inactivated during catalysis. Phospholipid hydroperoxides of membrane bilayers can be directly reduced by PHGSHPx (phospholipid hydroperoxide glutathione peroxidase), a monomeric Se-GSHPx which is partially bound to membranes and which is not strictly GSH-specific. However, the regulation of its biosynthesis, enzyme activity, intracellular compartmentation and tissue distribution is complex, and it is not clearly established that its antioxidant properties are the most important ones. In most animal cells, hydrogen peroxide is reduced by selenium-dependent GSHPx, but there are isoforms of glutathione S-transferases (GSTs) which exhibit non-selenium dependent GSHPx activity on organic hydroperoxides such as those derived from membrane lipids or from DNA bases. The kinetics of such enzymes are much slower than those of selenium-dependent GSHPx, but they are probably of biological significance in cells or cellular compartments where Se-GSHPx is normally not expressed, or in situation of selenium deficiency. Selenium-dependent glutathione peroxidases have not been found in plant cells, where hydrogen peroxide may be essentially degraded by ascorbate peroxidases. Such an enzyme activity has also been found in insects (Mathews et al., 1997), and may also be found in a few mammalian tissues.

1.6.4 Glutathione S-Transferases (GSTs) (EC 2.5.1.18)

Glutathione transferases (GSTs) play an important role in the detoxication and elimination of xenobiotics as the first step of the mercapturic acid pathway. This process involves conjugation of glutathione with electrophilic metabolites and extrusion of the conjugate out of the cell for further metabolism. Certain GSTs, including the microsomal GST (mGST), also function as glutathione peroxidases by reducing organic hydroperoxides to the corresponding alcohols, of importance for protection against oxidative stress and lipid peroxidation (Ji *et al*., 1996).

The liver plays a major role in the glutathione transferase-catalyzed formation of glutathione S-conjugates as the initial step of the mercapturic acid pathway, involved in detoxication and excretion of xenobiotics (Jösch *et al*., 1998). Under oxidative stress the microsomal GST in the cell can be activated through direct hydroperoxide-mediated S-thiolation of the enzyme with GSH, its reversal occurring via a thiol exchange-mediated dethiolation imposed by the intracellular glutathione redox state (Sies et al., 1998).

1.7 Basic Principles of Spectroscopy

Spectroscopy is defined as the study of the interaction of electromagnetic radiation with matter. Spectroscopic techniques involve irradiation of a sample with some form of electromagnetic radiation, measurement of the scattering, absorption, or emission in terms of some measured parameters, and the interpretation of these measured parameters to give useful information. Figure 1.14 represents many of the important regions of the electromagnetic spectrum.

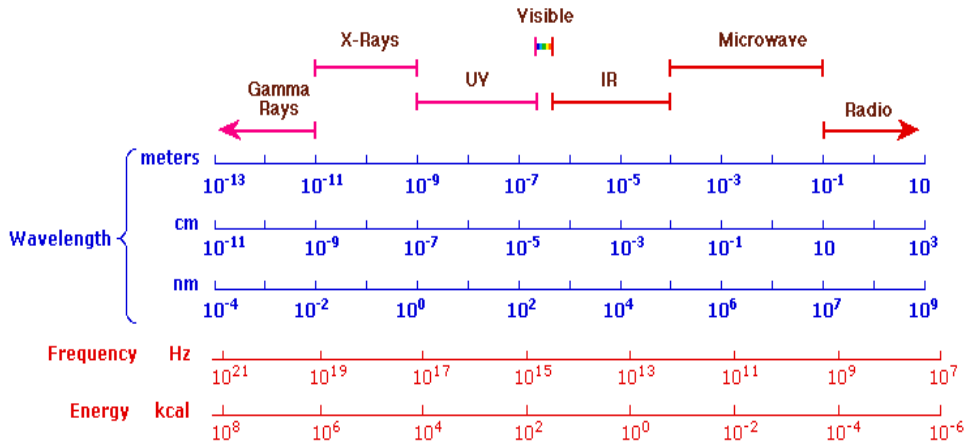


Figure 1.14. The Electromagnetic Spectrum

The interaction between electromagnetic radiation and matter can cause redirection of the radiation between energy levels of the atoms or molecules. In this phenomenon, the energy excites a molecule to a higher energy level. The type of excitation depends on the wavelength of the light. For example, the transition is from one vibrational energy level to another, the radiation will be from the infrared portion of the electromagnetic spectrum (Freifelder *et al.*, 1982). The energy levels are usually described by an energy level diagram shown in Figure 1.15.

For most purposes it is convenient to treat a molecule as if it possesses several distinct reservoirs of energy. The total energy is given by the following equation (Campbell and Dwek *et al.*, 1984).

$$E_{total}=E_{translation}+E_{rotation}+E_{vibration}+E_{electronic}+E_{electronicspinorientation}+E_{nuclearspinorientation}$$

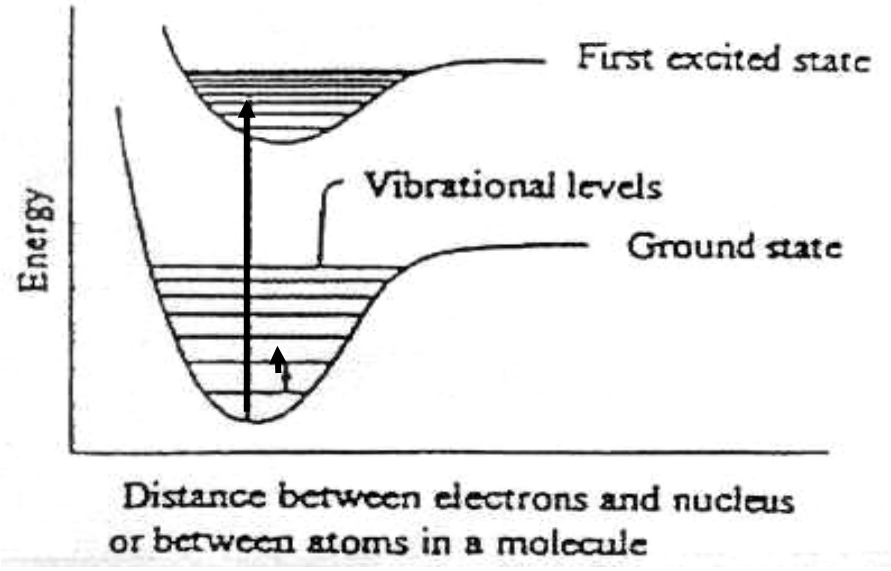


Figure 1.15. Energy level diagram illustrating the ground and first excited electronic energy level. Vibrational energy levels are represented as parallel lines superimposed on the electronic levels. The long arrows show a possible electronic transition from the ground state to the first excited state while the short arrow represents a vibrational transition within the ground electronic state.

The frequency dependence of absorption arises since energy is absorbed by transitions induced between different energy states of the molecules in the sample. The transitions occur only if there is a strong interaction between the incident radiation and the molecule. Absorption is most probable when the energy level separation matches the energy of the incident radiation as indicated below,

$$\Delta E = h\nu$$

where ΔE is the separation between the energy states of interest, ν is the frequency of the applied radiation and h is Planck's constant ($h = 6.6 \times 10^{-34}$ joule/second).

$$c = \lambda \nu$$

where c is the speed of light in vacuum (3.0×10^{8} ms^{-1}) and λ is the wavelength of light.

These two equations can be used to identify a common spectroscopic unit called wavenumber, which is denoted by ν. Wavenumber is defined as the reciprocal of the wavelength as follows;

$\overline{\nu}$ = wavenumber = (1/ λ) [has a unit of cm^{-1}]

Thus, $E = h \nu = h c \overline{\nu}$,

From these equations, it is clear that both wavenumber and frequency are directly proportional to energy (Volland; 1999).

1.7.1 Basis of Infrared Spectroscopy

The term "infrared" covers the range of the electromagnetic spectrum between 0.78 and 1000 μm. In the context of infrared spy, wavelength is measured in "wavenumber".

Infrared (IR) region is divided into three sub regions (Table 1.2) (Smith, 1999):

Table 1.2. Three subregions of infrared region

Region	**Wavenumber range (cm^{-1})**
Near	14000-4000
Mid dle	4000-400
Far	400-4

The atoms in a molecule are constantly oscillating around average positions. Bond lengths and bond angles are continuously changing due to this vibration. A molecule absorbs infrared radiation when the vibration of the atoms in the molecule produces an oscillating electric field with the same frequency as the frequency of the incident IR light. The molecule will only absorb radiation if the vibration is

accompanied by a change in the dipole moment of the molecule. A dipole occurs when there is charge separation across bond. If the two oppositely charged molecules get closer or move further apart as the bond bends or stretches, the moment will change.

All of the motions can be described in terms of two types of molecular vibrations. One type of vibration, a stretch, produces a change of bond length. A stretch is a rhythmic movement along the line between the atoms so that the interatomic distance is either increasing or decreasing. The second type of vibration, a bend, results in a change in bond angle. These are also called scissoring, rocking or wigwag motions. Each of these two main types of vibration can have variations. A stretch can be symmetric or asymmetric. Bending can occur in the plane of the molecule or out of plane; it can be scissoring, like blades of a pair of scissors, or rocking, where two atoms move in the same directions (Volland, 1999). Figure 1.16 demonstrates the main types of variations schematically.

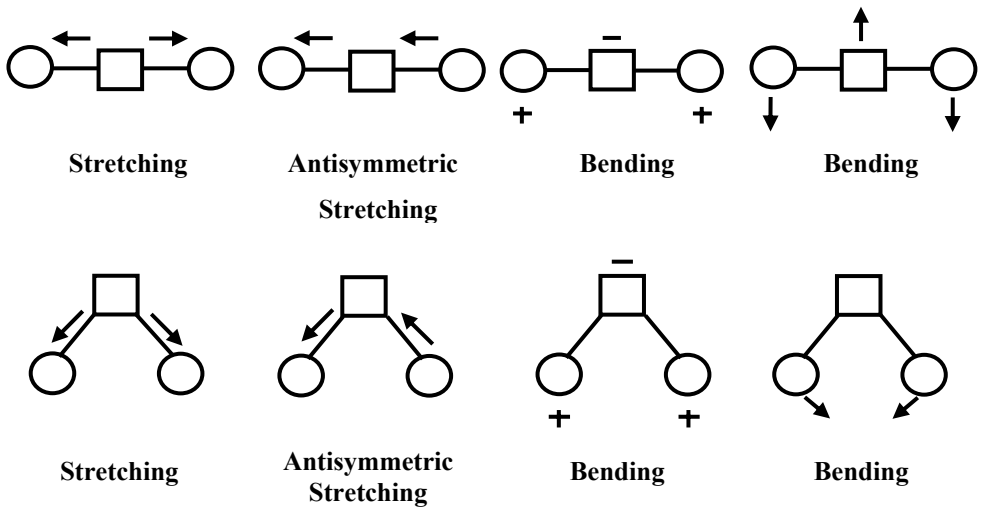


Figure 1.16. Types of normal vibration in a linear and non-linear triatomic molecule. (Arrondo *et al*., 1993).

1.7.2 The Fourier Transform Infrared Spectrometer (FT-IR)

General instrumentation of FT-IR spectrometer is shown in Figure 1.17.

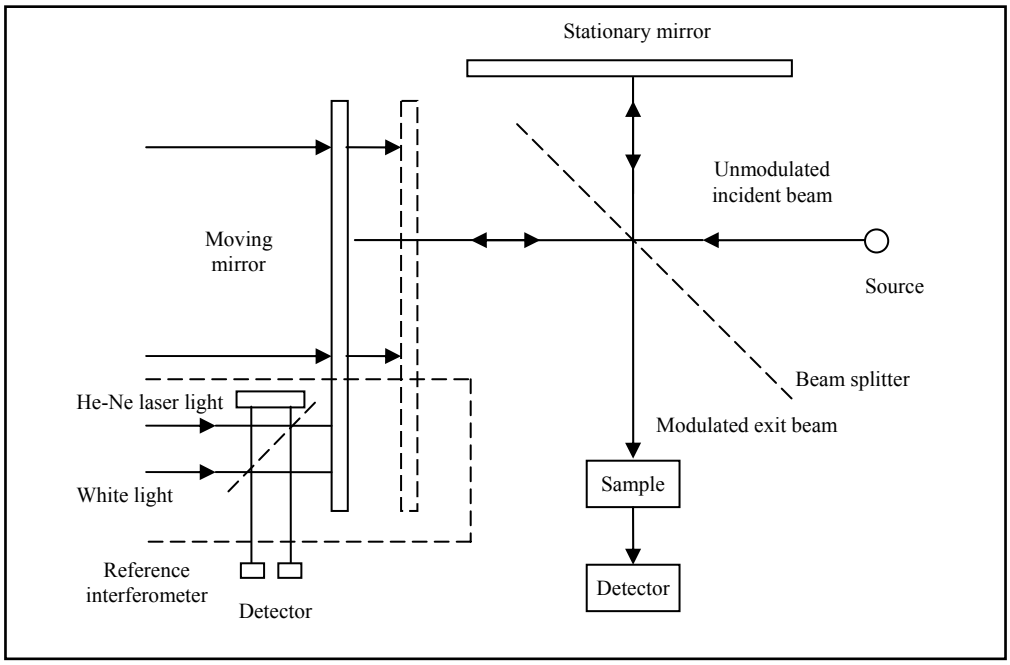


Figure 1.17. Instrumentation of FT-IR spectrometer

The pieces of FT-IR spectrometer and their functions are listed below;

- A source generates light across the spectrum of interest.
- A monochromater (in IR this can be either a salt prism or a grating with finely spaced etched lines) separates the source radiation into its different wavelengths.
- A slit selects the collection of wavelengths that shine through the sample at any given time.

- In double beam operation, a beam splitter separates the incident beam in two; half goes to sample, and half to a reference.
- The sample absorbs light according to its chemical properties.A detector collects the radiation that passes through the sample, and in double-beam operation, compares its energy to that going through the reference.
- The detector outputs an electrical signal, which is normally sent directly to an analog recorder. A link between the monochromater and the recorder allows you to record energy as a function of frequency or wavelength, depending on how the recorder is calibrated.
- A moving mirror allows the determination of the precision of the position of an infrared band, which is the precision with which the scanning mirror position is known. To determine this position exactly, a helium-neon laser beam is incorporated in the beam of the source. This laser beam produces standard fringes by interference which can line-up successive scans accurately and can determine the position of the moving mirror at all times (George and Mclyntyre, 1987).

The most important parameter in the FT-IR spectrometer is the interferometer, which is commonly a Michelson Interferometer. This is a device that splits an electromagnetic beam in two directions to recombine them later so that the intensity variation can be determined as a function of the path difference between them. The interferometer contains two orthogonal mirrors: one movable and the other fixed, as shown in Figure 1. The light passes through a beam splitter, which sends the light in two directions at right angles. One beam goes to a stationary mirror then back to the beam splitter. The other beam goes to a moving mirror.

The motion of the mirror makes the total path length variable versus that taken by the stationary-mirror beam. When the two meet up again at the beam splitter, they recombine, but the difference in path length creates constructive and destructive interference, which is called an interferogram.

The recombined beam passes through the sample. The sample absorbs all the different wavelength characteristics of its spectrum. The detector now reports variation in energy versus time for all wavelengths simultaneously. A laser beam is superimposed to provide a reference for the instrument operation.

Energy versus time is an odd way to record a spectrum until it is recognized the relationship between time and frequency was recognized: they are reciprocal. A mathematical function called Fourier transform allows us to convert an intensity-versus-time spectrum into an intensity-versus-frequency spectrum. The spectrometer computer is able to deconvolute (Fourier Transform) all the individual cosine waves that contribute to the interferogram, and so produce a plot of intensity against wavelength (cm), or more usually frequency cm^{-1}.

1.8. Scope and Aim of This Study

Free radicals are atoms or molecules that have an unpaired electron in their outermost shells. Having an unpaired electron makes them reactive. They are produced during the normal physiological processes. In the case of some diseases, it has been found that they can be either produced uncontrolled, or might not be removed. As a result of uncontrolled production of these reactive particles, they attack their own environments in the case that they can not be removed.

If the environment is a living organism, free radicals attack macromolecules and change their conformation, electronegativity and finally, their function. The most important species of these radicals are the moleculer oxygen and its derivatives.

Diabetes mellitus is a metabolic disease caused by the deficiency or impaired tissue responsiveness to pancreatic insulin. In both cases, high glucose levels are inevitable. Above a threshold glucose concentration, glycosylation of macromolecules which causes irreversible changes in their functions can occur. If the glycosylated macromolecule is an enzyme, especially an antioxidant enzyme which removes the excess reactive oxygen species (ROS), it would be impossible to transfom these reactive particles into harmless species. The result is a vicious circle that enhances the damage and this might be one reason for the complexity of diabetes mellitus.

Various mechanisms are developed by the body to protect itself from these toxic reactions. Reactive oxygen particles are mostly removed by molecules called "antioxidants" which might have endogenic and/or exogenic sources. Antioxidants are widely used in the medicine today because of their healing power.

In this study, the oxidative damage produced in the livers of CTZ- induced diabetic rats was studied. In terms of biochemical and biophysical parameters by looking at the membrane damage and antioxidant capacity of the tissues. The results were compared to control rats. Also the antioxidant effects of ALA and Vit C in decreasing the oxidative stress were studied. Individual and combined of these antioxidant effects may prevent the oxidative protein and lipid damage on the membrane which mostly contributed to the development of diabetic complications.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

Streptozotocin, copper sulfate, sodium potassium tartarate, sodium hydroxide, sodium bicarbonate, Folin–Ciocalteu phenol reagent, bovine serum albumin (BSA), sodium phosphate, hydrogen peroxide, Triton x-100, sucrose, tris-hydroxymethyl aminomethane (trizma base), ethylenediamine tetra acetic acid (EDTA), phenyl methyl sulphonyl floride (PMSF), dithiothreitol (DTT), potassium chloride, glycerol, ethanol, nitro-blue tetrazolium (NBT), xanthine, copper chloride, ammonium sulphate, xanthine oxidase, potassium phosphate, glutathione, nicotinamideadenine dinucleotide phosphate (NADPH), sodium azide, butylated hydroxy toluene (BHT), thiobarbutiric acid (TBA), ascorbic acid (Vitamin C), α-lipoic acid, hydrochloric acid were purchased from Sigma Chemical Company, Saint Louis, Missouri USA. All chemicals were obtained from commercial sources at the highest grade of purity available.

2.2 Animals and Feeding

36 male 3 months old Wistar rats (250-300g) were divided into 5 groups: Control (n=6), Diabetic (n=7), Diabetic + Vit C (n=7), Diabetic + α-Lipoic acid (n=6) and Diabetic Combination of Vit C and α-Lipoic acid (n=10). All the animals were fed with standard diet and water. Diabetes induction was made by a single intraperitoneal dose of streptozotocin (STZ) (50 mg/kg) dissolved in 0.05 M citrate buffer (pH 4.5). Control group received 50 mg/kg physiological saline solution. Diabetes was induced by an injection of 50 mg/kg STZ intraperitoneally (i.p.) in α-Lipoic acid, Vit C and Combination groups. Five days after the administration of STZ, α-Lipoic acid group was started to administer 50 mg/kg lipoic acid dissolved in minimum amount of ethanol:saline solution (1:1). Similarly, Vit C group was administered 100 mg/kg Vitamin C daily dissolved in neutral physiological saline solution after five days of STZ injection. The combination group was received 50 mg/kg lipoic acid plus 100 mg/kg Vitamin C. α-Lipoic acid injections were applied in the morning and Vitamin C injections were applied in the evening daily to the combination group. For all groups the injections were carried out daily. After two weeks, blood glucose levels were weekly checked and the rats who have blood glucose levels above 200 mg/dl were considered as diabetic. At the end of the eighth week, rats were weighed and decapitated, livers were removed and kept at $-80^{o}C$ until use.

2.3 Isolation of Rat Liver Microsomal Membranes

Livers were washed twice or more with cold water to remove blood, then dried on a filter paper, fatty tissue was removed, cut into small pieces and weighed. Homogenization solution containing 1.15 % KCl in phosphate buffer (pH 7.4), 1 mM EDTA, 0.2 mM PMSF, 2mM DTT, was added with a ratio of 1:4 g/mL (wet weight). All steps were carried at 0-4 ^{o}C.

The tissue was homogenized using Potter-Elvehjem glass homogenizer packed in crush ice, coupled motor – driven (Black & Decker, V850, multispeed drill) teflon pestle at 2400 rpm for 3x20 sec.

Homogenate was first centrifuged at 1000 *g* (Sorvall RC5Plus, Newtown St., USA) using SS-34 rotor for 10 min and the resulting nuclear pellet was discarded. From the supernatant small aliquot was removed for the determination of catalase (CAT) activity. Then, the rest of supernatant was centrifuged at 16000 *g* (Sorvall RC5Plus, Newtown St., USA) using SS-34 rotor for 20 min and the mitochondrial pellet was discarded. Then the supernatant was further centrifuged at 125000 *g* (Sorvall Combi Plus Ultracentrifuge, Newtown St., USA) using A-841 rotor for 60 min. The cytosolic fractions were stored as small aliquots at -80^{o}C, for determination of enzyme activities. The resulting pellet was suspended in 0.5 M Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and recentrifuged at 125000 *g* for 55 min. After discarding the supernatant, the microsomal pellet was suspended with 200 mM phosphate buffer (pH 7.4) containing 25% glycerol (v/v), 1.15 KCl (w/v) at a volume of 0.25 mL for each gram of primal liver tissue. The resuspended microsomes were homogenized manually using the teflon-glass homogenizer and

stored at -80^{o}C as small aliquots used for TBA test, total protein, total lipid, and FT-IR study immediately.

