The Electromagnetic Field as a Modulator of a Protein Activity, and the Resonant Recognition Model

Submitted in fulfilment of requirements for the degree of Doctor of Philosophy

Vuk Vojisavljevic
(Master of Multidisciplinary Studies)

School of Electrical and Computer Engineering
RMIT University
Melbourne, Australia

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DECLARATION

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not be submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and any editorial work, paid or unpaid carried out by a third party is acknowledged.

Vuk Vojisavljevic
15. 01. 2007.

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To all my colleagues from the RMIT, which share research experience with me.

ABSTRACT

In this study, it was experimentally proved, for the first time, that it is possible to predict the frequency of electromagnetic radiation that can modulate protein and more specifically enzyme activity. The prediction obtained using the computational model so called Resonant Recognition Model (RRM), was tested here experimentally using the reaction catalysed with the enzyme l-lactate dehydrogenase (LDH).

The RRM model was applied here to the group of the enzymes belonging to the sub-subclass EC 1.1.1.27 i.e. l-lactate dehydrogenase. And the wavelengths of the electro magnetic radiation proposed to alternate activity of l-lactate dehydrogenate were identified at λ=620 $\pm$ 25 nm and λ=840 $\pm$ 25 nm.

Enzyme activity was then measured after the exposure to the low-intensity, electromagnetic radiation (EMR) within the proposed EMR range [560-860 nm],

The experimental results have indeed shown that there is a significant increase in the activity of LDH only at radiation frequencies as predicted bi the RRM: 596nm (12%; $P < 0.001$) and 829 nm (11.8%, $P < 0.001$). These results prove successfully that activity of proteins and more specifically enzymes can be modified by EMR radiation of specific frequencies and even more that RRM computational model can successfully predict these frequencies.

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LIST OF ACRONYMS AND ABBREVIATIONS

EMF	Electromagnetic field
EMR	Electromagnetic radiation
LDHA	Lactate dehydrogenase type A
LDHB	Lactate dehydrogenase type B
LDHC	Lactate dehydrogenase type C
LDHBacterial	Lactate dehydrogenase from bacteria.
NIR	Near Infra Red light
VIS	Visible Light
EMER	The electromagnetic molecular electronic resonance
DFT	Discrete Fourier Transform
IR	Infra Red
E.C.	Enzyme commission
IR	Infra Red
FIR	Far infra red
UV	Ultra violet
UVA	Ultra violet range A
UVB	Ultra violet range B
NADH	Nicotinamide adenine dinucleotide, reduced form
NAD	Nicotinamide adenine dinucleotide
RRM	Resonant Recognition Model
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
EIIP	Electron Ion Interaction Potential
RF	Radio Frequency
ATP	Adenosine tri phosphate

Chapter 1

Introduction

Living organisms on the earth are under permanent exposure of the electromagnetic field (EMF). Both artificial and natural sources of Electromagnetic field are present permanently. In addition, the visible part of electromagnetic radiation (EMR) produced from the sun is the main source of the energy, supporting the life on the earth. Many living cells have developed mechanisms for acquiring and transforming the energy brought by EMR. The processes such as photosynthesis, photomorphogenesis and phototaxis are examples of the usage of the energy of the EMF within visible range. The number of medical and therapeutical applications that involve exposure of the tissues to the EMR within the range of the visible and near infrared (NIR) light, is in the constant increase. In addition, effects on metabolism of many cells, normally not exposed to the EMR within the range of visible light are well documented.

However, the lack in understanding of the mechanisms that lead to the stimulative or suppressive effect on living systems is limiting ability to predict characteristic of EMR that will have optimal effect.

Nearly every biochemical reaction inside the living organisms is controlled by enzymes. The enzymes are specific group of proteins with a catalytic role. Thus, if it is possible to predict the wavelength of the EMR that will have optimal effect on enzymatic reaction, then it is possible to predict selectively the wavelength of light that will have optimal effect on

biochemical process. As it will be shown later in this thesis. The evidence of the influence of EMR, which has selective wavelengths within visible range, on biological processes involving enzyme activation imply that enzyme activation engage energies of the same order and nature as the EMR. These results can lead to the conclusion that specificity of enzymatic interaction is based on the resonant electromagnetic energy transfer between interacting molecules, on a frequency specific for each observed function/interaction.

These phenomena are analysed in terms of the Resonant Recognition Model (RRM). The RRM proposes that protein activities (interactions) are based on resonant electromagnetic energy transfer within the range of infrared and visible light.

The RRM, is a physicomathematical method used to analyse protein and nucleic acid sequences. Its aim is to find possible significant patterns and assist in structure-function studies. The RRM is a model of protein-protein and protein-DNA interaction based on a significant correlation between spectra of the numerical presentation of amino acid or nucleotide sequences and their biological activity. The RRM is the only available model for the prediction of the wavelength of the light capable to affect enzymatic function that use distribution of the amino acids along the protein's backbone. The other models (Biscar 1976, Frolich 1986) are not involving actual distribution of amino acids along the protein's backbone.

In other to predict behaviour of enzymes upon exposure to the EMR, the computational model, so the Resonant Recognition Model (RRM), was applied on the group of the enzymes belonging to the sub-subclass EC 1.1.1.27 i.e. l-lactate dehydrogenase.

In this study it was shown for the first time that it is possible to predict a behaviour of proteins or enzymes after the exposure to the low-intensity, electromagnetic radiation (EMR) within the range [560-860 nm], using computational model. The prediction was tested experimentally using the reaction catalysed with the enzyme l-lactate dehydrogenase (LDH).

The accurate prediction of the wavelength of visible or NIR light that can activate the particular process, or stimulate proliferation of the particular cells, may improve significantly efficiency of corresponding therapeutical methods.

1.1 Research questions:

The following specific research questions are addressed in this thesis:

1. Whether low-intensity non-ionising, electromagnetic radiation from visible/ near infra red (NIR) region can affect catalytic activity of l-Lactate
2. Is it possible to predict the relationship between primary sequence of l-Lactate dehydrogenase and characteristics of electromagnetic field that affects kinetics (LDH) using the Resonant Recognition Model?
3. Are the experimental results within the range of computational prediction.

1.2 Aims of Research

This project will investigate effects of visible/near infra red light on particular enzymatic reaction, pyruvate to lactate conversion in the presence of LDH and NADH.

The computational prediction will be compared with the results obtained experimentally by radiating enzyme with the predicted wavelength of electromagnetic field.

Positive outcome of the research should confirm that protein activity can be altered by external EMR with a wavelength in the range of computationally determined value.

1.3 General Objectives

Objective of this work is to investigate the effect of the electromagnetic radiation in the range of [550-850nm] on the enzymes. This investigation consists of a) computational analysis; b) experimental design.

1.4 Specific Objectives

A very few studies have been undertaken to measure the enzymatic activities under influence of non-ionising, low intensity electromagnetic field, especially for the case of the radiation in visible/NIR range. However, a numerous studies demonstrated existence of effects on cells and tissue cultures after irradiation with visible/NIR electromagnetic radiation. Nevertheless, the mechanisms that cause those effects are not known, yet.

There is a need to do research to find out if it is possible to cause the change in activity of some of the common enzymes by external non-ionising, low intensity EMF.

This research study will investigate the variation in the kinetics of Lactate dehydrogenase after irradiation with EMR from the range (550-860nm). The computational predictions based on the RRM will be compared with experimental results. The outcomes will be discussed. Significance of the outcomes will be assessed by statistical analysis.

The system for measurement activity of lactate dehydrogense will include a design and development of:

1. A suitable source of radiation connected with the precise monochromators, with optical system, automatically controlled, that is able to produce high resolution monochromatic light bean.
2. A high resolution spectrophotometer suitable for continuous measurement and automatic recording of optical density during the wanted period of time

It is of great importance to mention that the following instruments, such as light source, monochromator, lenses, and spectrophotometer, as well as software that control spectrophotometer and light source were either commercial products or developed by other research institutions.

1.5 List of my Publications related to thesis

Articles

Vojisavljevic, V., Pirogova, E., Cosic, I. 2007 The effect of Electromagnetic Radiation (550 nm-850 nm) on l-Lactate dehydrogenase kinetics. Journal of Radiation Biology (accepted for publication)

Pirogova, E., Vojisavljevic, V., Fang, J., Cosic, I. 2006 Computational analysis of DNA photolyases using digital signal processing methods, 2007 Molecular simulations. Molecular simulations. Vol32, No 4, 1195-1203.

Vojisavljevic, V., Pirogova, E., Cosic, I. Investigation of the Mechanisms of Electromagnetic Field. Interaction with Proteins. Proceedings of the 2005 IEEE Engineering in Medicine and Biology 27th Annual Conference. Shanghai, China, September 1-4, 2005

Conferences:

Vojisavljevic V., Pirogova E., Cosic I. (2004) 'Possible functional classification of enzymes using the RRM multiple-cross spectral function' EPSM2004 Int. Conf. Engineering and Physical Sciences in Medicine, November, Geelong, Australia

Other Articles

K. Kalantar-zadeh, W. Wlodarski, V. Vojisavljevic, E. Pirogova, I. Cosic, S. Kandasamy, (2006) "Study of a ZnO/36º LiTaO3 SAW Biosensor with Gold Sensitive Layer", Rare Metal Materials and Engineering, Vol. 35, suppl.1.1 (In press)

Cosic, I., Pavlovic, M., Vojisavljevic, V., 1989, Prediction of "Hot Spots" in Interleukin-2, Based on Informational Spectrum, Characteristics of Growth Regulating Factors, Biochimie, 3, 333-342.

Cosic I., Vojisavljevic V., Pavlovic M., 1989, The Relationship of the Resonant Recognition Model to Effects of Low-intensity Light on Cell Growth, Int. J. Radiat. Biology, 56, 179-191.

Chapter 2

Biological realms

2.1 From cell to the organism

Living organisms are enormously complex. Cells are the basic morphological units of all living organisms. Therefore, understanding of the cell organization is the starting point for further investigation of cells response on the external electromagnetic field.

There are two major classifications of cells: the Eukaryotes, which have membrane-enclosed nucleus encapsulating DNA and the Prokaryotes, which lack nucleus (Vote and Vote 2004).

The Prokaryotes or **Moneras** are unicellular organisms bounded by cell membrane that consist of a lipid bilayer containing embedded proteins and controlling transport of materials in and out of the cell. The cells of most prokaryotic species are surrounded by rigid 3-25 nm thick polysaccharide cell wall.

The prokaryotic cytoplasm is, by no means, a homogenous liquid. Its single chromosome is condensed to form a body known as a nucleotide. The large numbers of proteins exist in prokaryotes' cytoplasm. For example approximately 4300 proteins are encoded by *Escherichia coli* DNA, with about 2000 of them presented in the cell at any time (Voet and Voet 2004).

Many Prokaryotes use at least the optical part of the EMR as an energy source (Xiong 2002) or as a signal for orientation (Armitage and Hellingwerf 2003). The main mechanisms in Prokaryotes to acquire and process EMR are detailed in the Chapters III.

Eukaryotic cells have membrane enclosed organelles, each of them with a specific function. Eukaryote's cells do not function independently, instead they are organized into groups to perform certain vital functions in the animal's life. A group of cells performing the specific function is called a TISSUE. The physiological or functional differences between various kinds of tissues are often accompanied by differences in physical appearance, since the cells of the tissues are adapted to the tasks they perform. Tissues do not function independently of each other. Several tissues are organized to work together as a group. The group of tissues is called an ORGAN. Tissues may be divided into 4 main groups (Gartner et al. 2002): **Epithelial tissue:** - cells are generally arranged into sheets, which cover the surfaces of the organs or body. **Muscular tissue**: - there are three main varieties of muscle, but all of them are specialized for contraction of skeletal, smooth and cardiac muscle. **Nervous tissue**: -cells are specialized for the conduction of electrical impulses. **Connective tissue:** - consists of several types of cell, usually embedded in a non-cellular substance. It includes blood cells, cartilage, bone, and the cells of "proper" connective tissue.

Most organs, of course, consist of more than one type of tissue. Generally, different tissues have a different dielectric and magnetic properties (Popp 1994).

2.2 From cells to molecules

Anatomical studies show that cells are composed of subcellular organelles **(Albert et al 1994)**; Organelles consist of supramolecular assemblies, such as membranes or fibbers, that are organised in clusters of macromolecules.

2.2.1. Cell organisation

There are a few differences between animal cell and plants cell. Plants cells have the cell wall, vacuoles and chloroplasts, while animal cells have not (Figure1, 2).

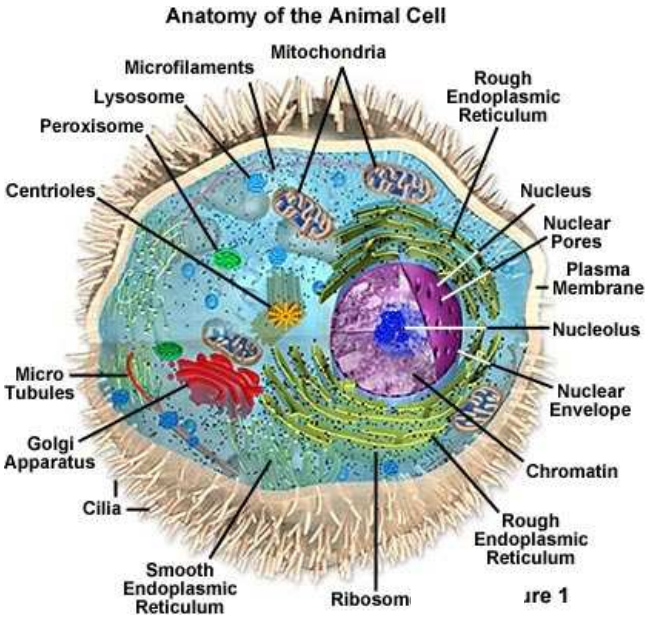


Figure 1. Animal cell with common organelles (http://micro.magnet.fsu.edu/)

The lack of a rigid cell wall allowed animals to develop a greater diversity of cell types, tissues and organs. The animal cell is unique among eukaryotic organisms because in the most animal tissues cells are bound together in an **extracellular matrix** by a triple helix of protein known as the **collagen** (Albert et al 1994). Plant and fungal cells are bound together in tissues or aggregations by other molecules, such as the **pectin**.

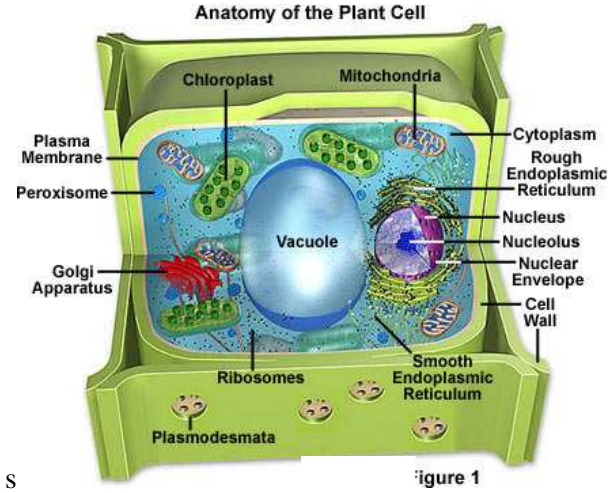


Figure 2. Typical plant cell image (http://micro.magnet.fsu.edu/cells

Plants are unique among the eukaryotes organisms, as they can synthetise their own food. Chlorophyll, which gives plants their green colour, enables them to use sunlight to convert the water and carbon dioxide into the sugars and carbohydrates, the chemicals the cell uses as a fuel. Plants cells have retained the protective cell wall structure of their prokaryotic ancestors (Gunning & Steer 1996). The basic plant cell has much in common with the typical eukaryote cell, but has not organelles such as centrioles, lysosomes, intermediate filaments, cilia, or flagella, all of which animal cell does have. Plant cell has, a number of other specific structures, including a rigid cell wall, central vacuole, plasmodesmata, and chloroplasts. The next level of the cell organisation are organelles. They are:

Mitochondria - Mitochondria are oblong shaped organelles that are found in the cytoplasm of every eukaryotic cell. Mitochondria are the sites of respiration, and generate chemical energy in the form of adenosine triphosphate (ATP). The ATP molecule is the main energy carrier in living cells. Cell synthetise the ATP by metabolizing sugars, fats, and other energy rich chemicals, with the assistance of the molecular oxygen. Many of mitochondrial proteins have chromophores and absorb light in visible part of the spectra (Karu et al. 2004, Menteifel and Karu 2005).

Centrioles- Centrioles are self-replicating organelles made up of nine bundles of microtubules and are found only in animal cells (Albert et al. 1994).

Cilia and Flagella For single-celled eukaryotes, cilia and flagella are essential for the locomotion of individual organisms (Albert et al 1994).

Endoplasmatic Reticulum- The endoplasmic reticulum is a network of structures that manufactures, processes, and transports chemical compounds for use inside and outside of the cell. It is connected to the double-layered nuclear envelope, providing a pipeline between the nucleus and the cytoplasm (Voet and Voet 2004).

Endosomes and Endocytosis - Endosomes are membrane-bound vesicles, formed via a complex family of processes collectively known as **endocytosis**, and are found in the cytoplasm of virtually every animal cell. The basic mechanism of endocytosis is the reverse of what occurs during exocytosis or cellular secretion. It involves the invagination (folding inward) of a cell's plasma membrane to surround macromolecules or other matter diffusing through the extracellular fluid.

Golgi Apparatus - The Golgi apparatus is the distribution and shipping department for the cell's chemical products. It modifies proteins and fats built in the endoplasmic reticulum and prepare them for export to the outside of the cell.

Intermediate filaments - Intermediate filaments are a very broad class of fibrous proteins that play an important role as both structural and functional elements of the cytoskeleton. Ranging in size from 8 to 12 nanometres, intermediate filaments function as tension-bearing elements to help maintain cell shape and rigidity.

Lysosomes- The main function of these microbodies is digestion of unwanted products of cell metabolisms

Microfilaments - Microfilaments are solid rods made of globular proteins called actin.

Microtubules- These straight, hollow cylinders carry out a variety of functions, ranging from transport to structural support.

Nucleus- The nucleus is a highly specialized organelle that serves as the information processing and administrative centre of the cell. This organelle has two major functions: it stores the cell's hereditary material, or DNA, and it coordinates the cell's activities, which include growth, intermediary metabolism, protein synthesis, and reproduction (cell division).

Plasma membrane- All living cells have a plasma membrane that encloses their contents. In prokaryotes, the membrane is the inner layer of protection surrounded by a rigid cell wall. Eukaryotic animal cells have only the membrane to contain and protect their contents. These membranes also regulate the passage of molecules in and out of the cells.

Cell Wall- Like their prokaryotic ancestors, plant cells have a rigid wall surrounding the plasma membrane.

Chloroplasts- The most important characteristic of plants is their ability to photosynthesize, or produce their own food by converting the light energy into the chemical energy. This process is carried out in specialized organelles called chloroplasts.

Chloroplasts are the sites of photosynthesis in eukaryotes (Figure 3). They contain chlorophyll, the green pigment necessary for photosynthesis to occur, and associated accessory pigments (carotenes and xanthophylls) in photosystem embedded in membranous sacs. Chloroplasts contain many different types of accessory pigments, depending on the taxonomic group of the organism being observed.

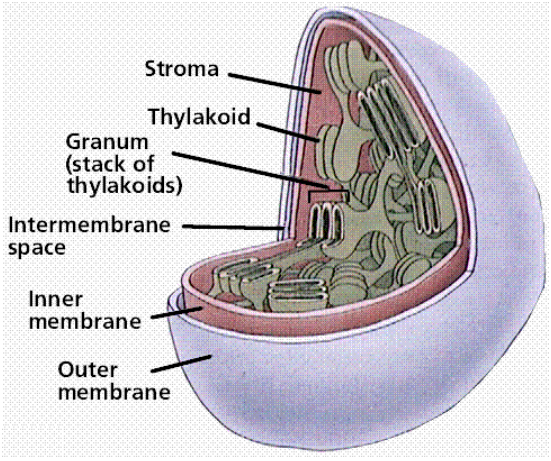


Figure 3. Structure of the chloroplast. (http://www.emc.maricopa.edu/faculty/farabee/biobk/BioBookCELL2.htm)

The many organelles are bounded with the membranes densely packed with proteins, which have a role in biochemical pathways. For example mitochondria and chloroplasts. Many of these proteins are enzymes that can absorb energy in visible range of the electromagnetic spectrum (Manteifel and Karu 2005).

2.3 Biomacromolecules

There is a structural hierarchy in the molecular organization of cells. Cells contain organelles, such as nuclei, mitochondria, and chloroplasts. The organelles contain supramolecular complexes, such as membranes and ribosome. The membranes and ribosome consist of clusters of macromolecules bound together by many relatively weak, non-covalent forces. Macromolecules consist of covalently linked subunits. The formation of macromolecules from simple subunits creates order (decreases entropy) and requires energy (Lehninger, 2004)**.**

Even though living organisms contain a very large number of different proteins and different nucleic acids, a fundamental simplicity underlies their structure. The simple monomeric units from which all proteins and all nucleic acids are constructed are few in number and identical in all living species (Vout & Vout 2004). Proteins, deoxyribonucleic acids (DNA) and ribonucleic acids (RNA) are linear polymers. Each protein, each DNA and RNA molecules has a characteristic information rich subunit sequence.

2.3.1 DNA and RNA

DNA carries the genetic information of a cell and consists of thousands of genes. Each gene serves as a code on how to build a protein molecule. The flow of information from the genes determines the protein composition and thereby the functions of the cell (Lehninger 2004).

The DNA is situated in the nucleus, organized into chromosomes (Figure 4). Every cell must contain the genetic information within the DNA. When proteins are required, the corresponding genes are transcribed into RNA by the process of transcription. The RNA is first processed and then transported out of the nucleus. Outside the nucleus, the proteins are built based upon the code in the RNA.

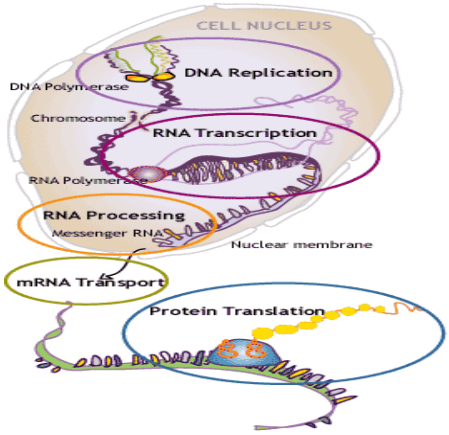


Figure 4. When proteins are requested, the corresponding genes are transcribed into RNA. The RNA is first processed and then transported out of the nucleus. Outside the nucleus, the proteins are built based upon the code in the RNA (http://nobelprize.org)

In proteins, DNA and RNA, the individual monomeric subunits are joined by covalent bonds. Supramolecular complex contain more then one macromolecule and they are held together by non-covalent interactions that are much weaker individually than covalent bonds (Daune 1999). Among noncovalent interaction are hydrogen bonds (between polar groups), ionic interactions, hydrophobic interactions (among nonpolar groups in aqueous solution), and Van Der Waals interactions, all of which have energies substantially smaller than those of covalent bonds. The large numbers of weak interactions between macromolecules in supramolecular complexes stabilize non-covalent assemblies, producing their unique “native” structures.

2.3.2 PROTEINS:

1.3.2.1 Amino acids

The particular function of a given protein is determined by the sequence of amino acids, which are sequentially linked in the protein molecule. Amino acids are organic acids that have an amino group on the alpha carbon - the carbon next to the carboxyl group. The general structure of an amino acid is as follow (Figure 5).

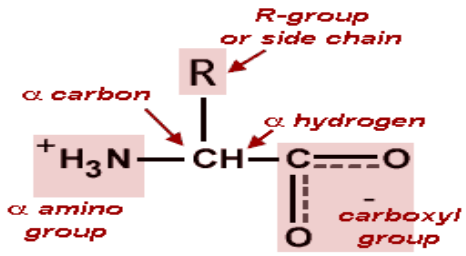


Figure 5. General structure of an amino acid. (www.biology.arizona.edu)

The different R-group side chains on the amino acids distinguish one amino acid from the other. Amino acids are so called because they contain at least one primary amino group in their molecular structure - NH (or cyclic imino group > NH) and one carboxylic group. Therefore, they have both *basic* and *acidic* characteristics.

Amino acids have many uses in the body. They are used by cells to synthesize tissue protein for use in the formation of new cells or the repair of old cells, to synthesize enzymes, to form non-protein nitrogen-contained compounds such as nucleic acids or heme groups, or to form new amino acids. The schematic representation of protein synthesis from amino acid is shown in Figure 6.

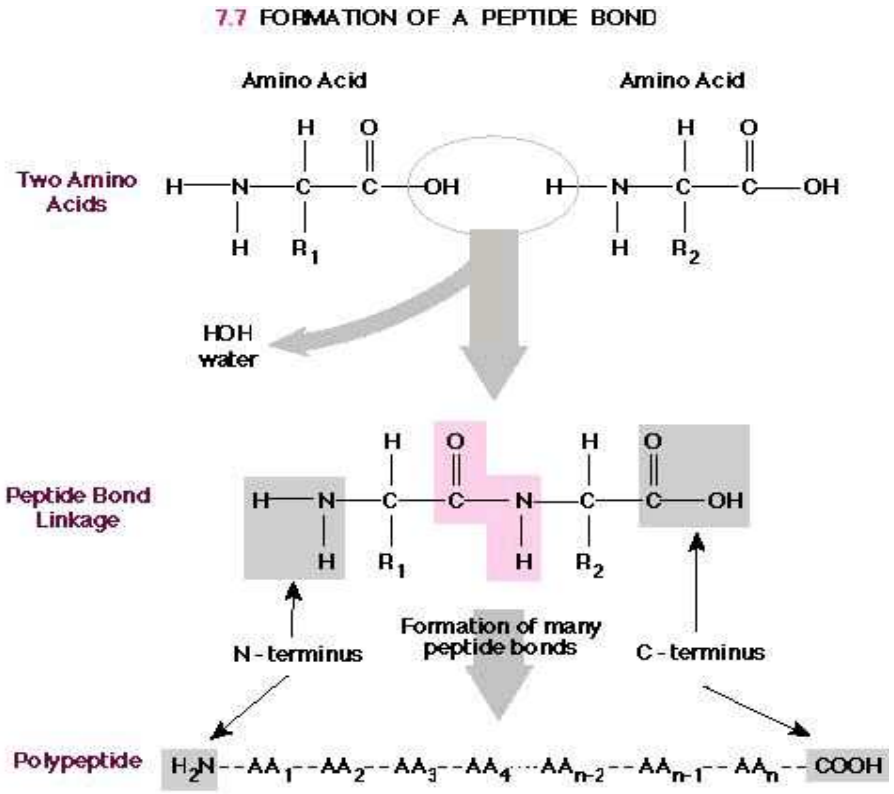


Figure 6. Formation of polypeptide bond during the process of polymerisation of amino acids (www.science.siu.edu)

Properties of amino acids

All amino acids are white crystalline solids that melt at high temperatures (>200°C). They are soluble in water and they vary considerably in their solubility. Furthermore, the solubility of any acid increases as the solution is made basic or acidic. Most of amino acids are insoluble in organic solvents. These properties are a direct consequence of the dual basic-acidic character of amino acids, which gives rise to ionic behaviour (Fasman 1989; Chan and Dill 1993).

Proteins' categorisation

Proteins may be generally classified into two categories: the *fibrous* proteins, which are insoluble in water and *globular* proteins, which are soluble in the water. Molecules of fibrous proteins tend to be long and thread-like. They may be set side by side to form fibres, which are often held together at many points by hydrogen bonds. Fibrous proteins often serve as

structural materials. Globular proteins are folded to form spheroids. The hydrophobic parts of the proteins are turned into the centre of the spheroid. The hydrophilic parts of the proteins are turned outward to the surface. Due to the shape of the globular protein, the contact area between molecules tends to be small. Hence, intermolecular forces also tend to be relatively weak and the proteins are relatively soluble. This solubility and resultant mobility are often exploited by biological systems to perform functions of the regulation and maintenance of biological processes.

It should also be noted that proteins along with nucleic acids exhibit the phenomenon of denaturation. Exposing a protein to heat or a strong reactive agent will cause it to lose its ability to perform physiological functions.

2.3.2.2 Protein structure

Proteins are complex molecules that can be classified according to specific primary, secondary, tertiary and quaternary structural characteristics (Figure 7).

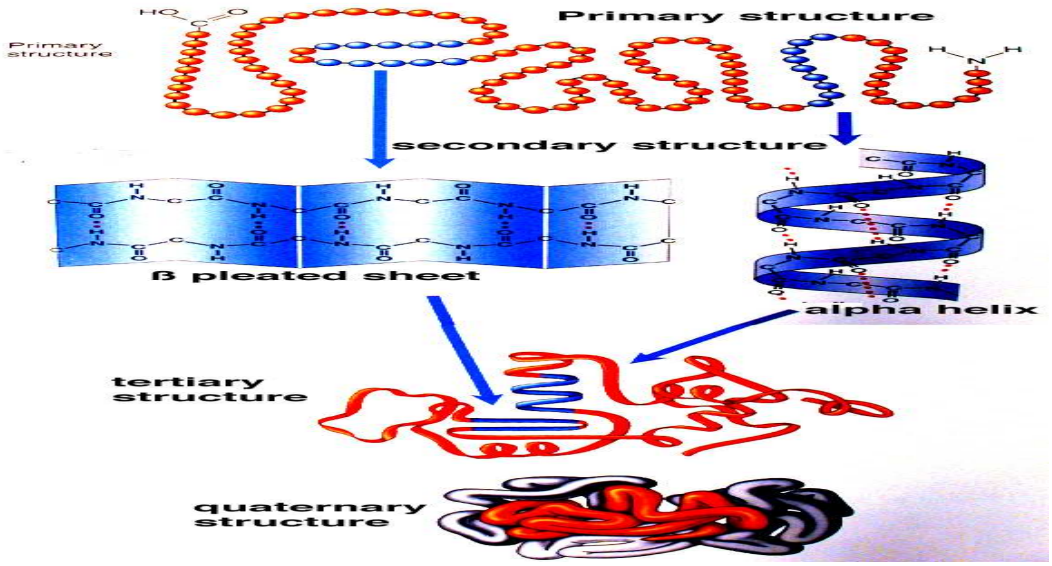


Figure 7. General presentation of 4 levels of the organization of molecules (http://academic.brooklyn.cuny.edu/biology/bio4fv/page/prot_struct-4143.JPG)

2.3.2.3. Primary Structure: The Amino Acid Sequence and the Peptide Bond

The primary structure of a protein is the sequence of amino acids in the protein molecule. These amino acids are coupled by covalent bonds called *peptide bonds.* The peptide bond is an amide linkage, formed by joining the carboxyl group of one amino acid to the amino group of a second amino acid through elimination of water (Figure 6).

The amino acids can be classified as being hydrophobic versus hydrophilic, and uncharged versus positively or negatively charged. Ultimately, the three dimensional conformation of a protein and its activity is determined by complex interactions amongst side chains. Some aspects of a protein structure can be deduced by examining the properties of clusters of amino acids (Harper 2003).

The peptide bond is stable if pH or salt concentration changes in solvents. It can be broken only by acid, base hydrolysis, or by specific enzymes. Two amino acids held together by a peptide bond are called dipeptide; three amino acids form a tripeptide and more than three form a polypeptide. One end of the protein will consist of an amino acid having a free amino group so called the N-terminal end. At the other end of the protein there will be an amino acid having a free carboxyl group. This is the C-terminal amino acid. The order of amino acids in a protein will determine its function, and is crucial to its biological activity. A change of just one amino acid in the sequence can disrupt the entire protein molecule (Nail and Dill 1991).

2.3.2.4. Secondary structure: Noncovalent Bonding

The secondary structure are local conformations of protein backbone stabilised by hydrogen bonds between carbonil oxygen and amil proton od adjacent portions of the peptide chain.
Two basic types of secondary structure are: $\alpha-$helix and β-sheet.

α - Helix

Pauling determined that the polypeptide chains in the protein keratin were curled in an arrangement called α - helix. In this configuration, the amino acids form loops in which the hydrogen bounded to the nitrogen atom in the peptide bond forms hydrogen bond with the oxygen attached to the carbon atom in a peptide bond farther down the chain. There are 3.6 amino acids in each turn of the α -helix, and the R-groups on these amino acids extend outward from the helix (Figure 8).

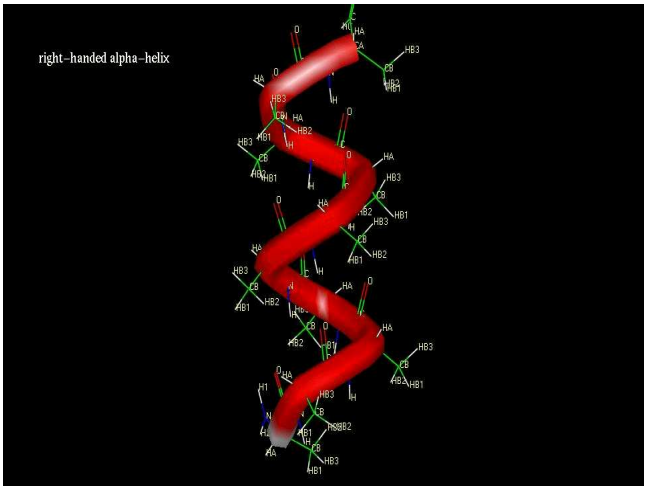


Figure 8. A right-handed α-helix. The α-helix has 3.6 residues per turn. The green dots show the hydrogen bond. (from:www.imb-jena.de)

β - Configuration, or Pleated Sheet

The fibrous protein of a silk has a secondary structure that is different then $\alpha - helix$. In silk, several polypeptide chains going in different directions are located next to each other; what gives the protein a zigzag appearance called β -sheet (Figure 9). The chains are held together by hydrogen bonds, and the R-groups extend above and below the sheet. Each protein will have a specific secondary structure depending on the amino acid sequence. It should be noted that the hydrogen bonding is weak non-covalent bonding, and is easily disrupted by changes in pH, temperature, solvents or salt concentrations (Daune 1999).

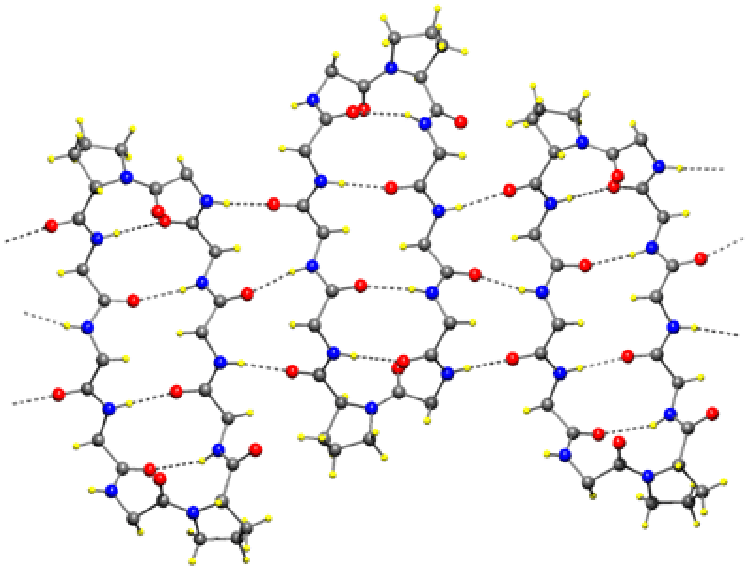


Figure 9. The five-stranded parallel beta sheet in thioredoxin (2TRX.PDB). The tree parallel strands are shown. The backbone is represented in stick form and contains atoms N, Cα, C (blue) and O (red). Hydrogen bond is represented by dashed line. (from: msc.oups.ac.jp)

2.3.2.5. Tertiary Structure: Globular Proteins

The tertiary structure refers to the total 3D structure of the polypeptide units of the protein. It includes the conformation relationships in space of the side chain groups to the polypeptide chain and the geometric relationship of distant regions of the polypeptide chain to each other. The native tertiary conformation is that of the lowest Gibbs free energy kinetically accessible to the polypeptide chains for the particular condition of ionic strength, pH and temperature of the solvent in which the folding process originates (Nail and Dall 1991).

Tertiary structure (3D) of globular proteins is presented in Figure 10. In general, globular proteins are very tightly folded into a compact spherical form (Fasman 1989).

These 3D surface features are fundamental to protein function as they allow protein molecules to interact with other molecules in specific ways. For example specific surface features are responsible for the binding of enzymes (Biswanger 1993, Ebbing 1987).

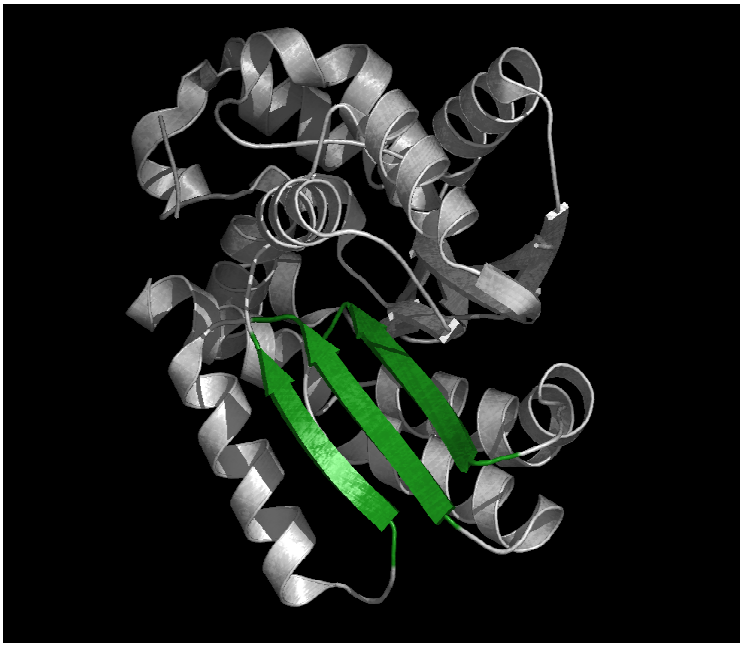


Figure 10. 3D structure of lactate dehydrogenase The image is made using X-ray cristalogtraphy data documented at Protein data Bank site (from ftp://ftp.rcsb.org/pub/pdb)

Proteins also form larger structures such as tubules or filaments. Therefore, knowledge of 3D structure of protein, either by prediction or experimental measurements, will contribute to the understanding of protein functions that are so crucial in living systems.

Disulfide Bridges

Disulfide bridges are interactions that include a disulfide linkage between molecules of the amino acid cysteine. Sulfhydryl groups (—S—H) are easily oxidised to form a disulfide (—S—S—). These bridges are covalent linkages that can be ruptured by reduction, but they are stable if pH or salt concentrations changes in solvents (Schulz 1979, King 1989).

Salt bridges

Salt bridges result from ionic interactions between charged carboxyl or amino side chains found on amino acids such as aspartic acid, glutamic acid, lysine and arginine. These linkages are particularly vulnerable to changes in pH (Darby and Creighton 1993).

Hydrophobic Interactions

Hydrophobic interactions occur between the nonpolar side chains of the amino acids in the protein molecule. Since the solvent water repulses nonpolar side chains, they tend to be on the inside of the molecule along with other hydrophobic groups.

At normal pH and temperature, each protein will take a shape that is energetically the most stable for the given specific sequence of amino acids, and for the various types of interactions, we have mentioned. This shape is called the native state or native configuration of the protein (King and Sternberg 1996; Bowie et al. 1991).

2.3.2.6. Quaternary Structure: Two or More Polypeptide Chains

The quaternary structure refers to the structure and interactions of the noncovalent association of discrete polypeptide subunits among themselves, creating a multisubunit protein. Not all of proteins have a quaternary structure. The quaternary structure of a protein is the way in which its polypeptide chains interconnect.

2.3.2.7. Factors, which may influence structure.

Various changes in the environment of a protein can disrupt the secondary, tertiary or quaternary structure of the molecule. Disruption of the native state of the protein is called **denaturation,** and will cause the loss of protein biological activity. Denaturation may or may not be permanent; in some cases the protein will return to its native state when the denaturing agent is removed. Denaturation may result in coagulation, with the protein being precipitated from solution. The process of denaturation involves the uncoiling of the protein molecule into a random state. Denaturing agents come in many forms, several of which are:

Heat

Heat causes an increase in thermal agitation of the molecule, disrupting hydrogen bonding and salt bridges. After gentle heating the protein can usually regain its native state, while violent heating will result in irreversible denaturation and coagulation of the protein.

pH

Changes in pH have their greatest disruptive effect on hydrogen bonding and salt bridges. For example the polypeptide polylysine, which has an amino group on its side chain, is composed entirely of the amino acid lysine. In acidic pH all of the side chains are positively charged and they repel each other, causing the molecule to uncoil. In basic pH, however, the side chains are neutral, they do not repel, and the molecule does coil into a α-helix.

Organic Solvents

Organic solvents such as alcohol or acetone are capable of forming hydrogen bonds, which then compete with the hydrogen bonds naturally occurring in the protein. This can cause permanent or temporary denaturation and coagulation.

Heavy Metal Ions

Heavy metal ions such as Pb^{2+}, Hg^{2+}, and Ag^{+} may disrupt the natural salt bridges of the protein by forming salt bridges of their own with the protein molecule. This usually causes coagulation of the protein. The heavy metal ions also bind with sulfhydryl groups, disrupting the disulfide linkages in the protein molecule and denaturing the protein.

Alkaloidal Reagents

Reagents such as tannic or picric acid affect salt bridges and hydrogen bonding causing proteins to precipitate.

Reducing Agents

Reducing agents disrupt disulfide bridges formed between the cysteine molecules. For example hair permanents work by reducing and disrupting the disulfide bridges in a hair.

Ionising radiation

Ionizing radiation is either particle radiation or electromagnetic radiation in which an individual particle or photon carries enough energy to ionize an atom or molecule by completely re-

moving an electron from its orbit. Radiation is similar to heat in its effect on proteins. The amount of energy required to ionize a molecule is different for different molecules. Gamma rays and X-rays will ionize almost any molecule or atom. Far ultraviolet, near ultraviolet and visible light have ionizing effect on some molecules. The radiation of lower energy such as microwaves and radio waves are non-ionizing radiations.

Interaction forces

The forces between the atoms in a molecule and between molecules themselves determine the molecule's structure. A variety of interaction forces are involved in the interactions of protein molecules: a resonance, a dipole, a polarisation, van der Waals' and electron exchange forces (Daune 1999). We can classify those interactions into strong and weak, with such a criterion of the classification, which will help to determine whether the thermal motion will be disrupted in the interaction. The weak interactions are of the same order as kT and they contribute mostly to forming of higher-order structures. Dipole-dipole interactions and hydrogen bonds are weak interactions. Bonds that are made through weak interactions are easily disruptive by heating, what results in the loss of quaternary, tertiary and secondary structure respectively.

1.4 Enzymes

Enzyme is a protein that speeds up a chemical reaction in a living organism (Bisswanger 2002). The very important moment of the enzyme's functioning is that it is possible to control the intensity of the enzyme's activity (by activators or inhibitors) without changing the

amount of the enzyme. This is crucial for functioning of any living organism. The regulation of the cell metabolism is one of the most important processes regulated by enzymes.

2.4.1. Enzymes nomenclature.

The enzymes are usually classified according to the reaction that they catalyse, with a particular emphasis on the direction of the reaction. The enzymes, which belong to the same class, always have the same direction of their reactions.

The standard classification in accordance with Enzyme commission (EC) contains four-digit code, separated by points (e.g., EC 1.1.1.27). Enzyme commission is the part of specialised international body with a name: Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NU-IAMB) (http://www.chem.qmul.ac.ak/iubmb/enzyme).

The first number is one of the six main classes of enzymes:

1. Oxidoreductase
2. Transferases
3. Hydrolases
4. Lyases
5. Isomerases
6. Ligases

The next two numbers indicate a subclass and a sub-subclass respectively. The last number is the serial number of the individual enzyme within its sub-subclass.

2.4.2 Enzymes and Chemical Catalysis.

It is known that enzymes have well defined binding sites for their substrates with which they sometimes form covalent intermediates. There are several theories of chemical kinetics. The

simplest theory is collision theory based on kinetics of enzymes and substrates (Cantor and Schimmel 1980).

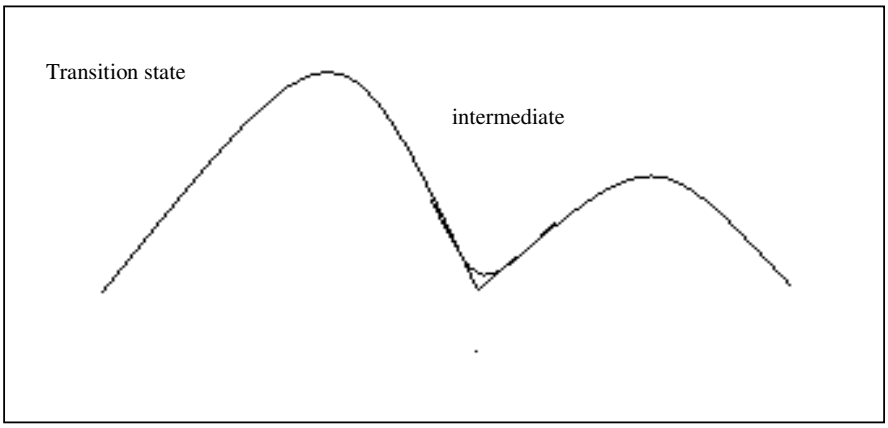


Figure 11. The transition state occurs at the peaks of the energy profile of a reaction and intermediates occupy the troughs.

A more sophisticated theory is *transition state theory*. The kinetics of the enzymes and substrates are ignored in *transition state theory.* Instead, the main attention is given to the most unstable structure on the reaction pathway so called *the transition state.*

In the reaction coordinate diagram (Figure 11), the peak is showing a transition state, in which the energy of the reaction is potted as the reaction proceeds. In the transition state, chemical bonds being made and broken. Intermediates, whose bonds are fully formed, occupy the troughs in the diagram. A simple way of deriving the rate of reaction is to consider that the transition state and the ground state are in the thermodynamic equilibrium and that the concentration of the transition state may be calculated from the difference in their energies. In general the presence of the enzyme stabilize charges in transition state by strategically placed acids, bases, metal ions or dipoles that are part of the structure of the enzyme (Freshet 1986).

2.4.3 The basic equations of enzyme kinetics

The concentration of enzyme bound intermediates is main variable in enzyme kinetics. When enzyme is mixed with a large excess of substrates, there is an initial period known as the *pre-steady state,* during which the concentration of these intermediates build up to their steady state. Once, the intermediates reach their steady state concentration, the reaction rate changes relatively slowly with time. It is during this steady state period that the rates of enzymatic activity are traditionally measured.

It is found experimentally in most cases that the rate of formation of product (or depletion of substrate) υ is directly proportional to the concentration of enzyme $[E]_0$. However, υ generally follows saturation kinetics in respect to the substrate concentration $[S]$ in the following way shown in Figure 12. At the sufficiently low $[S]$, υ increases linearly with $[S]$. However, as $[S]$ is increased, the relationship begins to break down and, υ increases less rapidly then $[S]$, until at sufficiently high or saturating $[S]$, tends toward a limiting value termed $V_{\max}$. This is expressed quantitavely in the Michaelis-Menten equation as follow (Fersht 1986):

$$\upsilon = \frac{[E]_0[S]k_{ca}}{K_M + [S]} \quad \text{where: } k_{cat}[E]_0 = V_{MAX}$$

The concentration of substrate at which $\upsilon = \frac{1}{2}V_{MAX}$ is termed K_M or *Michaelis constant.*

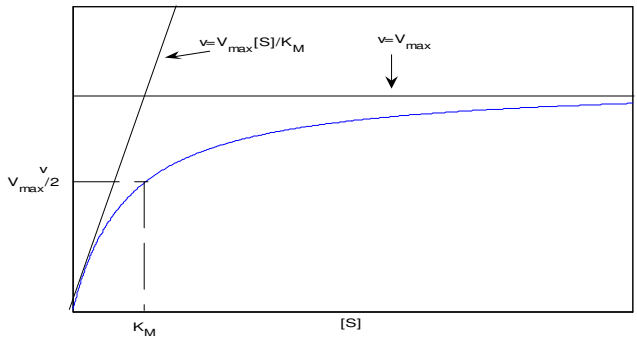


Figure 12. Reaction rate υ plotted against substrate concentration $[S]$ for the reaction obeying Michael-Menten kinetic.

The l-Lactate dehydrogenaze binds both pyruvate and the NADH. Many of the principles developed for single substrate systems may be extended to multi substrate systems. However, the general solution of the equation for such systems is complicated and beyond the scope of this thesis. In general, reactions in which all the substrates bind to the enzyme before the first product is formed, are called *sequential.*

Sequential mechanisms are called *ordered* if the substances combining with each other and the product dissociating from them, are in an obligatory order.

Ordered mechanism occurs in the NADH linked dehydrogenasys such as l-LHDB. The l-LDHB first binds coenzyme NADH, and then pyruvate. The molecular explanation for this is that the binding of the NADH causes a conformational change that increases the affinity of the enzyme to the pyruvate. The *ordered process* is shown in equation:

$$E \xleftrightarrow{A} EA \xleftrightarrow{B} EAB$$

As an analogy in this thesis E is l-LDHB; A is NADH and B is pyruvate. The more detailed analysis of dehydrogenaze reaction is presented in chapter IX.

Chapter III

Specialized photoreceptor systems

3.1 Photosynthesis

Photosynthesis is a light driven fixation of CO_2 which forms carbohydrates. (Vout and Vout 2004). In plants, photosynthesis takes place in the chloroplasts (see Chapter II). In plants, photosynthesis occurs in two distinctive phases: a) the light reaction, which uses light energy to generate chemical energy; b) the dark reactions, light-independent reactions, which use chemical energy to drive synthesis of carbohydrate.

The principal photoreceptors in photosynthesis are chlorophylls. The chlorophylls a, b, d are shown in Figure 13. Various chlorophylls strongly absorb visible light. The relatively small chemical differences among the various chlorophylls greatly affect their absorption spectra. After excitation, an electronically excited molecule of chlorophyll can dissipate its excitation energy in many ways (Vout and Vout 2004). The most important processes are:

1.) Internal conversion, as a common mode in which energy is converted to the kinetic energy of molecular motion or heat.

2.) Fluorescence, in which an electronically excited molecule decays to ground state by emitting photon. The fluorescently emitted photon has a lower energy then the initially absorbed photon.

3.) Exciton transfer (also known as resonance energy transfer), in which an excited molecule directly transfers its excitation energy to nearby excited molecules with similar properties. This exciton transfer is of particular importance in transport of light energy to the photosynthetic reaction centres.

4.) Photooxidation, in which a light excited donor molecule is oxidized by transferring its electron to the acceptor molecule. In photosynthesis, excited chlorophyll is such donor.

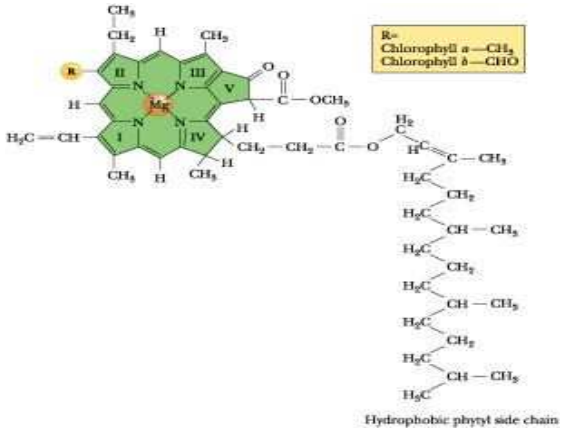


Figure 13. The molecular formulas of chlorophylls a, b (http//www.web.virginia.edu/Heidi/Chapter22.htm)

Chlorophylls and other pigments are bound to proteins, which provide the pigment molecules with the appropriate orientation and position with respect to each other. Light energy absorbed by individual pigments, is transferred to chlorophylls that are in a special protein environment.

In oxygenic photosynthetic organisms, two different reaction centres, known as photosystem II and photosystem I, work concurrently, yet in series (Vout and Vout 2004). In the light

phase photosystem II feeds electrons to photosystem I. The electrons are transferred from photosystem II to the photosystem I by intermediate carriers.

Pigments and proteins involved with this actual primary electron transfer event together are called the reaction centre. A large number of pigment molecules (100-5000), collectively referred to as antenna, "harvest" light and transfer the light energy to the same reaction centre (Figure 14). The absorption characteristic of some pigments are shown in Figure 15. In general the photosynthetic process involves the reduction of CO_2 to carbohydrates and the removal of electrons from H_2O, which results in the release of O_2 as shown:

$$CO_2 + H_2O \xrightarrow{light} (CH_2O) + O_2$$

In this process, known as oxygenic photosynthesis, the photosystem II reaction centre oxidizes water. The photosystem II is a name for a multi-subunit group of proteins located in the membranes. However, some photosynthetic bacteria, such as purple bacteria, green sulphur bacteria, green gliding bacteria, and gram positive bacteria can use light energy to extract electrons from molecules other than water (Govindjee 1996).

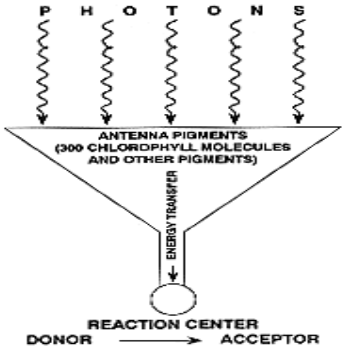


Figure 14. Simplified scheme showing light absorption in antenna pigments followed by excitation energy transfer to reaction centre chlorophyll. The antenna and reaction centre chlorophyll molecules are physically located in different proteins. Primary photochemistry (electron transfer from the primary electron donor to the primary electron acceptor) takes place in the reaction centre.

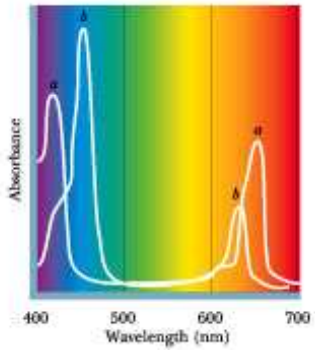


Figure. 15 Estimated absorption spectra of chlorophyll a and chlorophyll b (http//www.web.virginia.edu/Heidi/Chapter22.htm)

Algae

Algae are photosynthetic eukaryotic organisms that, like plants, evolve O_2 and reduce CO_2. They represent a diverse group that includes the dinoflagellates, euglenoids, yellow-green algae, golden-brown algae, diatoms, red algae, brown algae, and green algae. The photosynthetic apparatus and biochemical pathways of carbon reduction of algae are similar to plants. Photosynthesis occurs in chloroplasts. The pigments functioning as antenna are: chlorophyll b in green algae; chlorophyll c and fucoxanthin in brown algae and diatoms; phycobilins in red algae (Kingsley 1989). Green algae are thought to be the ancestral group from which land plants evolved (Lemieux et al. 2000).

Bacteria

Cyanobacteria are photosynthetic prokaryotic organisms. Fossil evidence indicates that cyanobacteria existed over 3 billion years ago and it is thought that they were the first oxygen evolving organisms on earth (Wilmotte, 1994). Cyanobacteria are presumed to have evolved in water in an atmosphere that lacked O_2. Initially, the O_2 released by cyanobacteria reacted with ferrous iron in the oceans and was not released into the atmosphere. Geological evidence

indicates that the Fe was depleted around 2 billion years ago, and earth's atmosphere became aerobic. The release of O_2 into the atmosphere by cyanobacteria has had a profound affect on the evolution of life.

The photosynthetic apparatus of cyanobacteria is similar to that of chloroplasts. The main difference is in the antenna system. Cyanobacteria depends on chlorophyll a and specialized protein complexes “phycobilisomes” to gather light energy (Willmotte, 1994).

Anoxygenic photosynthesis

Anoxygenic photosynthetic bacteria differ from oxygenic organisms as each species has only one type of reaction centre (Blankenship et al., 1995). In some photosynthetic bacteria the reaction centre is similar to photosystem II and in others, it is similar to photosystem I. However, neither of these two types of bacterial reaction centers are capable of extracting electrons from the water, so they do not release O_2. Despite these differences, the general principles of energy transduction are the same in anoxygenic and oxygenic photosynthesis. Anoxygenic photosynthetic bacteria depend on bacteriochlorophyll (a family of molecules that are similar to the chlorophyll) that absorbs strongly in the infrared range 700-1000 nm. The antenna system consists of bacteriochlorophyll and carotenoids that serve a reaction centre where primary charge separation occurs.

Purple Bacteria

There are two divisions of photosynthetic purple bacteria: the non-sulphur purple bacteria (e.g., *Rhodobacter sphaeroides* and *Rhodospeudomonas viridis*) and the sulphur purple bacteria e.g., *Chromatium vinosum* (Blankenship et al., 1995). Non-sulphur purple bacteria typi-

cally use an organic electron donor, such as succinate or malate, but they can also use hydrogen gas. The sulphur bacteria use an inorganic sulphur compound, such as hydrogen sulphide, as the electron donor. The only pathway for carbon fixation by purple bacteria is the Calvin cycle. Sulphur purple bacteria must fix CO_2 to grow, whereas non-sulphur purple bacteria can grow aerobically in the dark by respiration on an organic carbon source.

Green Sulphur Bacteria

The antenna system of the green sulphur bacteria is composed of bacteriochlorophyll and carotenoids and is contained in complexes known as a chlorosomes that are attached to the surface of the photosynthetic membrane (Blankenship et al., 1995).

Green Gliding Bacteria

Green gliding bacteria (e.g., *Chloroflexus aurantiacus*), also known as green filamentous bacteria, can grow photosynthetically under anaerobic conditions or in the dark by respiration under aerobic conditions. Like the green sulphur bacteria, green gliding bacteria harvest light using chlorosomes. The green gliding bacteria appear to have reaction centres similar to those of the purple bacteria, but there are several notable differences. For example instead of two monomer bacteriochlorophyll molecules, *C. aurantiacus* has one bacteriochlorophyll and one bacteriopheophytin

Heliobacteria

Bacteria *Heliobacterium chlorum* and *Heliobacillus mobilis* are in the phylum Gram Positive Bacteria that are strict anaerobes. Although the heliobacterial reaction centre is similar to photosystem I, it contains a different type of chlorophyll known as bacteriochlorophyll g (Nuijs et al. 1985).

Photosynthesys conclusionThe study of photosynthesys have important role in establishing new disciplines in photobiology and physics of proteins. The important processes such as electron and proton transfer are important part of photosynthetical mechanisms (Govindjee 1989). In addition, energy transfer between two proteins and energy transfer between proteins and chromophores are of particular importance in all photobiological processes.

3.2 Rhodopsins/Bacteriorhodospin

3.2.1 Rhodopsins

In all animals, specialized cells of retina "rods" (Figure 16) and "cons" have been evolved for the purpose of detecting light signals. These cells have numerous membrane folds that accommodate a high density of visual pigment molecules called rhodopsin (Avers 1976).

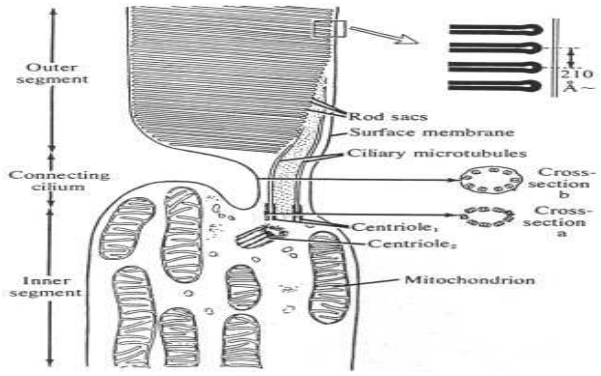


Figure 16 Rod-cell a large number of rod sacs that contain rhodopsins are visible in the top part of the cell. (Avers, 1976, p 387)

Rhodopsin consists of an **opsin** protein and a covalently bound cofactor, retinal. The structure of rhodopsin consists of a bundle of seven transmembrane helices (Figure 17) that surround the photoreactive chromophore, 11-cis-retinal. Isomerisation of 11-*cis*-retinal into all-*trans*-retinal by light induces a conformational change in the opsin that activates the associated G protein and triggers a second messenger cascade.

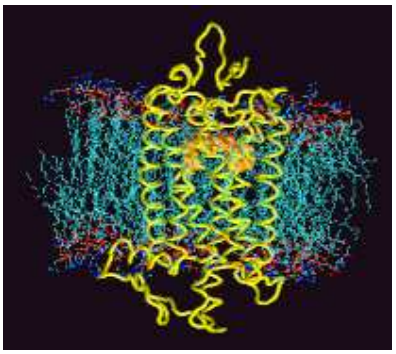


Figure 17. Rhodopsin molecule (yellow) with bound retinal (orange), embedded in a cell membrane (lipids shown as green, head groups as red/blue). (from http://www.ks.uiuc.edu/Research/rhodopsin/)

Opsin molecules that differ only by a few amino acids can show very different absorption characteristic. For example Humans have three different opsins, beside rhodopsin, the blue, green and red opsin, with absorption maxima at 420 nm, 534 nm and 564 nm respectively (Figure 18).