2.4 Protein Determination

Principle:

Protein concentrations of microsomes and fractions of differential centrifugation were estimated by using the method of Lowry *et al*. (1951). The principle of protein determination based on the reduction of Folin reagent (phosphomolybdate and phosphotungstate) by Cu^{++} treated protein's tyrosine and tryptophan residues. The color due to reduction of molybdate in Folin reagent is directly proportional to protein content and can be measured at 660 nm.

Reagents:

Reagent I: 2% $CuSO_4.5H_2O$ (w/v): 2 g $CuSO_4.5H_2O$ dissolved in 100mL distilled water

Reagent II: 2% Na-K Tartarate (w/v): 2 g Na-K Tartarate dissolved in 100mL distilled water

Reagent A: 2% Na_2CO_3 (w/v): 2 g Na_2CO_3 dissolved in 100 mL of 0.1 N NaOH

Lowry ACR Reagent (alkaline copper reagent): Reagent I, II and A were mixed respectively with a ratio of 1:1:100 (v/v/v)

Folin Phenol Reagent: 2N stock Folin Phenol Reagent was diluted to 1:1 ratio with distilled water.

Assay:

Prior to the preparation of reaction tubes initial dilutions were carried out. In addition to initial dilutions, dilution within the tube was prepared by taking 0.1 and 0.2 mL of initially diluted samples into the reaction tubes and completed to final volume of 0.5 mL with distilled water. Concentrations of bovine serum albumin (BSA) standards were shown in Table 2.1.

Table 2.1: Preparation of BSA standards

Tube number	**BSA standards**	**H_2O(mL)**
0-0'	0	0.5
1-1'	0.5 mL (0.02mg/mL)	0
2-2'	0.5 mL (0.05mg/mL)	0
3-3'	0.5 mL (0.10mg/mL)	0
4-4'	0.5 mL (0.15mg/mL)	0
5-5'	0.5 mL (0.20mg/mL)	0
	Samples	
6-6'	0.1	0.4
7-7'	0.2	0.3

Then, 2.5 mL Lowry ACR was added to each tube, mixed by vortex and let stand for 10 minutes at room temperature for copper reaction in alkaline medium.. 0.25 mL Folin Reagent was added to each tube and mixed within 8 seconds by vortex. The tubes were then incubated at room temperature for 30 min. The intensity

of color developed was measured at 660nm (Shimadzu 1601 UV/Vis Double-beam Spectrophotometer, Kyoto, Japan) against blank cuvette (1.0 cm light path). A standard BSA calibration curve was constructed from the absorbance readings of standards and used for the calculation of protein amounts of samples (Figure 2.1).

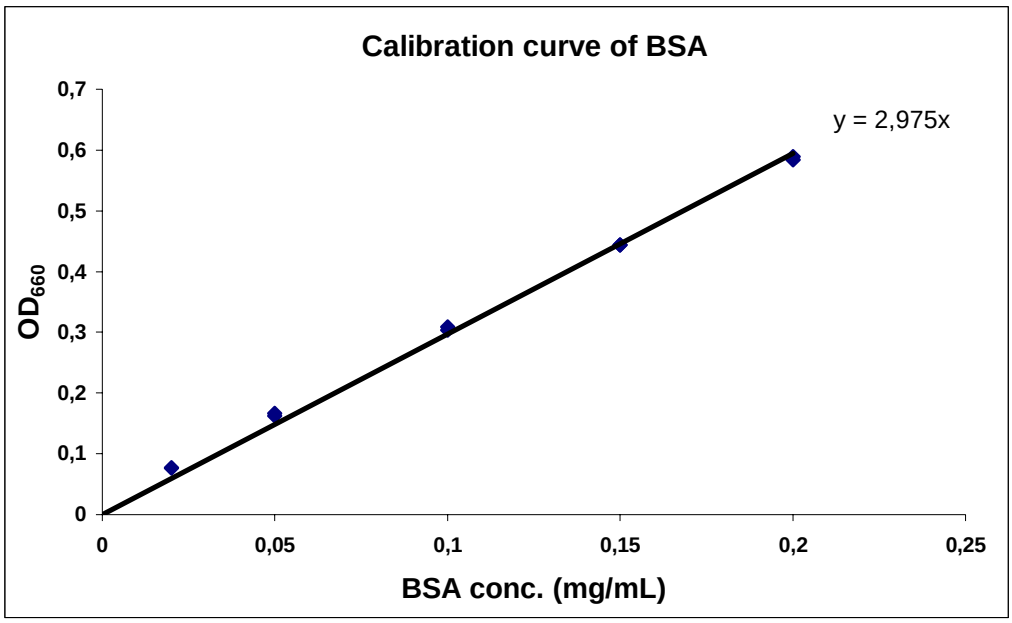


Figure 2.1. Standard calibration of BSA for Lowry Method

2.5 Lipid Peroxidation (Thiobarbituric Acid Reactive Substances) Test

The lipid peroxidation products were measured according to the method of Jain and Levine (1995), as described below.

Principle:

Malondialdehyde (MDA) which is an end product of lipid peroxidation, reacts with thiobarbituric acid (TBA) and forms a colored complex having a maximum absorbance at 532 nm.

Reagents:

0.018 M PBS (pH 7.4): 8.1 g NaCl, 2.302 g Na_2HPO_4 and 0.194 g NaH_2PO_4 dissolved and completed to 1 L. pH is adjusted to 7.4.

BHT: 88 mg BHT dissolved in 10 mL of absolute ethanol

30% TCA (w/v): 30 g TCA was dissolved in distilled water and completed to100 mL.

0.1 M EDTA: 37.224 g EDTA dissolved in dH_2O and completed to 1 L.

1 % TBA (w/v): 1 g TBA dissolved in 100 mL of 0.005 N NaOH

0.05 N NaOH: 2 g NaOH dissolved in dH_2O and completed to 1 L.

Reaction:

0.2 mL of homogenate was suspended in 0.8 mL of 0.018 M phosphate-buffered saline (pH 7.4) and 0.025 mL of butylated hydroxytoluene (BHT). Thirty percent trichloroacetic acid (0.5 mL) was then added. The tubes were mixed and allowed to stand in ice for at least two hours. The tubes were centrifuged at 2000 *g* for 15 min. One mL of each supernatant was then transferred to another tube to which 0.075 mL 0.1 M EDTA and 0.25 mL of 1% TBA in 0.05 N NaOH was added. The tubes were mixed and kept in boiling water bath for 15 min. The samples were cooled to room temperature and the absorbances were measured at 532 nm and 600 nm in Shimadzu 1601 UV/Vis Double-beam Spectrophotometer (Kyoto, Japan). The absorbances of the samples at 600 nm were subtracted from 532 nm. This process was applied for the deduction of free MDA in the sample. MDA values in nmole were determined with the extinction coefficient of MDA - TBA complex at 532 nm (ε_{532}= 1.56 x 10^5 L/mol.cm). TBA reactivity was expressed per mg of protein. TBARS test determines any compound that reacts with thiobarbituric acid, but the

most abundant product is malondialdehyde. Therefore, the test is named as thiobarbituric acid reactive substances (TBARS) test. Butylated hydroxytoluene (BHT) is used to prevent artificial increase of MDA during the experiment.

2.6 Total Lipid Determination

Colorimetric determination of total lipid content of microsomal suspensions was performed by the method of Frings *et al*. (1972).

Principle:

The principle of the method is based on the reaction of double bonds in lipids with phosphovanillin reagent in acidic conditions forming a colored complex that was read at 540 nm. Olive oil (700 mg/dL) was used as a standard.

Reagents:

H_2SO_4: Concentrated sulfuric acid

Standard Olive oil: 700 mg olive oil was dissolved in 100 mL absolute ethanol

Phosphovanillin reagent: 1.2 g of vanillin was dissolved in 200 mL water and 800 mL concentrated phosphoric acid was added and stored in dark bottle.

Reaction:

Hundreds microliters of microsomal suspension (or standard) and 2 mL of concentrated sulfuric acid were mixed in a test tube and heated in a boiling water

bath for 10min, cooled to room temperature, then 0.1 mL from each mixture was transfered to another test tube. At the same time 0.1 mL concentrated sulfuric acid was added to another test tube (blank) and to the all other tubes 6mL phosphovanillin reagent was then added. Absorbances were measured at 540 nm (Shimadzu 1601 UV/Vis Double-beam Spectrophotometer, Kyoto, Japan). Total lipid content was calculated as follows:

$$\text{Total lipid (mg/dL)} = (OD_{sample}/OD_{std}) \times 700\text{mg/dL}$$

2.7 Determination of Catalase Activity

Catalase activities of 1000 g samples were determined according to the method of Aebi (1964).

Principle:

In the UV range, H_2O_2 shows a continual increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240nm (ε_{240}=0.00364 L/mmol.mm). Catalase activity can be calculated from the difference in the absorbance at 240 nm. per unit time.

Reagents:

50 mM Phosphate buffer (pH 7.0): (a) 6.81 g KH_2PO_4 and (b) 8.9 g $Na_2PO_4.2H_2O$ dissolved in distilled water and make up to 1000 mL each. Solutions (a) and (b) are mixed in proportion of (1:1.5 (v/v)). pH of the buffer was confirmed.

30mM Hydrogen peroxide: (H_2O_2): 0.34 mL 30% H_2O_2 diluted to 100 mL with phosphate buffer.

Assay:

Two mL enzyme solution (1000 *g* supernatant) was pretreated with ten fold 1% Triton X-100 for 10 minutes, and then the mixture diluted further 300 fold to make a total dilution of 3000 with phosphate buffer, and 1mL 30mM H_2O_2 was further added. The reaction was started by the addition of H_2O_2 and followed by the decrease in absorbance at 240 nm for about 1min. The decrease in absorbance was recorded against a blank tube containing 1 mL of buffer instead of substrate H_2O_2. CAT activity was calculated from the equation 1. The results were given as specific activity (eq.2).

$$dA/min=(0.00364\ L/mmol.mm) \times c \times 10\ mm \quad \text{(eq. 1)}$$

$$\text{Specific Activity}= \mu mol\ H_2O_2/mg\ prot.min \quad \text{(eq.2)}$$

2.8. Determination of Superoxide Dismutase (SOD) Activitiy

Superoxide Dismutase (SOD) activity was determined according to the method of Marklund and Marklund (1974)

Principle:

The superoxide radical ($O_2^{-\cdot}$), substrate for SOD, was generated by the autooxidation of pyrogallol under illumination at alkaline pH with the action of atmospheric oxygen. As $O_2^{-\cdot}$ builds in the solution, the formation of yellow chromophore of oxidized pyrogallol accelerated because $O_2^{-\cdot}$ also reacts with pyrogallol. SOD reacts with the $O_2^{-\cdot}$ and therefore slows down the formation of oxidized pyrogallol. Because of this slowing process, SOD is said to inhibit the oxidation of pyrogallol. Decrease in absorbance was followed at 440 nm. Specific

activity units of SOD were given as the amount of SOD solution required to cause 50 % inhibition of the oxidation of pyrogallol.

Reagents:

Tris-EDTA buffer (50mM Tris, 10mM EDTA, pH 8.2). pH is important since autooxidation of pyrogallol follows linear form at that pH. Above this value, curvature was observed and below this value (< pH 8.0) there is no pyrogallol autooxidation.

Pyrogallol (15mM): 0.47 mg pyrogallol was dissolved in dH_2O and completed to 250 mL.

Assay:

To 2.8 mL of Tris-EDTA buffer varied volumes (5, 10, 15, 20, 25, 30, 40, 50 µL) of cytosolic fraction (125000 *g* supernatant) were added. The volume was further completed to 2.9 mL with buffer. Final volume of the cuvettes was completed to 3 mL by the addition of 100µL pyrogallol solution. Contents were mixed and read at 440 nm continuously for 5 min, against a reference cuvette containing buffer only. Percent inhibition of autooxidation of pyrogallol was calculated as follows:

$$\% \text{ Inhibition} = 100x\ [(\Delta OD/min)_{blank} - (\Delta OD/min)_{sample}] / (\Delta OD/min)_{blank}$$

dOD/min blank in above formula represents the absorbance difference per minute of non-inhibited pyrogallol autooxidation. Sample contains superoxide dismutase which inhibits pyrogallol autooxidation. Protein content of the diluted samples was calculated using predetermined protein concentrations of the cytosolic fraction. Percent inhibition versus mg protein in diluted sample graph was constructed and

converted to percent inhibition versus log protein graphs for each samples' each dilution. Figure 2.2 shows representative graphs for an individual of α-Lipoic acid group (LA_5).

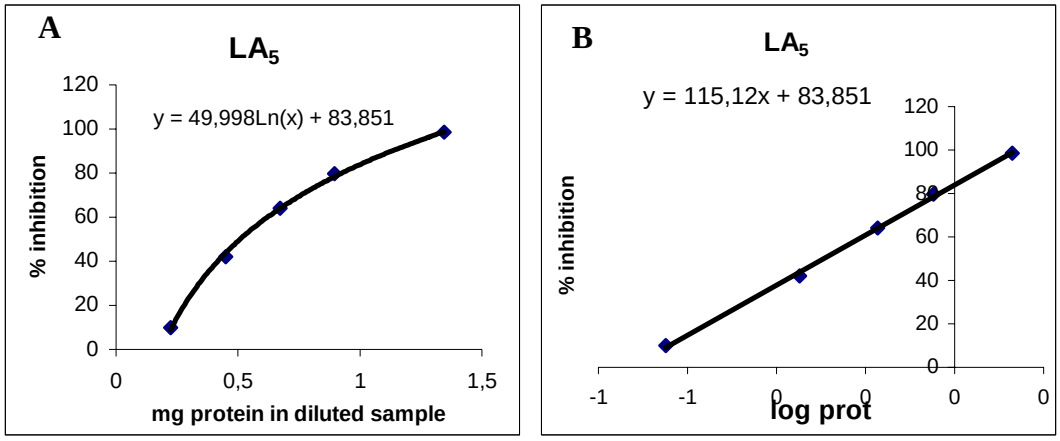


Figure 2.2. Percent inhibition versus mg protein in diluted sample (A) and log protein (B) graphs were poltted and used in order to determine the protein amount which inhibited pyrogallol autooxidation by 50%.

One unit SOD activity is described as the amount of protein that cause 50% inhibition of pyrogallol autooxidation. Specific activity units were expressed as:

$$1 / (\text{mg prot.}_{\text{50\% inhibition of autooxidation of pyrogallol}})$$

2.9 Determination of Glutathione Peroxidase (GSH-Px) Activity

GSH-Px activity of cytosolic fractions were determined according to the method of Paglia and Valentine (1967).

Principle:

Glutathione Peroxidase activity determination is based on the measurement of the rate of decrease in NADPH amount at 340 nm. Glutathione Reductase uses oxidized glutathione (GSSG) as a substrate and NADPH as a coenzyme. Since oxidized glutathione is produced by GSH-Px, the rate of decrease in NADPH amount is directly proportional to GSH-Px activity (Figure 2.3).

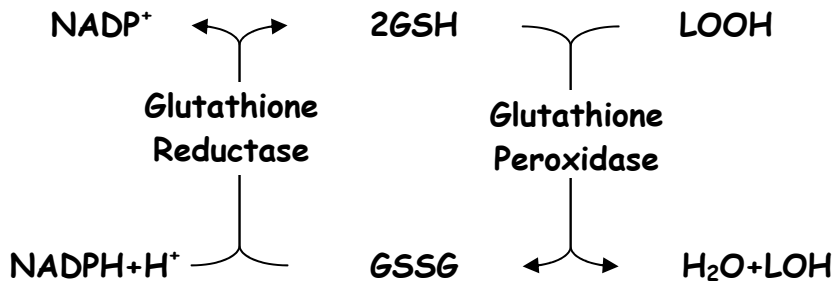


Figure 2.3. Glutathione cycle

Oxidized glutathione (GSSG) is converted to reduced form by glutathione reductase which uses NADPH (reduced from of puridine nucleotides) as a cofactor. Reduced glutathione is oxidized by glutathione peroxidase while lipid hydroperoxide is transformed to lipidalkoxyl and water.

Reagents:

0.1M Tris-HCl buffer (pH 8.0): 0.1 M Tris was prepared by mixing 2.422 g of Trizma base in 200 ml and pH is adjusted to 8.0 with 1M HCl.

80 mM Glutathione (GSH): 0.246 g GSH was dissolved in 10 ml dH_2O

2mM NADPH: 0.0017 g dissolved in 1 ml distilled water.

0.24 unit Glutathione Reductase (GSH-Red) dissolved in 100 µL distilled water. 28 µL of enzyme solution completed to 2.5 mL with 3.6 M Ammonium sulphate.

3.6 M Ammonium Sulphate: 1.19 g Ammonium Sulphate was completed to 2.5 mL with distilled water.

1.5 mM Hydrogen peroxide (H_2O_2): 42.5 µL 30% H_2O_2 was diluted to 250mL

30 mM Sodium azide (NaN_3): 0.195 g (NaN_3) dissolved in 100 mL distilled water.

Assay:

2.525 mL Tris-HCl buffer, 75 µL glutathione (GSH), 100 µL glutathione reductase (GSH-Red), 100 µL 40 fold diluted cytosolic solution (125000 *g* supernatant), 100 µL NaN_3 (sodium azide for catalase inhibition), were added to 3 mL quartz cuvettes and incubated for 5 min at room temperature. Then, 100 µL H_2O_2 was added to start the cyclic reaction and absorbance was followed at 340 nm for 5 min continuously. Rate of enzymatic reaction was calculated from the linear part of the record using an extinction coefficient of 6.22 L/mmol.cm given for NADPH. Specific activity units were expressed as; nmol NADPH produced/mg prot.min

2.10 Determination of Total Glutathione S-Tranferase (GST) Activity

Total GST activities were determined according to the method of Habig *et al*. (1974)

Principle:

1-chloro-2, 4-dinitrobenzene (CDNB) is a common substrate for all isozymes of GST. It can be used as a substrate for total GST catalyzed GSH oxidation which is monitored by the increase in the absorbance at 340nm due to the 1-glutathione 2,4-dinitrobenzene- (DNB-SG) formation (Figure 2.4).

Reagents:

20 mM 1-chloro-2,4-dinitrobenzene CDNB [in ethanol:dH_2O (3:2)]: 20.26 mg CDNB dissolved in 3 mL ethanol and added 2 mL distilled water

50 mM Potassium Phosphate (KP) Buffer (pH 7.0): 2.175 g K_2HPO_4 and 1.7 g KH_2PO_4 weighed and each was dissolved in 250 mL distilled water. Both components were mixed in approprate ratios to adjust pH to 7.0.

20 mM Reduced Glutathione (GSH): 0.0615 g dissolved in 10 mL distilled water.

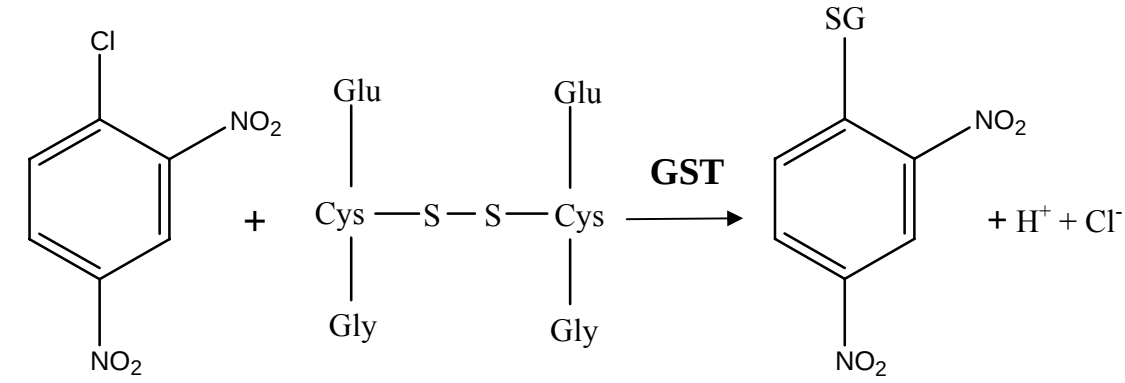


Figure 2.4. DNB-SG formation by GST catalyzed reaction

Assay:

To the 3 mL quartz cuvette (1.0 cm light path), followings were added with the order of 2500 µL potassium phosphate buffer, 200 µL GSH, 150 µL enzyme source (1/50 diluted 125000 g cytosolic supernatant [≈0.6mg/mL protein]) and the reaction started by adding 150 µL CDNB solution. Then, the thioether formation was followed at 340 nm for 2 minutes. Each time, there should be blank reading (reaction with no enzyme source) for subtracting non-enzymatic product formation from the GST assay.

The enzyme activity was calculated as the amount of thioether (nmol) formed by 1mg total protein containing cytosol in one mitute by using 0.0096 ($\mu M.cm^{-1}$) as an extinction coefficient of thioether formed by GST.

Specific activity was expressed as; nmol DNB-SG formed/mg prot.min

2.11 Determination of Reduced Glutathione (GSH) Concentration

Reduced glutathione concentration was determined by the method of Sedlak and Lindsey (1968).

Principle:

GSH determination is based on the oxidation of reduced GSH by 5,5'-dithiobis-2-nitrobenzoic acid [DTNB] to produce pale yellow color that gives its maximum absorbance at 412 nm. DTNB method measures the ability of SH groups to reduce DTNB to form 1 mole of 2-nitro-5-mercaptobenzoic acid (yellow) per mole of SH group (Figure 2.5.).