The mechanism of rhodopsin photoactivation has been also extensively studied through mutations in the rhodopsin gene. Researches revealed that absorption characteristics of rhodopsins are highly dependant on the primary structure of opsin molecule (Hwa et al., 1997).

The major difference between rhodopsin mechanism in vertebrates and invertebrates lies in the photo-regeneration process. In vertebrates, as the chromophore retinal is released in all-trans form, the process of regeneration, taking place in the dark, requires that all-trans retinal is isomerized to the 11 -cis form. Opsin will spontaneously recombine with 11-cis form to give rhodopsin.

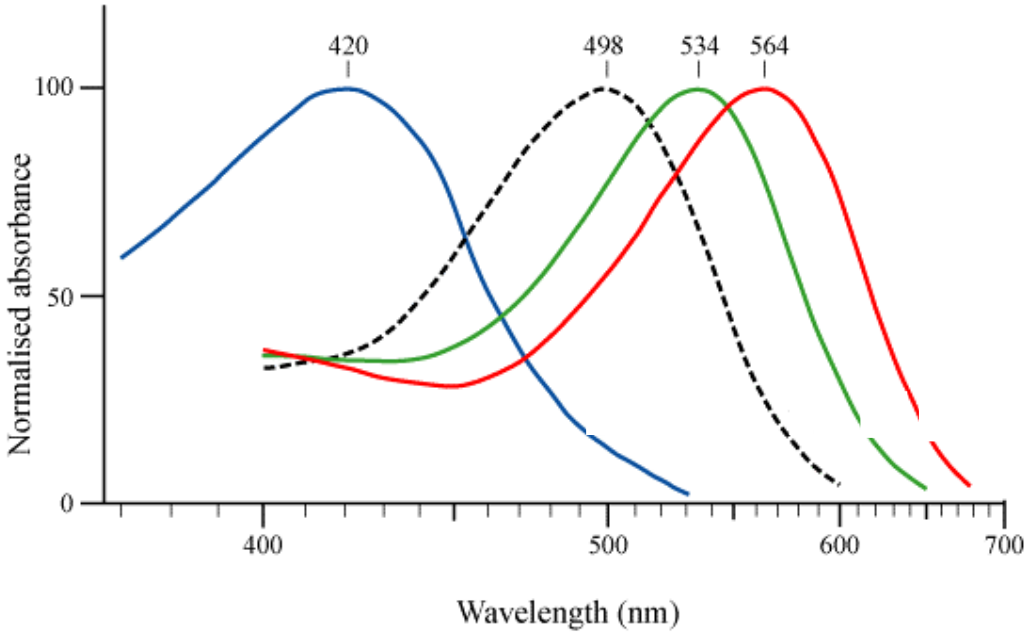


Figure 18. Normalised absorption spectra of four human rhodopsins.

3.2.2. Bacteriorhodopsin

The system similar to rhodopsin is found in purple bacteria. Bacteriorhodopsin - protein from *Halobacterium halobium*- folds into a seven-transmembrane helix topology with short inter-connecting loops. The helices (named A-G) are arranged in an arc-like structure and tightly surround a retinal molecule that is covalently to a lysine (Lys-216) on helix G. The cross-section of BR with residues important for proton transfer and the probable path of the proton are shown in Figure 19.

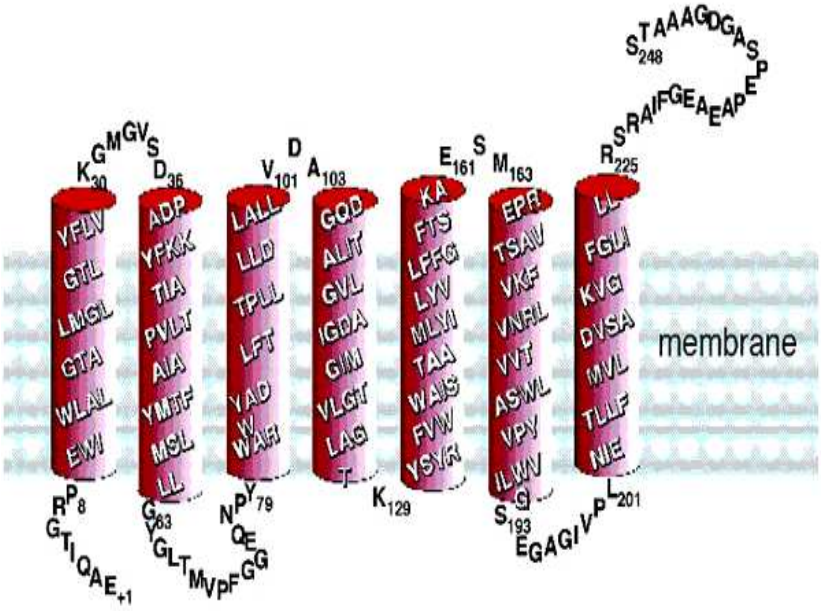


Figure 19. The Schematic presentation of the primary structure of Bacteriorhodopsin (www.boichem.mpg.de).

3.3 Rhodopsins and the RRM

It was mentioned above that rhodopsins are proteins, which consist of an apoprotein (opsin), and a retinal chromophore covalently bound to the apoprotein by a protonated Schiff base linkage to a lysine residue. While the protonated form of retinal Schiff base absorbs at about 440 nm in organic solvents, its maximal absorption is changed after binding to the apoprotein. This effect is known as 'opsin shift'. The mechanisms by which the protein matrix adjusts the maximal absorption of the chromophore, using the molecule retinal to detect light at different wavelengths, are still unknown. However, the RRM analysis demonstrates correlation between the RRM frequency of prominent peak in RRM spectra and wavelengths of maximal absorption of rhodopsins/bacteriorhodospins (Cosic et al.1989, 1994). Chapter VI discusses this phenomenon.

3.4 PHOTOMORPHOGENESIS

The plants are particularly sensitive to environmental EMR. A long list of processes is associated with light. Brief exposure of pants to the light cause internode's elongation (Taiz and Zeiger 1991), initiate leaves to expand and triggers chlorophyll synthesis to begin.

The best known plant photo regulating hormone is phytochrome. Phytochrome is a homodimer: two identical protein molecules, each conjugated to a light-absorbing molecule (Ni et al. 1999). The phytochrome has one chromophore per dimmer, The chomophores are open chain tetrapyrolle; covalently bonded to the protein via linkage involving a cysteine.

At least five types of phytochromes have been identified. There is some redundancy in function of the different phytochromes. In addition, it seems there are functions that are unique to one or another. The phytochromes also differ in their absorption spectrum (Sage 1992). The phytochromes exist in two inter-convertible forms: P_R (phytochrome form R as it absorbs red light at 660 nm) and P_{FR} (FR as it absorbs far red light at 730 nm). By changing its form, phytochrome is changing its catalytic function of activating or deactivating a chain of processes. Two inter-convertible forms of phytochrome are exchanging in the following way: a) Absorption of red light by P_R converts P_R into P_{FR}. b) Absorption of far red light by P_{FR} converts P_{FR} into P_R. c) In the dark, P_{FR} spontaneously converts back to P_R However, the phytochrome cycle is still not completely understood.

3.5 Cells without specialized receptor system

In the last 20 years it has been documented by many researches that low-intensity light irradiation in the visible wavelength range, can alter metabolic processes in both mammalian

cells and bacteria (Karu et al 2004, Manteifel and Karu 2005). Many of these cells are not exposed to light in their natural environment.

Effect	**Wavelength**	**Source**	Power/ Dose	Ref
Hela Proliferation	820nm,	Laser	1-5J/cm	Karu 2003, Mognato et al(2004)
E. coli growth	600-900nm	Led,Laser	1-5J/m	Nussbaum et al. 2002
Human neutrophils	830nm	Monochromators, Laser	N/A	Fujimaki et al. 2003
Streptococcus spp.	630 nm, 810	Led	N/A	Karu 2001
Fibroblast (rats)	Green light (560nm)	Monochromators, Lase	N/A	Poureau-Schneider et al 1990
Mitochondrial cell respiration	630nm	Monochromators Laser	N/A	Karu 2004

Table 1. Effects on sell, which not exposed to light in *in vivo* conditions

Light can accelerate or inhibit the cellular metabolism, depending on the wavelength, irradiation dose (explained in Chapter V) and treatment protocol (Karu 1995). Studies done *in vitro* have demonstrated effects of visible-light irradiation, such as changes in growth-factor production (Hsin-su et al. 2003); ATP synthesis (Passarella et al. 1984); phagocytic activity (Ju et al. 2006); altered production of collagen by fibroblasts (Anders 1995). In addition, Lubart at al. (1992) had found significant increase in count of fibroblast cells during mitosis after irradiation with laser diode at 780 nm ($7\mathrm{J}\,cm^{-2}$).

The electromagnetic radiation in the visible/near infra red range has application in wound healing, particularly in the treatment of venous, diabetic and pressure ulcers (Karu 1997). Use of visible light has also been extended to the treatment of inflammatory diseases (Sokolova and Erina 1995). Research has indicated that the biological effects, incited by exposure of the biological subject to the visible light, may cause changes in cell cycle progression. Several investigators have reported a reduction in cell proliferation following radiation treatment (Karu 1994).

Nussbaum et al (1994) have conducted the studious measurement of the effect of low intensity laser light on Escherichia coli. It has been found that the growth of the *Escherichia coli*

depends on intensity and length of the time of exposure. In addition; wavelength of EMR is proved to be influential on proliferation of bacteria (632 nm, 1066nm, 1286nm). The important role of metabolical enzymes *cytochrome d*, which is absorbing in visible part of electromagnetic spectra, have been suggested by Hallen et al. (1988, 1991). However, the role itself has not been explained.

Ostinia et al. (1992) monitored the change in activity of the purified glutamate dehydrogenase from beef liver (E.C. 1.4.1.3.) irradiated with either He-Ne laser (632.8 nm) or a red light-emitting diode (650 nm). In both cases, the energy dose was $0.24 \mathrm{J}\, m^{-2}$. In addition, the same activity has been monitored after irradiation with non-coherent Xe-Hg lamp under the same experimental conditions.

In research by (Kropat &Back 1998) has been found that low intensity light at 600 nm may induce the heat-shock gene *HSP70A* via the heat stress-independent pathway.

Nitzan et al (2004) measured photoinactivation when bacteria *A. baumanni* and *E. coli* cells were irradiated by blue light (400– 450 nm). Afterwards, the author suggested that these results could be regarded as a consequence of the membrane damage.

Taking in consideration the documented experimental data, there is a strong indication that low intensity light can affect cells and tissues, and most probably protein's function and enzyme activity.

Chapter VI will discuss the relationship between RRM frequencies of growth factors and wavelengths of EMR that activate change in the proliferation of the cells

Chapter IV

The Electromagnetic Field and its effects on the living matter.

4.1 The electromagnetic Filed

The source of visible light and all other forms of electromagnetic radiation is the atom. Electrons are moving in orbits around the nucleus of an atom, arranged in different energy levels within their electron clouds. These electrons can absorb additional energy from outside sources of electromagnetic radiation, which results in their promotion to a higher energy level. This promotion is an inherently unstable situation. The electron eventually loses the extra energy by emitting photons and, in doing so, the electron falls back into its original and stable energy level.

Figure 20 illustrates an electromagnetic wave that propagates, or travels, in a direction from upper left to lower right. This wave travels at the speed of light and is known as a transverse wave, in which the direction of wave energy lies at right angles to the direction of propagation. The wave in Figure 20 is generating both electric and magnetic oscillating fields that are oriented at 90-degree angles with respect to each other and also to the direction of energy.

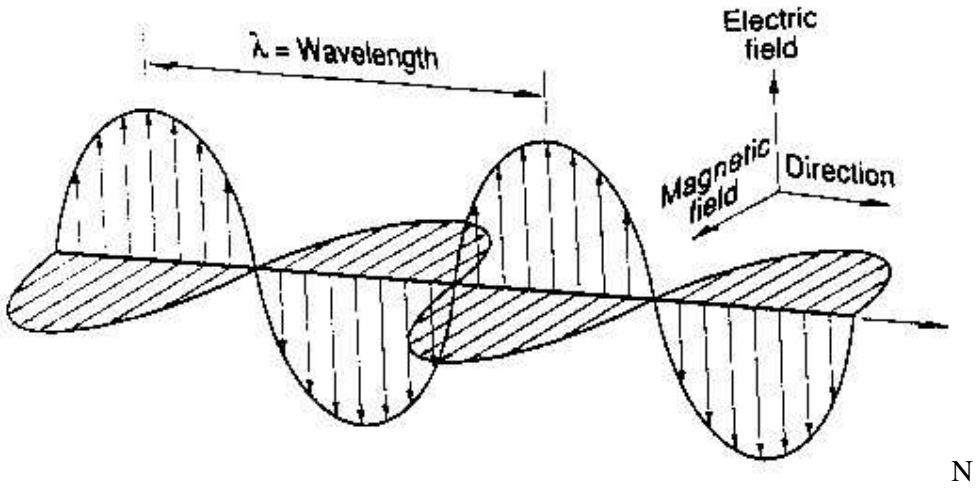


Figure 20. Schematic representation of an electromagnetic wave. The both electric and magnetic oscillating fields are oriented at 90-degree angles with respect to each other and to the direction of energy. The distance between two successive peaks of the wave is equivalent to the wavelength of the radiation.

The distance between two successive peaks of the wave is equivalent to the wavelength of the radiation. The frequency of the radiation, however, is determined by the number of oscillations per second, which is usually measured in hertz, or cycles per second.

The relationship between the wavelength of the light and its frequency in inversely proportional and can be illustrated by a simple equation:

$$\lambda = c / \upsilon$$

where c is the speed of the light $c \approx 3 \cdot 10^8 m\, s^{-1}$ in air, ν is the frequency of the light in hertz (Hz), and λ is the wavelength of the light in meters. After entering a new medium, such as glass or water, the speed and wavelength of the light is reduced, although the frequency remains unaltered.

The energy of a photon, however, is directly proportional to its frequency and inversely proportional to its wavelength. This relationship can be expressed by another simple equation:

$$E = h\nu$$

where E is the energy in kilo Joules per mole, *h* is Planck's constant. Thus, as frequency increases, the energy of emitted photons increases.

Rapid development in telecommunication and other technologies that deal with generating of electromagnetic field, affects already existing environmental level of EMR from natural sources from the Sun, Cosmos and from the Earth. The scientific interest for the interaction of EMF and living organisms unsurprisingly initiates a trend of a large number of experiments. The most important parameter that these experiments deal with is the wavelength of EMF. The classification of EMF in respect to frequency is given in Table 2.

In the last twenty years, a particular interest arose for the weak non-thermal and non-ionising EMF (Panagopoulos 2003). In many of those experiments, the biological effects cannot be simply explained by an increase of the temperature inside the exposed tissues. Due to enormous complexity of living organisms, it is very difficult to specify the particular process or the particular molecule that has been affected by the EMR.

In addition, even if the EMR alternates the specific physiological process, it is not always possible to detect each change in physiological parameters**.**

Abbreviation	Common name	Wavelength	Source
	IONISING		
Gamma rays		**<0.01nm**	**Atomic nuclei**
HX	**Hard X-rays**	**0.01-0.1nm**	**Electron pr.**
SX	**Soft X-Rays**	**0.1-10nm**	**Electron pr.**
F/EUV	**Far/Extreme ultraviolet**	**200nm-1nm**	**Sun**
NUV	**Near ultraviolet**	**200-380nm**	**Sun**
Visible light		**380-760 nm**	**Sun**
	NON-IONISING	**Frequency [Hz]**	
NIR	**Near infrared**	**30-300Thz**	**Heat (object)**
MIR	**Moderate infrared**	**3 THz- 30Thz**	**Heat (object)**
FIR	**Far infrared**	**300 GHz -3 THz**	**Heat (object)**
Radio waves:			
EHF	**Extremely high frequency(Microwave)**	**30-300GHz**	**Radar**
SHF	**Super high frequency (Microw.)**	**3-30 GHz**	**Radar**
UHF	**Ultrahigh frequency**	**0.3-3 GHz**	**Microwave**
VHF	**Very high frequency**	**30-300 MHz**	**Radio**
HF	**High frequency**	**3-30 MHz**	**Radio**
MF	**Medium frequency**	**0.3-3 MHz**	**Radio**
LF	**Low frequency**	**30000-300000**	**Radio**
VLF	**Very low frequency**	**3000-30000**	**Radio**
VF	**Voice frequency**	**300-3000**	**Acoustic**
ELF	**Extremely low frequency**	**0-300**	**Electricity lines**

Table 2. Frequency ranges and sources of EMF. First column represents common abbreviation, third column is the range. Fourth column are usual source. Radio means that sources are telecommunication tool that is emitting radio waves. The frequency higher then visual light are usually referred as a ionising. The frequencies lower then visual are usually called non-ionising.

The reason for this is the regulatory effect of "homeostasis" on organisms, or the regulation of the *steady state* (Vout and Vout 2004) which neutralises small alternations in the metabolic processes.

The biological response to the EMIR is usually the result of one or more physical mechanisms: *thermal, acoustics, optical and photochemical.*

An electromagnetic field is one of four fundamental fields. In terms of classical physics the electromagnetic field could be described with a complete system of Maxwell's equations, representing variations in time and space of the coupled electrical and magnetic fields E(t, x) and B(t, x.). As living organisms are very inhomogeneous complex systems that consist of a very

large number of different molecules, organised in various structures, it is practically impossible to solve the system of Maxwell's equations.

However, in cases where only the energy of the EMR is of interest, it is convenient to use a wave-particle duality or "quantum of electromagnetic radiation", where EMR may be regarded as a collective effect of an ensemble of photons.

The common specifications of EMR used photobiology are based on the physical characteristics of the EMF source.

- Wavelength or frequency of EMF in [m] or [Hz]
- Irradiance in [W m^{-2}] or in [W m^{-2} / nm]
- Radiant exposure that describes surface exposure dose in [J m^{-2}]

The EMR has been a crucial element in the development and evolution of living organisms since the very beginning of life, some 5 billions years ago. From that time on, living organisms developed many mechanisms for utilising environmental EMR. The effects of EMR in various ranges are shown at Table 2.

High energy EMF (gamma, X-rays, and UVA, UVB) with wavelength $\lambda <$ 360nm has enough energy to brake covalent bonds, to excite electrons above ionisation energy and to form very reactive free radicals. The development of nuclear power technology in 20^{th} century and consequent health hazard stimulate an enormous number of experiments that monitor effects of ionising irradiation on living matter.

The EMR of the following ranges: near infrared (NIR); infrared (IR) and far infrared (FIR), will excite vibrational spectra of many organic molecules presented in the cells. The main vibrational modes responsible for infrared spectra of proteins are out of this range and will not be addressed in this work.

The interest for the EMR within the range of radio frequencies (RF) and microwaves has been increase since the introduction of mobile telephony. Heat production due to conductivity loss, time varying B induces E-Field which transfers energy; Penetration depth to the organs or

bodies decreases by frequency and dielectric loses become more and more important (Popp 1994).

For lower energies of EMR it is accepted that fields at frequencies above about 1 MHz, have, as the main cause, movement of ions and water molecules through the medium, which results in the heating of a biological subject. Even very low levels of energy produce a small amount of heat. This heat is usually carried away by the body's normal thermoregulatory processes without change to the body temperature. Even static EMF can cause some effect such as polarization of cell membrane structures (Popp 1994).

Visible EMR within the range of the visible light have a specific role in the life of living organisms. In this thesis will be analysed effect of visible light on important reaction in every cell, pyruvate to lactate conversion.

Frequency	Physics	EFFECTS
Static	Ionic current and polarization of biological tissue is possible, Magnetic field penetrate practically without any absorbance (Popp 1994).	Sensitivity to static field is observed in plants (Galand & Pazur 2005) Increased cell proliferation and changes in gene expression compared with control groups. (Potenza et al. 2004)
Extremely low frequencies (0-300Hz)	ε is decrease, σ is increases with frequency, impedance of cell membranes is decrease, wall become charged, Loss possible by eddy currents, and it is due to conductivity loss (Popp 1994)	Various cellular components, processes, and systems can be affected by EMF exposure the possible correlation between immune cell system stimulation and EMF exposure (Simko & Mattsson 2004) All these results indicate that exposure to non-ionizing radiations contribute to a more rapid cell maturation. (c) (Lisi et at. 2005)
Radiofrequencies(300MHz-30GHz)	Heat production due to conductivity loss, time varyng B induces E-Field, which transfers energy; Penetration depth to the organs or bodies decreases by frequency, Dielectric loses become more and more important. (Popp 1996)	Changes on heat shock proteins in human electrocites are were observed (Lim et al. 2005 In a series of publications, it has been shown that exposure to mobile phone signals can influence cellular processes such as proliferation (Velizarov et al., 1999 and French et al., 1997), cell morphology (Donnellan et al., 1997), and the level of heat-shock protein expression (Leszczynski et al., 2002).
Microwaves	Dielectric loss of water becomes more and more significant	Exponentially growing S. cerevisiae yeast cells. A statistically significant enhanced growth rate was observed at 341 GHz.(Hadjiloucas et al 2002)
IR	Rotation and vibrations of molecules	Increase proliferation of E. coli (Karu, 2003). Can influence enzymatic activity (Biscar 1976),

Table 3. Effects detected after irradiation with EMR within the range (static to Infra red)

4.2 Visible light

The term visible light is rather physiological then physical term and usually is correspond to the frequencies of electromagnetic radiation that can be sensed with a human eye. Usually interval between 380 nm and 700 nm, is called visible light Figure 21. Some animals can visualise up to the wavelength of 1000 nm and more.

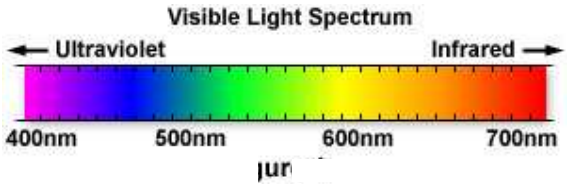


Figure 21. The EMF in the range 400-700nm as it is perceives by human eye

For each set of wavelengths in the visible spectrum, humans perceive certain colours, the distribution of which is outlined in Table 4. It is possible, however, for many different spectral distributions to produce identical colour sensations. A yellow colour sensation may be caused by a single wavelength of light, for instance 590 nm, or it may be the result of viewing two wavelengths, such as 590 and 600 nm. It is also possible to view the colour yellow as a narrow distribution involving all wavelengths between 590 and 600 nm. The same array of possibilities exists for all colours in the visible spectrum. Colours associated with different frequencies of visible light are shown in Table 4.

Wavelength range	Color Perceived
340-400	Near Ultraviolet (UV; Invisible)
400-430	Violet
430-500	Blue
500-560	Green
560-620	Yellow to Orange
620-700	Orange to Red
Over 700	Near Infrared (IR; Invisible)

Table 4 Common names of colours corresponding to the specific wavelengths

It is possible, however, for many different spectral distributions to produce identical colour sensations. A yellow colour sensation may be caused by a single wavelength of light, for instance 590 nm, or it may be the result of viewing two wavelengths, such as 590 and 600 nm. It is also possible to view the yellow as a narrow distribution involving all wavelengths between 590 and 600 nanometres. The same array of possibilities exists for all colours in the visible spectrum.

White light, however, does not appear in Table 4 because it is composed of a mixture containing all or most of the colours in the visible spectrum. White light is emitted by a variety of sources, such as tungsten lamps, which are frequently labelled “incandescent” because they radiate light when heated by electrical energy. The fluorescent source may produce the white light as a result of electrical current through a charged gas. The largest source of white light, is the sun.

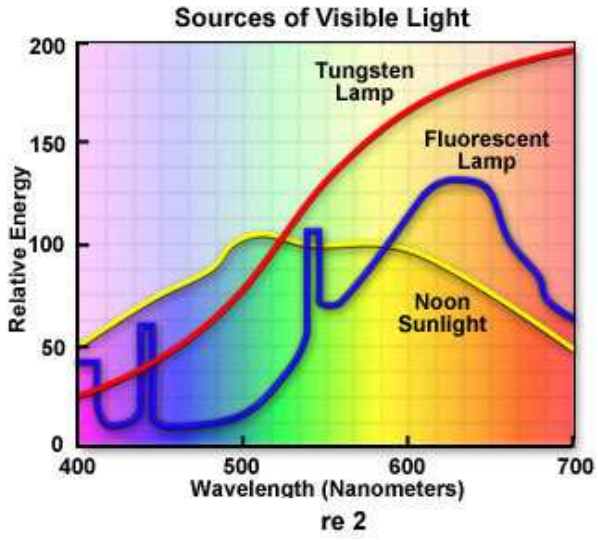


Figure 22. Spectral characteristics of sources of light. The red line represents the relative energy of tungsten light over the visible spectrum. The yellow line represents what humans see with a natural sunlight spectrum sampled at noon. The blue line illustrates what is seen with fluorescent light

Spectral distribution curves demonstrating the relative amounts of energy versus wavelength for the three most common sources of white light are illustrated in Figure 22. The red line represents the relative energy of tungsten light over the visible spectrum. As is apparent, the energy of tungsten light increases as wavelength increases, which dramatically affects the average colour temperature of the resultant light, especially when it is compared to that of natural sunlight and fluorescent light. The yellow line represents what humans see with a natural sunlight spectrum sampled at noon. Under normal circumstances, sunlight would have the greatest amount of energy, but the spectrum has been normalized in order to compare (Figure 22) it to the other two. The blue line illustrates what is seen with fluorescent light and contains some notable differences from the tungsten and natural sunlight spectra. Several energy peaks are present in the fluorescent light spectrum, which are a result of the superposed line spectrum of mercury vapour in a fluorescent lamp.

Since different sources of light exhibit different characteristics, the decision of which type of lighting should be utilized generally depends upon application. For instance, a variety of non-incandescent visible light sources is used for microscopy, indoor, and outdoor lighting. Most

of these are based on electronic discharge in a gas such as mercury or the noble gases neon, argon, and xenon. The generation of visible light in these devices relies on the collision of atoms and ions in the gas with the current that is discharged from the electrodes at the ends of the bulbs.

The laser is another important source of visible light. Lasers are unique in that they emit a continuous beam of light made up of a single wavelength that exits in a single phase, commonly termed coherent light. The wavelength of light emitted by a laser depends upon the material from which the laser crystal or gas is composed.

There are not experimental evidence that the polarizability (where the electric field vectors are restricted to a single plane) of the light has any effect on irradiated biological objects.

Chapter 5

THE RESONANT RECOGNITION MODEL

Chapter II detailed importance of proteins for functioning of any living organisms. A number of functions are connected with a protein activity (enzymatic, transport and storage, immune protection, intracellular communication and many other).

The proteins are linear macromolecules built up of sequentially linked amino acids. The information contained in the amino acid sequence determines the protein's chemical properties, chain conformation and protein's functions.

Although both function and structure of a large number of proteins are becoming known throughout projects like Human Genome Project led by the "The Nacional Human Genome Research Institute for the National Institute of Health" (official web site is http://www.genome.gov) and they are available through a number of data bases, the crucial problem of understanding how the biological function is "written" within the protein sequence still remains.

Once this understanding has been gained it should be possible to design peptides and even proteins *de novo* with the chosen biological function and thus to produce new and more efficient drugs and other biotechnological products.

With the rapid accumulation of databases of proteins' primary structures, there is an urgent need for theoretical approaches that are capable of analysing protein structure/function relationships. As described in the previous section, many attempts have been developed to predict the tertiary structure and biological function of protein from its sequence but only with limited success. These approaches have inherent limitations, i.e. they do not provide sufficient knowledge about the physical process for proteins folding and interacting. They also lack the informational, structural and physicochemical parameters crucial to the selectivity of protein interactions, which can be used for *de novo* design of peptide or protein analogous with the desired biological activity.

The **Resonant Recognition Model** (RRM) (Cosic 1997; Cosic 1994, Cosic et al 1989, Cosic et al 1986, Pirogova et al. 2003) views a protein as a string encoded with information. The positions of the protein's constituent amino acids together with the type form the signal. The RRM algorithm assigns electron ion interaction potential (EIIP) to each amino acid in the primary structure (see Chapter II).

The EIIP (Veljkovic and Slavic 1972; Veljkovic 1980), which describes the average energy states of all valence electrons in particular amino acid. The EIIP values for each amino acid were calculated using the pseudopotentials general model (Veljkovic 1972):

$$\langle k + q \,|w|\, k\rangle = 0.25\, Z \sin(\pi\, 1.04Z)/(2\pi)$$

where q is a change of momentum k of the delocalised electron in the interaction with potential w, while

$$Z = \frac{\sum_i Z_i}{N}$$

where Z_i is the number of valence electrons of the i^{th} component of each amino acid and N is the total number of atoms in the amino acid.

The EIIP values for 20 amino acids, as well as for five nucleotides (the whole procedure can be applied to the DNA and RNA), are shown in Table 5. A unique number can thus represent each amino acid or nucleotide, irrespective of its position in a sequence.

Nucleotide	**Nucleotide**	**EIIP**
A	A	0.1260
G	G	0.8606
T	T	0.1335
C	C	0.1340
Amino Acid	**Amino Acid**	**EIIP**
Leu	L	0.0000
Ile	I	0.0000
Asn	N	0.0036
Gly	G	0.0050
Val	V	0.0057
Glu	E	0.0058
Pro	P	0.0198
His	H	0.0242
Lys	K	0.0371
Ala	A	0.0373
Tyr	Y	0.0516
Trp	W	0.0548
Gln	Q	0.0761
Met	M	0.0823
Ser	S	0.0829
Cys	C	0.0829
Thr	T	0.0941
Phe	F	0.0946
Arg	R	0.0959
Asp	D	0.1263

Table 5. The Electron-Ion Interaction Potential (EIIP) Values for Nucleotides and Amino Acids

In previous studies (Cosic et al. 1986; 1989, Cosic 1990, 1994, 1997, 2004, 2005, 2006), EIIP of each amino acid residue was employed in the RRM approach. This numerical series was then converted into a discrete Fourier spectrum, which carried the same information content about the arrangement of amino acids in the sequence as the original numerical sequence (Cosic 1997).

Approaches similar to the RRM, based on the Fourier transform and physical characteristics of amino acids, have been successfully applied by Mandell who has shown that the characteristic hydrophobic mass energy Fourier modes are signatures of isomorphism and immu-

nological reactivates (Mandell 1988). Viari et al. (1990) have used the RRM approach with scale independent coding to localize biologically relevant patterns in calcium-binding proteins. Cosic (1993) shows that concept of RRM could be connected with concepts of resonance in living cells and organisms (Frolics 1985) and with a concept of soliton and exciton transfer in proteins (Ciblis and Cosic 1997). Furthermore it was shown (Cosic 1993) that the proteins and their targets (another protein) have the same characteristic frequency. Thus, it can be postulated that the RRM frequencies characterise not only general function but also recognition and interaction between a group of proteins. This interaction can be considered as a resonant energy transfer between interacting molecules. Cosic (1990,1994) estimated the frequency range for protein interactions as $10^{13} - 10^{15}$ Hz. Analysing group of light absorbing proteins it has been found a linear correlation between RRM frequencies and wave number of the light effecting monitored proteins. In the chapter VII this result will be analysed in deep.

5.1 Definition of Common Frequency Characteristics

The RRM is a physico mathematical model. It interprets the protein's sequence linear information using signal analysis methods. It comprises two stages. The first involves the transformation of the amino acid sequence into a numerical sequence. Each amino acid is represented by the value of the electron-ion interaction potential

Numerical series obtained in this way are then analysed by digital signal analysis methods in order to extract information pertinent to the biological function. The original numerical sequence is transformed to the frequency domain using the discrete Fourier transform (DFT). As the average distance between amino acid residues in a polypeptide chain is about 3.8 Å, it can be assumed that the points in the numerical sequence derived are equidistant. For further numerical analysis, the distance between points in these numerical sequences is set at an arbitrary value d=1. Then, the maximum frequency in the spectrum is $F = d/2 = 0.5$. The total number of points in the sequence influences the resolution of the spectrum only. Thus, for *N*-point sequence the resolution in the spectrum is equal to *1/N*. The n^{th} point in the spectral function corresponds to the frequency $f = n/N$ (Lyons 2004).

In order to extract common spectral characteristics of sequences having the same or similar biological function, the following cross-spectral function was used:

$$Sn = XnYn^* \quad n = 1,2,..,N/2$$

Where: *Xn* are the DFT coefficients of the series *x(m)* and *Yn** are complex conjugate DFT coefficients of the series *y(m).* Peak frequencies in the amplitude cross-spectral function define common frequency components of the two sequences analysed (Cosic 1993).

The block diagram of the whole procedure: **protein sequence → numerical series → amplitude spectra → cross-spectra** is shown in Figure 23:

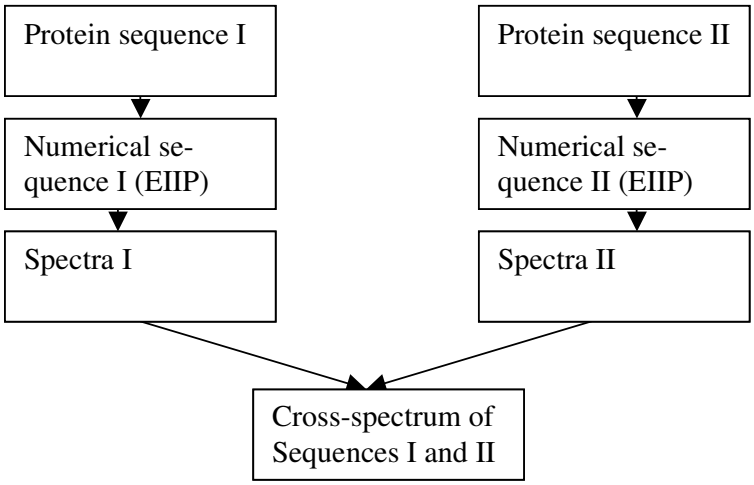


Figure 23. The simplified presentation of the algorithm for calculating resonant frequencies.

Figure 24 presents graphically the process of forming cross spectra using the sequences of α and β human haemoglobins; obtained by replacing every amino acid with its EIIP value. The prominent peaks denote common frequency components. The abscissa represents RRM frequencies, and the ordinate is the normalized intensity.

To determine the common frequency components for a group of protein sequences, the absolute values of multiple cross-spectral function coefficients *M* have been calculated as follows:

$$|M_n| = |X_{1n}| \cdot |X_{2n}| \Lambda \; |X_{Mn}| \quad n = 1,2,\mathrm{K}\; N/2$$

Peak frequencies in such a multiple cross-spectral function denote common frequency components for all sequences analysed. Signal-to-noise ratio (S/N) for each peak is defined as a measure of similarity between sequences analysed (Cosic 1997). The S/N is calculated as the ratio between signal intensity at the particular peak frequency and the mean value over the whole spectrum. The extensive experience gained from previous research suggests that an S/N ratio of at least 20 (Cosic 1997) can be considered significant. The multiple cross-spectral

function for a large group of sequences with the same biological function has been named "consensus spectrum". The presence of a peak frequency with a significant S/N ratio in the consensus spectrum implies that all of the analysed sequences within the group have one frequency component in common (Cosic et al. 1986; 1994; 1997). This frequency is related to the biological function provided the following RRM criteria are met:

A: One peak only exists for a group of protein sequences sharing the same biological function

B: No significant peak exists for biologically unrelated protein sequences

C: Peak frequencies are different for different biological functions.

In previous studies, the above criteria have been tested with over 1000 proteins from 25 functional groups (Cosic 1994). The regulatory DNA sequences were analysed in the same way. The following fundamental conclusion was drawn from these studies: each specific biological function of protein or regulatory DNA sequence is characterised by a single frequency.

HUMAN α-HEMOGLOBIN
VLSPADKTNVKAAWGKVGAHAGEYGAEALER
MFLSFPTTKTYFPHFDLSHGSAQVKGHGKKVAD
ALTNAVAHVDDMPNALSALSDLHAHKLRVDPV
NFKLLSHCLLVTLAAHLPAEFTPAVHASLDKFLA
SVSTVLTSKYR

HUMAN β-HEMOGLOBIN
VHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVV
YPWTQRFFESFGDLSTPDAVMGNPKVKAHGKKVL
GAFSDGLAHLDNLKGTFATLSELHCDKLHVDPEN
FRLLGNVLVCVLAHHFGKEFTPPVQAAYQKVVAGV
ANALAHKYH

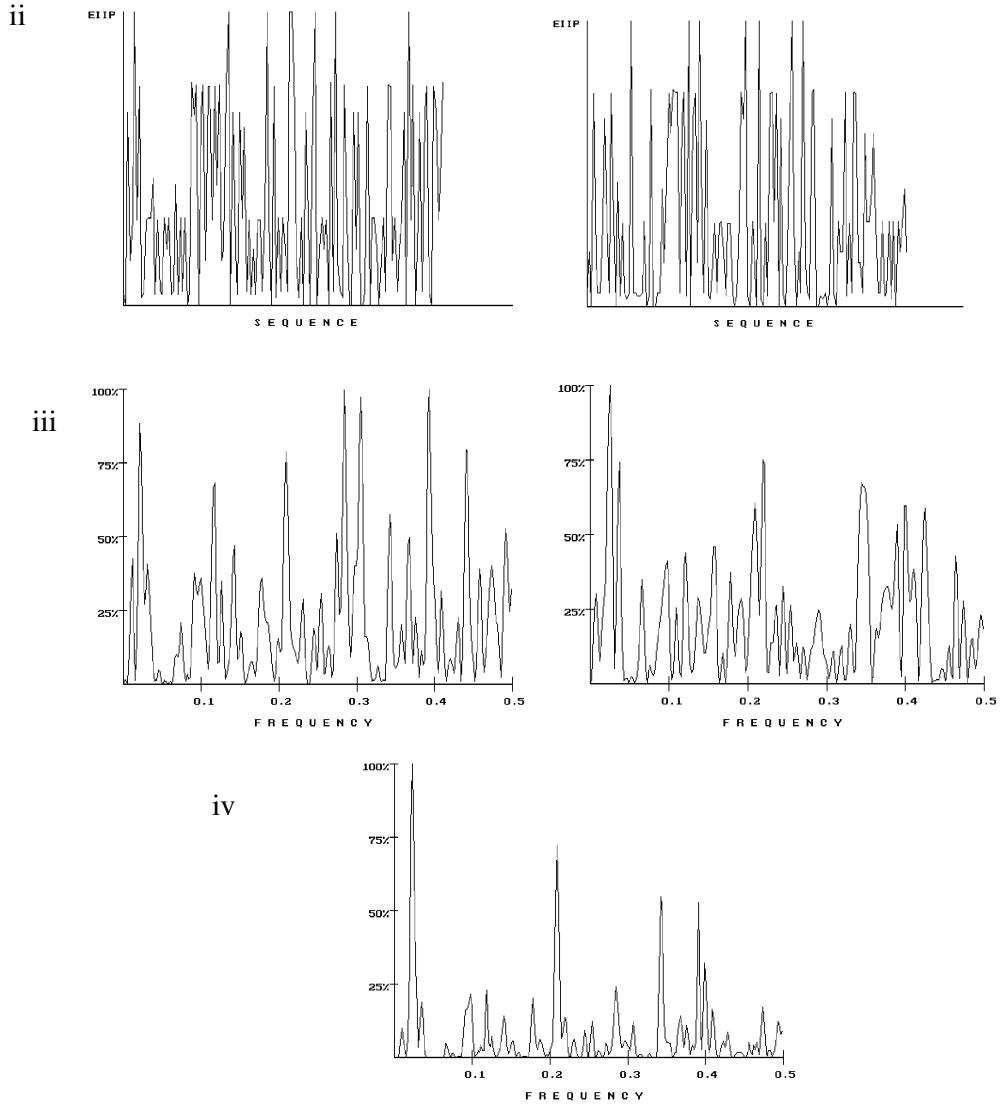


Figure 24. Graphical presentation of the RRM procedure in calculation of the common frequencies for alpha and beta chain of haemoglobin (i) is the letters presentation of amino acid sequences; (ii) Signal form from the EIIP values of amino acids, (iii) Fourier transforms of a signal; (iv) Cross spectral function calculated for analysed haemoglobin chains. (Cosic 1997).

The RRM also provides a method to identify the individual amino acids that contribute mostly to a protein's specific biological function. Since the characteristic frequency correlates with the biological function, the positions of the amino acids that are most affected by the change of amplitude at the frequencies and consequently to the corresponding biological function. The inverse Fourier transform is used in this determining process. This "hot spots" identification method has been applied already to a number of examples. The previous studies with interleukin 2, SV40 enhancer, Ha-ras p21 oncogene product, glucagons, Cytochrome C, haemoglobins, myoglobins and lysozymes all have documented evidence that such predicted amino acids are found to be spatially clustered in the protein tertiary structure and to be positioned in and around the protein active site in some of those examples (Cosic 1997).

Protein Group	f_{RRM}	Protein-Group	f_{RRM}
ACH receptors	.4922	NGF	.4040
Grow Factors	.4922	Proteases	.3770
Actins	.4800	Myosins	.3400
Cytochrome	.4765	Lysozymes	.3281
Protease	.4609	Glukagon	.3203
FGF	.4512	Chymotripsin	.2363
Fibrogenes	.4423	IGF	.1602
Kineases	.4297	Signal proteins	.1406
Amilases	.4121	Represors	.0990
NGF	.4040	Interferons	.0820
Proteases	.3770	Myoglobins	.0820
Myosins	.3400	Neurotoxins	.0703
Lysozymes	.3281	Cytochrome B	.0590
Glukagon	.3203	Phospholipase	.0430
Chymotripsin	.2363	Hemoglobins	.0234

Table 6. The frequency of the most prominent peaks calculated for the various groups of proteins

It was postulated (Cosic 1997) that difference in the potentials between NH2 and COO terminal in proteins can cause harmonical oscillations in a protein chain. The maximal speed of electrons bounded in π orbital belonging to the peptide bonds may be calculated as follow:

$$W = W_{COOH} - W_{NH_2} = 0.13Ry$$

$$eW = \frac{mV_{\max}^2}{2} \Rightarrow V_{\max} = \sqrt[2]{\frac{2eW}{m_e}}$$

$$\Rightarrow V_{\max} \leq 10^5 \, m\,s^{-1}$$

W is the difference in potentials between the ends of the protein

V is the velocity of the electron m_e is the mass of electron

Ry is a unit of energy in Rydbergs equal to the ionization potential of hydrogen (13.5978eV)

Assuming that distance between subsequent $C\alpha$ atoms along peptide chain is 3.8A, it is possible to calculate wavelength of EMR that will be absorbed the best.

As follow:

$$f_{\max} \leq \frac{V_{\max}}{2d} \cong 10^{15} \, Hz$$

or in wavelength presentation:

$$\lambda_{\min} \geq 330 \text{ nm}$$

Similarly $\lambda_{\max} < 30000$ nm

These frequencies cover UV, Visible and IR range (Cosic 1994, 1997).

5.2 Signal-Noise Ratio Normalization

The frequency characteristic for an observed biological function is defined as the prominent peak frequency in the multiple cross-spectral function of the family of proteins having this function in common. This prominent peak denotes a common frequency component for all analysed protein sequences. Any common frequency component can be considered as a characteristic of the observed function providing certain criteria be satisfied. To quantify those criteria it is important initially to define the index, which will be a measure of the level of commonality for any frequency component in the cross-spectral function. However, this definition of S/N is sensitive to the protein signal length and the number of sequences that computed in

the multiple cross-spectral function. Thus, it is not quite suitable for the development of the general criteria for a variety of protein families. There was a need to define S/N as a general comparable index that could be consistent and universally applied to the system under examination. This normalized S/N index has to be independent of the sequence length and the number of sequences analysed. This index should measure the confidence of determining the characteristic frequency of a biological function. After an empirical and statistical testing, a new normalized S/N ratio has been introduced to avoid the problem discussed above (Cosic 1994, 1997).

All these results have shown, the RRM represents a whole new view to protein activity, in particular protein-protein and protein-DNA interactions. The underlying hypothesis of this model is that certain periodicities in the distribution of energy of delocalised electrons along the protein molecule are critical parameters for protein biological function. This model allows extract the linear information contained in amino acid sequence and also provides a physical explanation of macromolecule's interaction processes. With a characteristic frequency identified by the RRM, it is possible then to design peptides of different length having desired periodicities in their distribution of energies of delocalised electrons along their sequence (Cosic 1994). Thus, the RRM can identify signals that characterize protein biological functions. Applications of the RRM are mostly in the area of biotechnology, drug design and pharmacology.

5.3 Coherent excitations.

Most large physical systems have vibrational modes characterized by a coherent motion of many constituent parts of the system. (Frohlich 1986, Popp 1994). It is very likely that biological systems such as cell membranes, macrobiomolecules, also have vibration modes, which will couple with EMR.

Primarily Frohlich considered the cell membranes, which display an electric potential of about 100 mV across a thickness of about 10^{-6} *cm.* This gives field strength of about 10^{5} V/cm, and

it is of the order of the break-through amplitude of an electric field in air. Frohlich suggested that by means of strong fluctuations of this field and its coupling with the highly polarisable membrane, typical oscillations with frequencies equal ***d/v*** where ***d*** is thickness of the membrane (about $10^{-6} cm$) and ***v*** is velosity of sound wave or $10^{11} - 10^{12} Hz$.

Regarding a protein as a large set of semi ordered dipoles (C-N part of peptide bond). In organised protein's structure such as $\alpha - helix$ or $\beta - helix$. System of dipoles will be arranged on ordered way. Then, it is possible that the interaction between dipoles manifest itself in vibration excitations. In addition, each dipole also interact with all other dipoles via electromagnetic forces. Other types of interaction mediated by that structure are also possible. The net result of these mutual interactions is to spread the frequency into narrow band. Frohlich (1986) the most probably.

Chapter 3

Electromagnetic radiation, RRM and Enzyme activity

6.1 Biscar's Experiment

Until now only a few documented experiments have been published regarding the change of the activity of enzymes (that are naturally not bounded with a chromophores or metal ions) measured upon irradiation with the external EMF in the range (400-1000 nm). Biscar (1976) attempted to confirm experimentally that the $\alpha-$chymotrypsin, which was exposed to the EMR within the range [800-900 nm], changed the activity. Biscar developed the model called "The electromagnetic molecular electronic resonance" (EMER), to predict the wavelength of the EMR capable of alternating the $\alpha-$chymotrypsin's function. A quantum electronics phenomenon "Electromagnetic Molecular Electronic Resonance" (EMER) is due to a molecular electronic density perturbation wave propagating along bonding electrons of a long chain polymer (Biscar 1974). The $\alpha-$chymotrypsin consist of two chains. Biscar claimed that the relation between chain's lengths and coupling positions suggest a possible energy transfer, at the EMER frequencies, from one chain to the other.

Biscar speculated that the evidence of $\alpha-$chymotrypsin, being activated by a photon, indicates a possibility that enzymes use the light energy, which is corresponding to EMER frequencies, for their function. By contrast to the molecular vibration modes that

are due to the motion of the nuclei with respect to the bonding electronic system (Daune 1999), EMER is the phenomenon where the bonding electronic orbitales oscillate in a collective electromagnetic mode with respect to the nuclei.

For practical calculations, the EMER frequencies are given by the standing wave equation:

$$P_r P_c P_{ol} = m\frac{\lambda}{2}$$

in which P_0 is the propagation constant for a stretched out polymer, *Pc* is the relative propagation constant (*Pc* = 1 for the stretched out form) introduced by the conformation; P_r is the relative propagation constant describing the effect of added adjacent molecules (such as water, etc.) to the propagation; λ is the vacuum wavelength corresponding to the EMER frequencies obtained for m= 1,2,3 …N; L is the stretched chain length of the molecule. The factor ½ indicates that free end boundary conditions are considered in such a way that the whole chain resonates as $\lambda/2, 2\lambda/2, 3\lambda/2\text{K}\ m\lambda/2$. Biscar calculated wavelengths of EMR, which could activate enzymes such as polyglutamic acid (Biscar, 1973a) and bovine serine albumin. However, he experimentally investigated only $\alpha-$chymotrypsin.

Applying the concept of the standing wave, and using only the length and weight of the polypeptide chain as the parameters in calculation, Biscar found that the third harmonic in EMER spectrum should be within the range [800-900nm]. He found that the activity of the $\alpha-$chymotypsin is unaffected by EMR with wavelength outside of the 850-860 nm range, while, inside this range, the activity was increased by more than a factor of 2 (at 855 nm). The activation peak has a line width of approximately 10 nm at half amplitude as it is shown in Figure 25.

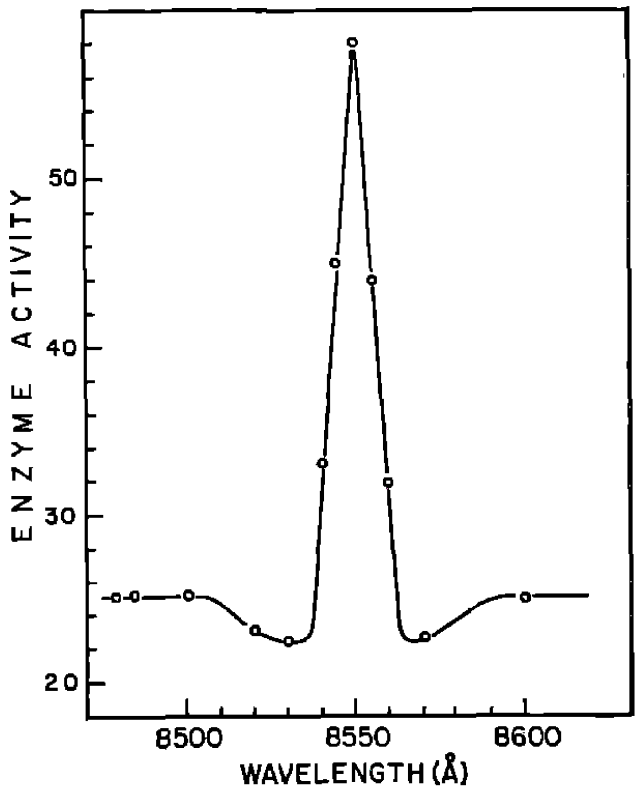


Figure 25. The activity of α-chymotrypsin is displayed versus the wavelength of the exciting near infrared light. It was established an activity increase by more than a factor of 2 at 855nm (Biscar 1976).

6.2 Biscar experiment and Resonant Recognition Model

Biscar consider dynamical resonant behaviour of a protein as a function of its mass and length. The specific characteristics of the amino acids are disregarded. Nevertheless, the RRM (Chapter IV) does regard both the physical parameters of the amino acids and the allocation of the amino acids along the chain. In the articles (Cosic 1993, 1994) is shown that cross spectral analysis of five $\alpha-$chymotypsins (bovine, human, rat, hornet and vasp) revealed common RRM frequency (Figure 26) at $f_{RRM}=0.2363\pm0.004$. This frequency component can be considered as a specific characteristic of chymotrypsin activation rather than of its protease activity. In comparison with the other proteases a different frequency $f_{RRM}=0.377\pm0.004$ was obtained as general protease characteristic (Cosic 1993, 1994). Ac-

cording to the RRM the wavelength of the EMF corresponding to the RRM frequency f_{RRM} =0.377 is 851 ± 15 nm. This result explicitly supports the idea that the RRM characteristic frequencies represent specific electromagnetic oscillations within the near infrared and visible range, which are crucial for the protein activity and interactions.

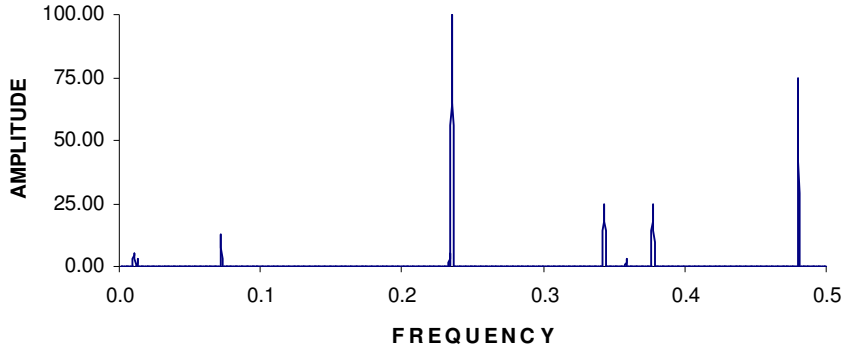


Figure 26. Multiple cross spectral function calculated for a group of five chymotripsins (Cosic 1993).

6.3 The RRM and photosensitive proteins

The evidence of the relationship between the absorption characteristics of the photosensitive proteins and their RRM spectral functions where revealed in the comparative studies. (Cosic 1989). The Table 7 shows absorption maxima for the group of photosensitive proteins together with their RRM frequencies corresponding to the prominent peaks in RRM spectra. Moreover, the investigations demonstrated similarities in the response of the cell cultures to EMR at selective wavelength in the range (400-1000nm) and the response of the cell cultures to the specific growth factors, on normal diploid cells (Cosic 1989,1994, 1997).

The correlation between RRM frequencies and wavelength of EMR that activate photosensitive proteins, can be even used to predict wavelength of EMR that has stimulating effects on cells proliferation.

The RRM spectra calculated for the growth regulation factor i.e. IGF-Is, FGF, CFS, EGF, GHs, Insuline, PDGF_B and PDGF_A are shown at Figures 27a,b,c,d,e. The RRM frequencies of the prominent peaks of the same proteins are shown at Table 8, together with experimentally predicted activating wavelengths of EMR.

Protein Group	**Wavelength (nm)**	**RRM frequency**	**K**
Cyt .C	**415 ± 20**	**0.437 ± 0.003**	**188-209**
Absorbing blue	**430 ± 25**	**0.475 ± 0.004**	**188-215**
Absorbing Green	**540 ± 30**	**0.355 ± 0.004**	**177-203**
Absorbing Red	**570 ± 30**	**0.346 ± 0.004**	**185-210**
Haemoglobins	**14770 ± 30**	**0.023 ± 0.007**	**235-400**
Absorbing Purple	**860 ± 35**	**0.281 ± 0.02**	**212-258**
Flavo	**470 ± 30**	**0.379 ± 0.004**	**165-192**

Table 7. The groups are formed according to their characteristic absorption maxima: cyt C (cytochromes C); blue (group of proteins absorbing blue light: blue-light absorbing rhodopsins and bioluminescent proteins); green (green-light absorbing chlorophylls and rhodopsin); red (red-light absorbing rhodopsin); haem (haemoglobins); purple (purple-light absorbing pigments from purple bacteria); flavo (flavodoxins). The second column represents the wavelengths (nm) of characteristic absorption of the groups of photosensitive proteins, while the third represents the same data in cm -1 units. The fourth column contains characteristic RRM frequencies f of the same groups of proteins and the fifth column shows the range of coefficients K (nm), the scaling factors between the numerical frequency space of RRM and the wavelength A (nm) of real frequency space: A=k/f. The last column gives the mean values of the coefficient K for each group of proteins (K). The mean value of these coefficients is Km=201 with a standard deviation of 15 per cent. This compares with a coefficient of 196 derived from the linear relation plotted in figure 28, and leading to a maximum frequency $f_{\max}$., =25,500 cm^{-1}, corresponding to a minimum effective wavelength of 392 nm (Cosic 1989)

In the Table 8 is shown Correlation between characteristic RRM frequencies of five groups of growth regulating factors and the low-intensity light irradiation frequencies, which produce significant effects on cell growth. The wavelengths of low-intensity light that would affect the cell growth are calculated as: $\lambda = K/f_{RRM}$, where K is the coefficient relating the inverse of the numerical RRM spectral frequencies, while f_{RRM} is the RRM frequency characteristic of the particular group the of growth factors. It is evident that the wavelengths predicted by the RRM, are experimentally verified, within calculation and experimental errors, as it is shown in the Table 8.

Growth factor	Effect observed	Charact. RRM frequency	Theoretical/ Expected	Experimental Value
IGFs	DNA syn. (HeLa)	0.492 ± 0.008	406.5(6.5)	400
FGFs	DNA syn. (E.coly)	0.453 ± 0.004	441	441
Insulins	DNA syn. (E.coly)	0.344 ± 0.16	581(10)	552
(EGF, SCF, GHs)	DNA syn. (E.coly)	0.293 ± 0.016	686(35.4)	650
PDGFs	Therapeutic effects	0.242 ± 0.008	826(26)	830

Table 8. Correlation between characteristic RRM frequencies of five groups of growth regulating factors and the low-intensity light irradiation frequencies, which produce significant effects on cell growth. The fourth column lists the wavelengths related to the RRM frequencies given in column three which was calculated according to the correlation shown on table 8 and figure 28: $\lambda \approx 201/f$. The effects of low-intensity laser radiation on cell proliferation in vitro and in vivo (via DNA synthesis or the therapeutic effect in wound healing), as well as the effect observed on ATP synthesis in bacterial cells, are indicated in the second column, while the wavelength and frequency for maximum effect in each case are given in the last two columns (Cosic 1989)

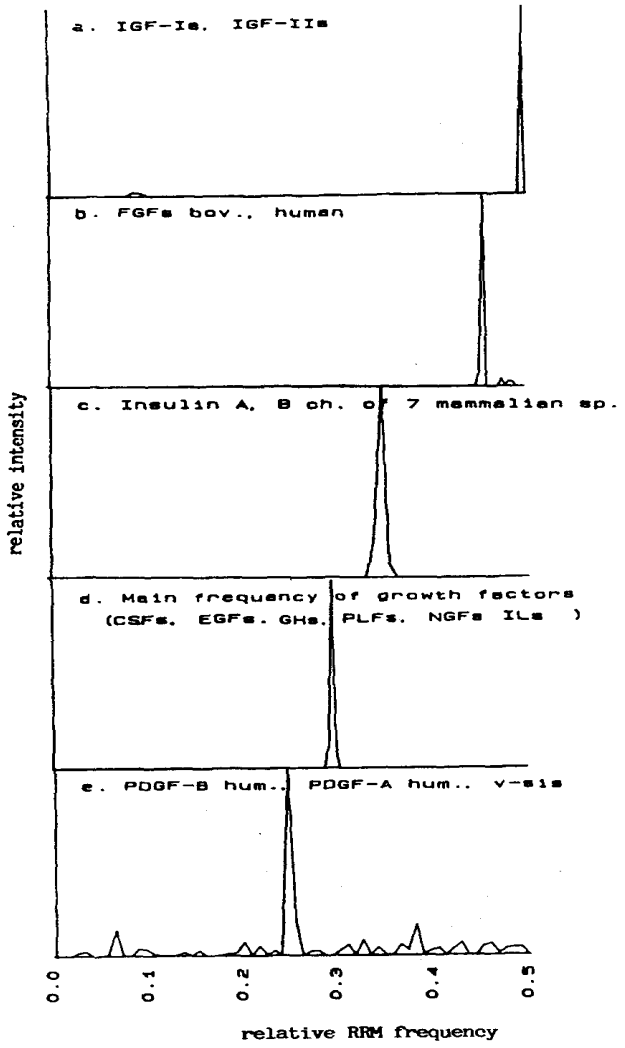


Figure 27. Consensus spectra of the five groups of growth-regulating factors. For each spectrum the abscissa represents the RRM frequency (in the range 0-0.5), while the ordinate is the relative intensity of the spectral features (in arbitrary units). (a) Consensus spectrum of IGFs (IGF-Is and IGF-II of human, bovine, mouse and rat origin); (b) consensus spectrum of acidic and basic form of FGFs of bovine and ' human origin; (c) consensus spectrum of insulin A and B chains from seven mammalian species; (d) consensus spectrum of CSFs, EGFs, GHs, PLFs, NGFs IL-2s, IL-4s, BSF-2, and IL-Is from several species (the main RRM frequency characteristic of the growth factors); (e) consensus spectrum of human PDGF B and A chains and v-sis. a. IGF-XS. IGF-11M p. FGFS bovine (Cosic 1989)

A summary of the results given in the Table 8 and 9 is presented graphically in Figure 28, where the abscissa represents RRM frequency (dimensionless), and the ordinate is real frequency space (cm^{-1}).

Figure 29 present the correlation between RRM frequency space and real frequency space. The photosensitive proteins are denoted by a square. The grow factors are represented by

filled diamond. A linear correlation between RRM frequency space and real frequency space is evident (Cosic 1989, 1994, 1997).

A linear correlation between real frequency space and RRM frequency space is evident. Thus the correlations established between RRM numerical spectra and the biological function of peptide growth factors on the one hand, and RRM numerical spectra and light absorption characteristics of photosensitive proteins on the other, appear to be subsume in the correlation between RRM frequencies and spectra of low-intensity light-induced biological growth stimulation.