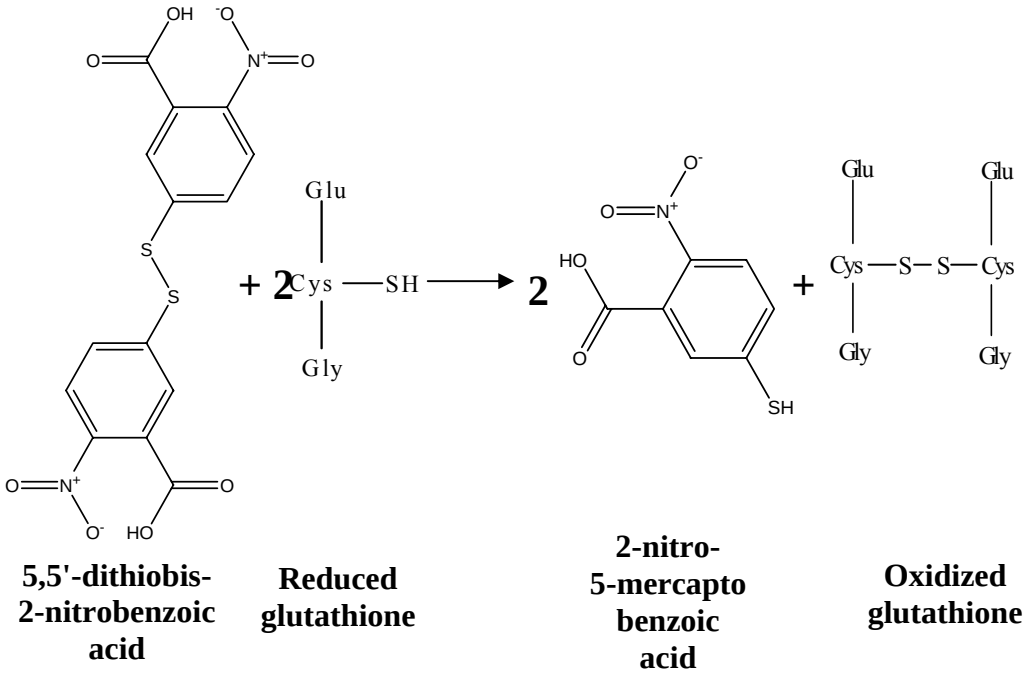


Figure 2.5. Oxidation of glutathione while forming yellow 2-nitro, 5-mercaptobenzoic acid.

Reagents:

0.2 M Tris-HCl buffer (pH 8.2) containing 20 mM EDTA

10 mM DTNB

CH_2OH (Methanol) (pure)

Assay:

To the 0.25 mL appropriately diluted tissue supernatant (1/5 and 1/10), 0.75 mL Tris-HCl, 0.05 mL DTNB and 3.95 mL methanol were added, then incubated at room temperature for 30 min with occasional shaking. After incubation, the samples were centrifuged at 3000g for 15 min to remove proteins having SH group and reacting with DTNB. The absorbance of supernatant is directly proportional to the cytoplasmic GSH concentration. GSH standard solutions ranging between 0.1 to 1 mM were used. GSH standard calibration curve was drawn and slope was used to determine the GSH concentration (Figure 2.6). GSH level was expressed as μmol GSH/mg protein.

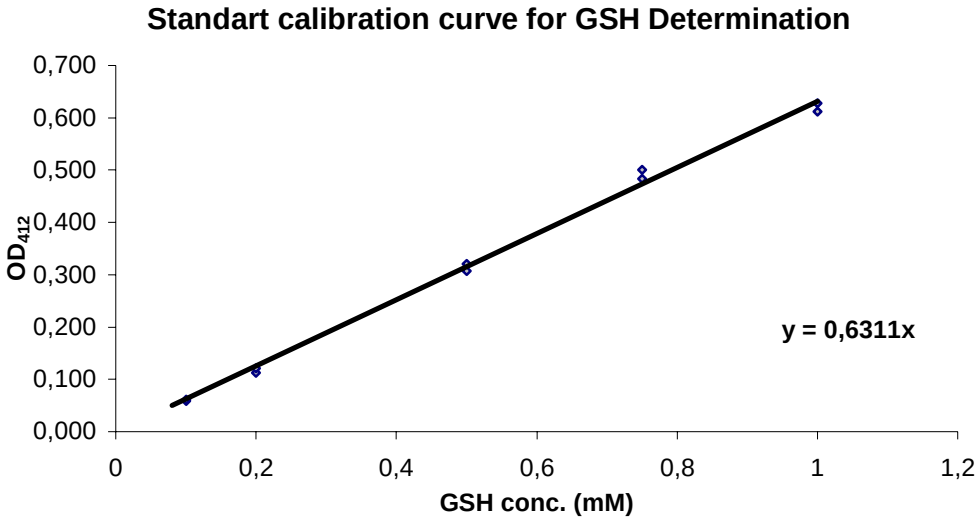


Figure 2.6. Standard calibration curve for GSH determination

2.12 Fourier Transform Infrared (FT-IR) Study

The spectral analysis was carried out using a Bomem 157 FT-IR Spectrometer equipped with DTGS detector (MB Series, Canada). The sample compartment was continuously purged with dry air to minimize atmospheric water vapor absorbance, which overlaps in the spectral region of interest, and carbon dioxide interference. To overcome this problem, the spectrum of air was recorded as background and subtracted automatically by using appropriate software. Figure 2.7 shows the infrared spectrum of air used as a background in this study. Spectra of membrane samples were recorded in the 4000-1000 cm^{-1} region with CaF_2 window using 12 μm path length. Interferograms were accumulated for 100 scans at 2 cm^{-1} resolution. Temperature regulation was performed by Graseby Specac digital temperature controller unit. Samples were incubated for 10 min at each temperature before data acquisition. Spectra were taken for temperatures between 6-60^{o}C. Data analysis and calculations were carried out with Grams/386 Converter.

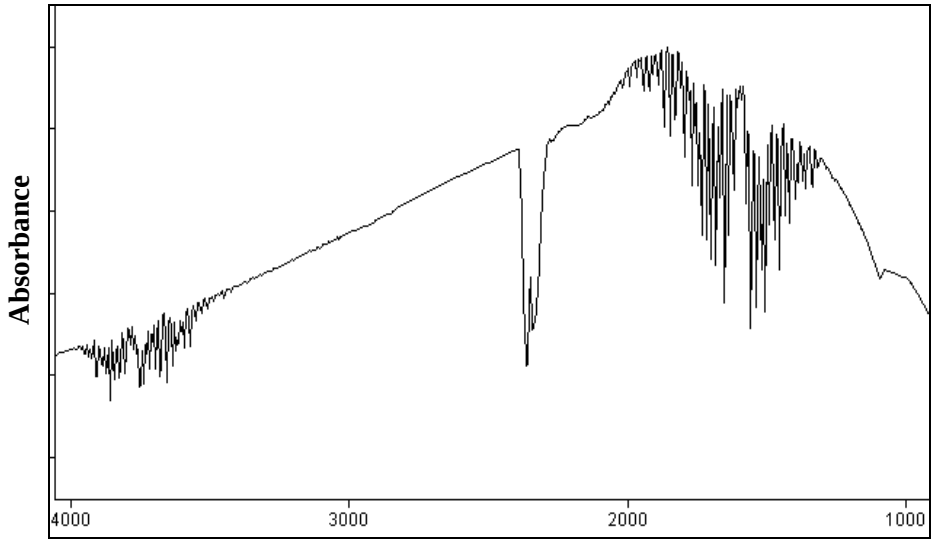


Figure 2.7. The Infrared Spectrum of air

2.13 Statistical Analysis

The results were expressed as mean ± standard deviation (SD). Data were analyzed statistically by using Mann-Whitney Test and the *p* values less than 0.05 were considered as statistically significant.

CHAPTER III

RESULTS

Five groups of Wistar rats namely; Control (n=6), Diabetic (n=7), α-Lipoic acid [α-lipoic acid treated diabetic (n=6)], Vit C [vitamin C treated diabetic (n=7)] and Combination [both α-lipoic acid and vitamin C treated diabetic (n=10)] groups were used in this study. The effects of eight week diabetes were evaluated by the changes in diabetic rats' antioxidative capacity. The presence of oxidative stress was shown by thiobarbituric acid reactive substances (TBARS) test. Activities of enzymatic antioxidants; superoxide dismutase (SOD), glutathione peroxidase (GSHPx), catalase (CAT) and glutathione S-transferase (GST) were measured. Non-enzymatic antioxidant, reduced glutathione (GSH) levels were compared.

Total protein and total lipid values were also compared in both control, diabetic and treatment groups. The effect of diabetes mellitus on rat liver microsomal membranes were also studied and FT-IR results were compared in terms of lipid peroxidation, total lipid values, lipid to protein ratio, phase behaviour, dynamic and order of the acyl chains. The changes in secondary structure of control and diabetic microsomal membrane proteins were also compared by the curve fitting analysis of amide I band at 1653 cm^{-1}.

3.1 Body Weights and Blood Glucose Levels of Rats

Animals were weighed both before teratment and before decapitation. They were given in Table 3.1. Diabetic animals' body weights were decreased while controls kept their prior values. The decrease in the body weight was related with the disease conditions, which is a characteristic symptom of type I diabetes mellitus (Champe and Harvey., 1994). Blood glucose levels were measured weakly; started after STZ treatment till decapitation. The results were given in Figure 3.1. As seen from the Figure, blood glucose levels of diabetic rats were increased after STZ injections as expected. The occurance of STZ induced diabetes was confirmed by establishing weekly blood glucose levels in diabetic groups. In all diabetic groups, there was a big variation (higher standard deviation) in the individual blood glucose levels compared to controls (lower standard deviation). The results might indicate the antioxidant capacity of each individual, in terms of their endogenous systems, and the physiological instability effect of the disease.

Table 3.1. Average body weights of rats used in this study

Groups	**Average body weights** (g)	
	Before Treatment	**Before Decapitation**
Control (n=6)	225.34±23.04	289.60±41.15
Diabetic (n=7)	252.37±18.75	232.87±18.56
α-Lipoic acid (n=6)	207.70±12.6	192.00±22.22
Vitamin C (n=7)	271.25±42.23	220.87±12.03
Combination (n=10)	237.50±25.00	212.50±14.28

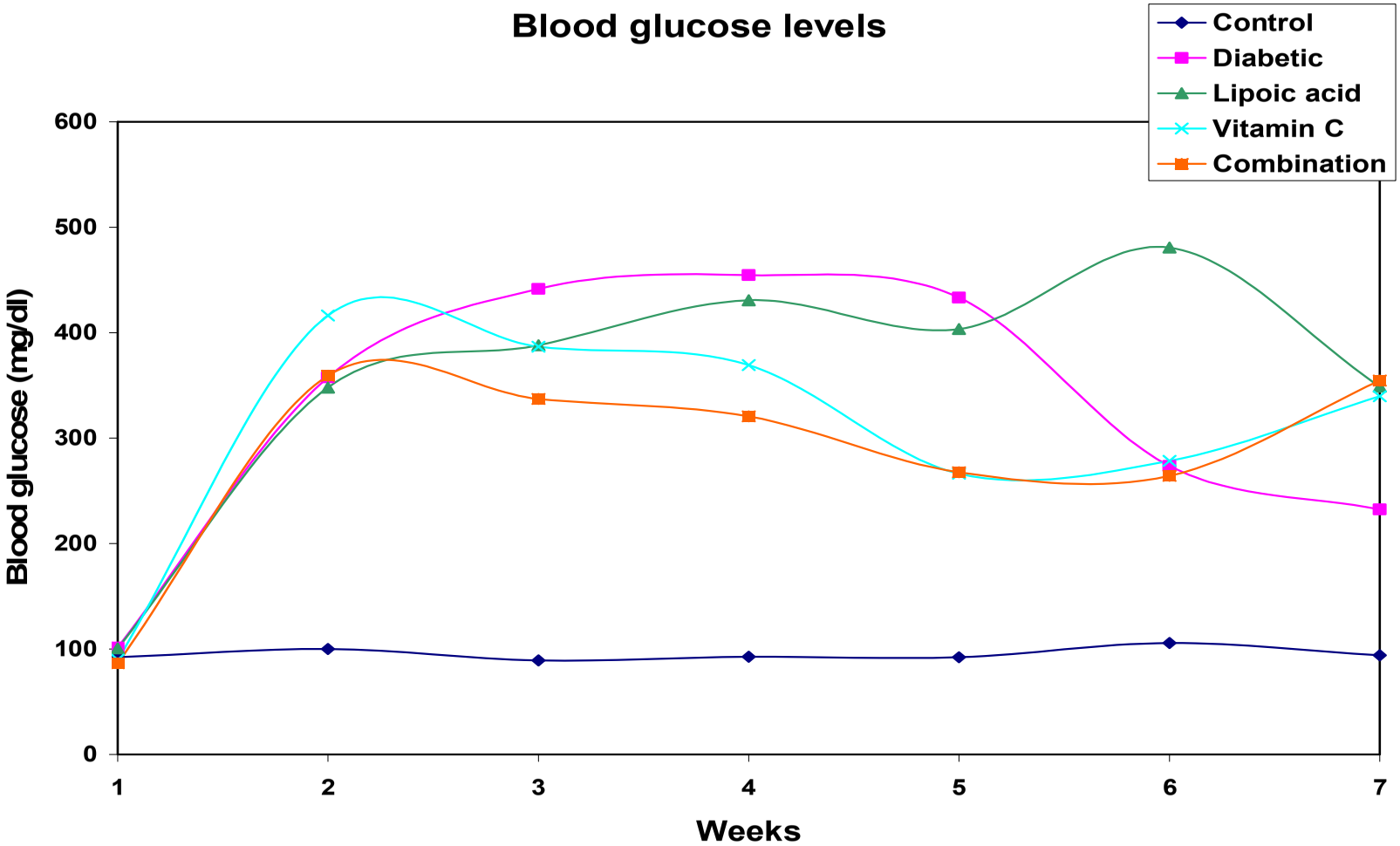


Figure 3.1. Changes in blood glucose levels of rats during eight weeks

3.2 Protein Determination

Total protein content of rat liver tissues were determined from the crude homogenate and approximate values were defined as mg protein per gram of wet tissue. As seen in Figure 3.1, total protein values of Diabetic group were significantly decreased ($p<0.05$) compared to controls. In addition, total protein values of α-Lipoic acid group were significantly increased ($p<0.0.5$) compared to Diabetic group (Figure 3.2).

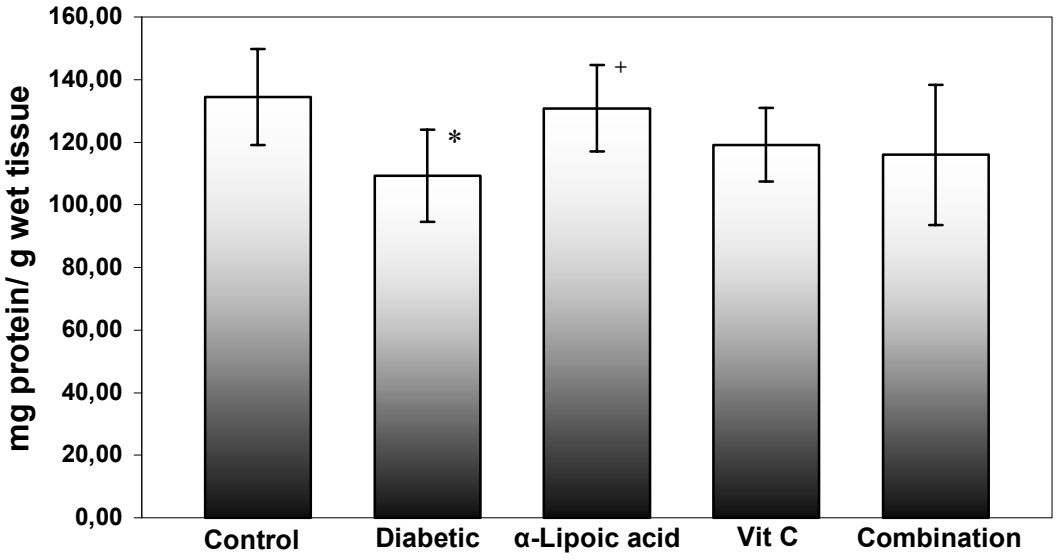


Figure 3.2. Comparison of total protein in crude homogenate of rat liver (mean±SD). "*" and "+" indicates the level of significance at 95% confidence interval compared to Control and Diabetic groups, respectively.

3.3 Total Lipid Content of Microsomal Membranes

Total lipid contents of microsomal membranes of Diabetic group slightly increased compared to control group. On the other hand, as seen from Figure 3.3, in treatment groups, namely α-Lipoic acid, Vit C and Combination groups lipid content approaches to control's values.

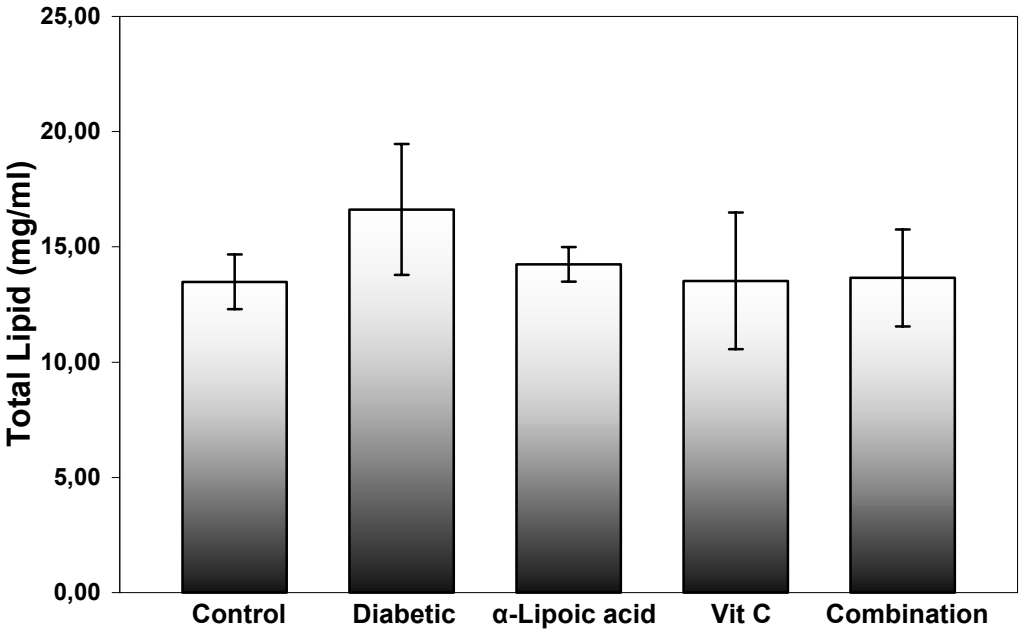


Figure 3.3. Comparison of total lipid levels of all groups.

3.4 Antioxidant Enzyme Activities

Antioxidant enzymes, namely catalase (CAT), glutathione peroxidase (GSHPx), superoxide dismutase (SOD) and glutathione S-transferase (GST) activities were determined. Each of these enzymes are a part of antioxidant network and should be evaluated together since SOD removes superoxide radical ($O_2^{\cdot -}$) while CAT and GSHPx removes hydrogen peroxide (H_2O_2) which is an end product of SOD catalyzed reaction. GSTs have the Se-independent GSHPx activity against lipid hydroperoxides.

3.4.1 Catalase Activity

Catalase (CAT) activities were determined in 1000 *g* supernatant fraction. The results are shown in Figure 3.4. Catalase activity decreased significantly for diabetic group compared to controls ($p<0.05$). α-Lipoic acid, Vit C and Combination groups' catalase activities increased significantly ($p<0.05$) compared to both Control and Diabetic groups. However, the change between control and antioxidant treated (α-lipoic acid, vitamin C and combination of both) diabetic groups were not statistically significant which shows the restoring effect of these antioxidants.

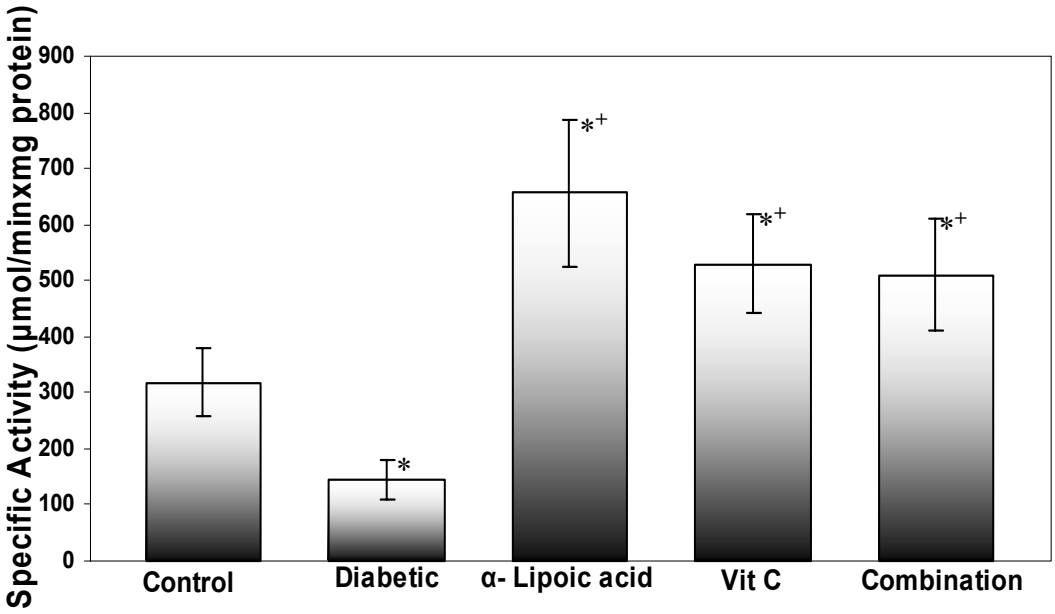


Figure 3.4. CAT activity (µmol/mg prot.min) in rat livers of all groups. Bars indicate the mean values ± SD. "*" and "+" indicate the level of significance in 95% confidence interval, compared to controls and diabetics, respectively.