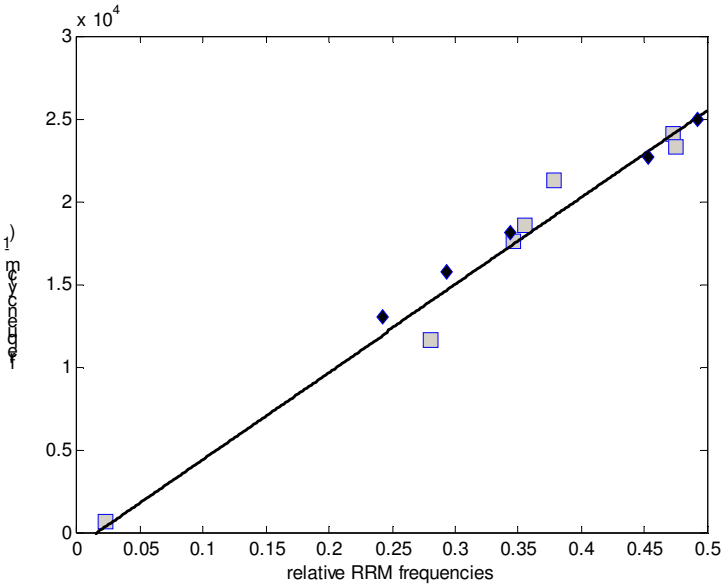


Figure 28. Graphical presentation of the correlation between RRM frequency space and real frequency space. The abscissa represents the dimensionless RRM frequencies (in the range 0-0.5) while the ordinate represents real frequencies (cm^{-1}) in the visible and near-infrared region of the electromagnetic spectrum. The photosensitive proteins are denoted by a square. The grow factors are represented by filled diamond. A linear correlation between RRM frequency space and real frequency space is evident. (Cosic et al. 1989)

Chapter VII

ABSORPTION SPECTROSCOPY

7.1 Absorption spectroscopy

The electronic structure of an atom can be described by quantum mechanical wave equation, in which electrons are considered to occupy orbital around nucleus. Covalent bond is formed when an electron pair is shared between atoms. According to the valence bond theory (Atkins 1983), electron sharing among atoms occurs by overlapping of two atomic orbital. According to the molecular orbital (MO) theory, bonds result from the combination of atomic orbital to give molecular orbital, which belong to the entire molecule. Bond that have circular cross-section and are formed by head-on interaction are called **sigma** (σ) **bonds**; bond formed by sideways interaction of p orbital are called **pi** (π) **bond**. Valence electrons that are not used for bonding are called **nonbonding electrons.**

The visible and ultraviolet spectra of organic compounds are associated with transition between electronic energy levels. The transitions are generally between a bonding or lone-pair electrons, and an unfiled non-bonding orbital. The wavelength of the absorption is then measure of the separation of the energy levels of the orbitals concerned (Williams and Fleming 1973).

Two empirical laws have been formulated about the absorption intensity. Lambert's law stated that the fraction of incident light absorbed is independent of the intensity of source.

Beer's law states that the absorption is proportional to the number of absorbing molecules (Williams and Fleming 1973).

It is useful to define absorbance of light of wavelength λ by a sample in terms of the percent transmittance. Since the amount of radiation absorbed can vary over an extremely wide range, it is useful to define absorbance logarithmically. The absorbance of a sample is defined in terms of percent transmission as follows:

$$\log_{10} \frac{I_0}{I} = \varepsilon l c$$

Where I_0 and I are the intensities of the incident and transmitted light respectively, l is the path length of the absorbing solution in centimetres, and c is the concentration in moles/litre. $Log_{10}(I_0 / I)$ is called the absorbance or optical density; ε is molar extinction coefficient.

The irradiation of organic molecules may or may not give rise to excitation of electrons from one orbital (usually bonding) to another orbital (usually non-bonding). It can been shown that

$$\varepsilon = 0.87 \times 10^{20} Pa$$

where P is called the transition probability and ***a*** is the target area of the absorbing system. The absorbing system is usually called a chromophore. There are many factors, which affect the transition probability of any particular transition. In the first place, there are rules about which transitions are allowed and which are forbidden.

The linearity of the Beer-Lambert law is limited by chemical and instrumental factors. Causes of nonlinearity include:

- deviations in absorptivity coefficients at high concentrations (>0.01M) due to electrostatic interactions between molecules in close proximity
- scattering of light due to particulates in the sample
- fluorescence or phosphorescence of the sample

- changes in refractive index at high concentration
- shifts in chemical equilibrium as a function of concentration
- non-monochromatic radiation, deviations can be minimized by using a relatively flat part of the absorption spectrum such as the maximum of an absorption band
- stray light

The absorption of UV or visible radiation corresponds to the excitation of outer electrons. There are three types of electronic transitions, which can be considered

1. Transitions involving π, σ, and *n* electrons
2. Transitions involving charge-transfer electrons
3. Transitions involving *d* and *f* electrons (Those transition are specific for metal bounding proteins and they will not be discussed in this thesis).

The transitions involving valence electrons are shown in Figure 29.

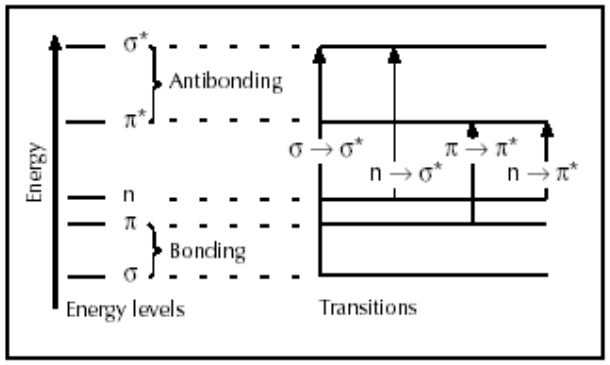


Figure 29. Possible *electronic* transitions in molecules. The $\sigma - \sigma^*$ requests the highest energy, and is not seen in typical UV-VIS spectra. (Faust 1992)

$\sigma \rightarrow \sigma^*$ Transitions

Absorption maxima due to $\sigma \rightarrow \sigma^*$ transitions, which appear usually at $\lambda \leq 200\ nm$, are not seen in typical UV-Vis. spectra (200 - 700 nm).

$n \rightarrow \sigma^*$ Transitions

Saturated compounds (Those that have only single C-C and C-H bond) containing atoms with lone pairs (non-bonding electrons) are capable of $n \rightarrow \sigma^*$ transitions. These transitions usually need less energy than $\sigma \rightarrow \sigma^*$ transitions. They can be initiated by light which wavelength is in the range of 150 - 250 nm. The number of organic functional groups with $n \rightarrow \sigma^*$ peaks in the UV region is small.

$n \rightarrow \pi^*$ Transition

This transition is usually very weak comparing with previous ones and this band is characteristical for peptide group peptide group.

$\pi \rightarrow \pi^*$ Transition

Transition is characteristic for aromatic amino acids: Phe, Tyr and Trp.

Charge - Transfer Absorption

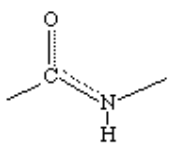
Many inorganic species show charge-transfer absorption and are called *charge-transfer complexes*. For a complex to demonstrate charge-transfer behaviour, one of its components must have electron donating properties and another component must be able to accept electrons. Molar absorbtivities of charge-transfer absorption are large (greater that 10,000 L mol^{-1} cm^{-1}).

The absorption of radiation then involves the transfer of an electron from the donor to an orbital associated with the acceptor.

7.2 Absorbance of proteins (UV-Visible) –electron excitations.

Protein absorbance comes from three sources**:**

Peptide Bond



π ⟶ π* transition is very intense at 190 nm (ε_{max} ~ 7000)

n ⟶ π* transition is weak as it is symmetry forbidden; it forms a shoulder on the π ⟶ π* transition (ε_{max} ~100) at 210-220 nm

Peptide bond has a very intense $\pi \rightarrow \pi^*$ transition at 190 nm and much weaker $n \rightarrow \pi^*$ transition at 210-220nm. The side chains formed from the amino acid: Asp, Glu, Asn, Gln, Arg, and His have absorption within the region (190-230 nm) but in this case, absorption is very weak compared to peptide bond $\pi \rightarrow \pi^*$. Absorption in the 190-230 nm region is used to quantitate protein and peptide concentrations

Aromatic Amino Acids

The most important chromophores amongst the amino acids are the amino acids having the aromatic rings: Phe, Tyr and Trp. The molar absortivity of them is shown in Figure 30.

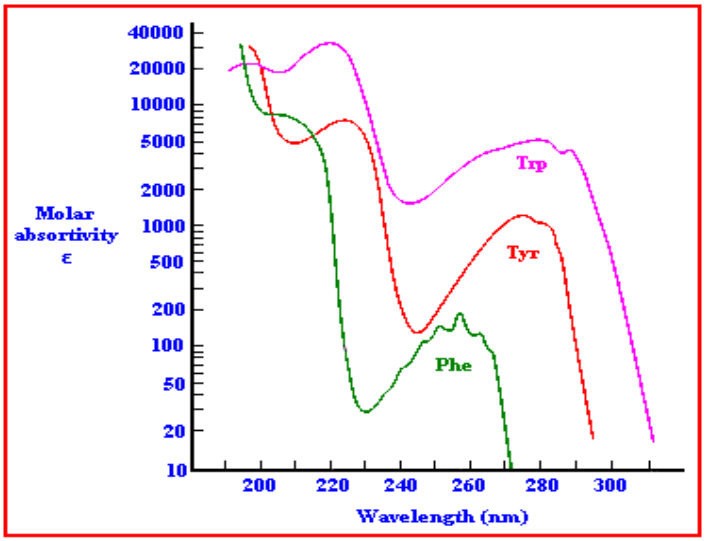


Figure 30. Molar absorptiveness for the aromatic amino acids

Chromophores and metal bounding proteins

Generally, polypeptide chains do not absorb visible light (360-700nm). However, a large number of proteins consist of polypeptide chain covalently bounded with a prosthetic group such as porphyries, metals, flavines, retinols etc. One example is rhodopsin and 11-cis retinol, which already was detailed in Chapter III. Long conjugated and polycyclic aromatic molecules can stretch a protein spectrum with many bands of proteins into the visible region.

It is important to notice that those absorption spectra can change due to solvent effects (Williams and Fleming 1973). This is for the reason that chromophores display shifted spectra upon increasing or decreasing polarity of their environment, with changes in wavelength of maximum absorbance and its molar extinction coefficient.

I

7.3 **ACTIVITY SPECTRA**

Action Spectroscopy.-problematic

Since the 19th century "Action spectroscopy" (AS) has been an important tool for investigations in the field of photobiology, (Kiefer 1986) particularly in investigation of the mechanism of plant physiology.

The AS can be defined as the process of measuring a biological effect caused by irradiation by an external EMF as a function of wavelength or frequency. Although in most of cases it is impossible to identify the molecules involved in the process being studied, the AS can be useful in obtaining important information about photoresponse.

For the successful design of the AS experiment the following steps have to be considered:

Preparation of samples containing a living matter i.e. molecules, cell organelles, cells, cultures or even organisms.

Selection of experimental variables whose alteration has to be investigated.

Design of the methodology for measurement of experimental variables.

If during the experiment the alteration of variables was detected it suggests that there are specific chromophores that can absorb in the tested region. However, the affected chromophore, which has an energy level that matches the energy of the incident photons, is not necessarily the one that might cause the final effect (Kiefer 1986).

One of the limiting factors in the design of AS experiments is potentially a very large number of measurements. Due to overlapped energy zones the molecular absorption peaks usually have "half-width" ranging from 1nm up to the tents of nanometres. Thus, a resolution of 1nm is reasonably sufficient for the monitoring. Even at this resolution, the number of measurements required to perform the study of protein activity and EMF in a range of (360-760nm) is about 400. This number is only for a single setting of a single variable. For the purpose of significance each of those measurements has to be repeated several times. The measured ac-

tivity is usually determinated by either the change of the concentration of a product or a yield of the product in a unit of time.

The slow reaction will severely affect the experimental time. For example, a single measurement of activity requires 6 hours, than the total time required for the experiment is 2400 hours or 300 full time working days. Even if a laboratory is equipped with more than one set of light sources the conduct of such an experiment would be very impractical.

The experiment designed in this thesis for the first time undertakes measurements of the AS using exposure by the EMR in the range (560-860 nm) with a resolution of 1-3nm.

Chapter VIII

LACTATE DEHYDOGENASE NADH AND PYRUVATE_LACTATE TRANSFORMATION

8.1 l-Lactate dehydrogenase

The choice of the enzyme is very important for the experimental design. Therefore, the following must be considered:

1. what is the availability of the enzyme for purchasing;
2. how well is the enzyme assay documented;
3. how many primary structures of the particular enzyme are sequenced from different sources, (prerequisite for the RRM computational analysis);
4. how well are know kinetics of the particular enzyme;

One of the enzymes suitable for the experiment was l-lactate dehydrogense (LDH). The characteristics of LDH considered are:

1. The kinetic of the LDH and conformation of an active site are very well known.
2. The LDH, substrates NADH, and pyruvate are not expensive and they are commercially available for purchasing.

3. The half-time of a degradation of the LDH and substrate is long enough not to 0influence the kinetics of the reaction.
4. The influence of all controllable experimental variables (concentrations, pH, and temperature) is well documented elsewhere.

As many as 178 different enzymes were sequenced from different organisms, ranging form bacteria to the animals. The number of different structures of LDH with a common function form very solid base for theoretical calculations using Resonant Recognition Model.

LDH is an efficient enzyme that catalyses the direct transfer of a hydride ion H^+ from the reduced nicotinamide group of NADH to the C_2 carbon of pyruvate. The LDH is accelerating the solution reaction by 14 orders of magnitude. Studies have shown that the reaction mechanism of LDH follows an ordered sequence (Chapter II). To reduce lactate, NADH must be bind to the LDH first and then pyruvate must be bind to the LDH as well.. Transfer of a hydride ion then happens quickly in either direction giving a mixture of the two ternary complexes, enzyme-NAD+-lactate and enzyme-NADH-pyruvate. The rate limiting step in this reaction is the rate of dissociation of NADH. (McClendon et al. 2005). Figure 31 shows the general reaction catalysed by l-lactate dehydrogenase (LDH).

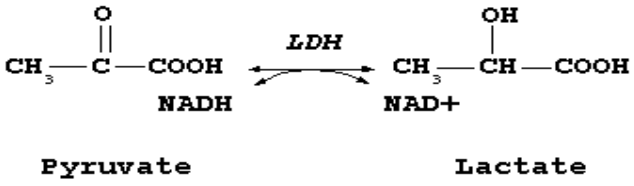


Figure 31. Reaction catalysed by LDH. Different LDH molecules favourite different direction of the reaction. LDH from rabbit hurt is dominantly oriented to left side of equilibrium.

The initial step in monitoring of the enzyme activity is the measurement of the rate of change of the concentration of the NADH. The measurement is performed by continuous spectrome-

try. The minimum exposure time necessary to achieve a response to EMR was defined upon completion of the pilot experiments.

The differences in the primary structure of LDH molecules have been investigated intensively. Some investigators thought that all LDH isozymes have arisen from a single LDH-A like enzyme (Tsuji et al. 1994). Tsuji found that the number of nucleotide substitutions, between various LDHB molecules, that leads to the change in amino acids, is ranging from two (between human and mouse LDH B) up to 23 (between human LDHB and African fog LDHB). For LDHA the number of nucleotide substitutions is up to 30 between human and dogfish LDHA. The number of nucleotide substitutions, between various bacteria, which lead to the change in amino acids in a LDH, is up to the hundred.

Primary structure of LDHB from the rabbit heart is taken from "SWISS PROT" (www.expasy.org/sprot). Initially sequence was published by Sass (1989). The primary sequencies of other LDH molecules analysed in this thesis are given in Appendix I.

***Primary structure*:**

ATLKEKLIAP VAEEEATVPN NKITVVGVGQ VGMACAISIL GKSLADELAL VDVLEDKLKG
EMMDLQHGSL FLQTPKIVAD KDYSVTANSK IVVVTAGVRQ QEGESRLNLV QRNVNVFKFI
IPQIVKYSPD CIIIVVSNPV DILTYVTWKL SGLPKHRVIG SGCNLDSARF RYLMAEKLGI
HPSSCHGWIL GEHGDSSVAV WSGVNVAGVS LQELNPEMGT DNDSENWKEV HKMVVESAYE
VIKLKGYTNW AIGLSVADLI ESMLKNLSRI HPVSTMVKGM YGIENEVFLS LPCILNARGL
TSVINQKLKD DEVAQLKKSA DTLWDIQKDL KDL

Tertiary structure of LDHB-from rabbit heart.

Tertiary structure data were calculated theoretically using X-rays crystallography with a resolution of 0.16 nm (http://www.rcsb.org/pdb/). Four units of LDHB are shown in Figure 32.

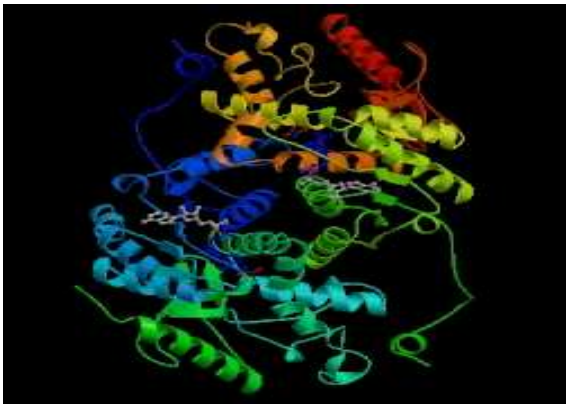


Figure 32. Lactate Dehydrogenase from biosys. The four units of LDH are presented spiral image represent alpha helixes.

The LDH is usually made of four subunits in the form of tetramer. The LDH subunits exist as two major structural forms, usually referred to as M (muscle) and H (heart) or A and B, respectively, which give rise to five different isozymes of the tetrameric molecule (Eventoff et al 1977) in higher vertebrates. The differences in the properties of the LDH isozymes are dependent on their subunit composition and are most exaggerated between the homotetramers M4 (LDH-5) and H4 (LDH-1). In addition, a recent (Zakhartsev et al. 2004) study reveals a negligible difference in their kinematic properties, for example maximal activity (Vmax) and Michaelis-Menten constant (Km). The active site of LDH-from rabbit heart is shown in Figure 32. Active ternary complex are the form shown in Figure 33. The carboxilate group of the substrate forms a salt bridge with the side chain of Arg-171. In addition, carbonyl group of pyruvate forms a hydrogen bond with the ring of His 195. The His-195 orients the pyruvat and stabilise the negative charge that develops on the oxygen atom on pyruvate. (Fersht 1985).

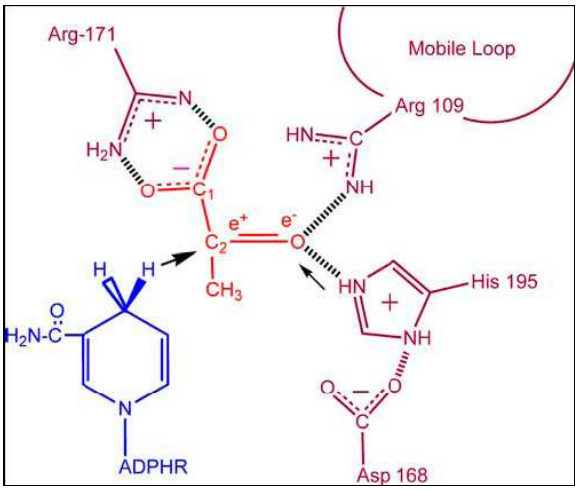


Figure 33. Active site of lactate dehydrogenase showing the relative arrangements of reacting groups. (McClendon, 2005).

Coenzymes NADH and NAD

Nicotinamide adenine dinucleotide (NAD+), shown in Figure 35, and reduced nicotinamide adenine dinucleotide (NADH), shown in Figure 34, are two important cofactors found in cells. The NADH is reduced form of NAD^+, and NAD^+ is the oxidized form of NADH. The NAD^+ is used extensively in glycolysis and the citric acid cycle of cellular respiration. The chemical energy stored in the NADH can be converted to adenosine tri-phosphate (ATP) through the electron transport chain or used for anabolic metabolism.

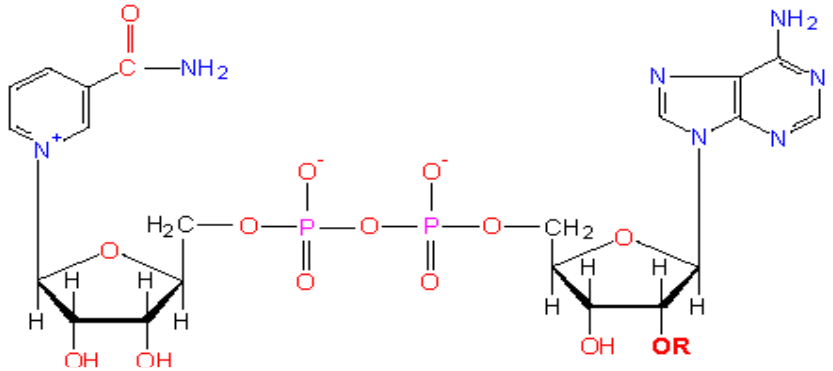


Figure 34. reduced form of NADH (Harris 1996)

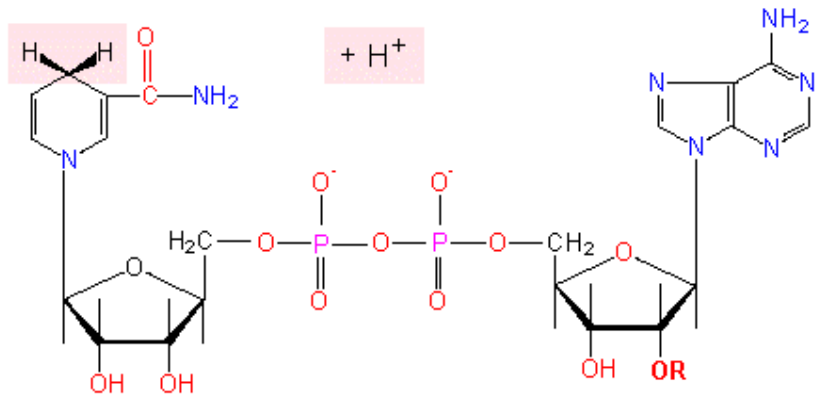


Figure 35. NAD or oxidised form of NADH (Harris 1996).

Pyruvate

Pyruvate is the anionic form of the three-carbon organic acid, pyruvic acid (CH3COCO2H). Pyruvate is a key intermediate in the both glycolytic and pyruvate dehydrogenase pathways, which are involved in biological energy production. Pyruvate is widely found in living organisms. It can be synthesized in the cells of the body.

8.2 ASSAY

The assay was chosen to satisfy following criteria:

- The stored enzyme does not change its activity during the conduction of the experiment.
- Time needed for catalysing of 90% of the substrate should not be longer then 10 min.
- Buffers do not include any substance that absorbs in visible/IR range and does not absorb at 340nm.

In accordance with above-mentioned prerequisites, the SIGMA assay (SIGMA No 63336) was chosen.

CONDITIONS: T = 27°C, pH = 6.0, A340nm, Light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

100 mM Sodium Acetate Buffer, pH 6.0 at 27°C (Prepare 100 ml in deionised water using Sodium Acetate, Trihydrate, Prod. No S-8625. Adjust to pH 6.0 at 30°C with 1 M Acetic Acid.)

250 mM Pyruvate Solution (Pyruvate) (Prepare 10 ml in deionised water using Pyruvic, Acid, Sodium Salt, Prod. No. P-2256)

13 mM ß-Nicotinamide Adenine Dinucleotide, Reduced Form Solution (ß-NADH) (Prepare 1 ml in cold deionised water using ß-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt, Prod. No. N-8129**).**

50 mM Potassium Phosphate Buffer, pH 7.5 at 30°C (Enzyme Diluent) (Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Prod. No. P-5379. Adjust to pH 7.5 at 30°C with 1 M KOH.)

PROCEDURE

1. Appropriate volumes of previously prepared solution of NADH, pyruvate and buffer were added to cuvette making 1.5 mL
2. Buffer solution with previously irradiated LDH 0.5 mL was added (to start reaction)
3. The content of cuvette was quickly mixed
4. Absorption at 340 nm was determined at 30 sec intervals for 600 sec.

*Final assay concentration in moles***.**

In a reaction mix, the final concentrations are 93 mM sodium acetate, 9.7 mM pyruvate, 0.25 mM ß-nicotinamide adenine dinucleotide, reduced form, 0.81 mM potassium phosphate, and 0.05 unit L-lactic dehydrogenase.

The Absorption experiment

The LDH kinetic was measured by a continuous monitoring of NADH absorption at 340nm. The protein concentration was determined by the extinction coefficient.

During the continuous monitoring of 10 minutes, each 10 sec the data from the detector of spectroscopy were registered to be used for the calculation of the absorption coefficient at 340 nm by using the following formula:

$$A_\lambda = -\log_{10}\left(\frac{S_\lambda - D_\lambda}{R_\lambda - D_\lambda}\right)$$

where A_λ is the absorption, S_λ is the sample intensity at wavelength λ, D_λ is the dark intensity at wavelength λ. R_λ is the reference intensity at$\tilde{}$ wavelength λ

CHAPTER IX

Experimental equipment and procedure

9.1 Experimental procedure

The experiment was designed to measure activity of LDH in several steps:

1) Irradiation of the solution containing the LDH and the buffer.
2) Preparation of the solution of NADH and pyruvate (Chapter VIII).
3) Mixing or irradiated LED with the solution from the step two.
4) Measurement of the optical density of the solution from the step III on 340 nm in time steps of 10 sec
5) Calculation of the rate of change of optical density for each wavelength of applied EMR.
6) Formation of Activity spectra and statistical analysis

The **equipment** and **optical** apparatus required to produce the monochromatic light suitable for irradiation is shown in Figure 36. The distance from the slit and the sample was 50 mm. During the measurement all other light's sources in the laboratory were switched off. The detector was connected with voltmeter (HP 36253) and it was used for additional control of beam characteristics. For example for the wavelengths $\lambda \geq 700\,nm$, it would not be possible to test just visually presence or not presence of the light beam, so a detector was used.

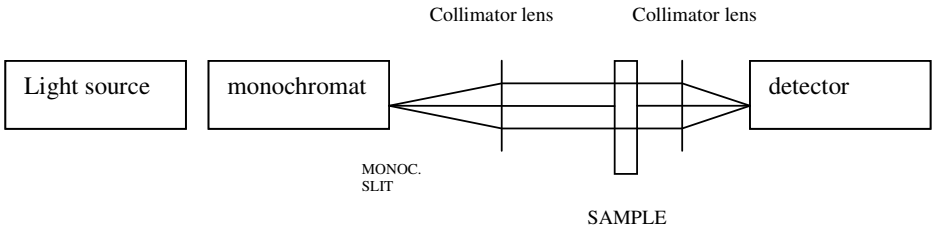


Figure 36. Laboratorial equipment. Source of EMR is tungsten lamp beam was directed to the monochromator SPEX 240. Beam was collimated and focused by lens *(f=50 mm).*

The power of EMR, is also a parameter of EMR that should be controlled in experiments with biogical objects. The standard radiometry term correlated to the power is an *Irradiance*. The *Irradiance* measure the power of electromagnetic radiation at a surface, per unit area. The term *irradiance* is used when the electromagnetic radiation is incident on the surface. The SI unit for irradiance is watts per square metre (W/m^2). The irradiance is a total amount of the radiation present, at all frequencies. It is also common to consider the irradiance for each frequency in the spectrum separately. In this case the irradiance is called *spectral irradiance*, and has SI units W/m^3, or commonly $W \cdot m^{-2} \cdot nm^{-1}$. In Figure 37 is shown the spectral irradiance by the tungsten lamp (OSRAM 6332), used in the experiment, at the distance of 5 cm from the source

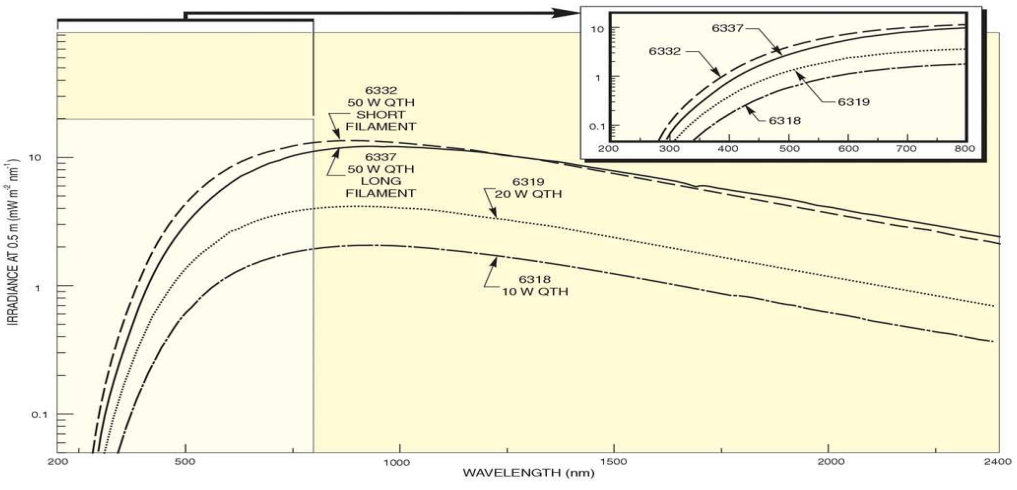


Figure 37. Spectral characteristic of tungsten lamp used for irradiation (6332). (Manual Osram 1997)

8.2 THE OPTICAL SYSTEM AND THE MONOCHROMATOR

The Monochromator and The Grafting

As a source of VIS/near IR it has been used monochromator SPEX 270M (Figure 38): 1200g/mm grating, focal length -270 mm, resolution - 0.1nm on 500nm, dispersion - 3.1 nm/mm, , RS232 connection with HP 34001A, controlled by LabView 6.1 (National Instruments).