3.4.2 Glutathione Peroxidase Activity

Glutathione peroxidase activity observed in Diabetic group were higher compared to controls ($p<0.005$). Increase in glutathione peroxidase activity for diabetic group treated with α-lipoic acid and vitamin C treated diabetic group were also statistically significant as ($p<0.05$) and ($p<0.005$), respectively. However, the Combination group treated both with α-lipoic acid and vitamin C showed no significant difference compared to controls (Figure 3.5).

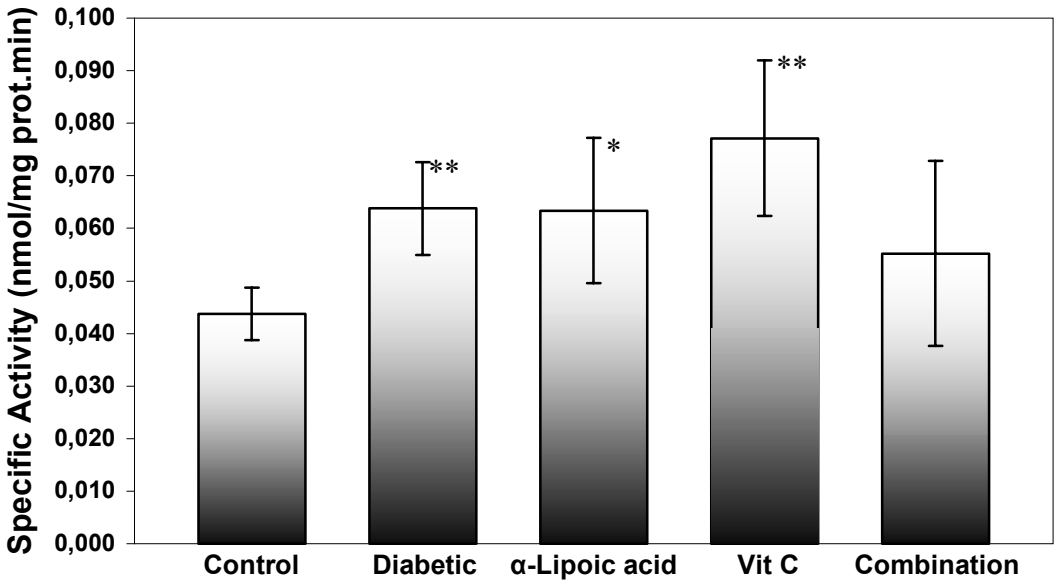


Figure 3.5. GSHPx activity (nmol/mg prot.min) in rat livers of all groups. Error bars indicate the mean values ± SD. "*" and "**" indicate the level of significance compared to control group in 95% and 99.5% confidence interval, respectively.

3.4.3 Superoxide Dismutase Activity

Superoxide dismutase activities were determined in all groups and the results were given in Figure 3.6. SOD activity was increased significantly for Diabetic, α-lipoic acid, vitamin C treated groups compared to Control group ($p<0.05$, $p<0.05$ and $p<0.005$, respectively). SOD activity of Vit C group were increased significantly compared to Diabetic group ($p<0.05$). However, Combination group, who received both α-lipoic acid and vitamin C showed no significant change compared to both Control and Diabetic groups.

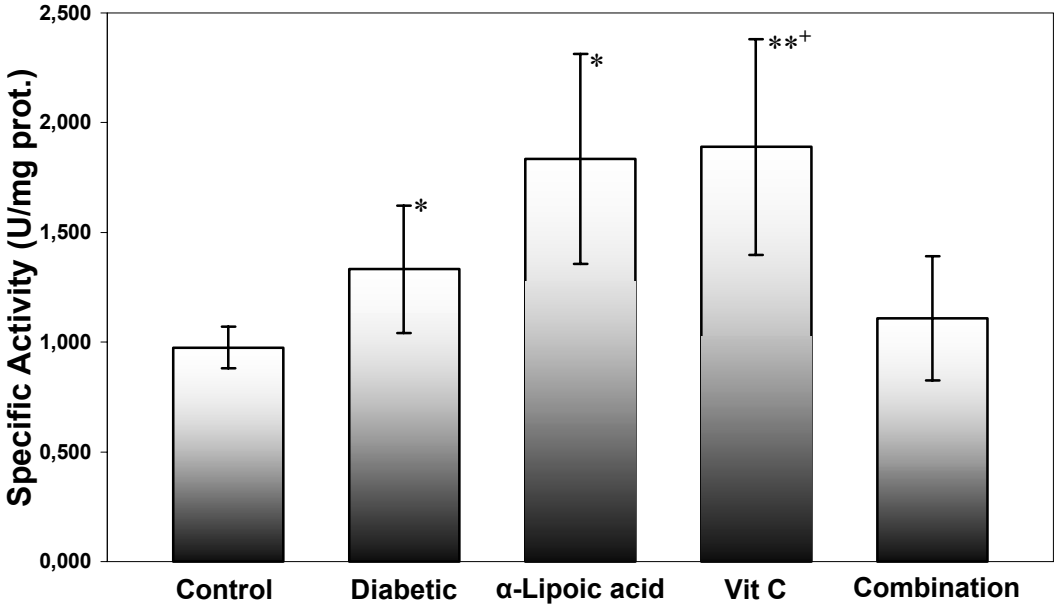


Figure 3.6. SOD activity (units/mg prot.min) in rat liver of all groups. Error bars indicate the mean ± SD. "*" and "+" indicate the level of significance in 95% confidence interval compared to Control and Diabetic groups, respectively.

3.4.4 Glutathione S-Transferase Activity

Glutathione S-transferase activities in different groups were also compared. No significant change was observed in total GST levels of both Diabetic and treated groups namely, α-lipoic acid and vitamin C treated groups, compared to controls. Only in the Combination group there was a slight increase, but this increase was not significant statistically (Figure 3.7).

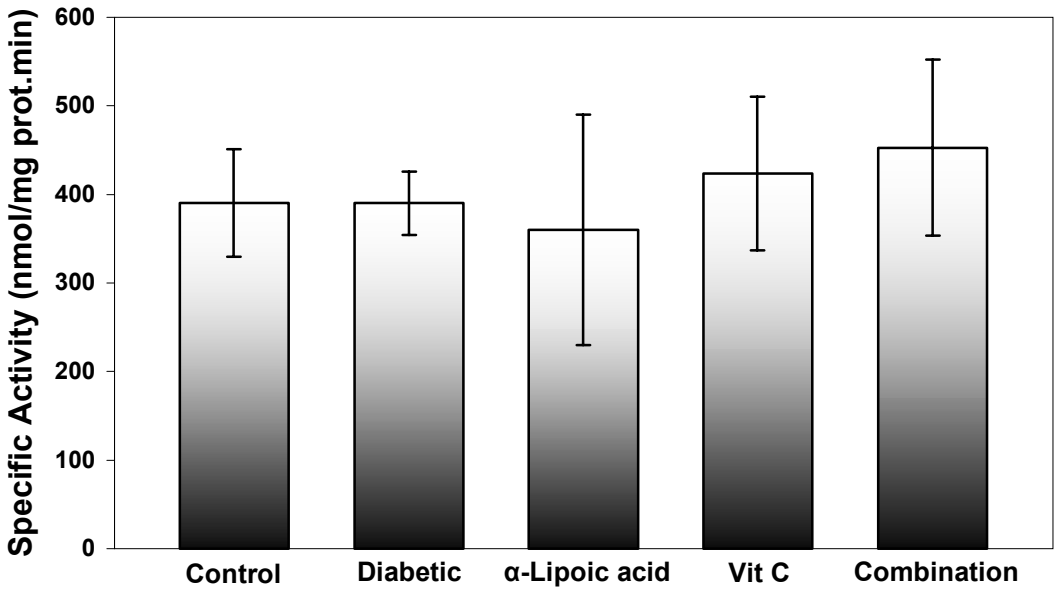


Figure 3.7. GST activity (nmol/mg prot.min) in rat livers of all groups. Error bars indicate the mean values ± SD.

3.5 Reduced Glutathione (GSH) Levels

The results of total reduced glutathione (GSH) levels were given in Figure 3.8. As seen from the Figure, both diabetic and treated groups' reduced glutathione (γ-glu-cys-gly) levels were decreased significantly ($p<0.05$) compared to control group.

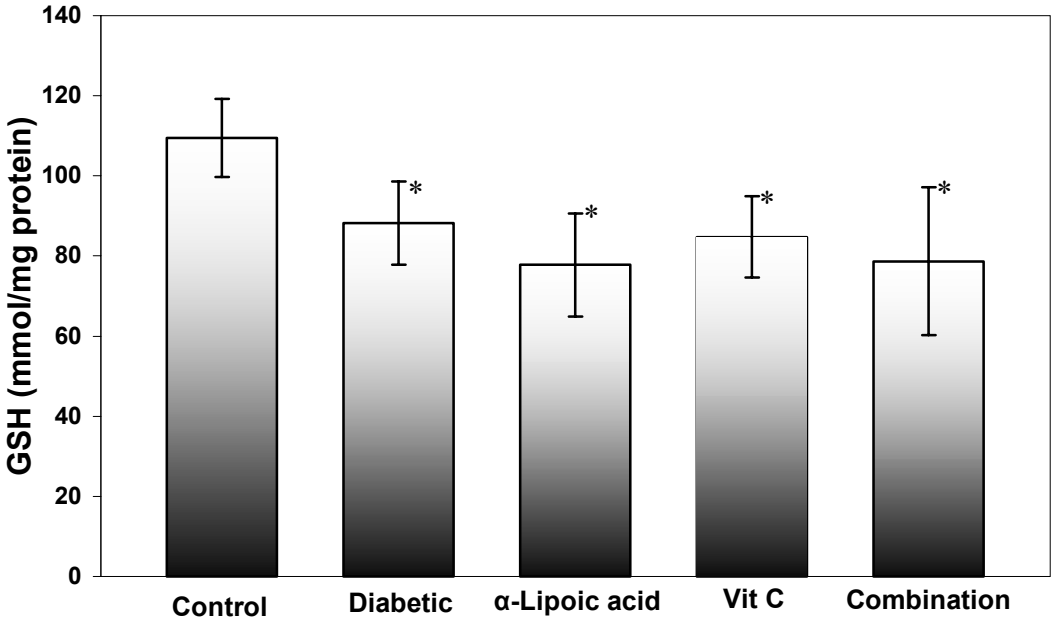


Figure 3.8. Comparison of reduced glutathione levels of all groups. Error bars indicate the mean values ± SD.

3.6 Lipid Peroxidation of Microsomal Membranes

Malondialdehyde (MDA) levels were measured by TBA-reactivity test. Figure 3.9 illustrates TBA-reactivity (MDA) levels for all Control, Diabetic and treated groups. Both Diabetic and treated groups namely, α-Lipoic acid, Vit C and Combination groups showed a significant increase in TBA-reactivity (MDA) levels ($p<0.005$, $p<0.005$, $p<0.005$, $p<0.005$, respectively) compared to Control group. In addition, MDA levels of Combination group were decreased significantly ($p<0.005$) compared to Diabetic group. The decrease observed in both α-Lipoic acid and Vit C group was not significant, statistically compared to Diabetic group.

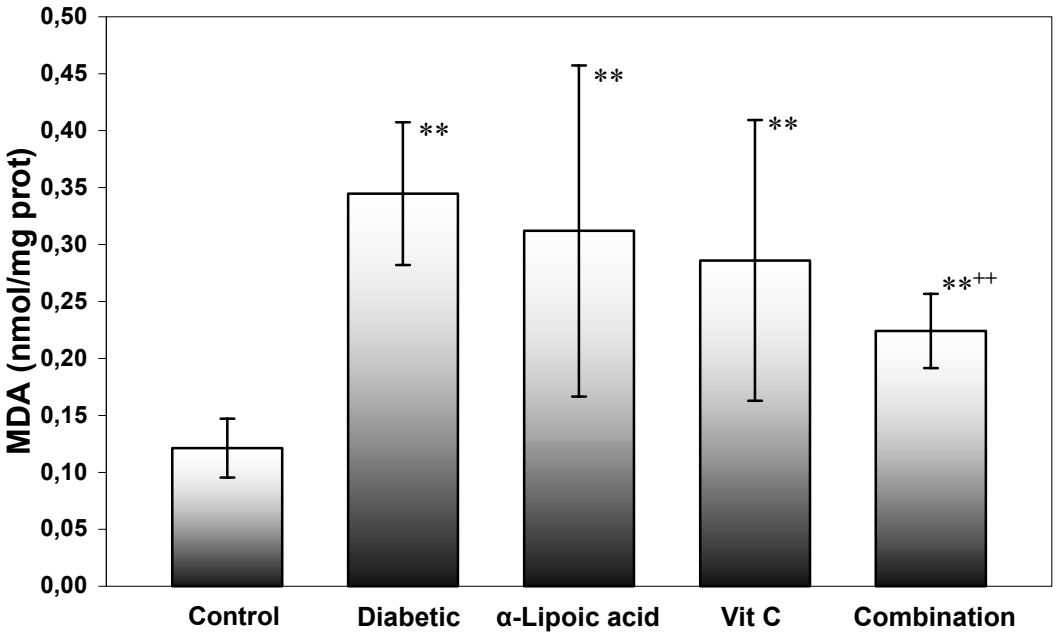


Figure 3.9. Comparison of TBA-reactivity (MDA) for all groups. Error bars indicate the mean values ± SD.

All the antioxidant enzyme activities (CAT, GSHPx, SOD and GST) and total protein, total lipid, TBA-reactivity (MDA) and reduced glutathione (GSH) determinations were given in two separate tables, Table 3.2 and Table 3.3, respectively. The results were discussed in " Discussion" section.

Table 3.2. Antioxidant enzyme activities (Mean±SD)

Groups	Specific activities of CAT (µmol/mg prot.min)	Specific Activities of GSHPx (nmol/mg prot.min)	Specific Activities of SOD (U/mg prot.)	Specific Activities of GST (nmol/mg prot.min)
Control (n=6)	114.97±19.98	0.044±0.005	0.976±0.095	390.55±60.44
Diabetic (n=7)	83.06±14.21*	0.064±0.009**	1.332±0.29*	390.05±90.72
α-Lipoic acid (n=6)	151.14±48.37*$^{+}$	0.056±0.018*	1.835±0.478*$^{+}$	360.16±49.62
Vitamin C (n=7)	115.79±30.21*$^{+}$	0.077±0.015**	1.889±0.491**$^{+}$	423.53±61.74
Combination (n=10)	102.24±17.75**$^{+}$	0.055±0.018	1.109±0.284	452.87±91.51

* : $p<0.05$ compared to control group

** : $p<0.005$ compared to control group

$^{+}$: $p<0.05$ compared to diabetic group

Table 3.3. Total lipid, total protein, TBA-reactivity (MDA) and reduced glutathione levels of all groups (mean±SD)

Groups	Total Lipid (mg/ml)	Total protein in crude homogenate (mg protein / g of wet tissue)	TBA-reactivity [MDA] (nmol/mg protein)	GSH (µmol/mg protein)
Control (n=6)	13.48±1.18	134.43±15.34	0.121±0.026	113.57±5.22
Diabetic (n=7)	16.62±2.84	109.22±14.69*	0.345±0.063**	88.19±10.39*
α-Lipoic acid (n=6)	14.24±0.75	130.78±13.81^{+}	0.312±0.145*	77.76±12.88*
Vit C (n=7)	13.53±2.96	119.14±11.80	0.286±0.123*	85.99±10.15*
Combination (n=10)	13.65±2.10	115.98±22.39	0.224±0.032** $^{++}$	78.67±18.47*

* : $p<0.05$ compared to control

** : $p<0.005$ compared to control

$^{+}$: $p<0.05$ compared to diabetic group

$^{++}$: $p<0.005$ compared to diabetic group

3.6 Fourier Transform Infrared Spectroscopy (FT-IR) STUDY

3.6.1 General FT-IR Spectrum and Band Assignment of Rat Liver Microsomal Membranes

The present FT-IR study primarily based on the investigation of the changes in structure and composition, phase behaviour and dynamics of the control and diabetic rat liver microsomal membranes. In addition, the biophysical effects of antioxidants α-lipoic acid and/or Vitamin C were investigated.

Main absorptions observed in the spectra were labelled in Figure 3.10 and detailed band assignment based upon work from this and other studies (H.H. Mantsch, 1984; Sills *et al.*, 1994; Jackson *et al.*, 1998; Severcan *et al.*, 2000; Toyran *et al.*, 2004; Cakmak *et al.*, 2003) were given in Table 3.4.

Water is the most frequently used environment, which is a strong infrared absorber (Mantsch, 1984). It gives strong bands around 3409 cm^{-1} and 1645 cm^{-1}, which interferes with the C-H stretching modes of lipids in the 3000-2800 cm^{-1} region and the C=O stretching (~ 1735 cm^{-1}).

As the infrared absorption of water masks several lipid bands, it is often required to subtract the water bands from the sample spectra by using the appropriate software. However, two main precautions should be considered, namely that: i) the water spectra are recorded with cells of identical pathlength and window material, and ii) they are recorded at the same temperature as that of the sample in order to have into account temperature-induced spectral changes in the water spectrum (Mantsch, 1984).

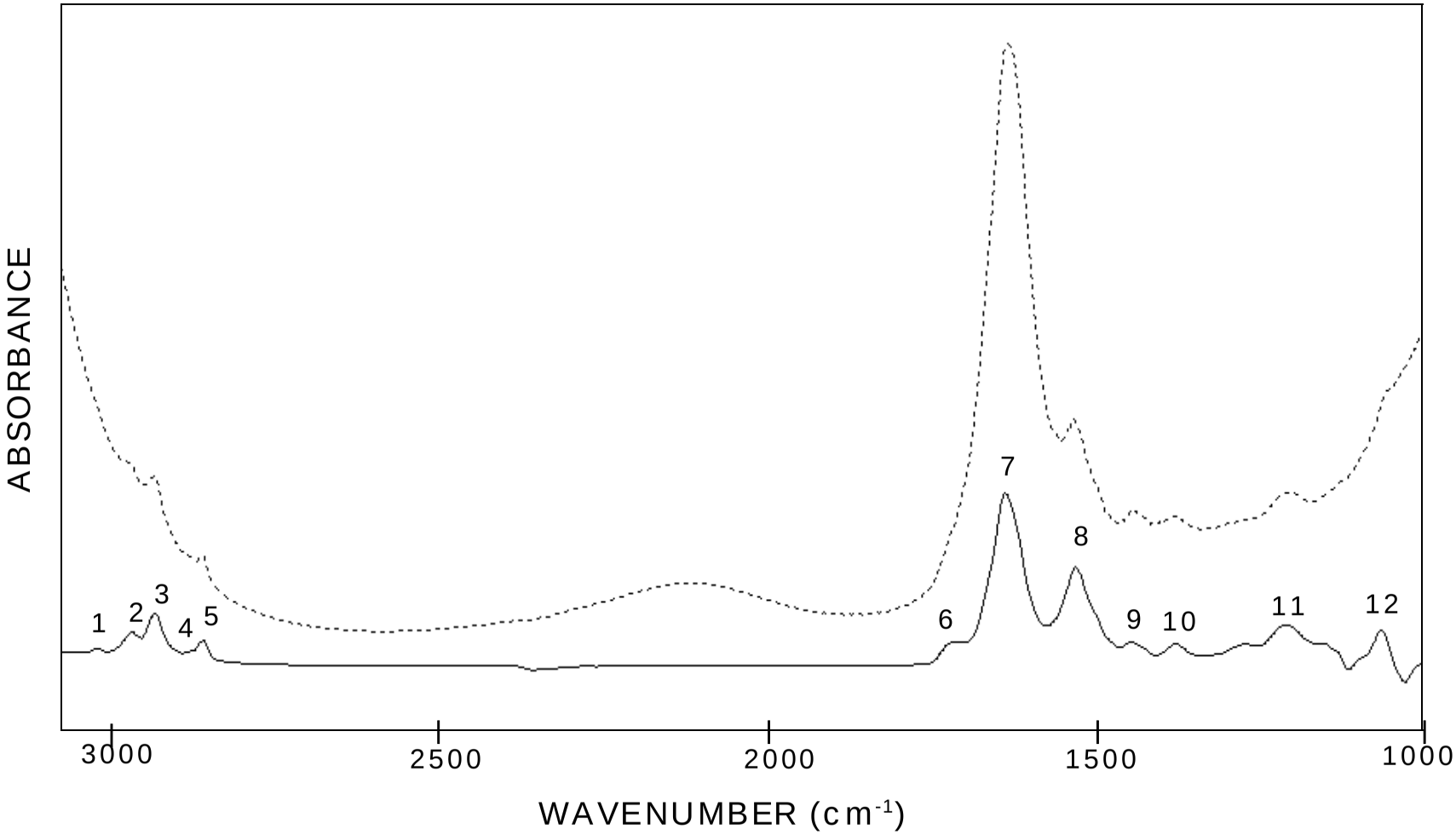


Figure 3. 10. A representative FT-IR spectra of rat liver microsomal membranes before (·····) and after (—) water subtraction in the region between 3030 – 1000 cm^{-1}.

Table 3.4. FT-IR spectral band assignments of rat liver microsomal membrane in the region of 3030-1000 cm^{-1} (Mantsch, 1984, Toyran and Severcan *et al.*, 2004, Severcan *et al.*, 2000).

#	**Wavenumber (cm^{-1})**	**Definition of the assignment**
1	3012	Olefinic =CH : unsaturated lipids
2	2958	CH_3 asymmetric stretching: lipids
3	2924	CH_2 asymmetric stretching :lipids
4	2873	CH_3 symmetric stretching: mostly proteins with little contribution from lipids
5	2853	CH_2 symmetric stretching: lipids
6	1741	Saturated ester C=O stretching: lipid
7	1653	Amide I (80% protein C=O stretching, 10% protein N-H bending, 10% C-N stretching)
8	1547	Amide II (60% protein N-H bending, 40 % C-N stretching
9	1466	CH_2 scissoring: lipids
10	1400	COO^- symmetric stretching (fatty acids)
11	1232	PO_2^- asymmetric stretching (phospholipids)
12	1090	C–C/C–O stretching vibrations (sugar)

As seen from Figure 3.10, the spectrum of rat microsomal membrane is quite complex consisting of several bands which arises with the contribution of different functional groups belonging to lipids, proteins and polysaccharides. Therefore, for

further studies, the spectra were investigated in detail in two regions namely; the C-H stretching region (3500-2800 cm^{-1}) and finger print region (1800-1000 cm^{-1}).

The spectrum shown in Figure 3.10 was investigated in two regions, the C-H stretching region (3500-2800 cm^{-1}) and finger-print region (1800-1000 cm^{-1}).

3.6.2 Effect of Diabets on the Microsomal Membranes in the C-H Region

Alterations in lipid content and structure can be obtained by examining the dominant lipid bands in the range 2800–3050 cm^{-1} that originate from CH stretching vibrations of the fatty acyl chains of all cellular lipids.

Figure 3.11 shows a representative absorbtion (A) and second derivative (B) spectra of rat liver microsomal membrane in the C-H region for both diabetic and control samples. As seen from the figure, the bands are better resolved in the second derivative spectra. The vibrational bands were sufficiently seperated after careful water subtraction procedure and therefore, it was not necessary to use band deconvolution or fit routines to evaluate their signal intensities for relative measurements for this study as reported by others (Harrison *et al*., 2000, Toyran and Severcan, 2003). The spectra were normalized according to the CH_2 asymmetric stretching band. The figure also indicates that the area of =CH olefinic band at 3012 cm^{-1} was increased in diabetic sample compared to control sample. The other bands of interest are CH_2 asymmetric and CH_2 symmetric stretching bands located at 2924 cm^{-1} and 2853 cm^{-1}, respectively. These bands manily monitor lipids.

Bandwidth values of these bands will be compared to obtain dynamic

information about microsomal membranes (Severcan *et al*., 1995). As can be seen from the figure the bandwidth of the CH_2 asymmetric stretching band decreased for diabetic samples.

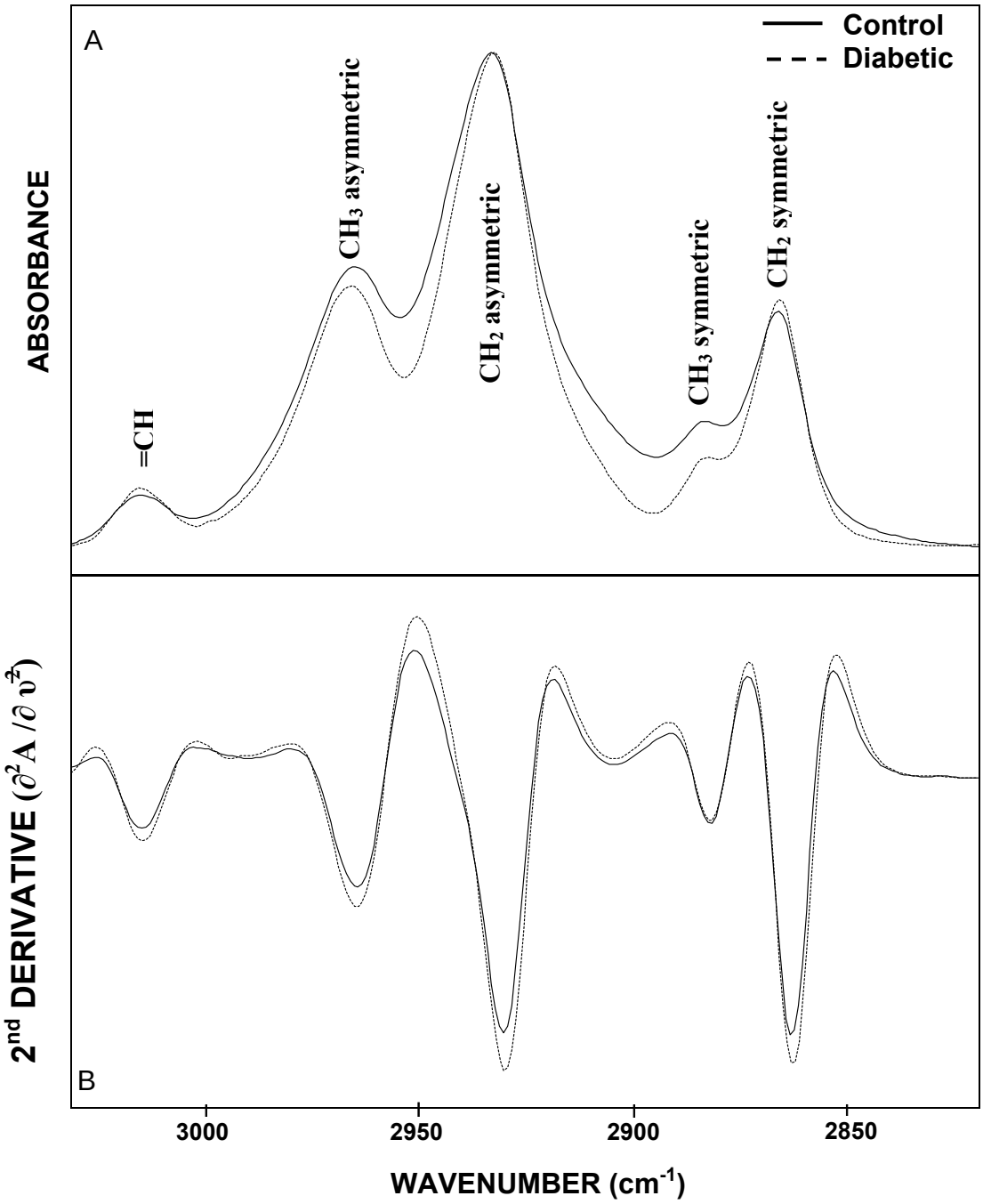


Figure 3. 11. FT-IR absorption (**A**) and second derivative (**B**) spectra of control and diabetic rat liver microsomal membranes.

In order to find out the effect of diabetes on phase transition behaviour, lipid order and dynamics temperature dependent studies were carried out.

Two bands in C-H region, namely; CH_2 asymmetric (2924 cm^{-1}) and CH_2 symmetric stretching (2853 cm^{-1}) bands were analyzed according to their frequency and bandwidth values between 12°C and 55°C. The temperature dependent behavior of the frequency of average CH_2 antisymmetric (Figure 3.12) and CH_2 symmetric (Figure 3.13) bands showed that the frequency shifted to lower values for diabetic samples compared to controls. The band shift became more evident as the temperature increases.

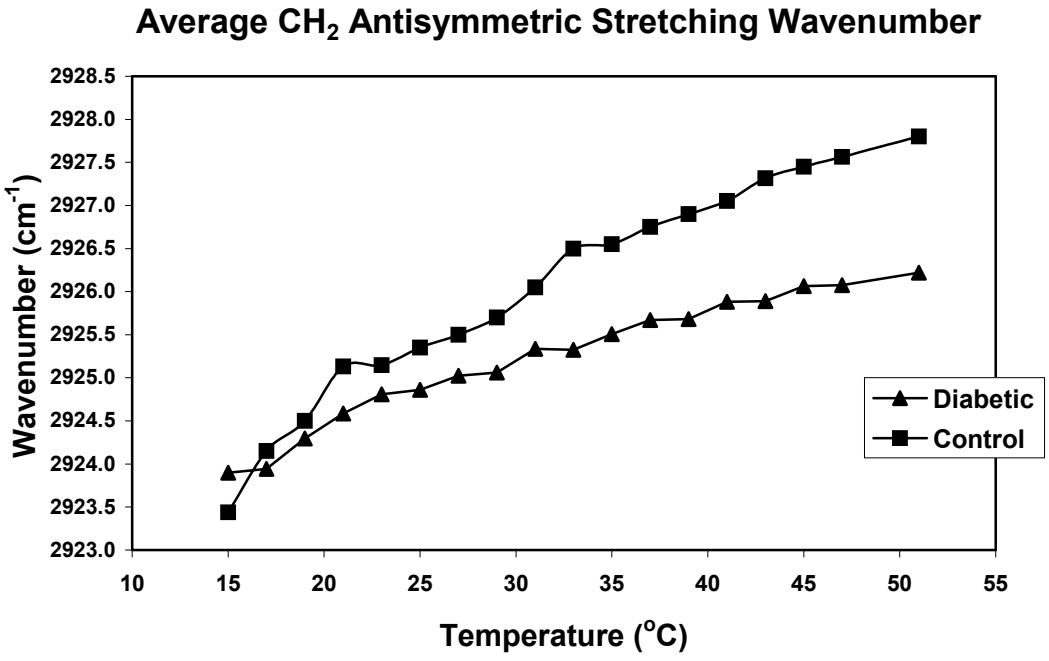


Figure 3.12. Temperature dependent variation of the frequency of mean CH_2 antisymmetric stretching modes for Control (n=6) and Diabetic (n=7) rat liver microsomal membrane.

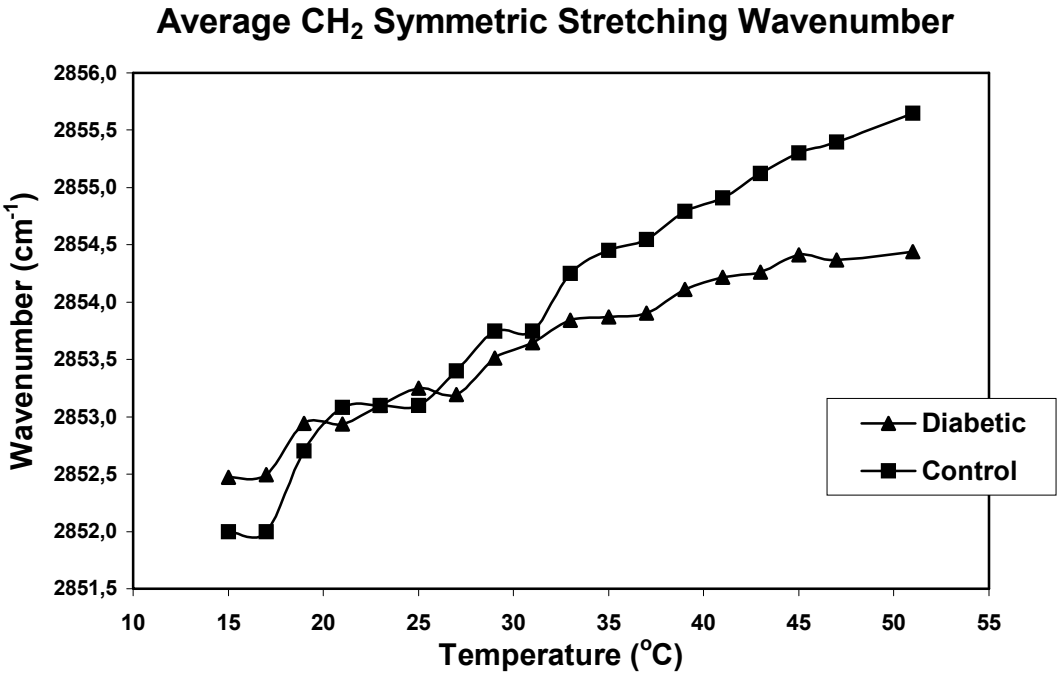


Figure 3.13. Temperature dependent variation of the frequency of mean CH_2 symmetric stretching modes for Control (n=6) and Diabetic (n=7) rat liver microsomal membranes.

Figure 3.14 shows the average bandwidth values for Control and Diabetic groups as a function of temperature. Diabetic microsomal membranes showed also remarkable decrease in the CH_2 antisymmetric stretching bandwidth values. As seen from the Figure, the bandwidth values dramatically decrease as temperature increase for diabetic samples compared to controls.

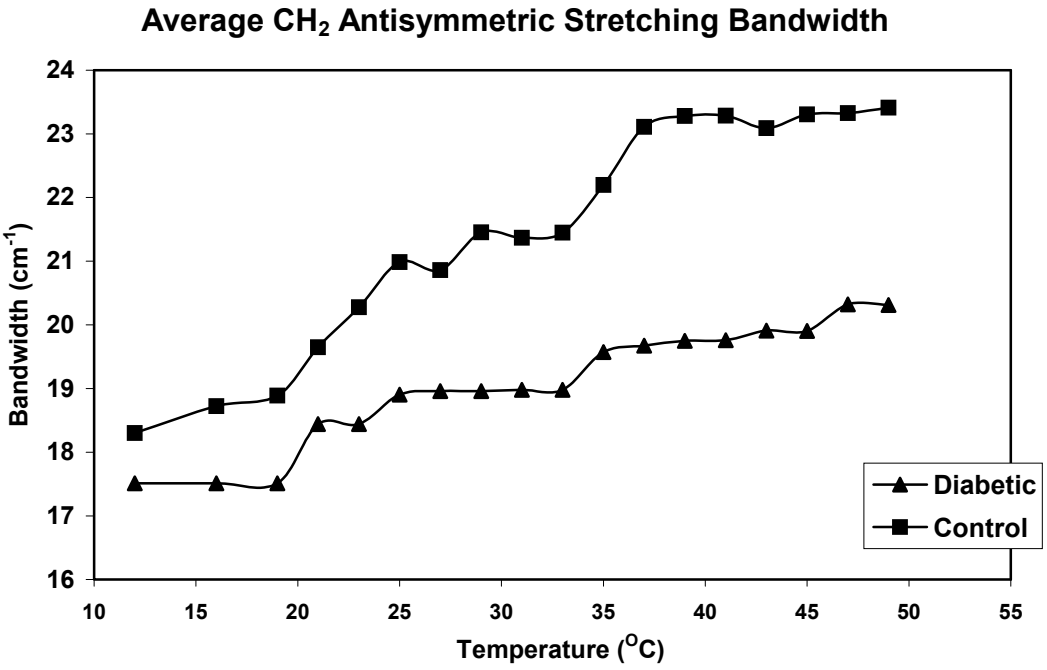


Figure 3.14. Temperature dependent variation of CH_2 antisymmetric stretching bandwidths of Control and Diabetic rat liver microsomal membranes.

3.6.3 Effect of Antioxidants on Diabetic Rat Liver Microsomal Membranes

The effects of α-Lipoic acid, vitamin C and combination of both to the diabetic rat liver microsomal membranes were investigated by analyzing the CH_2 symmetric stretching band frequency, CH_2 antisymmetric stretching bandwidth and (=CH) olefinic band (3012 cm^{-1}) area. Both bands correspond to lipids and give information about acyl chain order (frequency), dynamics (bandwidth) and lipid peroxidation status (olefinic band area) of phospholipids of microsomal membranes, respectively. Bandwidths were calculated from 3/4 peak hight. Half-maximal hight was interfered with the adjacent CH_3 antisymmetric stretching band.

Table 3.5 shows the frequency values of CH_2 symetric stretching band. The changes between groups and the level of significances were displayed in Figure 3.15 and 3.16.

Table 3.5. The frequency values of CH_2 symmetric stretching bands in the C-H region for all groups at 25°C and 37°C.

GROUPS	WAVENUMBER cm^{-1}	
	CH_2 SYMMETRIC	
	25°C	37°C
Control	2853.31±0.11	2853.63±0.11
Diabetic	2853.22±0.08	2853.43±0.06*
α-Lipoic acid	2853.16±0.11*	2853.42±0.16*
Vit C	2853.38±0.07^{+}	2853.49±0.12
Combination	2853.47±0.08*$^{++}$	2853.55±0.10^{+}

* : $p<0.05$ significantly different from control group

** : $p<0.005$ significantly different from control group

$^{+}$: $p<0.05$ significantly different from diabetic group

$^{++}$: $p<0.005$ significantly different from diabetic group

The frequency values of the CH_2 symmetric mode were displayed as bar graphs for a better perception and comparison in Figure 3.15 and 3.16. Since the changes in the frequency of the the CH_2 asymmetric and symmetric stretchnig mode give the same information, only the results of the CH_2 symmetric stretching mode will be presented here.

As seen from the Figure 3.15, the frequency decreased slightly but not significantly for diabetic samples compared to controls. This decrease in the frequency indicates that lipid order was increased in diabetic samples (Toyran and Severcan, 2003, Cakmak *et al*., 2003; Toyran *et al*., 2004). α-Lipoic acid supplemented diabetic group's CH_2 symmetric stretching frequency values significantly shifted to lower wavenumbers compared to control group ($p<0.05$). However, in Vit C tretaed diabetic samples the CH_2 symmetric stretching band shifted significantly ($p<0.005$) to higher values apporaching to those of control group. Similar effect was also observed for the combination group except that the frequency values were significantly higher ($p<0.05$) than those of controls (Figure 3.15).

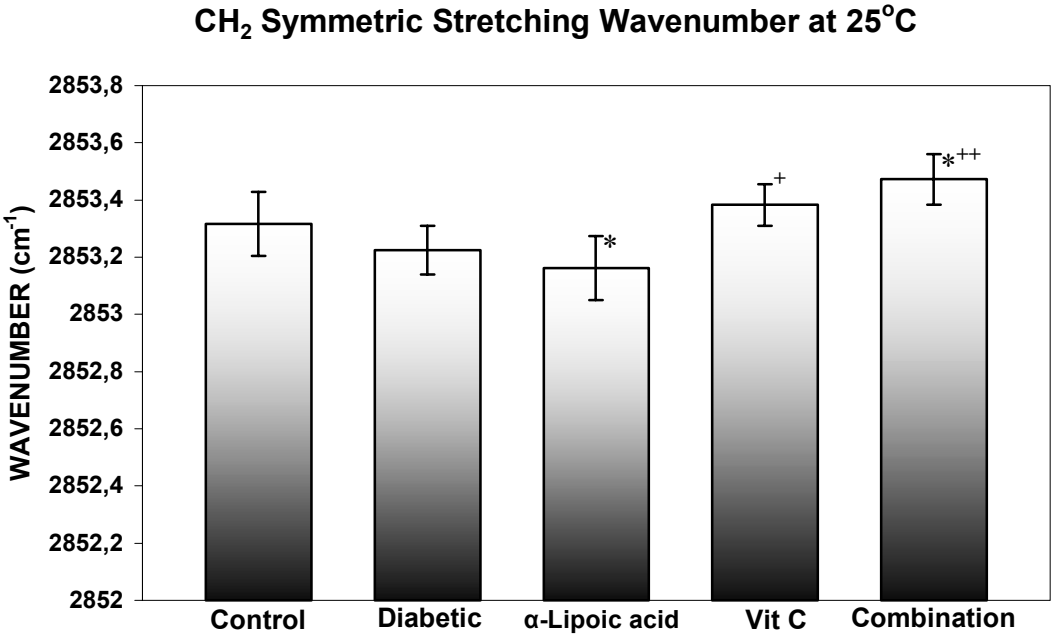


Figure 3.15. Comparison of CH_2 symmetric stretching band frequency values at 25°C

As seen in Figure 3.16, the frequency of the CH_2 symmetric stretching band at 37°C showed a significant decrease for diabetic samples compared to controls ($p<0.05$). α-Lipoic acid did not cause any restoring effect. But frequency values started to rise for Vit C and Combination group. The most significant increase was observed for combination group ($p<0.05$) compared to diabetics.