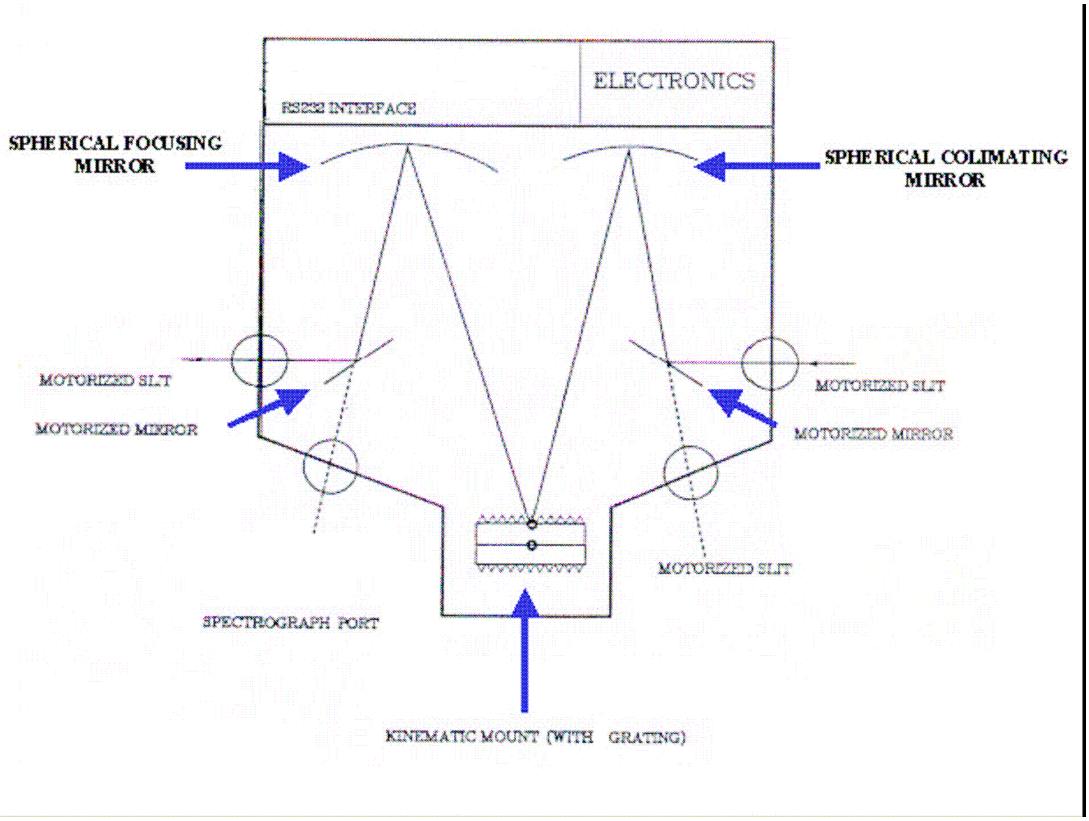


Figure 38. Monochromator SPEX 240 used in experiments.

Table shows the properties of the spectrometer.

Focal Length	0.27 m
Aperture	f=1/4
Mechanical scanning range	0-1100 nm (for a groove density of 1200 gr/mm)
Flat field	25 x 13mm (width x height)
Dispersion	3.1 nm/mm (for a groove density of 1200 gr/mm)
Resolution	0.1 nm at 546.07 nm
Accuracy	0.1 nm
Repeatability	0.05 nm
Drive step size	0.03125 nm
Scan Rate	70 nm/sec
Dimensions	39.4 x 41.4 x 19 cm (width x length x height)

At a groove density of 1200 gr/mm (grooves per mm), the scanning range would be 0-8800 nm and the dispersion would be 24.8 nm/mm. To achieve the resolution of approximately 0.1

nm 1200 gr/mm grating is required. For the given grating angle, the light of a very narrow bandwidth, centred at the given wavelength, can pass the exit slit. As additional equipment the collimator lens has been used and positioned at 5cm from the exit slit of the monochromator.

8.3 Spectrometer used in the experiment

To estimate the activity of the particular enzyme accurately, the rate of a change in the optical density of the solution was monitored for the period of time of 600 sec. During this time the absorption spectra were scanned each 10 seconds. In this experiment, the spectrometer USB2000 has been used (Figure 39). Spectrometer USB2000 has following characteristic: CCD detector with 2048 pixels; USB-2 connection with Pentium IV (Windows XP); controlled with OOIBase32 software. This spectrometer was connected with the light source USB-ISS-UV/VIS (Ocean Optics, Inc.) of a range 190nm-870nm,

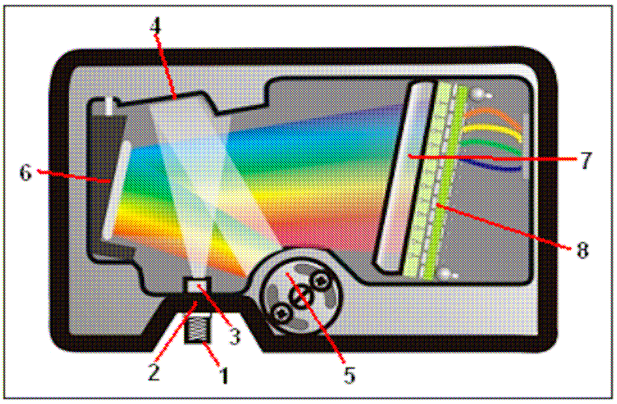


Figure 2-1: USB2000 Spectrometer with Components

Figure 39. Spectrometer USB2000 for measuring optical density of NADH in substrate (Ocean Optics manual)

The function of the particulars component are explained in Table 9

1	SMA Connector	The **SMA Connector** secures the input fiber to the spectrometer. Light from the input fibber enters the optical bench through this connector.
2	Slit	The **Slit** is a dark piece of material containing a rectangular aperture, which is mounted directly behind the SMA Connector. The size of the aperture regulates the amount of light that enters the optical bench and controls spectral resolution.
3	Filter	The **Filter** is a device that restricts optical radiation to pre-determined wavelength regions. Light passes through the **Filter** before entering the optical bench.
4	Collimating Mirror	The **Collimating Mirror** focuses light entering the optical bench towards the Grating of the spectrometer. Light enters the spectrometer, passes through the SMA Connector, Slit, and Filter, and then reflects off the **Collimating Mirror** onto the Grating.
5	Grating*	The **Grating** diffracts light from the Collimating Mirror and directs the diffracted light onto the Focusing Mirror. **Gratings** are available in different groove densities, allowing you to specify wavelength coverage and resolution in the spectrometer.
6	Focusing Mirror	The **Focusing Mirror** receives light reflected from the Grating and focuses the light onto the CCD Detector or L2 Detector Collection Lens (depending on the spectrometer configuration).
7	L2 Detector Collection Lens*	The **L2 Detector Collection Lens** (optional) attaches to the CCD Detector. It focuses light from a tall slit onto the shorter CCD Detector elements.
8	CCD Detector (UV or VIS)	The **CCD Detector** collects the light received from the Focusing Mirror or L2. Detector Collection Lens and converts the optical signal to a digital signal. Each pixel on the **CCD Detector** responds to the wavelength of light that strikes it, creating a digital response. The spectrometer then transmits the digital signal to the OOIBase32 application.

Table 9. Components of the spectrometer USB 2000

Chapter X

The RRM and l-LDH

The roles of the LDH in metabolic processes and the reasons for selecting of the LDH as the representative enzyme were detailed in the chapter IV. Despite the differences in the primary structures of analysed LDH sequences, LDH sequences have a similar active site and the common function (LDH catalyse the pyruvate to the lactate transformation and *vice versa*)

The primary structure is known for 176 different LDH molecules. (Swiss prot 2005). After filtering sequences that are non complete and sequences with ambiguities (missing amino acids etc.), the data set of 107 sequences was formed.

The list of the LDH molecules suitable for RRM analysis is given in Table 10 (46 LDHA, 24 LDHB, 7 LDHC, and 30 LDH from bacterial sources). The groups of LDH molecules are formed in regard to their structure, origin and function. Those groups are LDHA, LDHB, LDHC and bacterial LDH and they were made in accordance with standard practice (Tsuji 1994)

LDHA	LDHB	LDHC	LDHBacterial
LDHA_ALLMI	LDHB_ALLMI	LDHC_FUNH	LDH_ALCEU
LDHA_AMBMEt	LDHB_BRARE	LDHC_HUMAN	LDH_BACCL
LDHA_ANGRO	LDHB_CAICA	LDHC_MOUSE	LDH_BACHD
LDHA_BOVIN	LDHB_CHICK	LDHC_PIG	LDH_BACME
LDHA_BRARE	LDHB_COLLI	LDHC_RAT	LDH_BACST
LDHA_CAICA	LDHB_FUNHE	LDHC_VULVU	LDH_BACSU
LDHA_CHAAC	LDHB_FUNPA	LDHC_XENLA	LDH_BORBU
LDHA_CHAGU	LDHB_HORVU		LDH_CLOAB
LDHA_CHICK	LDHB_HUMAN		LDH_CLOPE
LDHA_COLLI	LDHB_MONDO		LDH_CORGL
LDHA_CORNI	LDHB_MOUSE		LDH_DEIRA
LDHA_CYPCA	LDHB_PELS		LDH_LACHE
LDHA_DISEL	LDHB_PIG		LDH_LACLC
LDHA_DISMA	LDHB_RABIT		LDH_LACPE
LDHA_ELEMC	LDHB_RAT		LDH_LACSK
LDHA_FUNHE	LDHB_RHIOR		LDH_LISIN
LDHA_GILMI	LDHB_SCEUN		LDH_LISMO
LDHA_GOBGI	LDHB_SCEWO		LDH_MYCGA
LDHA_HARAN	LDHB_SQUAC		LDH_MYCGE
LDHA_HORVU	LDHB_TRAS		LDH_MYCHY
LDHA_HUMAN			LDH_MYCPN
LDHA_LEPNU			LDH_MYCPU
LDHA_MACFAl			LDH_PEDAC
LDHA_MONDO			LDH_STRBO
LDHA_MOUSE			LDH_STRMU
LDHA_NOTAN			LDH_STRPN
LDHA_NOTCO			LDH_STRPY
LDHA_PARCR			LDH_STRTR
LDHA_PARMG			LDH_THEAQ
LDHA_PATTE			LDH_THECA
LDHA_PELSJ			
LDHA_PIG			
LDHA_PYTRG			
LDHA_RABIT			
LDHA_RAT			
LDHA_RHIDE			
LDHA_RHIOR			
LDHA_SCEUNl			
LDHA_SCEWO			
LDHA_SPHAG			
LDHA_SPHID			
LDHA_SPHLU			
LDHA_SQUAC			
LDHA_TRASC			
LDHA_XENLA			

Table 10. Enzymes used in calculation of RRM frequency

All of these sequences were analysed using RRM model as explained in the Chapter 5. The first step in RRM is translation of protein sequence into a sequence of numbers that are values of EIIP for each amino acid. For example for enzyme belonging to the group LDHB extracted from rabbit heart the corresponding numerical sequence is shown in Figure 40.

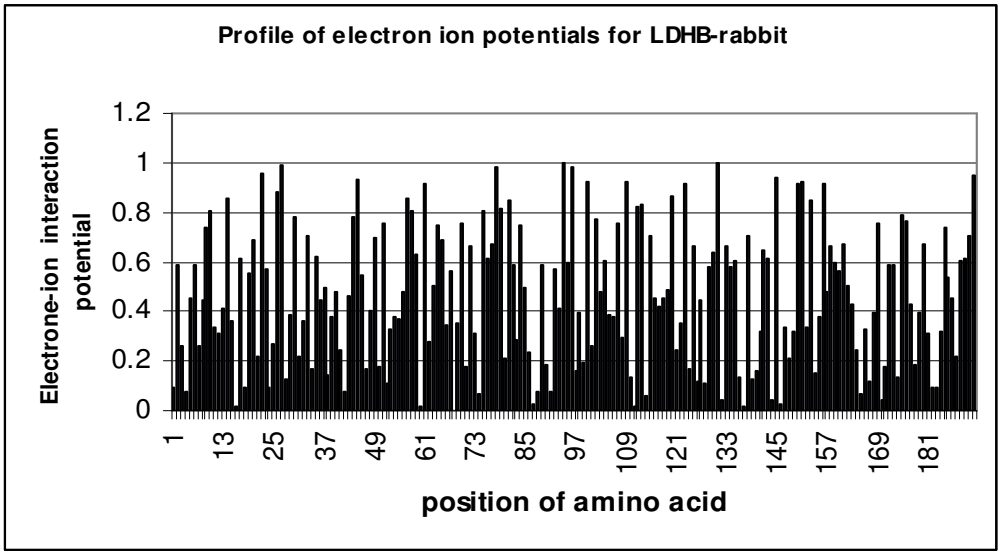


Figure 40. Values of EIIP calculated for the amino acids belonging to the LDHB-rabitt.

As RRM methodology uses FFT, it requests that analysed numerical sequences are normalised to the same length equal power of two. As LDH molecules do varies in size, from 210 to 380 amino acids so the length of sequences were adjusted to the size of 512, by standard procedure of zero padding (Lyons 2004).

The RRM multiple cross spectral function calculated for each group of the LDH enzymes are shown in figure 42a,b and Figure 43a,b. The RRM multiple-cross spectral function can be regarded as a measurement of the similarity among different protein's sequences in the frequency domain when each protein sequence is treated as a numerical series (Cosic 1994, 1997). Thus, the most prominent peak in the RRM multiple-cross spectral function illustrate the spectral similarity of the protein sequences. In addition, it has been suggested that prominent peaks in multiple cross spectral function point towards the common function of the whole group of proteins (Cosic et al. 1989, Cosic 1993, Cosic et al. 1997; Pirogova et al. 2003, 2006). Within the RRM, it has been proposed (Cosic 1997) that prominent peaks in such spectral function are related to the optical characteristics of the proteins (Cosic et al 1989, Cosic 1993, Vojisavljevic et al 2006, 2007). Thus, it may be expected that selective frequencies of the EMF can initiate the change in internal dynamics of irradiated proteins. For

enzymes, such as the LDH, the change in internal dynamics can lead to the change in speed of the reaction (The speed of the reaction can be portrayed by the number of pyruvate to lactate conversions per molecule per second).

Cosic et al (1989, 1997, and 2003) and Vojisavljevic et al. (2007) did show that it was possible to connect RRM frequency of the LDH enzyme with the frequency of the EMR, which affects the activity of the LDH. The correlation between the RRM frequencies and wavelengths of EMR was demonstrated also for rhodopsins, flavoproteins, haemoglobins, α-chymotripsin (Cosic 1989).

Table 11 and Figures 41a,b and 42a,b show prominent peaks in the RRM spectra together with the wavelengths of EMR calculated using the following relationship (Cosic et al. 1989):

$$\lambda_{EMR} = 201 / f_{RRM} ;$$

A multiple cross-spectral analysis was performed for each group of proteins and resulted in the few specific features:

a) LDHA, LDHC and LDHBacterial have frequency identified at f_{RRM} =0.1688 in common. This frequency is related to the biological activity of LDH as it was found in previous investigations (Cosic 1994, Cosic 1997). The LDHB has very weak peak at f_{RRM} =0.1688 in RRM multiple spectral function. The role of LDHB to converse pyruvate to lactate is much more likely to happen then the role of conversing lactate to pyruvate, what is opposite with LDB-A, LDHC and LDHBacterial.

b) Multiple cross spectral function for the LDHC shows five prominent peaks. The reason is having only seven LDHC molecules included in calculation of RRM spectra.

c) Multiple cross spectral function calculated for the group of The LDHB molecules is shown in Figure 41b. There are only two prominent peaks at $f_{RRM} = 0.239$ and $f_{RRM} = 0.323$. Having the existence of the two prominent peaks inside the RRM mul-

tiple spectral function it was possible to predict that EMR, which has the particular wavelengths of $\lambda = 840$ nm and $\lambda = 620$ nm, would influence the activity of the LDH.

d) LDHC, also have a weak peak at $f_{RRM} = 0.239$, what is interesting for experimental analysis together with the LDHB. However, it was not possible to find LDHC, suitable for the experiment, on the market.

The absence of the peaks in RRM spectra that correspond to the wavelengths of EMR at $\lambda =$ 840 nm and $\lambda = 620$ nm in LDHA and LDHBacterial, imply usage of LDHA and LDHBacterial as a control samples in experiment of LDHB activity.

LDH groups	The RRM frequencies	Corresponding wavelengths (nm)	Errors
LDHA	0.1688	1190 nm	±25*nm*
	0.2735	740 nm	
LDHB	0.2390	840 nm	±25*nm*
	0.3230	622 nm	±25*nm*
LDHC	0.1688	1190 nm	±25*nm*
	0.0730	2753 nm	
	0.2735	740 nm	±25*nm*
LDHBact	0.1688	1190 nm	±25*nm*

Table 11. The frequencies calculated multiple cross-spectral function of LDHA, LDHB, LDHC and LDHBacterial. Second column represent corresponding wavelengths of electromagnetic radiation calculated using formula $\lambda = 201/f_{rrm}$, the third columns represent combined error from the RRM calculations and prediction

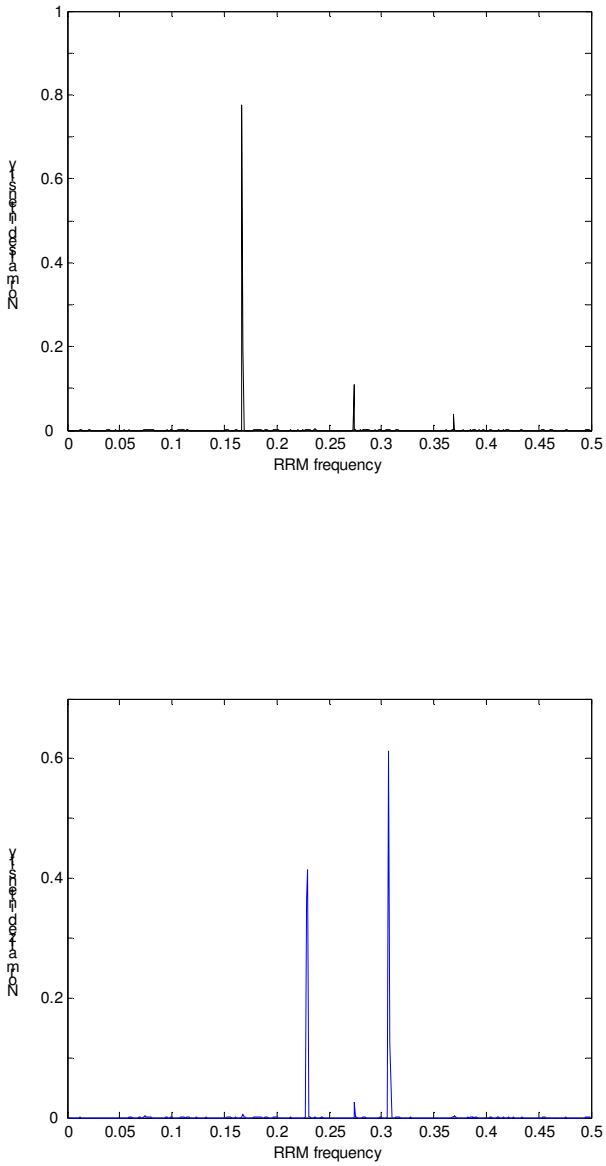


Figure 41. a) Multiple cross-spectral functions of 46 different proteins (belonging to the LHA group b) Multiple cross spectral function of 24 proteins, belonging to the group LDHB. The multiple cross-spectral function of each group of proteins has a prominent peak representing the frequency characteristic for the biological function.

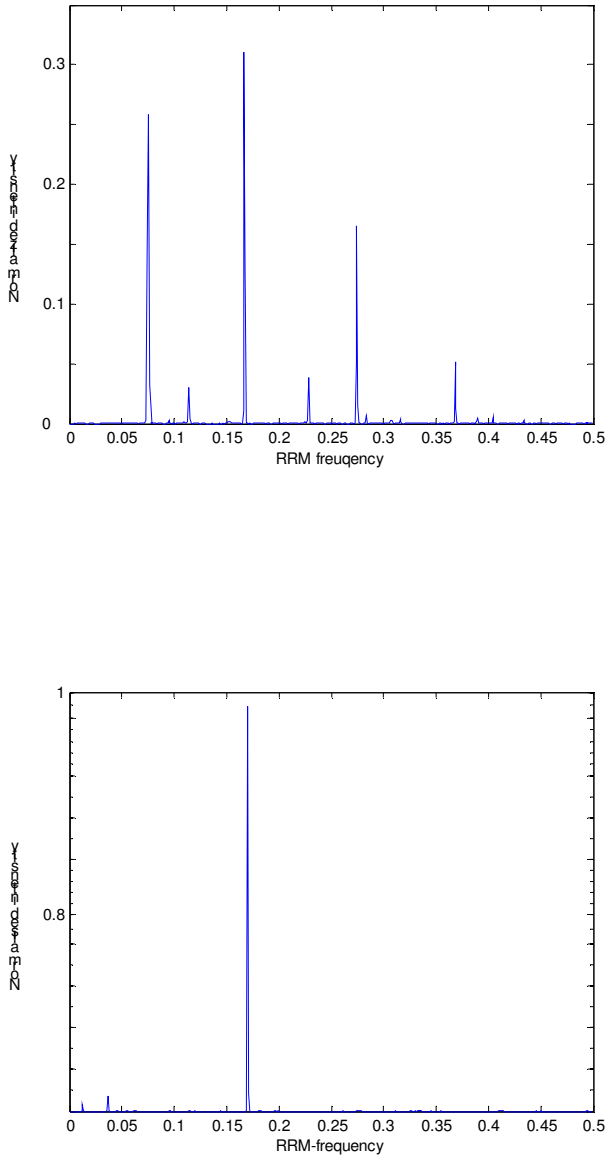


Figure 42. a) Multiple cross spectral functions of 7 different proteins (belonging to the LHC group b) Multiple cross-spectral function of 30 proteins, belonging to the group LDHBacterial. The multiple cross-spectral function of each group of proteins has a prominent peak representing the frequency characteristic for the biological function.

Chapter XI

EXPERIMENT AND DISCUSSION

The aim of this experimental design was to find out whether the EMR within the range [560-860nm] can modify the LDH activity. The outcome of the experiment was the comparison of the activity of LDH measured before and after the exposure to the EMR. The outcome will be compared to the theoretical predictions as presented in chapter X.

The single independent variable was the wavelength of the applied EMR. The influence of the additional uncontrolled variables (small variability in temperature < 0.05K; small variability in pH < 0.1; small variability in concentrations of chemicals < 0.1%;) on the final result will be discussed later in this chapter.

The variables such as the length of time of the exposure and the length of time of the measurement were determined in the pilot experiment and they were kept constant during the experimental procedure. The schematic representation of the experimental design is show in Figure 43.

Using the terminology from the Festinig (2001) guidelines for the design of biological experiments, the set of samples that were treated equally was called the *experimental unit*. Thus, all samples with the LDH exposed to the same wavelength will belong to the same experimental unit. The term experimental unit was also used through the process of statistical analysis.

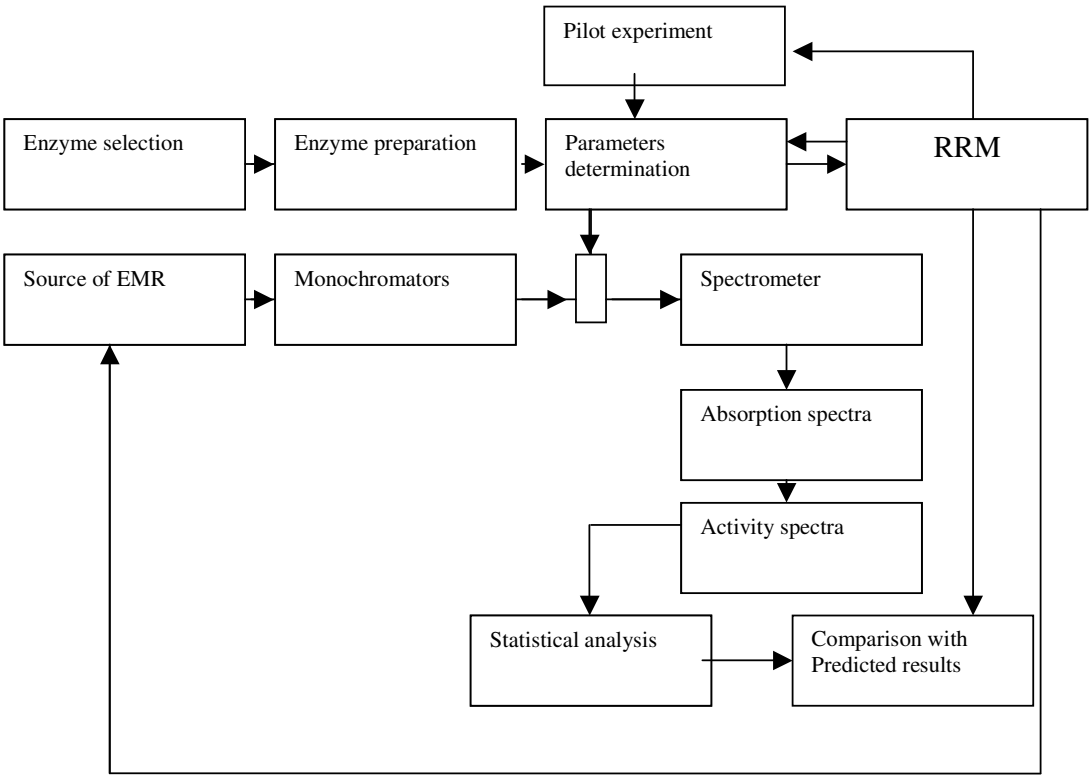


Figure 43. The schematic representation of the experimental design. Enzyme selection is made on the basis of criteria (Chapter V); enzyme preparation is based on industrial assay (Sigma No 633030); parameters determination include independent variables (time of irradiation and wavelengths of EMF); source of EMR, monochromators/optics and spectrometer are commercial equipments; absorption spectra were recorded at regular intervals, and used for the calculation of activity spectra. Statistical analysis was performed to analyse significance of results.

To avoid bias, due to unknown uncontrolled variables, the random generator (EXCEL©-Microsoft office) was used to determine the order in which the samples were exposed. For example the first sample was irradiated at 623 nm, the next one at 754 nm and so on.

Activity spectra were calculated from the optical density of the NADH that had been recorded every 20 seconds during the time period of ten minutes (One record is given in Appendix II). Following data analysis was undertaken using MatLab™ VI software package.

The pilot experiment was used to provide estimation of the mean, standard deviation, sample size for the treated and non-treated *experimental units* and wavelengths of used EMR.

Determination of the sample size of *experimental unit* is very complex problem. It depends on (Everit 1995):

a) standard deviation of the *experimental unit*;

b) chosen significance level usually $p < 0.01$;

c) distribution of data for *the experimental unit*.

To determine the sample it often convenient to use the measure called "effect size" (D). When only two experimental units are to be compared, the "effect size" is the difference in means that the investigator wants the experiments to be able to detect. Usually "D" is expressed in units of standard deviations by dividing through by the standard deviation. In addition, the *experimental unit* that is too small may miss biologically important effects, whereas the *experimental unit* that is to large need a lot of the measurement time (Festing 2002).

11.1 Experimental settings

Spectral characteristic of the irradiating light is predetermined by technical specifications of the used lamp and the monochromators optics (Figure 37, 38). It is possible to control intensity of the light by adjusting power of the lamp from 0 W to 60W. For all experiments the power of the lamp was adjusted to the 60W (maximal power). In addition, the distance from the slit of the monochromator (Figure 38) to the sample was fixed to 5 cm. The pilot experiment was designed to estimate the optimal length of the exposure time to the EMR.

Factors linked with a sample preparation.

Several variables had to be controlled during the preparation of the sample.

- Concentration of enzyme (Lactate dehydrogenase)
- Concentration of NADH
- Concentration of pyruvate

- Buffer characteristics
- pH value
- Temperature

The variability of these parameters was mostly known from the literature, therefore the influence of these parameters on the outcome of the experiment could be predicted (Fersht 1985). The concentrations of all substances were calculated in accordance with industrial assay (SIGMA 633063, Chapter IX). Still, due to characteristics of the scale (the accuracy 0.001g) and the pipettes (the accuracy 2 μl), same variation in concentration of the solvents was present. All buffers used in the experiment were previously prepared using Sigma assays (Chapter V). Every time when the new sample was prepared the pH value was checked. The accuracy of used pH meter was 0.1. The temperature was kept at constant value.

Procedure with outliners:

In the case when any of samples in experimental unit had unusual outcome (the activity of the sample differs for more then 3 standard deviation from the mean of the activity of the experimental unit), all the other samples of the experimental unit were discarded. The new experimental unit was created and measured.

11.2 The pilot experiment

The pilot experiment had to provide an estimation of following parameters: wavelength, mean, standard deviation, sample size and the length of the time of the irradiation. The Chapters X and XI detailed the linkage between RRM frequencies f_{RRM} of an enzyme and the wavelength of the EMR known to activate the particular enzyme.

The computational predictions based on the RRM revealed two particular wavelengths of the EMR, that could affect the enzymatic activity of the LDHB, at 840 nm and 620 nm (Figure 42b, Table 12). Unlikely, the calculation done for the LDHA from Bovine muscle and the LDHBacterial from *Bacillus thermophilus,* did not show any existence of prominent peaks at 820 nm and 620 nm (Figure 41a, 42b).

All previously discussed parameters regarding sample size were taken in account for the irradiated samples. Using the method for the two experimental units using T-comparison, (with a significant level of 5% and two sided tests) given by Festing (2002), it was possible to detect the "effect size" that equals two, with the sample size that equals eight.

In other to find out the optimal length of the exposure time, the experimental unit was exposed to EMR for various lengths of time ranging from 300 sec to 3600 sec with the steps of 300 sec.

For each of various lengths of exposure time eight experimental units were selected for the pilot studies:

a) two of them were irradiated with the wavelengths of the EMR calculated using the RRM model (620 nm and 840 nm);
b) three were irradiated with random wavelengths (EXCEL number generator) at 700 nm, 760 nm and 810 nm;
c) one non irradiated experimental unit of LDHB (for each length of the exposure time one unit of LDHB was prepared, not exposed to the EMR and kept as a control experimental unit)
d) one experimental unit with LDHA;
e) one experimental unit with LDHBacterial.

Immediately after the irradiation, the solution containing LDHB and the buffer were mixed with already prepared solution containing the NADH and pyruvate. The absorp-

tion spectra of so prepared sample were monitored for the following 600 sec. The optical density of NADH was measured at 340 nm.

Figure 44 shows how the concentration of the NADH decreases in time. The absorption spectra within the range 280-380 nm was measured after 30, 250 and 600 sec.