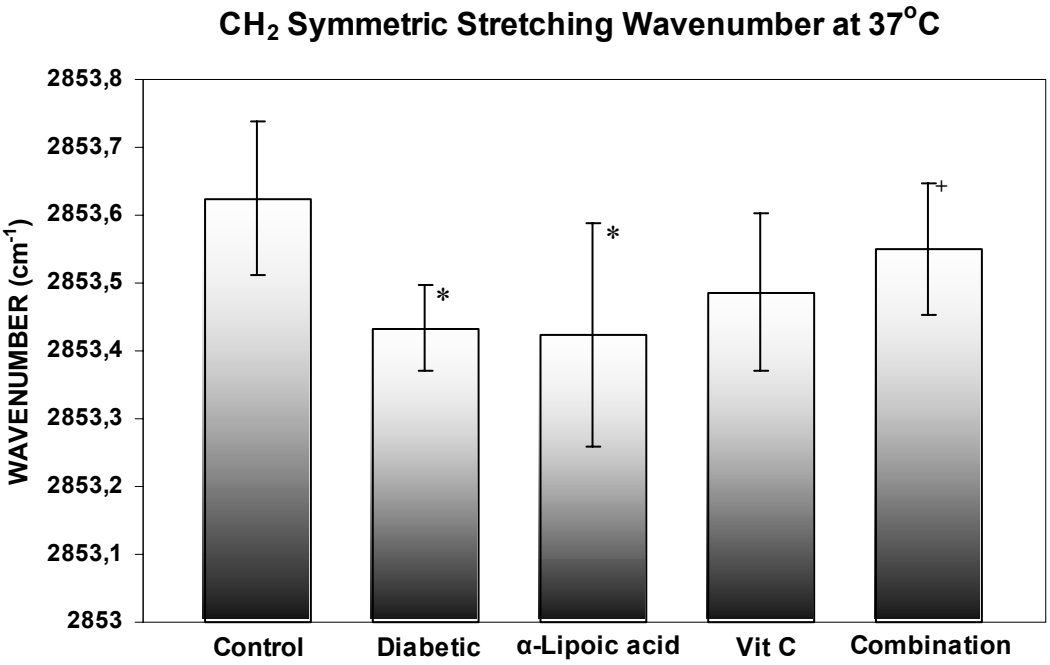


Figure 3.16. Comparison of CH_2 symmetric stretching band frequency values at 37°C

Table 3.6 shows the average bandwidth values at 0.75 peak hight of CH_2 antisymmetric stretching band at 25°C and 37°C. The comparison of these values are

better displayed in Figure 3.17 and 3.18, respectively. As clearly seen in Figure 3.14, Diabetic groups' CH_2 antisymmetric bandwidth values decreased significantly ($p<0.05$) at both temperatures compared to the controls. α-Lipoic acid, Vit C and Combination groups showed a significant increase ($p<0.05$) in bandwidth values compared to Diabetic group (Figure 3.17). The frequency values approach to control values which show the restoring effect of anitoxidant in diabetic samples.

Table 3.6. CH_2 asymmetric stretching band's average bandwidth values

GROUPS	**AVERAGE CH_2 ANTISYMMETRIC STRETCHING BANDWIDTH (cm^{-1})**	
	25°C	**37°C**
Control	16.98±0.44	17.17±0.52
Diabetic	16.25± 0.52*	16.37± 0.48*
α-Lipoic acid	16.75±0.54	$17.20\pm0.55^{+}$
Vit C	$17.21\pm0.23^{+}$	$17.24\pm0.46^{+}$
Combination	$17.27\pm0.74^{+}$	$17.48\pm0.67^{+}$

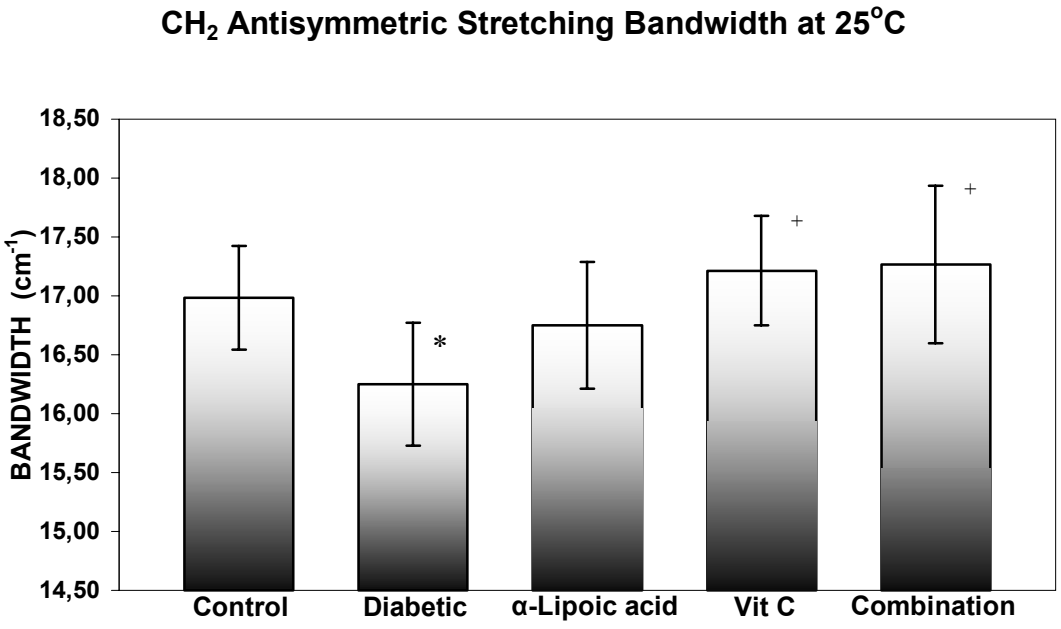


Figure 3.17. Comparison of CH_2 asymmetric stretching bandwidth values at 25°C

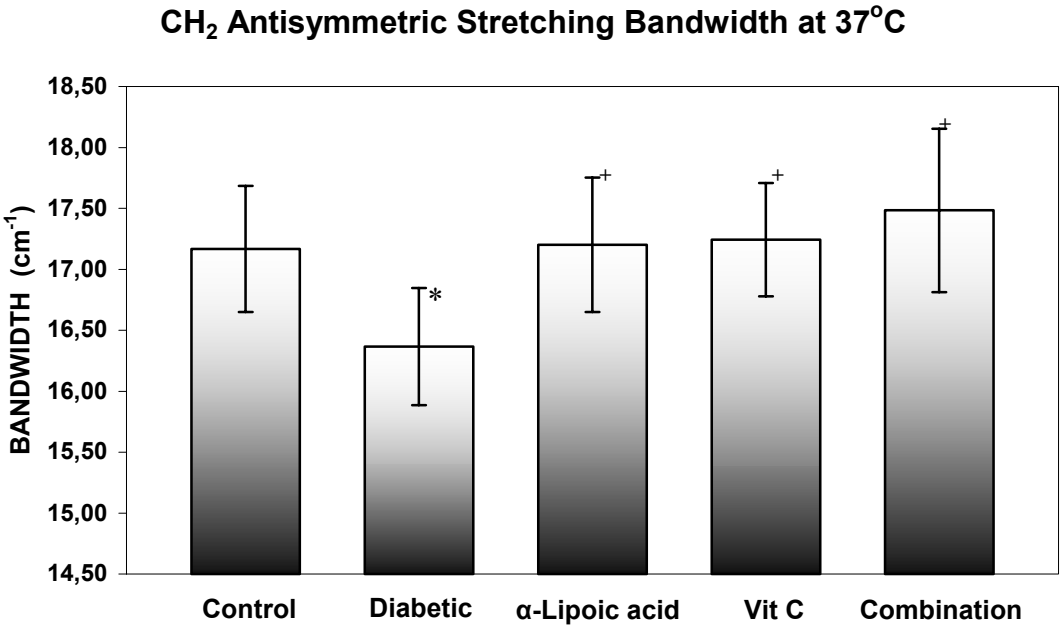


Figure 3.18. Comparison of CH_2 asymmetric stretching bandwidth values at 37°C

In Figure 3.19 and 3.20, =CH olefinic bands at (3012 cm^{-1}) of all groups at 25^{o}C and 37^{o}C are shown in different colors. Diabetic group occupied the highest integrated area of all. Subsequent groups were α-Lipoic acid, Control, Vit C and Combination, respectively. The spectra at 25^{o}C and 37^{o}C were identical. Bands were baseline-corrected and integrated areas were calculated. The quantitative evaluation of the band was given in Table 3.6.

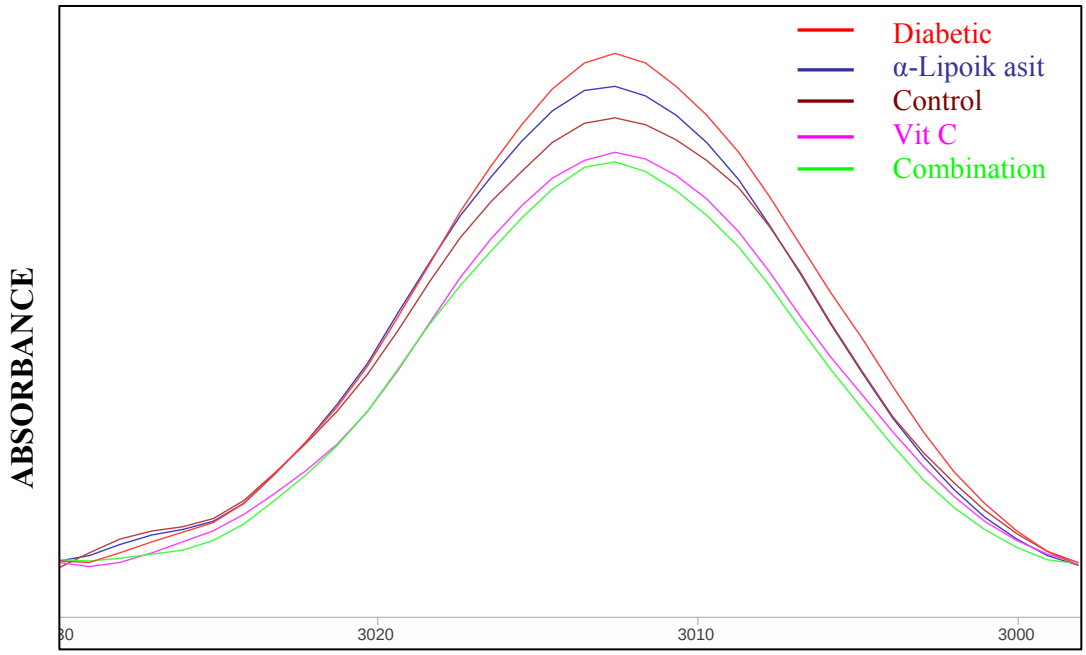


Figure 3.19. Mean Spectra of =CH olefinic bands (3012 cm^{-1}) for all groups at 25°C

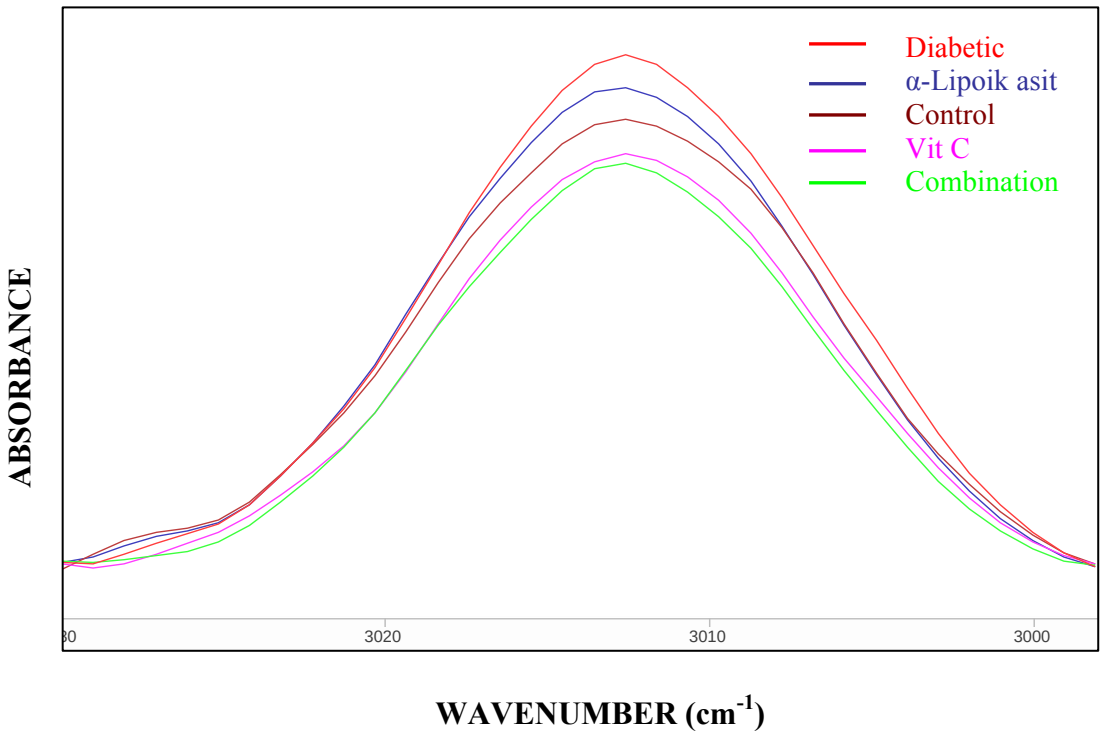


Figure 3.20. Mean Spectra of =CH olefinic band (3012 cm^{-1}) for all groups at 37°C

Table 3.7 shows the integrated area of =CH olefinic band (3012 cm^{-1}) for all groups. As seen from the table the =CH olefinic band area for Diabetic group increased significantly ($p<0.05$) compared to controls at both temperatures. In the case of antioxidant treatment bandwidth values decreased. Vit C and Combination group showed a significant decrease ($p<0.005$) in =CH olefinic band integrated area compared to Diabetic group. These results indicate the restoring effect of antioxidant on diabetic samples.

Table 3.7. Comparison of mean =CH olefinic band (3012 cm^{-1}) area

Groups	**3012 cm^{-1} AVERAGE BAND AREA**	
	25°C	**37°C**
Control	1.085±0.044	1.071±0.051
Diabetic	1.249±0.090*	1.234±0.096*
α-Lipoic acid	1.152±0.149	1.139±0.137
Vit C	$1.003\pm0.083^{++}$	$0.992\pm0.086^{++}$
Combination	$0.951\pm0.226^{++}$	$0.945\pm0.209^{++}$

* : $p<0.05$ significantly different from control group

$^{++}$: $p<0.005$ significantly different from diabetic group

In Figure 3.21 and 3.22 band areas of all groups at 25°C and 37°C are compared.

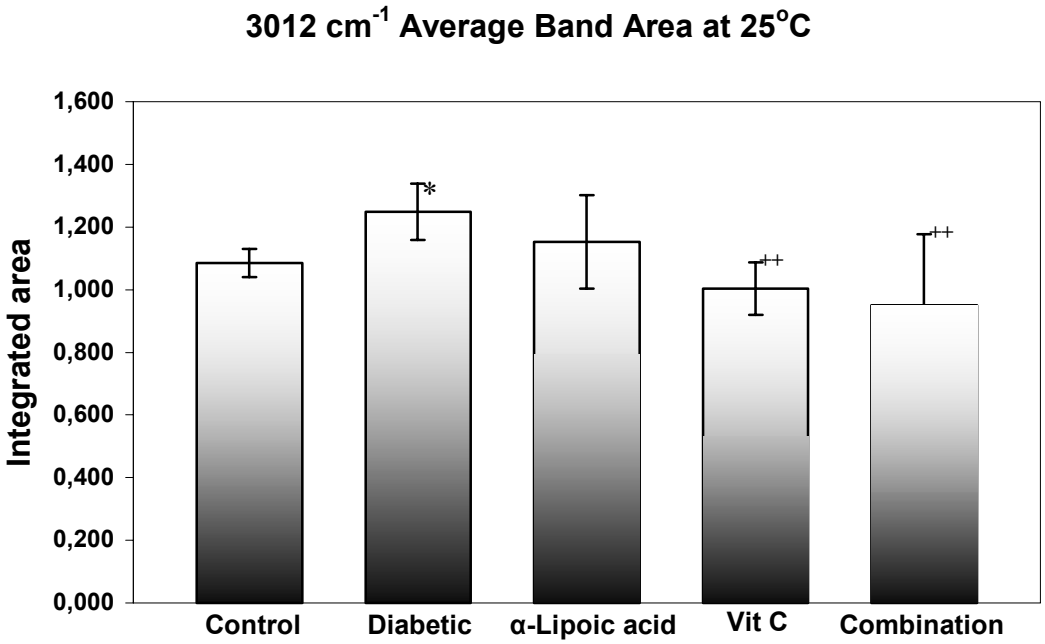


Figure 3.21. Comparison of =CH olefinic band area for all groups at 25°C

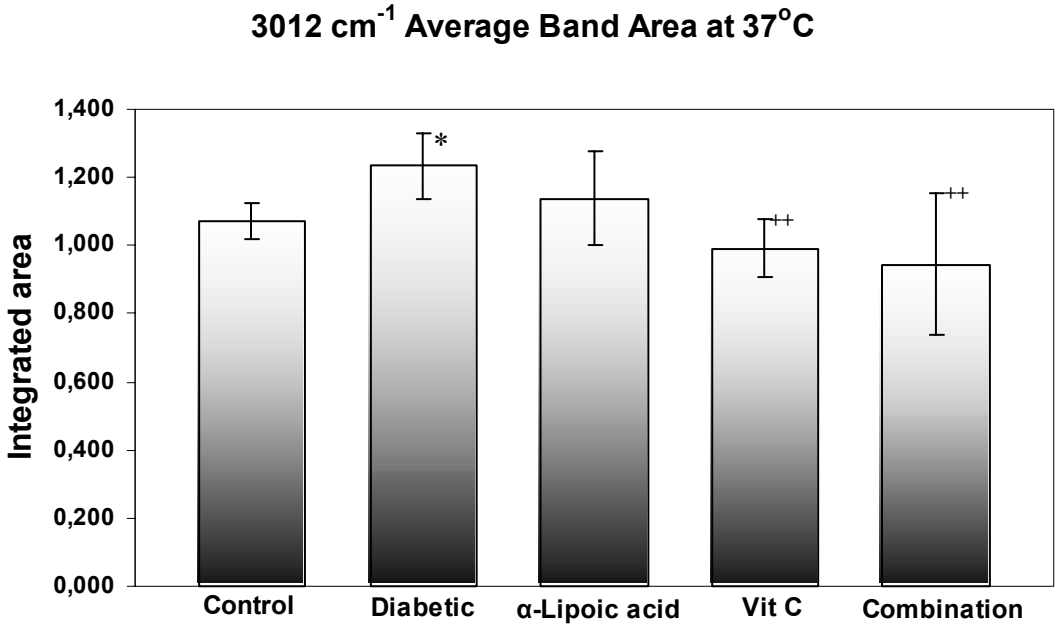


Figure 3.22. Comparison of =CH olefinic band area for all groups at 37°C

3.6.4 FT-IR Analysis of Amide I Region

Amide I band analyzed for the determination of secondary structure changes of microsomal protein content. Curve fitting analysis was carried on to determine the sub-bands under the major amide I band. The placement of the sub-bands were carried out according to second derivative of the spectrum. Previous band assignments were considered.

Figure 3.23-3.27 shows absorbtion and second derivative spectra of amide I band located in between 1700cm^{-1} and 1600 cm^{-1} for all groups at 37^{o}C. Resolution of overlapping bands were also displayed in the Figure. Table 3.8 shows the sub-band assignments and corresponding secondary structures of the amide I band (1653 cm^{-1}).

Table 3.8. Frequency ranges of sub-bands under amide I band and corresponding secondary structures (Arrondo *et al*., 1993; Severcan *et al*., 1999; Nolan et al., 2003).

Wavenumber (cm^{-1})	Secondary Structure
1683-1688	Antiparallel β Sheet
1672-1678	Turn, Bend, β Turn
1666-1668	Turn, Bend, β Turn
1653-1657	α Helix
1639-1642	Random coil
1634-1638	β Sheet
1620-1625	Aggregated β Sheet
1615-1616	Tyrosine residue

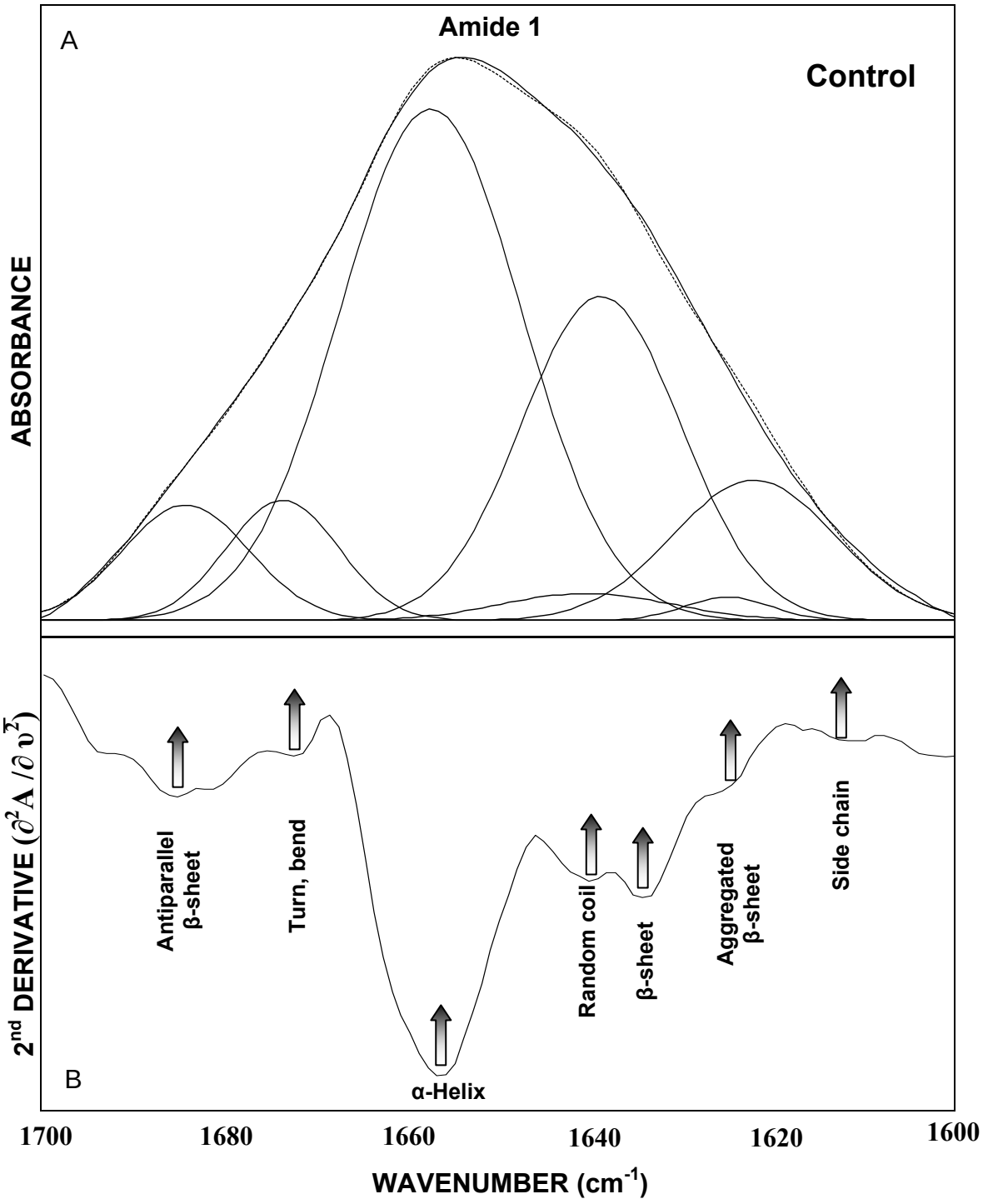


Figure 3.23. Curve fitting analysis (A) and second derivative spectrum (B) of Control group mean amide I band. Arrows in (B) indicate the sub-bands corresponding secondary structures in (A). Seven sub-bands can be recognized.

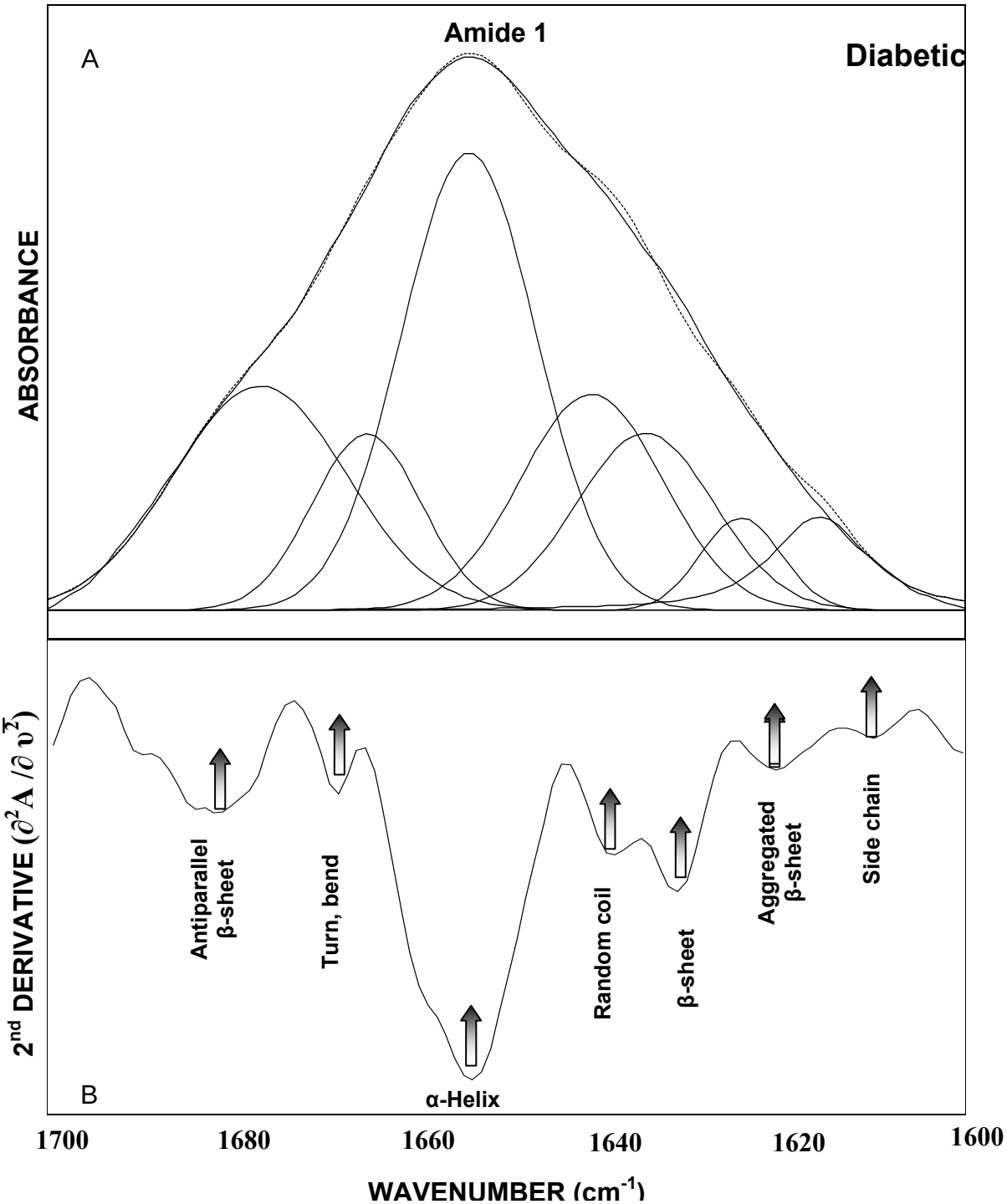


Figure 3.24. Curve fitting analysis (A) and second derivative spectrum (B) of Diabetic group average amide I band. Arrows in (B) indicate the sub-bands corresponding secondary structures in (A).

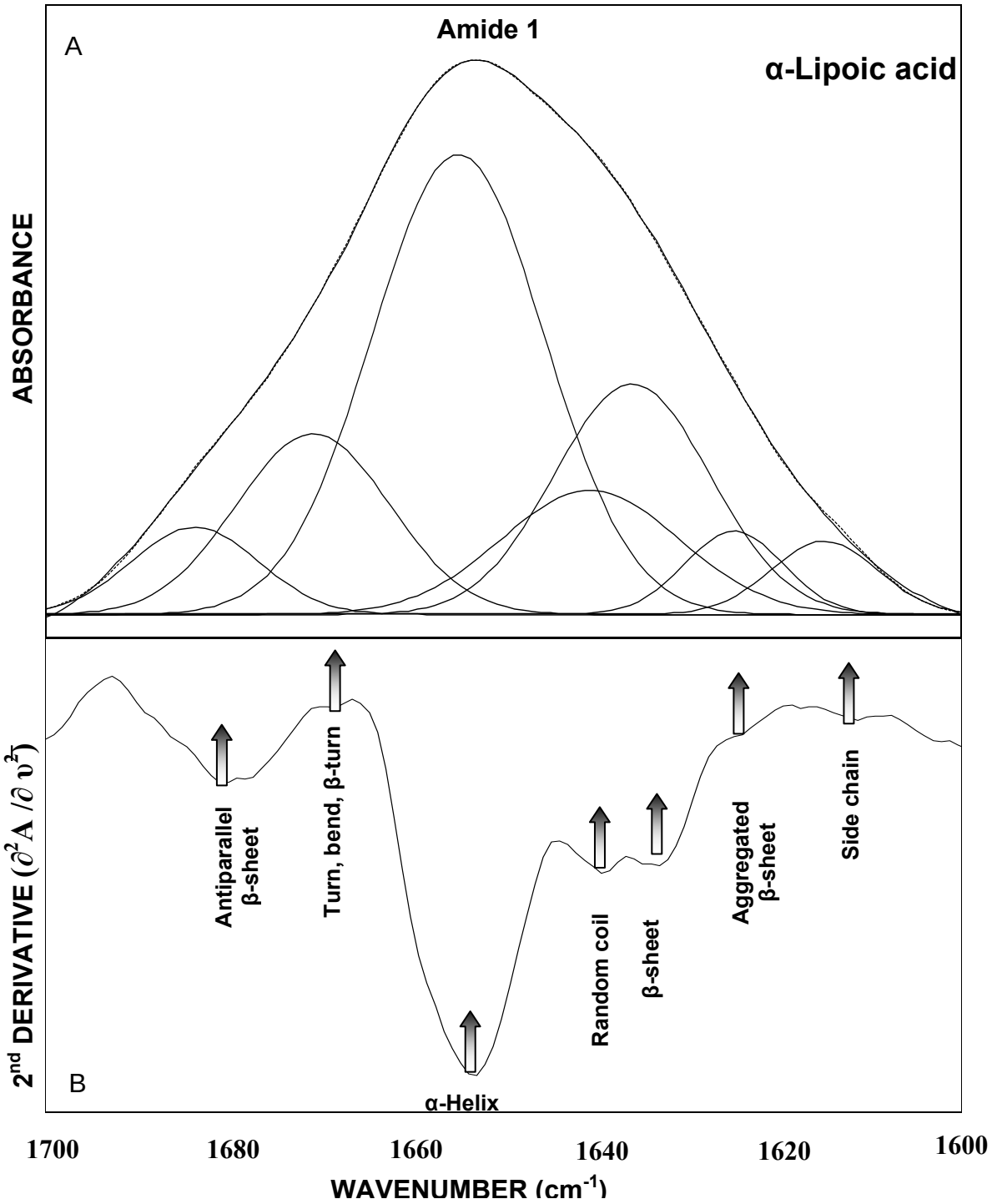


Figure 3. 25. Curve fitting analysis (A) and second derivative spectrum (B) of α-Lipoic acid group average amide I band. Arrows in (B) indicate the sub-bands corresponding secondary structures in (A).

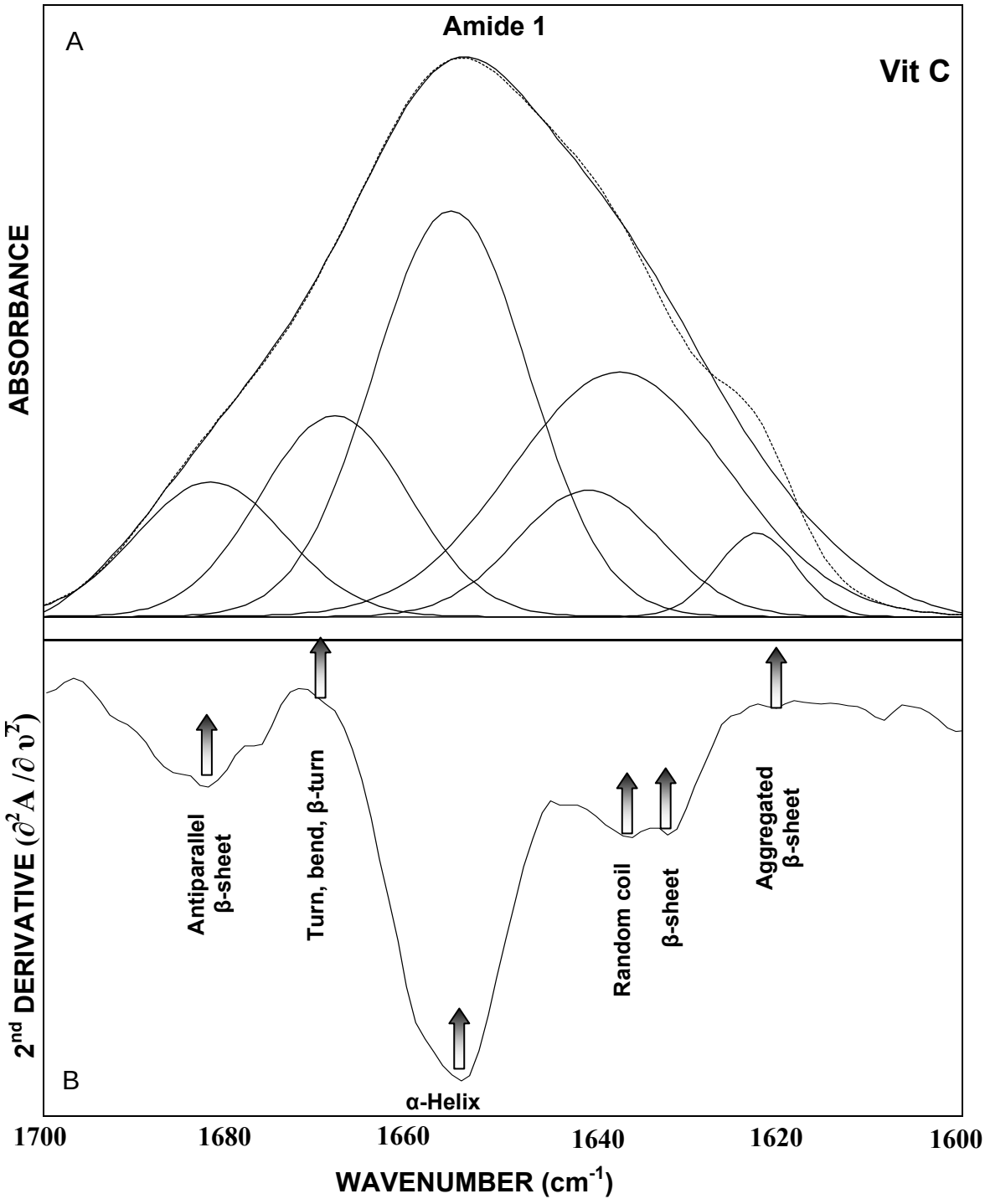


Figure 3. 26. Curve fitting analysis (A) and second derivative spectrum (B) of Vit C group average amide I band. Arrows in (B) indicate the sub-bands corresponding secondary structures in (A).

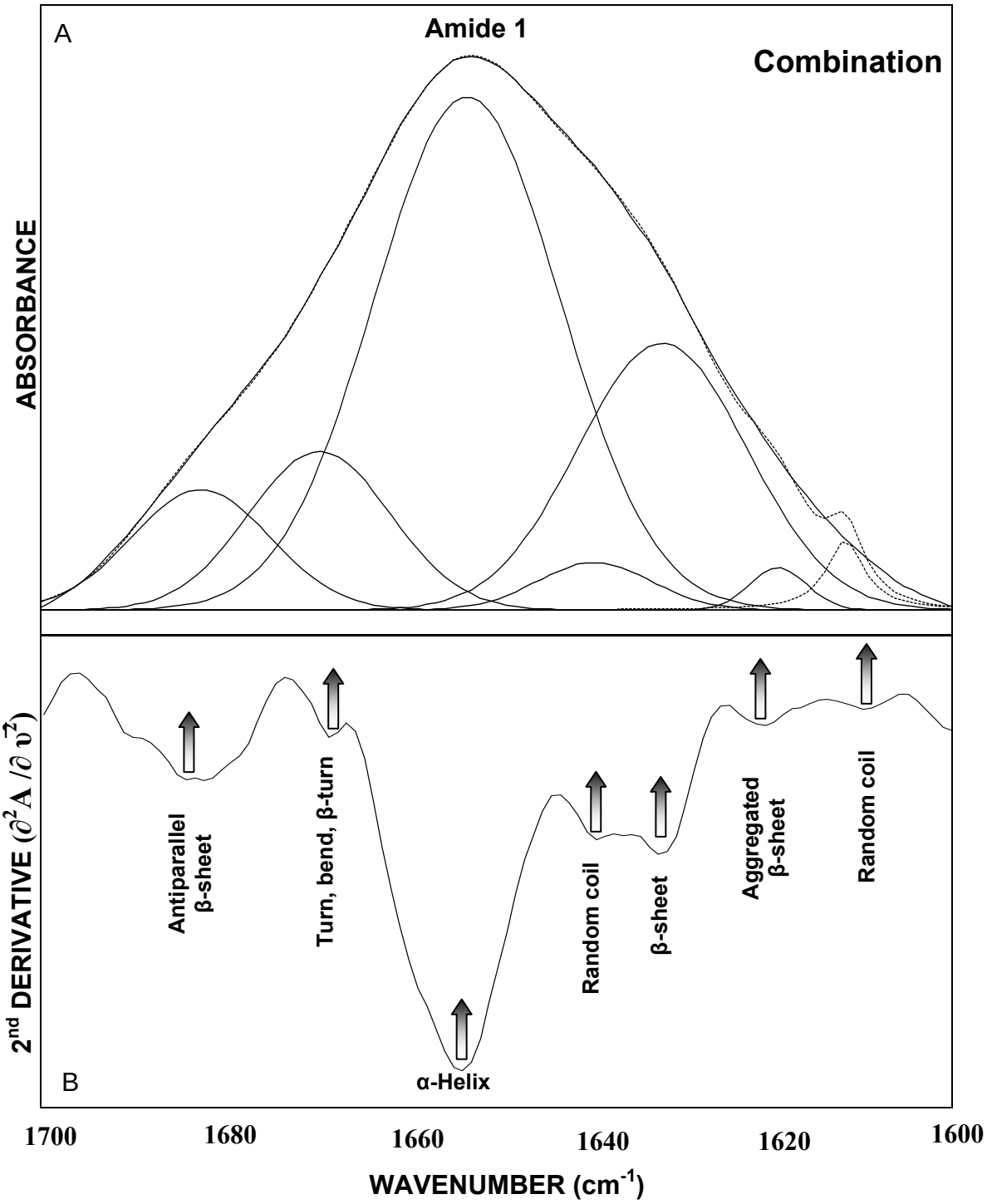


Figure 3. 27. Curve fitting analysis (A) and second derivative spectrum (B) of Combination group average amide I band. Arrows in (B) indicate the sub-bands corresponding secondary structures in (A).

Figure 3.28 shows percent area of sub-bands under average amide I band and corresponding secondary structures. As seen from the Figure, six different types of secondary structures can be recognized. Diabetic group showed apparent structural differences compared to Control group nearly at all types of secondary structure content. Antiparallel β-sheet content of the Diabetic group was the highest of all groups. α-helix percentage of the Diabetic group was the lowest of all groups. β-Sheet percentage of the Diabetic group was also the highest.

Alpha-lipoic acid has been reported as an efficient scavenger of reactive oxygen species (ROS) (Dincer *et al*., 2002). Somehow, in this study it was failed to decrease =CH olefinic band area, indirectly failed to scavenge lipid peroxidation products alone while Vit C achieved to decrease the integrated area even administered alone without any other agent. Combination of α-Lipoic acid and Vit C decreased the integrated area as expected. However, o additive effect of these two antioxidants were observed for the reduction of the integrated area of =CH olefinic band.

Decreased percent integrated area of α-helix of Diabetic group, compared to control, was restored by α-lipoic acid and Vit C supplementation to diabetic rats. α-Helix content was reached to control levels. Numerical values were shown in Table 3.9 .

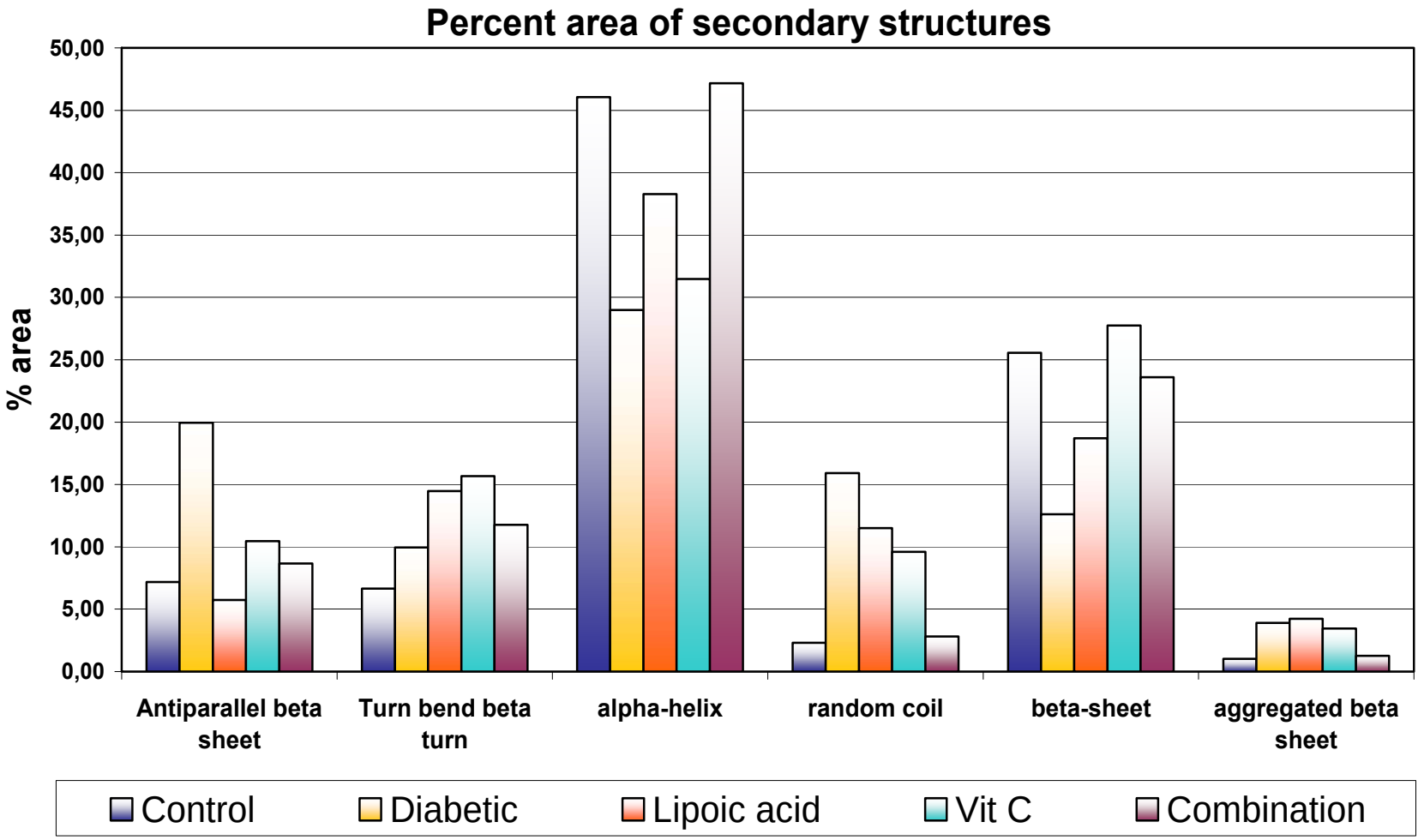


Figure 3.28. Comparison of average amide I sub-bands' percent area of all groups.