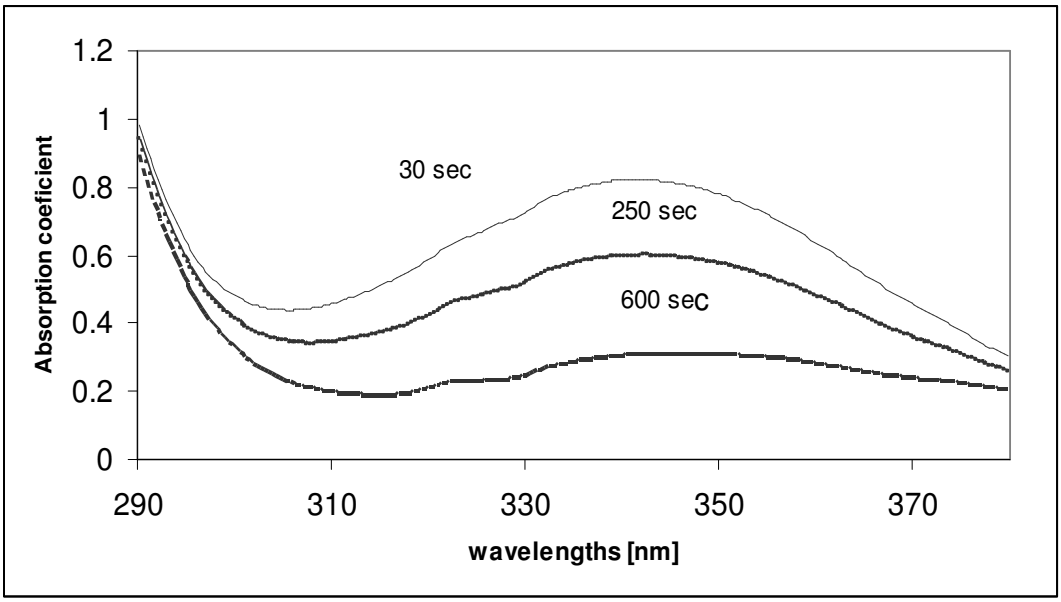


Figure 44. Absorptions spectra in the range [290-380 nm] of the solution of LDHB, NADH and pyruvate. The time in seconds represent time after mixing LDH with previously prepared solution of NADH and pyruvate

The absorption coefficient of the solution was measured at 340 nm. Spectra were recorded each 20 sec. From the absorption data, using the rate of change in optical activity of the NADH in time, the activity was calculated. Figure 45 represents the activity versus the length of the exposure time calculated for:

a) the LDHB, at five selected wavelengths of EMR;

b) for non-irradiated experimental unit;

c) for LDHA;

d) LDHBacterial.

Each point in Figure 45 represents mean value calculated for the particular experimental unit.

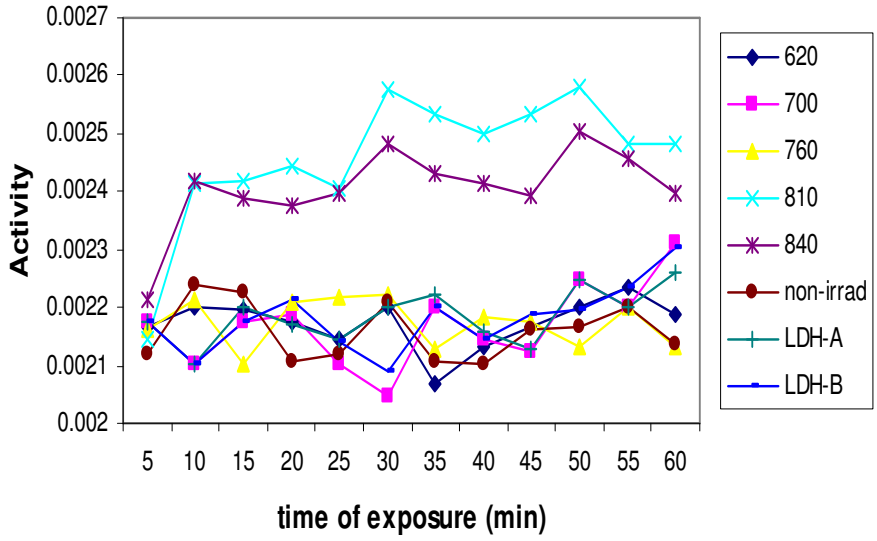


Figure 45. Change in activity of Lactate dehydrogenase measured after irradiation and for non irradiated sample. It can be noticed that 5 min exposure did not cause any effects. The difference in effects upon exposure at different wavelengths for a period of time between 300 minutes and 3600 minutes is not statistically significant (t-test, $P < 0.05$).

Finally, the outcome of the final experiment was the optimal length of the exposure time of 900sec. In addition, the pilot experiment did not show any significant change in activity of LDHA and LDHBacterial after the exposure of (Figure 45). The data for non irradiated LDHA and LDHBactrial had the same statistical values as data for non irradiated LDHB.

11.3 Experimental Measurement of the Activity spectra of LDHB

It is known that NAD^+ and NADH serve to remove or add 2 H atoms from/to molecules, i.e. oxidise or reduce a substrate molecule or Pyruvate/Lactate. (Ward 1988). Moreover, the NAD^+ does not absorb at 340 nm whereas NADH does absorb strongly, facilitating to monitor the concentration of NADH, [NADH] by spectrophotometry. The NAD^+ has an intensive peak at 260 nm, but it is not convenient for measurement of the concentration of the NAD^+ in the solution. As the absorption from LDH and NADH at the 260 nm are overlapping peak due

to absorption of NAD^+, it is much more convenient and accurate to measure change of the optical density of NADH at 340nm.

It has been mentioned previously that the absorption of the light by the solution depends on the absorption coefficient $\varepsilon\,(mol^{-1}\,cm^{-1})$, path length of the cuvette, (1cm in this case) and concentration of the substance **c**. Extinction coefficient for NADH = 6.3 mM/cm at 340 nm i.e. 1 μ mol NADH/m at 340 nm. For practical purposes the concentration of the NADH has been calculated to make the absorbance equal one, before introduction of the LDH.

To determine reaction velocity it is necessary to generate a progress curve. For the conversion of substrate (S) to product (P) , the general shape of the progress curve is that of a first order exponential decrease in substrate concentration as follow (Fleche 1993):

$$[S - S_{\min}] = [S_0 - S_{\min}]e^{-kt}$$

Where $[S_0]$ is top initial substrate concentration, $[S_{\min}]$ is minimum substrate concentration when $t \to \infty$, and $\lfloor S \rfloor$ is substrate concentration at time t.

The rate of the reaction correspond to the instantaneous slope of the progress curve as

$$v = -\frac{dS}{dt}$$

Where S is substrate concentration at time t and v is rate of the reaction.

For each molecule of pyruvate to be converted to lactate it is necessary that one molecule of the NADH is converted to the NAD^+. The measurement of the optical density of NADH as a function of time was undertaken using spectrometer (Chapter IV detailed construction and characteristics of USB 2000 spectrometer).

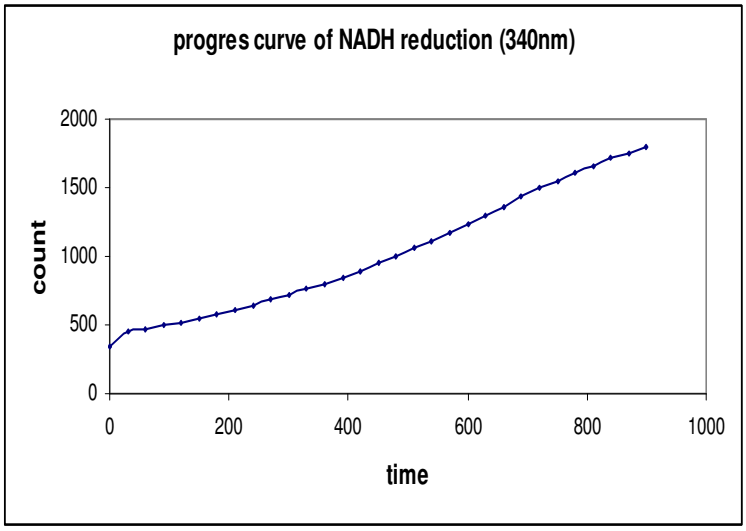


Figure 46. Progress curve measured as a number of counts (for the wavelength of 340nm) on CCD detector, after removing dark signal, and making border correction.

As a first step of the amount of transited light was measured (Figure 46). The next step was calculation of the coefficient of the absorption vs. time (Figure 47) using equation 1 from chapter VII. The very weak tendency for deviation from linearity make an error much smaller then standard deviation of measurements and it can be disregarded. Rate of change of activity has been measured using gradient of absorption coefficient vs. time curve. The rate of change of the activity was calculated using the following simple equation:

$$\frac{Absoption(100\text{sec}) - Absorption(600\text{sec})}{number\ of\ \ steps\ (20\text{sec})}$$

Absorption(100sec) and Absorption(600sec) are absorption coefficients measured at 340nm after 100 sec and 600sec respectively. The number of steps was 20.

The graph of absorption coefficient versus time is shown at (Figure 47). In this case the sample, having LDH enzyme, was previously exposed to 610nm.

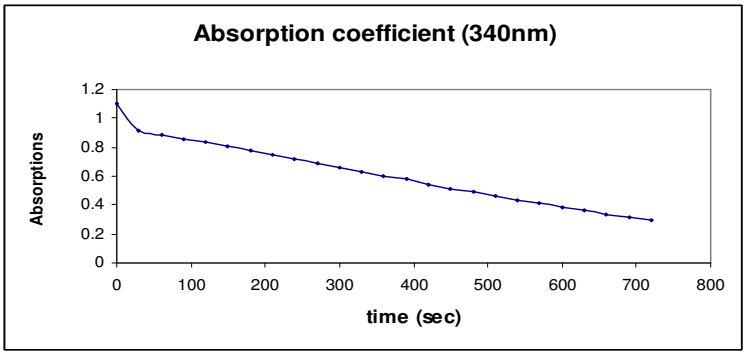


Figure 48. Absorption coefficient at 340 nm of the solution as a function of time. Very strong linear correlation is present. The activity can be calculated from the gradient of absorption characteristics.

Statistical consideration

The research question in this thesis was: if there is significant change in activity of the LDH after irradiation with the EMF. The null hypothesis, denoted H_0, was that the mean of activity of previously irradiated LDH does not differ from the mean of previously non-irradiated LDH. The alternative hypothesis, denoted H_1, was that difference does exist. The T-test (for comparing each of irradiated experimental units with non irradiated experimental unit) was used for accepting or rejecting the hypothesis.

If the activity of previously non irradiated experimental unit is independent random variable of size n_1, sample variance s_1 and a mean μ_1 and activity of previously irradiated experimental unit of size n_2, sample variance s_2 and a mean μ_2, then, if data in the experimental units had normal distribution, the statistics T has an approximate "*t*-distribution" and T is determined as (Walpole et al. 2002):

$$T = \frac{(\overset{\mu}{X}_1 - \overline{X}_2) - (\mu_1 - \mu_2)}{\sqrt{s_1^2/n + s_2^2/n_2}}$$

The two sided hypothesis on the difference between two means can be written as

$H_0 : \mu_1 - \mu_2 = d_0$

In this case, the H_0 hypothesis stated that there was not difference between means $d_0 = 0$; Hypothesis H_0 can be rejected in favour of H_1: $H_1 : \mu_1 - \mu_2 \neq 0$ if $t > t_{\alpha/2}$ or $t < -t_{\alpha/2}$;

where $t_{\alpha/2}$ represents the t-value above which the area equals to α; $t_{-\alpha/2}$ represents the t-value bellow which the area equals to α. α is probability of rejecting null hypothesis when it is true (Walpole 2002). The used t distribution has approximate degrees of freedom:

$$\upsilon = \frac{(s_1^2/n_1 + s_2^2/n_2)^2}{(s_1^2/n_1)^2/(n_1-1) + (s_2^2/n_2)^2/(n_2-1)}$$

Where n_1 and s_1 are the size and sample variance of the experimental unit 1; n_2 and s_2 are the size and sample variance of the experimental unit 2. In Table 12 are shown mean values for the activities of irradiated experimental units and the significance of the results that are presented as a p value (probability that both experimental units come from the same distribution). Example of data calculated for one experimental unit is given at Appendix III page 156.

Wavelength	Mean	P-value	Wave-ngth	Mean	P-value
580	0.02467	0.76	710	0.02345	0.01
585	0.02393	0.3	715	0.02235	0.39
590	0.02407	0.35	720	0.02305	0.09
595	0.02322	0.01	725	0.02391	0.16
596	0.0262	0.99	730	0.02386	0.13
600	0.02192	0.63	735	0.02246	0.59
605	0.02354	0.05	740	0.02391	0.27
610	0.02305	0.06	745	0.02255	0.52
615	0.02391	0.12	750	0.02288	0.26
620	0.02336	0.00	755	0.02376	0.11
625	0.02345	0.02	760	0.02303	0.13
630	0.02391	0.21	765	0.02345	0.02
635	0.02255	0.36	770	0.02235	0.61
640	0.02264	0.31	775	0.02305	0.10
645	0.02376	0.11	780	0.02282	0.17
650	0.02200	0.56	785	0.0233	0.00
655	0.02205	0.38	790	0.02298	0.14
660	0.02235	0.25	795	0.02391	0.21
665	0.02305	0.17	800	0.02255	0.33
670	0.02391	0.23	805	0.02288	0.21
675	0.02336	0.01	810	0.02376	0.15
680	0.02404	0.27	815	0.02467	0.56
685	0.02391	0.23	820	0.02555	0.87
690	0.02255	0.34	825	0.02588	0.93
695	0.02308	0.18	828	0.0261	0.99
700	0.02376	0.19	830	0.02608	0.99
705	0.02435	0.56	832	0.02576	0.91
			836	0.0245	0.51

Table 12. The mean values measured for each experimental unit. First column represent wavelength of light used for irradiation, the second column represent rate of change of optical density of NADH. Third column is P value. P value is related to the comparison between experimental unit of non-irradiated samples and irradiated samples. Only experimental units with size of 8 samples are represented in the table. It should be noticed that real data have negative value.

Results of a study of the interaction between EMR and the reaction catalysed by LDHB- rabbit heart are summarized in Figure 48.

The bold horizontal line represents the average activity measured for the experimental unit of 35 non irradiated samples. The dashed line represents one standard deviation up and down from the average value.

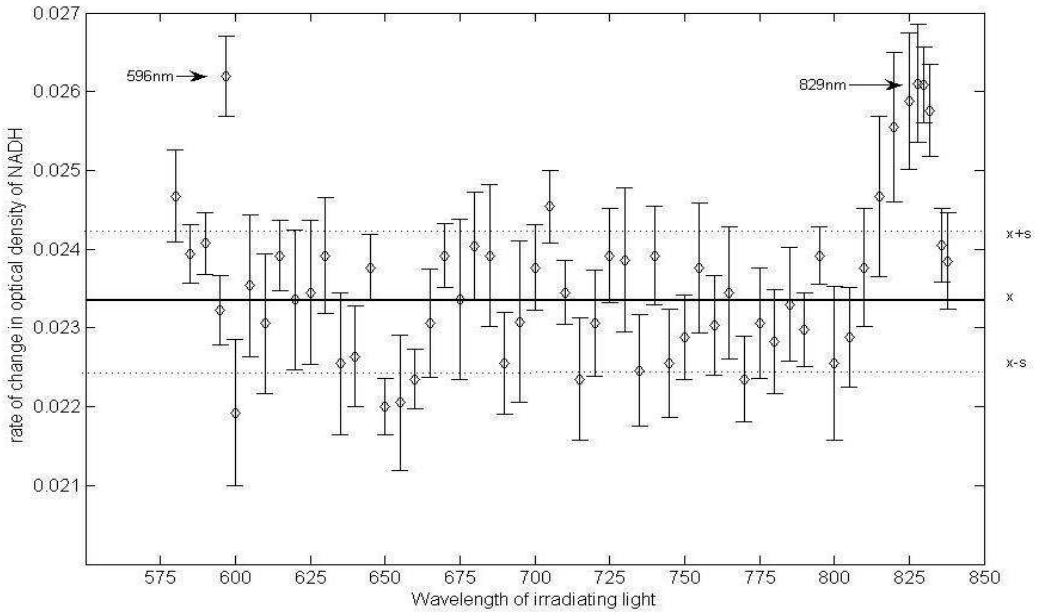


Figure 48. The rate of change in optical density of NADH as a function of the wavelength of light used for pre-treatment of LDH. The vertical line represent one standard deviation. Horizontal line represent the average value of measurement. Dashed lines are mean value plus one standard deviation and mean value minus one standard deviation for non irradiated experimental unit. Data shown in the table are multiply with (-1). Real data are negative.

A few points about the results have to be noted here:

a) The experimental unit, which was irradiated at 596 nm, had signicant difference in mean values from the mean values of non-irradiated experiment (11.6%, $p < 0.001$). Half width of that peak was only 1nm. (The peak of the similar width was found for α-chymotrypsin at 855 nm by Biscar (1976). Another significant peak was at 829 nm (10.9%, $p < 0.001$). The peak at 829 nm in activity spectra was much wider then the peak at 596 nm, and was covering interval for 815-840 nm.

b) The significance for a few other peaks can be discussed if it was used P value of 0.1 or 0.15 instead 0.01.

c) There was not significant difference in activity between control non-irradiated experimental unit of LDHB and the experimental units irradiated with EMR at wavelengths other then 596 nm and 829 ± 15 nm. The rate of change in the optical density (activity) was 0.0262 (units) at 829 nm with standard deviation of the experimental unit $\sigma = 0.0007$, while the activity was 0.0261 (units) at 596 nm with standard deviation $\sigma = 0.009$. These activity values were significantly different ($P < 0.01$) from the activity values of the experimental unit with previously non-treated LDHB and which was 0.0234 (units) with a standard deviation of ± 0.001.

d) Some level of variability was evident inside each of experimental units as well as between different experimental units (As it was defined, the experimental unit was set of samples of Lactate dehydrogenase irradiated with the light of the same wavelength). In Figure 48, only the results for the experimental units of 8 samples are represented. Yet, the experimental units which had only 3 samples of LDHB were investigated after irradiation with resolution of 1 nm. No additional peaks were found but this information will not be a part of a conclusion due to incomplete size of the experimental unit for the statistical analysis.

The light-induced biological growth stimulation with wavelength of 596 nm and 829 nm have been documented in the literature. For example Naum (1993) reported that the red light with wavelength of 596 nm produced by light emitted diodes (LED) had an effect on burn healing in-diabetic rats. For the EMR in the proximity of 829 nm the following effects have been documented: a change of pain intensity in patient with Hand-Foot diseases using low-level laser at 830 nm (Toida et al 2003); a change of Acetylcholinesterase activity of human erythrocytes (Kujawa, 2003); A change of concentration of LDH molecules after irradiation of the cells involved in inflammatory processes at 810 nm (Toida et al. 2001).

11.4 Temperature effect

The temperature of the solution was controlled and kept at the constant value. Nevertheless, a local increase in temperature of a very short life is still possible inside the sample due to absorption characteristics of the water, or in the case that some chromophores (the molecules with absorption band in visible part of the spectra) were present. For specific solution of the LDHB, NADH and pyruvate where chromophores absorbing in the range of applied EMR were absent, only water could contribute to the heating. According to Sogandares (1997) and Karu (2003) the maximum possible increase in the temperature for so called "averaged heating" (at a radiation intensity of $1\times10^{1}Wcm^{-2}$ and with the radiation completely absorbed at a depth of 1 mm during 10 s) does not exceed 0.1 K. Massener (1982) shown that an increase of temperature from 300K to 300.1K will change activity for less then 0.1%. Therefore, the increase of the temperature will produce changes that are very much smaller then measured differences in means of experimental units. Convincingly, the temperature is not the factor affecting the outcome of the experiment.

11.5 Internal dynamics of the enzymes

It is widely recognized that the function of enzymes is crucially dependent on their dynamic nature (Marcus 2005, Warshel 2003). For instance, an atomic motion is needed for the proteins to bind ligands and to carry out enzymatic catalysis. Otherwise, truly rigid structures, as suggested in static pictures of protein structure, would not be functional (Marcus 2005).
Furthermore, extensive theoretical studies on enzyme activity have been devoted to known enzyme structures with the aim to calculate the reaction rate numerically (Warshel 2003). Among the factors contributing to the reaction rate in a bound substrate-cofactor enzyme complex are:

a) the work required for the reactants in the bound complex to approach each other closely enough to optimize the H-transfer rate,

b) an additional energy barrier accompanying the bond rupture and bond formation in the substrate-cofactor complex, and
c) fluctuations in the structure of the enzyme and in solution (in electron transfers called "reorganization").

Another important influence on the dynamics of the enzymes appear to be the existence of de-localised π electrons along the peptide bond. Delocalised electrons are moving freely and they are in the close proximity of atoms or clouds of electrons belonging to the neighbouring residuals. Little (1967) calculated theoretically the possibility of the formation of Cuper's pairs due to the attracting forces from the polarons belonging to the residuals. The polaron is an electron in a crystal lattice together with a cloud of phonons that result from the deformation of the lattice produced by the interaction of the electron with ions or atoms in the lattice (Little 1964).

By the best knowledge of the author there is no published work in theoretical enzymology that is analysing influence of the EMR, within the range of 560-850 nm, on the activity of LDH or the activity of other enzymes.

However, the given experiment proves that it is possible to predict the wavelength of EMR, which will be influential on enzyme activity, even in the enzymes that do not have chromophores with absorption bends within the range of visible and infrared EMR.

Even though the relationship between RRM frequencies and wavelengths of electromagnetic field affecting the enzyme is not clear, the outcome of the experiment presented in this thesis and in article (Vojisavljevic et al. 2007) is prominent. The same RRM was applied by Cosic (1993) on the experiments done by Biscar (1976) with similar outcome.

11.6 Future experiments and theoretical considerations.

A very small number of experiments on influence of EMF on enzyme activity has been published. There are many reasons for that. One of the main reasons is the lack of the theoretical

explanations for small fluctuations of the enzyme structure in the presence of EMF or without a presence of EMF. The enzyme function is heavily dependant on geometrical positions of the residuals involved in the enzymatic process. Despite of the known structures of enzymes with a resolution smaller than 0.12 nm, it is very difficult to simulate theoretically enzymes kinetics (Gao and Truhlar 2002). More research needs to be done on the effects of visible light on various classes of enzymes. Unfortunately, the measurement of the activity spectra are very time consuming. For many enzymes there is not easy and short way to detect activity with a high accuracy. In addition it is not clear how many ezymes would be affected by EMR within the visible range. Having this in mind, future work should incorporate investigations of interaction between EMR and living systems. This could be organised on three levels:

a) The measurement of influence of EMR within the range [400-2000nm] on the activity of enzymes, belonging to the different classes. Those experiments have to establish: the classes of enzymes that would be affected after exposure to the EMR in the range. In addition, the accuracy of RRM prediction will be tested on larger set of enzymes.
b) Designing of experiments linked to the cells proliferation. There is a possibility to selectively activate the particular biochemical process by affecting the enzyme included in process. The wavelengths of applied EMR would be predicted using RRM methodology.
c) Developing of applications for selective irradiation of tissues *in vivo* or even whole organisms targeting activity of specific enzyme.

Chapter 12

Summary of Research and Conclusions

In this study, it was experimentally proven, for the first time, that it is possible to predict the frequency of electromagnetic radiation that can modulate protein and more specifically enzyme activity. The prediction obtained using the RRM, was tested here experimentally using the reaction catalysed with the enzyme l-lactate dehydrogenase (LDH).

Chapters 2 of the thesis reviewed the main features of biology from the organisms to the biomacromolecules.

Chapter 3 considered photobiology and main processes that include interaction of living matter with a light.

Chapter 4 overviewed the electromagnetic field, radiation and visible light

Chapter 5 detailed the RRM. This model uses computational techniques to reveal information from the primary structure of proteins. The strong correlation between RRM frequencies and protein function was found in previous investigations.

Chapter 6 considered previous investigations of relation between RRM frequencies and Activity spectra of proteins.

Chapter 7 introduced the absorption spectroscopy. The absorption spectroscopy is the main experimental tool used in this thesis.

Chapter 8 detailed Lactate dehydrogenase.

Chapter 9 outlined experimental equipment, monocromator, light source and spectrometer.

Chapter 10 reports results of RRM analysis applied for on the group of LDH molecules from different sources.

Chapter 11 present experiment, experimental results and discussion.

12.2 Conclusion

The RRM model was applied to the group of the enzymes belonging to the sub-subclass EC 1.1.1.27 i.e. l-lactate dehydrogenase. The prominent peaks in RRM multiple cross spectral function at $f_{RRM} = 0.239$ and $f_{RRM} = 0.323$ were used for the prediction of wavelengths of EMR at 620 ± 25 nm and 840 ± 25 nm respectively.

Enzyme activity was then measured after the exposure to the low-intensity EMR within the proposed EMR range [560-860 nm],

The experimental results were indeed shown that there is a significant increase in the activity of LDH only at radiation wavelenghts as predicted by the RRM: 596nm (12%; $P < 0.001$) and 829 nm (11.8%, $P < 0.001$). These results prove successfully that activity of proteins and more specifically enzymes can be modified by EMR radiation of specific wavelengths and even more that RRM computational model can successfully predict these frequencies.

This study is an attempt to shed a new light on possible deeper physical grounds that lead to understanding of protein interactions. Preliminary results obtained reveal that the frequencies obtained for dehydrogenase enzymes ($f_{RRM} = 0.239$ and $f_{RRM} = 0.323$) using the RRM ap-

proach can be directly related to the resonances in these macromolecules. The presented methodology may allow the generalization of the main advantages of the RRM in the case when the space structure of macromolecules is taken into account in a more realistic way. Based on the RRM spectral characteristic we can calculate the wavelength of electromagnetic energy that can be used to modulate the protein activity hence giving rise to an innovative efficient methodology to program, predict, design and modify proteins and their bioactivity. This could have major implications in drug design, medicine, agriculture, pharmacology and biotechnology.

Effects of EMR (with selective wavelength within visible range) on biological processes involving enzyme activation imply that protein activation involves energies of the same order and nature as the EMR. These results lead to the conclusion that specificity of enzymes interaction is based on the resonant electromagnetic energy transfer between interacting molecules, on a frequency specific for each observed function/interaction.

The results presented in this thesis are solid basis to encourage investigation on behaviour of other enzymes and proteins under the influence of EMR. The enzymes as a proteins are responsible for activation and acceleration of the most of the biochemical reactions. In many cases it is possible to measure activity of a particular enzyme *in vitro*. To generalise this finding over larger group of enzymes it will be necessary to design, for each of them, specific experiment in order to measure activity spectra

The possibility of controlling some of the important biochemical pathways with a selective EMR could significantly improve existing applications of visible light in medicine and pharmaceutical industry.

The therapeutic effects of EMR in the visible and infrared range are already known. The problem is that there are not real criteria for selecting the optimal wavelength of light. In most cases the effects were investigated using only few different wavelengths. In previous investi-

gations the choise of wavelengths was basid only on available light source (lasers and light emitted diodes). As a result, many potential effects were missed.

Due to recent developments in nanotechnology, photoelectronics and medica engineering, today, it is easy to bring EMR within the visible range to the many tissues inside the body. In addition, stimulative effect on culture of bacteria can increase productivity of many products.