Table 3.9. Percent integrated area values of groups and corresponding secondary structures.

Secondary Structures	Control (n=6)	Diabetic (n=7)	α-Lipoic acid (n=6)	Vit C (n=7)	Combination (n=10)
Antiparallel β-sheet	7.17	19.95	5.74	10.45	8.66
Turn, bend	6.65	9.96	14.47	15.68	11.76
α-Helix	46.05	29.00	38.27	31.48	47.16
Random coil	2.31	15.91	11.51	9.60	2.82
β-sheet	25.55	12.60	18.70	27.75	23.61
Aggregated β-sheet	1.01	3.90	4.23	3.45	1.27
Side Chain	11.25	5.71	4.02	-	1.94

CHAPTER IV

DISCUSSION

Oxidative stress is the result of an imbalance between ROS (e.g. superoxide anion, hydroxyl radicals, peroxynitrite, hydrogen peroxide) and antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx), glutathione S-transferase (GST) and compounds like vitamin C, vitamin E, α-lipoic acid, or reduced glutathione (GSH). Therefore, increased oxidative stress, which contributes to the pathogenesis of diabetic complications, is the consequence of either enhanced ROS production or attenuated ROS scavenging capacity, resulting in tissue damage, which is in most instances assessed by the measurement of lipid peroxides. Several mechanisms, including auto oxidative glycosylation, formation of advanced glycation end products (AGEs), and increased polyol pathway activity contribute to increased oxidative stress (Halliwell and Gutteridge, 1989).

Protein glycation has been shown to be partly an end product of glucose auto oxidation, which is a process mediated by reduced metal ions in the presence of free radicals and monosaccharide. The increased availability of glucose induces enhanced production of AGEs. This process has been described as auto oxidative glycosylation, and is probably the major cause of increased ROS production in

diabetic patients (Wolff, 1993). While the formation of AGEs primarily leads to irreversible tissue damage, hyperglycemia also leads to glycation of antioxidant enzymes (Blakytny *et al*.1992; Kawamura *et al*., 1992). Therefore, the process of glucose auto oxidation might not only be responsible for increased ROS production, but also for decreased availability or activity of antioxidant compounds.

An important feature of the antioxidant network is that its components act in cooperation to destroy activated oxygen species. Such synergistic interactions are reinforced by mutual protections of antioxidant enzymes. For example, superoxide dismutase protects glutathione peroxidase and catalase from inactivation by superoxide radical (Blum and Fridovich, 1985; Kono and Fridovich, 1982; Shimizu *et al*., 1984), while glutathione peroxidase and/or catalase protect superoxide dismutase from inactivation by hydro peroxides (Sinet and Garber, 1981).

There are two types of studies forming the evidences for an association between reactive oxygen species and diabetes: studies showing that oxygen radical damage are significantly increased and studies that find abnormalities in the antioxidant defenses of diabetic samples relative to matched controls. Both types were combined in our study; either the increased oxidative damage or the abnormalities of antioxidant defense of diabetic samples were investigated.

Lipid peroxidation is a free radical induced process leading to oxidative damage of unsaturated lipids. Under normal physiological conditions, low levels of lipid peroxides are found in tissues. However, free radicals which react with lipids can cause peroxidative change that result in enhanced lipid peroxidation. Hence, lipid

peroxidation is an important marker of early and reversible tissue damage caused by diabetes mellitus and of decrease in antioxidant defense of tissues.

In the present study, diabetes-induced lipid peroxidation in rat liver microsomal membranes has been investigated by both TBARS test and Fourier Transform Infrared (FT-IR) spectroscopy at different temperatures. The area values of the olefinic (=CH) band at 3012 cm^{-1} of diabetic samples were found to be increased significantly ($p<0,05$) at each temperature, compared to control samples. The signal intensity and more accurately the band area, give information about the concentration of related functional groups (Toyran and Severcan, 2003; Toyran *et al*., 2004) . Increase in the =CH olefinic band area is mainly attributed to the lipid peroxidation end products. Those results are also confirmed by the significant increase in malondialdehyde (MDA) levels of diabetic samples compared to controls ($p<0,05$) using thiobarbituric acid (TBA) test indicating an increase in lipid peroxidation in diabetic samples compared to control samples (Jain and Levine, 1995).

Loss of unsaturation during lipid peroxidation reactions, has been compensated by the presence of double bonds in the lipid peroxidation products such as malondialdehyde, lipid aldehydes and alkyl radicals. Therefore, instead of observing a decrease in the intensity of the olefinic band, we observe an increase due to the accumulation of end products of lipid peroxidation. Our results are in accordance with Liu *et al.* (2002) in which an increase in the olefinic band was also observed in diabetic patients' platelets. On the other hand, Sills *et al*. (1994) who

studied erythrocyte ghosts, reported a decrease in the 3012 cm^{-1} olefinic band intensity. The differences observed in the variation of the olefinic band as a result of lipid peroxidation, may be due to the structural differences in the samples. The reason for the increase in the olefinic band is due to the lipid peroxidation end products. These products, above a threshold concentration, are released into the extra or intracellular site of the cell. Platelets have the boundaries and a cytoplasm that can bear these end products giving rise to an increase in the olefinic bands (Liu *et al*., 2002). Microsomal samples may also resemble the structure of platelets since during the homogenization process, parts of the endoplasmic reticulum are mostly converted into spherical liposomes surrounding a part of the cytoplasm containing lipid peroxidation end products. Therefore, in the FT-IR results, the olefinic band increased in area in diabetic samples compared to control samples. In contrast, erythrocyte white ghosts, having pure membrane phospholipids without cytoplasm, do not contain released lipid peroxidation end products, so that Sills *et al.* (1994) observed a decrease in the olefinic band pattern.

Lipids are the major components of the biological membranes. They act as vital substrates for lipid peroxidation, and the increase in lipid content during diabetes may be the cause of increased lipid peroxidation. Total lipid levels were found to be increased in diabetic rats compared to controls (Table 3.3, Figure 3.3). Both α-lipoic acid and vitamin C restored the control levels preventing the increase in lipid peroxidation observed in diabetic rats.

Lipids also play a key role in membrane order and fluidity. In addition they govern the exposure and the diffusion of membrane proteins by affecting the

conformation of membrane proteins. By analyzing the methylene stretching IR bands of the lipid hydrocarbon chains as a function of temperature phase transition behavior, lipid acyl chain flexibility and membrane fluidity can be detected. As seen in Figure 3.13, lipid phase transition is broad which is different than those of model membranes. This kind of broad phase transition was previously reported (Zeron *et al*., 1998; Tsvetkova *et al*., 1999). In our samples pre-transition is observed around 18^{o}C and main transition occurs in between 30-35^{o}C.

We observed a decrease in the frequency of the CH_2 stretching bands (Fig 3.12 and 3.13). A shift in the position of the C-H stretching bands towards higher frequency indicates an increase in the percentage of gauche conformers and hence a more nonlinear geometry (Casal *et al*., 1984). Therefore, the shift to lower frequencies of the symmetric and asymmetric CH_2 stretching band, which is the case in our study, can be used as a marker for the detection of increased lipid order (Liu *et al*. 2002; Mantsch 1984; Severcan *et al*. 1997). Increase in the order of the system indicates a decrease in the number of gauche conformers resulting in more rigid membranes. A possible explanation for the membrane rigidity is the free radicals, and consequently the lipid peroxidation, which modifies the lipid composition of the membranes. After oxidative stress, a decrease in the membrane unsaturated/saturated fatty acid ratio is detected along with the formation of cross-linked lipid–lipid and lipid–protein moieties (Curtis *et al*., 1984; Levin *et al*., 1990; Niranjan and Krishnakantha, 2000).

Membrane fluidity is detected by monitoring the bandwidth of the C-H stretching bands (Mantsch 1984; Severcan *et al*. 1997). The increase in the

bandwidth corresponds to an increase in mobility of the phospholipids acyl chains and membrane fluidity (Levin *et al*., 1990; Severcan et al., 1999). Microsomal membranes from diabetic rats showed a decrease in the CH_2 antisymmetric stretching bandwidth values indicating an increase in lipid fluidity.

There is a very limited number of studies on the effect of diabetes on lipid fluidity. One of these studies which belong to Liu *et al*., (2002) reported a decrease in membrane fluidity in diabetic platelets by monitoring frequency shift of the CH_2 stretching modes. As mentioned before the shift of the frequencies of these bands do not give fluidity information, instead gives order-disorder information. Although Liu *et al.* did not report in their papers, but as seen from the figure in the paper they observed a decrease in the bandwidth which indicates a decrease in membrane fluidity in diabetic human platelets. Effect of diabetes on lipid fluidity is different for different systems. For examples one of our previous FTIR study we observed an increase in the fluidity of diabetic rat heart homogenate membranes (Severcan *et al*., 2000). However our recent FTIR microspectroscopy study reported no significant change in the lipid fluidity in rat heart tissues (Toyran 2003, PhD thesis). The different behavior of lipid fluidity in different systems in comparison of the present study is expected since in the present study, we studied pure microsomal membranes. As mentioned before platelets resemble microsomal liposome which gives identical effect on membrane fluidity. Whilst homogenates contain lipids from various membranes together with other impurities.

The biochemical aspect of membrane rigidity is more complicated. To give a better explanation to our results, two points should be mentioned which were not

investigated in this thesis study. First, the effects of diabetes on desaturase activities and second, the amount and type of unsaturated fatty acids namely, the (polyunsaturated fatty acid) PUFA and monounsaturated fatty acid (MUFA) in the membrane should be mentioned.

Experimental diabetes has a depressing effect on Δ9, Δ6 and Δ5 desaturases (Imai., 1961; Gellhorn and Benjamin., 1964; Mercuri *et al*., 1967; Poissson *et al*., 1979). The Δ9 desaturase is considered a 'lipogenic' enzyme mainly involved in the regulation of monounsaturated/saturated acid ratio in membranes. The Δ6 and Δ5 desaturases are involved in the production and regulation of polyunsaturated fatty acids of n-6 and n-3 series. These fatty acids are, in addition, involved in more gentle and specific functions in brain and other tissues, as well as regulatory mechanisms at the nuclear level. Eicosanoids, hepoxilines and lipoxines are some of their products (Brenner *et al*., 2000). Although, the mechanism of insulin action on the fatty acid desaturases is still not so apparent, there is a general consensus that insulin-dependent diabetes evokes a decrease of the three Δ9, Δ6 and Δ5 desaturase activity. The putative effects in the fatty acid composition of tissue lipids would be a decrease of palmitoleic and oleic acids due to the decrease of Δ9 desaturase activity, increase of linoleic acid and decrease of arachidonic and docosahexaenoic acids due to the decrease of Δ6 and Δ5 desaturases (Brenner *et al*., 2000). Synthesis of these unsaturated fatty acids is depressed by the diabetes resulting in the exchange of these fatty acids with the saturated ones.

Secondly, the polyunsaturated fatty acid (PUFA) and composition of membrane phospholipids are important. Unsaturated fatty acids are easily oxidized,

besides their beneficial effects (Suzuki *et al*., 1999). In diabetic conditions, where lipid peroxidation increased and desaturases are depressed by insulin deficiency, oxidized unsaturated fatty acids are replaced with adipocyte released saturated fatty acids. The altered unsaturated/saturated fatty acid ratio of microsomal membrane phospholipids evidently result with the increase in rigidity of microsomal membrane.

As seen from the Figures 3.15-3.22 and tables 3.5-3.7 we observed a significant restoring effect of both α-lipoic acid and vitamin C in lipid peroxidation, membrane order and fluidity. This effect was seen to be more profound for the combination group.

Diabetes related increase in lipid peroxidation might be the reflection of a decrease in enzymatic and non-enzymatic antioxidant defense system of the tissue (Jain and Levine, 1995; Kesavulu *et al*., 2001; Dincer *et al*., 2002; Cho *et al*., 2002). SOD, CAT, GSHPx and GST activity measurements, determination of reduced glutathione levels were carried out in order to understand the current status of the antioxidant defense system.

Non-treated diabetic group showed drastic changes in antioxidant enzyme activities. We observed a significant decline in the CAT activity while SOD and GSHPx activities were both increased significantly compared to Control group (Table 3.2, Figures 3.4-3.6). GST activity showed no significant change compared to Control group (Table 3.2, Figure 3.7). Reduced glutathione (GSH) levels decreased significantly ($p<0.05$) compared to Control group.

The decrease in CAT activity causes an imbalance between antioxidant enzymes. CAT activity has been shown to be responsible for the detoxification of

hydrogen peroxide. In diabetic rats, the decrease in CAT activity occurs probably due to increased free radical production. The decrease in activity may cause ineffective scavenging of hydrogen peroxide resulting in increased levels which can react with superoxide radical to give hydroxyl radical through Fenton reaction (Figure 1.1) and increase lipid peroxidation and tissue damage. The decreased CAT activity and increased GSHPx activity (as in our case) can suggest, thereby, compensatory mechanism among the antioxidant enzymes in response to increased oxidative stress so that tissues lacking significant CAT activity may be more dependent on the activity of GSHPx (Kakkar *et al*., 1997). However, in most of the pathological cases, hydrogen peroxide level is far from being removed by GSHPx alone. GSHPx catalyze the reduction of hydrogen peroxide into water and oxygen at the expense of GSH. Reduced glutathione levels in our study decreased in diabetic rats compared to controls. The decrease in tissue thiol status may cause an additive effect on lipid peroxidation.

SOD protects the cells against radicals by catalyzing the removal of superoxide radical ($O2^{\cdot-}$). SOD activity increased significantly in diabetic rats compared to controls, hence preventing the damage of the radicals.

GSTs are a group of isozymes capable of detoxifying various exogenous and endogenous substrates, which also play important role in the antioxidant defense system. However, total GST activities are almost same in both control and diabetic groups. In future studies, individual isozyme activities can be determined in order to see their roles in this system.

Treatment of rats with α-lipoic acid, which is a non-protein thiol, shows its effectiveness in protection of cell membranes by a possible interaction with non-enzymatic antioxidants GSH and ascorbic acid (vitamin C).

In our study, α-lipoic acid group showed increased levels of SOD activity compared to control group indicating that α-lipoic acid supplementation alone has failed to reduce the increased SOD activity. However, this increase in SOD activity can not prevent the damage produced by diabetes. Our results are partially in accordance with Dinçer *et al*., (2002) who has reported a significant increase in STZ-induced diabetic rat liver SOD activity. In contrary to our results, α-lipoic acid supplementation was diminished the increased activity of SOD. Increased SOD activity in diabetic rat livers may be due to the increased levels of superoxide radical. α-Lipoic acid restored the decreased CAT activity even administered alone. This restoring effect of α-lipoic acid might be related to its strong scavenging capacity. α-Lipoic acid treatment of diabetic rats showed slight however, statistically not significant decreasing effect on total GST activity. The most striking effect of α-lipoic acid supplementation to diabetic rats was the increased CAT activity. In non-treated diabetic case, decreased CAT activity formed an imbalance of antioxidant network. This imbalance was reversed by α-lipoic acid supplementation. Decreased GSH levels were remained unchanged compared to diabetic group. GSH, which also play role in the regeneration of dihydrolipoic acid (DHLA), might probably occupied by high doses of administered α-lipoic acid.

Vitamin C supplementation decreased CAT activity by approaching it to the control values compared to non-treated diabetics. The effects of vitamin C were observed on both SOD and GSHPx activities. These two enzymes showed the highest activities in vitamin C supplemented group. Vit C group's GST activity remained unchanged. Higher antioxidant enzyme activities may be the result of pro-oxidant effect of vitamin C which catalyzes the conversion of Fe^{+3} to Fe^{+2} (substrate for the Fenton reaction) and normally may produce hydrogen peroxide (H_2O_2) by the oxidation to dehydroascorbic acid (DHAA). Although, these pro-oxidant effects are not expected at higher concentrations, they might have some contribution on the increased activities of SOD and GSHPx.

A number of reports suggest that ascorbic acid metabolism is altered in diabetics (Gumieniczek *et al*., 2001). The active transport of ascrobate appears to be decreased by hyperglycemia and insulin deficiency. Hyperglycemia has also been shown to inhibit uptake of dehydroascorbic acid, the oxidized specie of ascorbic acid. On the other hand, the findings concerning plasma and tissue ascorbate levels in diabetes are somewhat controversial. Akkus *et al*. (1996) reported that in leukocytes and plasma of non-insulin dependent diabetes mellitus (NIDDM) patients, the levels of ascorbic acid were not changed.

In the present study, combination of α-lipoic acid and vitamin C showed some stabilizing effects which affected all antioxidant enzyme activities in a similar way; by approaching the values near the control levels. CAT activities of combination group decreased significantly compared to diabetics after

supplementation. The results reveal that the combination of α-lipoic acid and vitamin C shows significant restoring effect on the changes occurring in diabetic rat liver. Furthermore, although the sole effect of α-lipoic acid or vitamin C was not effective in reducing the lipid peroxidation levels in diabetic rat liver microsomal membranes, the combination of both significantly decreased the levels of peroxidation compared to diabetic group. The results show that, in the Combination group the enzymatic antioxidant defense system enzymes CAT, SOD, GSHPx and GST activities were restored and as a result, diabetes induced increase in lipid peroxidation was reduced. According to these results, the combined treatment of α-lipoic acid and vitamin C can prevent the tissue damage to some extent. The cooperation of these two antioxidants in the removal of reactive oxygen species in diabetes mellitus is quite effective. However, the Combination group was failed to restore the reduced glutathione levels.

Recently Cho *et al*. (2002) have been reported a significant increase in STZ-induced diabetic rat liver SOD activity compared to controls which are in agreement with our results. However, in contrary to our findings, they have reported an increase in CAT activity while GSHPx activity was decreased, compared to control group. However, mRNA expressions of the antioxidant enzymes were not changed significantly in both control and diabetic groups. Researches have been concluded that antioxidant enzymes were not induced at the level of transcription.

This study reveals that STZ-induced diabetes causes an increased level of lipid peroxidation due to antioxidant/pro-oxidant imbalance. Increased lipid peroxidation decreases the fluidity level of rat liver microsomal membranes.

The total protein concentrations of crude homogenate of diabetic rats were decreased significantly compared to controls. This can be the result of oxidative stress, generating protein oxidation.

In order to understand the changes in the secondary structures of the membrane proteins FT-IR studies were carried out. Curve fitting analysis of amide I band at 1653 cm-1 was carried out in order to determine the effects of STZ-induced diabetes on the secondary structures of rat liver microsomal membrane proteins. Apparent increase in antiparallel β-sheet content (from 7.17% to 19.95%) and the decrease in α-helix (from 46.05% to 29.00%) of diabetic rat liver microsomal membranes were pointed out a conversion of α-helix to β-sheet. α-helix to β-sheet transition has been described by Pan *et al*. (1993) as the fundamental cause of prion infection in scrapie disease. These conversions at least may result with a loss in the function of microsomal membrane proteins which may play a role in the secondary complications of diabetes mellitus. Diabetic group also showed a dramatic increase in random coil structure which indicated that at least some of the membrane proteins are denaturated in diabetic samples. This is confirmed with the apparent increase in the aggregated structure which appears when proteins denaturated. As seen from Figure 3.28 and Table 3.9, these damages occurred in diabetic samples were restored in the presence of antioxidants. Similar to our previous findings the most profound effect was observed for the combination group.

The combined effect of both α-lipoic acid and vitamin C, probably by preventing the protein oxidation which was produced by oxidative stress, restored the

total protein levels in the membrane. Also, the changes in the secondary structure of the membrane proteins and denaturation was reversed by the action of both antioxidants.

CHAPTER V

CONCLUSION

In conclusion, antioxidant network composed of enzymatic and non-enzymatic components work in synergy for the removal of free oxygen radicals and other reactive species. Any defect in the system causes the accumulation of one or more of intermediates which may start a chain reaction giving rise to irreversible damage to the organism. Diabetes, as a metabolic disease, causes the increase in reactive oxygen species by some mechanisms which are not known exactly today, but it is known that diabetes causes either the increase in reactive oxygen species or cause a failure in the removal system of those species.

In our study, antioxidant enzyme behavior to STZ-induced oxidative stress was various. SOD and GSHPx acticities were increased while CAT activity was decreased causing the accumulation of H_2O_2 that causes the formation of the most reactive particle hydroxyl radical.

In the non-enzymatic case, antioxidant reduced glutathione which is in the middle of the oxidative traffic, was decreased in all diabetic groups indicating the increased levels of utilization of reduced glutathione. Existance of oxitative stress in rat liver microsomes was established by TBA-reactivity (MDA) test.

Diabetes also caused a decrease in microsomal membrane order and dynamics. Decreased frequency, increased order, decreased acyl chain flexibility result in more rigid membranes.

Diabetic rat liver microsomal membranes also showed apparent changes in protein secondary structure content. Changes in α , β and random coil structures were restored back to control values by α-lipoic acid and vitamin C supplementation. Not in every case but in most instances, combination of these two amtioxidants yielded better results.

Supplementation of antioxidants (α-lipoic acid and vitamin C) to the diabetic rats, showed great differences in many cases. Use of those antioxidants together were given better results.

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