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Appendix I

Code used in thesis

a) **for calculation of RRM multiple cross spectra function**
b) **for extracting primary sequences**

The followinc code was used for extraction of primary sequences from data base:

```
//Author Vuk Vojisavljevic 2005.

#include "stdafx.h"
#include <stdlib.h>
#include <stdio.h>
#include <string.h>
#include <math.h>
#include  <assert.h>
#include <malloc.h>
#include <ctype.h>
#define MAX 60000
#define EMAX 550
#define DV 205
#define MAKS_L 3000

FILE *dumy_file;
int main(int argc, char* argv[])
{
int DUZINA_SEKVENCE(0), SeqPower, stepen_dvojke, stepen;
FILE* stream_seq[5];
FILE* stream_names;

char *buffer;

char  prop[120],test[60];
printf("dosam do ovde");

        int *pozicije_enzima; pozicije_enzima = (int*)calloc(EMAX, sizeof(int));

        char **sekvenca; sekvenca=(char **)calloc(MAX, sizeof(char*)); if(sekvenca==NULL)
{printf("problem sa memorijom1"); exit(1);}
        for (int ire(1); ire<MAX; ire++)
        {
                sekvenca[ire-1]=(char*)calloc(MAKS_L,sizeof(char));
                if (sekvenca[ire-1]==NULL) { printf("problem sa memorijom2\n"); exit(1);
        }}
printf("dosao sam");
// Privremeno iskljuceno

        char **enzyme_sekvenca; enzyme_sekvenca=(char **)calloc(EMAX, sizeof(char*));
if(enzyme_sekvenca==NULL) {printf("problem sa memorijom3\n"); exit(1);}
        for (int ir(1); ir<EMAX; ir++)
        {enzyme_sekvenca[ir-1]=(char*)calloc(MAKS_L,sizeof(char));  if (enzyme_sekvenca[ir-1]==NULL) {
printf("problem sa memorijom 3\n"); exit(1);}}

        char **ime_f; ime_f=(char **)calloc(EMAX, sizeof(char*));
        if(ime_f==NULL) { printf("problem sa memorijom"); exit(1);}
        for (int irs_E(1); irs_E<EMAX; irs_E++)
        {ime_f[irs_E-1]=(char*)calloc(20,sizeof(char)); if (ime_f[irs_E-1]==NULL) { printf("problem sa
memorijom 1\n"); exit(1);}}
        char **ime_fajla; ime_fajla=(char **)calloc(MAX, sizeof(char*));
        if(ime_fajla==NULL) { printf("problem sa memorijom"); exit(1);}
        for (int irs(1); irs<MAX; irs++)
        {ime_fajla[irs-1]=(char*)calloc(80,sizeof(char)); if (ime_fajla[irs-1]==NULL) { printf("problem sa
memorijom 1\n"); exit(1);}}
FILE* stream_seqa;
        if((stream_seq[0] = fopen("d:/SWISS_TOTAL/compress/Sw_I", "r" ))==NULL)
                printf("nemogu da otvorim atom 1 file");
        if((stream_seq[1] = fopen("d:/SWISS_TOTAL/compress/Sw_IIa", "r" ))==NULL)
```

```
		printf("nemogu da otvorim atom 1 file");
	if((stream_seq[2] = fopen("d:/SWISS_TOTAL/compress/Sw_IIb", "r" ))==NULL)
		printf("nemogu da otvorim atom 1 file");
	if((stream_seq[3] = fopen("d:/SWISS_TOTAL/compress/Sw_IIIa", "r" ))==NULL)
		printf("nemogu da otvorim atom 1 file");
	if((stream_seq[4] = fopen("d:/SWISS_TOTAL/compress/Sw_IIIb", "r" ))==NULL)
		printf("nemogu da otvorim atom 1 file");
	if((stream_names = fopen("d:/sredjeni_enzimi/E_2-8-3.txt", "r" ))==NULL)
		printf("nemogu da otvorim 2BBBBBB atom file");
if((dumy_file = fopen("d:/rezultati/E283seq", "a" ))==NULL)
		printf("nemogu da otvorim 1BBBBBB atom file");
	int c(0), l(0), tt(0), red(0),duzina_reda;
int Broj_enzima(0);		for(int hh(0);fgets(test,60,stream_names)!=0; hh++) {Broj_enzima++;
strcpy(ime_f[hh], test);}
	for(int hh_E(0);fgets(prop, 60, stream_seq[2])!=NULL; hh_E++)
	{


		duzina_reda = strlen(prop); buffer=(char*)calloc(duzina_reda+1,sizeof(char));
		strcpy(buffer, prop);

		if((buffer[4] == '_')||(buffer[3] == '_'))
		{
				tt++;strcpy(ime_fajla[tt-1], buffer);
				l=0; free(buffer);
				for (int no_enz(0); no_enz < Broj_enzima; no_enz++)
					if(_strnicmp(ime_f[no_enz], ime_fajla[tt-1], 8) == 0)
					{pozicije_enzima[red] = tt; red++;printf("%s\n",ime_f[no_enz]);
continue;free(ime_fajla[tt-1]);}
		}
		else
		{
			for (int jj(0); jj < duzina_reda; jj++)
			{
					{ sekvenca[tt-1][l] = buffer[jj]; l++;}
			}
			free(buffer);
		}
	}


	for (int broj(0); broj < red; broj++)
	{
    enzyme_sekvenca[broj] = sekvenca[pozicije_enzima[broj]-1];
		fprintf(dumy_file, "%s\n", ime_fajla[pozicije_enzima[broj]-1]);
		fprintf(dumy_file, "%s\n", sekvenca[pozicije_enzima[broj]-1]);
	}
 ////////////////////////////////////////////////////////////////////////////////
fclose(dumy_file);fclose(stream_seq[0]);fclose(stream_seq[1]);fclose(stream_seq[2]);fclose(stream_seq[3]);fclo
se(stream_seq[4]);

	return 0;
}
```

Code used for Computational analysis of protein's primary structures:

```
//Author Vuk Vojisavljevic 2005


#include "stdafx.h"
#include <stdlib.h>
#include <stdio.h>
#include <string.h>
#include <math.h>
#include <assert.h>
#include <malloc.h>
#include <ctype.h>
#define MAX 160000

#define DV 1000
#define BROJ_PIKOVA 10
#define MAKS 50000
#define MAKS_L 2000
#define BROJ_ENZIMA_U_FAJLU 200
FILE *dumy_file;
int* clasify(int*, int);
int* clasify(int* m_v, int stepen_dvojke)
{
int *tacke; tacke = (int*)calloc(BROJ_PIKOVA, sizeof(int));
for (int tacka(0); tacka < BROJ_PIKOVA; tacka++)
for (int point(0); point < 256; point++)
        {
                double p = point/256.+1./512.; double q = point/256.-1./512.; double r = 1./stepen_dvojke;
                if ((m_v[tacka]*r < p)&&(m_v[tacka]*r > q))
                        tacke[tacka] = point;
        }
return tacke;
free(tacke);
}
int MaxP(float*, int);
int MaxP(float* amplitude, int broj_tacaka)
{
float max(0.); int position(0); max=amplitude[0];

 for(int i(0);i<broj_tacaka;i++)
        if(amplitude[i]>=max)

                {max=amplitude[i]; position=i;}
        else
                continue;

 return position;
}

float Sum_ampl(float*, int );
float Sum_ampl(float* amplitude, int broj_tacaka)
{
float SUMA(0.); for (int i(0); i< broj_tacaka; i++) SUMA = SUMA + amplitude[i];
return SUMA;
}

int* sign_peaks(float* ,  int);
int* sign_peaks(float* amplitude,  int duzina_sekvence)
{

        float z;  z = 1-pow((1.2)/duzina_sekvence,2./(duzina_sekvence-2)); int lsp(0);
```

```
		float* am1; am1= (float*)calloc((duzina_sekvence/2+1),sizeof(float)); am1 = amplitude;
		int *Pix; Pix = (int*)calloc(BROJ_PIKOVA,sizeof(int));
		while((am1[MaxP(am1, duzina_sekvence/2)]/Sum_ampl(am1, duzina_sekvence/2) > z)&& (lsp<=4))
		{
			Pix[lsp]=MaxP(am1,duzina_sekvence/2); lsp++;
			am1[MaxP(am1,duzina_sekvence/2)]= 0;
		}
		Pix = clasify(Pix, duzina_sekvence/2);
		free(am1);
		return Pix;
}


int* sign_peaks_max(float* ,  int);
int* sign_peaks_max(float* amplitude,  int duzina_sekvence)
{
		float* am1; am1= (float*)calloc((duzina_sekvence/2),sizeof(float)); am1 = amplitude;
		int *Pix; Pix = (int*)calloc(BROJ_PIKOVA,sizeof(int)); int l(0);
		while(l < BROJ_PIKOVA) {Pix[l]=MaxP(am1,duzina_sekvence/2);
		l++;am1[MaxP(am1,duzina_sekvence/2)]=0;}
		return Pix; free(am1); free(Pix);
}
float* rmmean(float*,int);
float* rmmean(float* gEiip, int duzina_proteina)
{
float meanvl; meanvl=0;

for (int i(0);i<duzina_proteina-1;i++)
		{
		 meanvl=meanvl+gEiip[i];
		}
  meanvl=meanvl/duzina_proteina;
  for(int ia(1);ia<duzina_proteina;ia++)
  {
		(gEiip[ia]=gEiip[ia]-meanvl);
  }
  return gEiip;
}

////////////////////////////////////////////////////////////////////////////
float* cfft(float*, int, int);
float* cfft(float* gEiip,int SeqPower, int length)
{
int a = pow(2,SeqPower);
float* FFTm  =new float[a/2+1];
float* FFTp=new float[a/2+1];
  int n,nv2,nm1,k,j,l,le,le1,ip;
  float tr,ur,ui,wr,wi,ti,ur1,ui1;
  float* xr=new float[2048];
  float* xi=new float[2048];
  n=pow(2,SeqPower);
  for (int i(0);i<length;i++) xr[i]=gEiip[i];
const float pi=3.1415926535897932;
  nv2=n/2;
  nm1=n-1;
  j=0;
  for (int i1(length);i1<n+1;i1++)
		xr[i1]=0;
  for(int i2(0);i2<length;i2++) xr[i2]=xr[i2+1];

  for (int i3(0);i3<n;i3++) xi[i3]=0.0;
  for(int i4(0);i4<nm1;i4++)
  {
```

```
            if (i4<=j)
            {
            tr=xr[j];
            xr[j]=xr[i4];
            xr[i4]=tr;
            }
            k=nv2;
            while(k<=j)
            {
                        j=j-k;
                        k=k/2;
            }
                        j=j+k;
}
 for(l=1;l<SeqPower+1;l++)
 {
                                    le=pow(2,l);
                                    le1=le/2;
                                    ur=1.0;
                                    ui=0.0;
                                    wr=cos(pi/(double)(le1));
                                    wi=-sin(pi/(double)(le1));
                                    for (j=0;j<le1;j++)
                                    {
                                              for (i=j;i<n;i=i+le)
                                              {
                                                        ip=i+le1;
                                                        tr=xr[ip]*ur-xi[ip]*ui;
                                                        ti=xr[ip]*ui+xi[ip]*ur;
                                                        xr[ip]=xr[i]-tr;
                                                        xi[ip]=xi[i]-ti;
                                                        xr[i]=xr[i]+tr;
                                                        xi[i]=xi[i]+ti;
                                              }
                                               ur1=ur*wr-ui*wi;
                                               ui1=ur*wi+ui*wr;
                                               ur=ur1;
                                               ui=ui1;
                                    }

            }


 for(i=0;i<n/2;i++) {  FFTp[i]=atan(xi[i]/xr[i]);
                        FFTm[i]=(xr[i]*xr[i]+xi[i]*xi[i]);
                           }

 for(i=n/2;i>0;i--) {
                                                                  FFTm[i]=FFTm[i-1];
                        FFTp[i]=FFTp[i-1];
                                                          }

 FFTm[0]=n/2+1;
 FFTp[0]=n/2+1;
 printf("zavrsio sam");
            return FFTm;
 delete[] xr;
 delete[] xi;
 delete[] FFTm;
 delete[] FFTp;

 }
                        float *param1(const char* param);
```

```
float *param1(const char* param)

{
	int ak(-1);
	FILE *stream3;
	float koeficijent, value;
	float  *f; f=(float *)calloc(20, sizeof(float));
	if((stream3 = fopen("d:/Proteini/Amino/H3790", "r" ))==NULL) printf("xxx");
	for (int i(0); fscanf(stream3, "%f\n", &koeficijent) != EOF; i++)
		f[i] = koeficijent;
	fclose(stream3);
	float *parametar; parametar=(float*)calloc(strlen(param), sizeof(float));
	const int
kiseline[20]={'A','R','N','D','C','Q','E','G','H','I','L','K','M','F','P','S','T','W','Y','V'};
	for(int n_ta_aa(0); n_ta_aa < strlen(param); n_ta_aa++)
	{
		for (int ii1(0); ii1<20; ii1++)
		{
			if((kiseline[ii1] == param[n_ta_aa])) {ak = ii1; break;}

		}

		switch(ak)
		{
			case 0: value = f[0]; break;
			case 1: value = f[1]; break;
			case 2: value = f[2]; break;
			case 3: value = f[3]; break;
			case 4: value = f[4]; break;
			case 5: value = f[5]; break;
			case 6: value = f[6]; break;
			case 7: value = f[7]; break;
			case 8: value = f[8]; break;
			case 9: value = f[9]; break;
			case 10: value = f[10]; break;
			case 11: value = f[11]; break;
			case 12: value = f[12]; break;
			case 13: value = f[13]; break;
			case 14: value = f[14]; break;
			case 15: value = f[15]; break;
			case 16: value = f[16]; break;
			case 17: value = f[17]; break;
			case 18: value = f[18]; break;
			case 19: value = f[19]; break;
			default: {value = 100; printf("problem na vezama");}
		 }

		parametar[n_ta_aa] = value;
	}
	free(f);

	return parametar;
	free(parametar);
}

int main(int argc, char* argv[])
{
float *amplitude, *gEiip, *rmEiip;
int DUZINA_SEKVENCE(0), SeqPower, stepen_dvojke;
FILE* stream_seq;
FILE* stream_names;
FILE* sekvence;
const char ime_file[] = "H3670";
```

```
char *buffer;
int kii(0);
char  prop[150],test[61];
float* crossspectra;

char **sekvenca; sekvenca=(char **)calloc(200, sizeof(char*));
if(sekvenca==NULL) { printf("problem sa memorijom"); exit(1);}

for (int irs_EE(0); irs_EE<150; irs_EE++)
{
	 sekvenca[irs_EE]=(char*)calloc(MAKS_L,sizeof(char));
		if (sekvenca[irs_EE]==NULL) { printf("problem sa memorijom2\n"); exit(1);}
}
int *Pk;
int *His; His = (int*)calloc(512, sizeof(int));
	if((stream_seq=fopen("d:/Pr/LDHC.seq", "r" ))==NULL)printf("PROBLEM WITH OPENING 1 file");
	if((dumy_file = fopen("d:/TEST0001", "w" ))==NULL) printf("PROBLEM WITH OPENING 2 file");

	int c(0), l(0), tt(0), red(0),duzina_reda(0);
	printf("point      2 file");


	char* ime_sekvence;


	for(int hh_E(0);fgets(prop, 68, stream_seq)!=NULL; hh_E++)

	{
		printf(" point 3   %i  \n",hh_E);

		duzina_reda = strlen(prop)+1; buffer=(char*)calloc(duzina_reda,sizeof(char));
		ime_sekvence=(char*)calloc(duzina_reda+1,sizeof(char));
		strcpy(buffer, prop);
		printf("  %s  ",buffer);

		if((buffer[4] == '_')||(buffer[3] == '_'))
		{
			tt++;
			printf("    %i   \n",tt);



			l=0;
				strcpy(ime_sekvence,buffer);
				free(buffer);
				printf("idemo dalje\n");
				continue;
		}
		else
		{

			for (int jj(0); jj < duzina_reda+1; jj++)

			{
			printf(" ma sta je to    %i   \n",tt);
				if(isupper(buffer[jj]))
				{
					sekvenca[tt][l] = buffer[jj]; ++l;
				}
					else
				{if(buffer[jj]=='`')
					{
						break;
```

```
                                        }
                                        else if(buffer[jj]=="")  break;
                                }
                        }
                }
        }

printf("sta sad\n");
        float **amp11; amp11=(float **)calloc(16, sizeof(float*)); if(amp11==NULL) {printf("problem sa
memorijom1"); exit(1);}


        for (int iref(0); iref<512; iref++)
        {
                amp11[iref]=(float*)calloc(MAKS_L,sizeof(float));
                if (amp11[iref]==NULL) { printf("problem sa memorijom2\n"); exit(1);}}
                printf("memorija");

                for(int broj_sek(0); broj_sek < 100; broj_sek++)

                {
                {
                                Pk=(int*)calloc(BROJ_PIKOVA,sizeof(int)); if (Pk==NULL) printf("  jebi
se ");}
                                amp11[broj_sek] = cfft(rmmean(param1(sekvenca[broj_sek]),
strlen(sekvenca[broj_sek])),10,strlen(sekvenca[broj_sek]));
                                amp11[broj_sek][0]=0;
                                kii++;
                }
                crossspectra=(float*)calloc(1024,sizeof(float)); if (crossspectra==NULL) exit(1);
                fprintf(dumy_file, "%s  ", sekvenca[2]);
for (int point1(0); point1 < 512; point1++)
        {
                fprintf(dumy_file,"%f \n", amp11[2][point1]);
        }
                for (int cfreq(1); cfreq < 512; cfreq++)
                {
                        crossspectra[cfreq]=1;
                        for (int protein(2); protein < 4; protein++)
                {
                        crossspectra[cfreq]=crossspectra[cfreq]*amp11[protein][cfreq];

                        crossspectra[0]=0.;
                }
                }
 for (int point11(0); point11 < 512; point11++)
        {
  fprintf(dumy_file,"%f \n", crossspectra[point11]);
        }
 fclose(stream_seq);
fclose(dumy_file);
return 0;
}
```

Appendix II

Primary sequences of LDH molecules used in thesis:

Support data:

Primary sequencies used in thesis:

```
LDHA_ALLMI   .
SVKEHLIHNVHKEEHGHAHNKITVVGVGAVGMACAISILMKDLADELALVDVVEDKLRGE
MLDLQHGSLFLRTPKIVSGKDYSVTANSKLVIITAGARQQEGESRLNLVQRNVNIFKFII
PNVVKHSPDCKLLVVSNPVDIWTYVAWKISGFPKHRVIGSGCNLDSARFRYLMGERLGIH
SLSCHGWIVGEHGDSSVPVWSGVNVAGVSLKALHPELGTDADKEHWKEVHKQVVDSAYEV
IKLKGYTSWAIGLSVADLAETVMKNLRRVHPISTMVKGMYGIKDDVFLSVPCVLGYHGIT
DVVMMTLKSEEEEKLRKSADTLWGIQKELQF`
LDHA_AMBME   .
MSCMKEQLIINILKDEHAPAQNKITVVGVGAVGMACAMSILMKDLADELALVDVIEDKLK
GEMMDLQHGSLFLRTPKIVSGKDYSVTANSKLVIVTAGARQQEGESRLNLVQRNVNIFKF
IIPNVVKYSPDATLLVVSNPVDVLTYVAWKISGFPKHRVIGSGCNLDSARFRYLMGEKLG
VHAQSCHGWVVGEHGDSSVPVWSGVNVAGVSLQTLNPELGTDADKENWKEVHKQVVESAY
EVIKLKGYTSWAIGLSVADLAETIMKNLRRVHPVSTKVKGLYGVHEDVFLSVPCVLGNQG
ITDVVKMTLKPEEEDRLRKSSDTLWGIQKELHF`
LDHA_ANGRO   .
ASVMQKLITPLCSGPQRPRNKVTVVGVGQVGMACAVSILMRELADELALVDVIEDKLKGE
MMDLQHGSLFLKTSKIVADKDYSVSANSRIVVVTAGVRHREGESRLNLVQRNVNIFKHII
PQIVKYSPDCILVVVSNPVDVLTYVTWKLSGLPKHRVIGSGTNLDSARFRYLMAEKLGIH
SSSFNGWILGEHGDSSVPVWSGANVAGVNLQKLNPDIGTDADKENWKDAHKMVVESAYEV
IRLKGYTNWAIGLSVADLAETLIKNLNRIHPVSTMVKGMYGIGDEVYLSLPCVLNNGGVN
SVVNMTLTDEEIAQLKKSADTLWGIQKDLKDL`
LDHA_BOVIN   .
ATLKDQLIQNLLKEEHVPQNKITIVGVGAVGMACAISILMKDLADEVALVDVMEDKLKGE
MMDLQHGSLFLRTPKIVSGKDYNVTANSRLVIITAGARQQEGESRLNLVQRNVNIFKFII
PNIVKYSPNCKLLVVSNPVDILTYVAWKISGFPKNRVIGSGCNLDSARFRYLMGERLGVH
PLSCHGWILGEHGDSSVPVWSGVNVAGVSLKNLHPELGTDADKEQWKAVHKQVVDSAYEV
IKLKGYTSWAIGLSVADLAESIMKNLRRVHPISTMIKGLYGIKEDVFLSVPCILGQNGIS
DVVKVTLTHEEEACLKKSADTLWGIQKELQF`
LDHA_BRARE   .
ASTKEKLIAHVSKEQPAGPTNKVTVVGVGMVGMAAAVSILLKDLTDELALVDVMEDKLKG
EAMDLQHGSLFLKTHKIVADKDYSVTANSKVVVVTAGARQQEGESRLNLVQRNVNIFKFI
IPNIIKYSPNCILLVVSNPVDILTYVAWKLSGLPRNRVIGSGTNLDSARFRYLMGEKLGI
HPSSCHGWVVGEHGDSSVPVWSGVNVAGVSLQALNPDLGTDKDKEDWKSVHKMVVDSAYE
VIKLKGYTSWAIGMSVADLCESILKNMHKCHPVSTLVKGMHGVNEEVFLSVPCILGNNGL
TDVVHMTLKPEEEKQLVKSAETLWGVQKELTL`
LDHA_CAICA   .
SVKEHLIHNVHKEEHGHAHNKITVVGVGAVGMACAISILMKDLADELALVDVVEDKLRGE
MLDLQHGSLFLRTPKIVSGKDYSVTANSKLVIITAGARQQEGESRLNLVQRNVNIFKFII
PNVVKHSPDCKLLVVSNPVDILTYVAWKISGFPKHRVIGSGCNLDSARFRYLMGERLGIH
PLSCHGWIVGEHGDSSVPVWSGVNVAGVSLKALHPELGTDADKEHWKEVHKEVVDSAYEV
IKLKGYTSWAIGLSVADLAETVMKNLRRVHPISTMVKGMYGIKDDVFLSVPCVLGYHGIT
DVVMMTLKSEEEEKIRKSADTLWGIQKELQF`
LDHA_CHAAC   .
STKEKLISHVMKEEPVGSRNKVTVVGVGMVGMASAISILLKDLCDELAMVDVMEDKLKGE
VMDLQHGSLFLKTKIVGDKDYSVTANSKVVVVTAGARQQEGESRLNLVQRNVNIFKFIIP
NIVKYSPNCILMVVSNPVDILTYVAWKLSGFPRHRVIGSGTNLDSARFRHLIGEKLHLHP
SSCHAWIVGEHGDSSVPVWSGVNVAGVSLQGLNPQMGTEGDGENWKAIHKEVVDGAYEVI
KLKGYTSWAIGMSVADLVESIIKNMHKVHPVSTLVQGMHGVKDEVFLSVPCVLGNSGLTD
VIHMTLKAEEEKQLQKSAETLWGVQKELTL`
LDHA_CHAGU   .
STKEKLISHVMKEEPVGSRSKVTVVGVGMVGMASAISILLKDLCDELAMVDVMEDKLKGE
VMDLQHGSLFLKTKIVGDKDYSVTANSKVVVVTAGARQQEGESRLNLVQRNVNIFKFIIP
NIVKYSPNCILMVVSNPVDILTYVAWKLSGFPRHRVIGSGTNLDSARFRHLIGEKLHLHP
SSCHAWIVGEHGDSSVPVWSGVNVAGVSLQGLNPQMGTEGDGENWKAIHKEVVDGAYEVI
KLKGYTSWAIGMSVADLVESIIKNMHKVHPVSTLVQGMHGVKDEVFLSVPCVLGNSGLTD
VIHMTLKAEEEKQLQKSAETLWGVQKELTL`
LDHA_CHICK   .
SLKDHLIHNVHKEEHAHAHNKISVVGVGAVGMACAISILMKDLADELTLVDVVEDKLKGE
```

MLDLQHGSLFLKTPKIISGKDYSVTAHSKLVIVTAGARQQEGESRLNLVQRNVNIFKFII
PNVVKYSPDCKLLIVSNPVDILTYVAWKISGFPKHRVIGSGCNLDSARFRHLMGERLGIH
PLSCHGWIVGEHGDSSVPVWSGVNVAGVSLKALHPDMGTDADKEHWKEVHKQVVDSAYEV
IKLKGYTSWAIGLSVADLAETIMKNLRRVHPISTAVKGMHGIKDDVFLSVPCVLGSSGIT
DVVKMILKPDEEEKIKKSADTLWGIQKELQF`
LDHA_COLLI .
SLKDQLIHNVHKEEHSHAHNKISVVGVGAVGMACAISILMKDLADELALVDVVEDKLKGE
MLDLQHGSLFLRTPKIVSGKDYSVTAHSKLVIVTAGARQQEGESRLNLVQRNVNIFKVII
PNVVKYSPDCKLLIVSNPVDILTYVAWKISGFPKHRVIGSGCNLDSARFRHLMGERLGIH
PLSCHGWIVGEHGDSSVPVWSGVNVAGVSLKALHPDMGTDADKEHWKEVHKQVVDSAYEV
IKLKGYTSWAIGLSVADLAETIMKNLRRVHPISTVVKGMHGIKEDVFLSVPCVLGSSGIT
DVVKMILKPEEEDKLRKSADTLWGIQKELQF`
LDHA_CORNI .
STKEKLISHVSKEEAVGSRNKVTVVGVGMVGMASAISILLKDLCDELALVDVMEDKLKGE
VMDLQHGSLFLKTHKIVGDKDYSVTANSRVVVVTAGARQQEGESRLNLVQRNVNIFKFII
PNIVKYSPNCILMVVSNPVDILTYVAWKLSGFPRHRVIGSGTNLDSARFRHIMGEKLHLH
PSSCHGWIVGEHGDSSVPVWSGVNVAGVSLQTLNPKMGAEGDSENWKAVHKMVVDGAYEV
IKLKGYTSWAIGMSVADLVESIVKNLHKVHPVSTLVKGMHGVKDEVFLSVPCVLGNSGLT
DVIHMTLKADEEKQLVKSAETLWGVQKELTL`
LDHA_CYPCA .
ASTKEKLITHVSKEEPAGPTNKVTVVGVGMVGMAAAISILLKDLTDELALVDVMEDKLKG
EAMDLQHGSLFLKTHKIVADKDYSVTANSKVVVVTAGARQQEGESRLNLVQRNVNIFKFI
IPNIIKYSPNCILLVVSNPVDILTYVAWKLSGLPRNRVIGSGTNLDSARFRHLMGEKLGI
HPSNCHGWVIGEHGDSSVPVWSGVNVAGVFLQGLNPDMGTDKDKEDWKSVHKMVVDSAYE
VIKLKGYTSWAIGMSAADLCQSILKNLRKCHPVSTLVKGMHGVNEEVFLSVPCILGNSGL
TDVVHMTLKSDEEKQLVKSAETLWGVQKDLTL`
LDHA_DISEL .
STKEKLISHVMKEEPVGSRNKVTVVGVGMVGMASAISILLKDLCDELAMVDVMEDKLKGE
VMDLQHGSLFLKTKIVGDKDYSVTANSKVVVVTAGARQQEGESRLNLVQRNVNIFKFIIP
NIVKYSPNCILMVVSNPVDILTYVAWKLSGFPRNRVIGSGTNLDSARFRHLIGEKLHLHP
SSCHAWIVGEHGDSSVPVWSGVNVAGVSLQGLNPQMGTEGDGENWKAIHKEVVDGAYEVI
KLKGYTSWAIGMSVADLVESIIKNMHKVHPVSTLVQGMHGVKDEVFLSVPSVLGNSGLTD
VIHMTLKAEEEKQLQKSAETLWGVQKELTL`
LDHA_DISMA .
STKEKLISHVMKEEPVGSRNKVTVVGVGMVGMASAISILLKDLCDELAMVDVMEDKLKGE
VMDLQHGSLFLKTKIVGDKDYSVTANSKVVVVTAGARQQEGESRLNLVQRNVNIFKFIIP
NIVKYSPNCILMVVSNPVDILTYVAWKLSGFPRNRVIGSGTNLDSARFRHLIGEKLHLHP
SSCHAWIVGEHGDSSVPVWSGVNVAGVSLQGLNPQMGTEGDGENWKAIHKEVVDGAYEVI
KLKGYTSWAIGMSVADLVESIIKNMHKVHPVSTLVQGMHGVKDEVFLSVPSVLGNSGLTD
VIHMTLKAEEEKQLQKSAETLWGVQKELVL`
LDHA_ELEMC .
STKEKLISHVMKEEPVGSRNKVTVVGVGMVGMASAISILLKDLCDELAMVDVMEDKLKGE
VMDLQHGSLFLKTHKIVGDKDYSVTANSKLVVVTAGARQQEGESRLNLVQRNVNIFKFII
PNIVKYSPNCILMVVSNPVDILTYVAWKLSGFPRHRVLGSGTNLDSARFRHLIGEKLNLH
PSSCHAWIIGEHGDSSVPVWSGLNVAGVSLQGLNPQMGTEGDSENWKAIHKEVVDGAYEV
IKLKGYTSWAIGMSVADLVESILKNLHKVHPVSTLVQGMHGVKDEVFLSVPCVLGNSGLT
DVVHMTLKAEEEKQVQNSAETLWGVQKELTL`
LDHA_FUNHE .
STQEKLISHVMKEEPVGCRNKVTVVGVGMVGMASAISVLLKDLCDELALVDVMEDKLKGE
AMDLQHGALFLKTHKIVADKDYSVTANSKVVVVTAGARQQEGESRLNLVQRNVNIFKFII
PNIVKYSPNCILLVVSNPVDILTYVAWKLSGFPRHRVIGSGTNLDSARFRHLMGEKFHLH
PSSCHGWIVGEHGDSSVAVWSGVNIAGVSLQTLNPNMGADGDSENWKELHKKVVDGAYEV
IKLKGYTSWAIGMSVADLVESIVKNLHKVHPVSTLVQGMHGVKDEVFLSIPCVLGNSGLT
DVIHMTLKPEEEKQLVKSAETLWGVQKELTL`
LDHA_GILMI .
STKEKLISHVSKEEAVGSRNKVTVVGVGMVGMASAISILLKDLCDELALVDVMEDKLKGE
VMDLQHGSLFLKTHKIVADKDYSVTANSRVVVVTAGARQQEGESRLNLVQRNVNIFKFII
PNIVKYSPNCILMVVSNPVDILTYVAWKLSGFPRHRVIGSGTNLDSARFRHIMGEKLHLH
PSSCHGWIVGEHGDSSVPVWSGVNVAGVSLQTLNPKMGAEGDSENWKAVHKMVVDGAYEV
IKLKGYTSWAIGMSVADLVESIVKNLHKVHPVSTLVKGMHGVKDEVFLSVPCVLGNSGLT
DVIHMTLKADEEKQLVKSAETLWGVQKELTL`
LDHA_GOBGI .
STKEKLISHVMKEEPVGSRNKVTVVGVGMVGMASAISILLKDLCDELAMVDVMEDKLKGE
VMDLQHGSLFLKTKIVGDKDYSVTANSKVVVVTAGARQQEGESRLNLVQRNVNIFKFIIP

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NIVKYSPNCILMVVSNPVDILTYVAWKLSGFPRHRVIGSGTNLDSARFRHLIGEKLHLHP
SSCHAWIVGEHGDSSVPVWSGVNVAGVSLQGLNPQMGTEGDGEDWKAIHKEVVDGAYEVI
KLKGYTSWAIGMSVADLVESIIKNMHKVHPVSTLVQGMHGVKDEVFLSVPCVLGNSGLTD
VIHMTLKAEEEKQVQKSAETLWGVQKELIL`
LDHA_HARAN   .
STKEKLISHVMKEEPVGSRNKVTVVGVGMVGMASAISILIKDLGDELAMVDVMEDKLKGE
VMDLQHGSLFLKTKIVGDKDYSVTANSKVVVVTAGARQQEGESRLNLVQRNVNIFKFIIP
NIVKYSPNCILMVVSNPVDILTYVAWKLSGFPRHRVLGSGTNLDSARFRHLIGEKLHLHP
SSCHAWIVGEHGDSSVPVWSGVNVAGVSLQNLNPQMGTEGDGENWKAIHKEVVDGAYEVI
KLKGYTSWAIGMSVADLVESIIKNMHKVHPVSTLVQGMHGVKDEVFLSVPCVLGNSGLTD
VIHMTLKAEEEKQVQKSAETLWGVQKELTL`
LDHA_HORVU   .
MHKASSLSELGFDAGGASSGFFRPVADGCPATPTSSAVPHRRLTKISVIGAGNVGMAIAQ
TILTQNLADEIALVDALPDKLRGEALDLQHAAAFLPRVRISGTDAAVTKNSDLVIVTAGA
RQIPGETRLNLLQRNVALYRKIVPPVAEHSPDALLLVVSNPVDVLTYVAWKLSGFPASRV
IGSGTNLDSSRFRFLIAEHLDVNAQDVQAYMVGEHGDSSVAIWSSISVGGMPAFKSLRDS
HRSFDEAALEGIRRAVVGGAYEVIGLKGYTSWAIGYSVASLAASLLRDQRRVHPVSVLAS
GFHGISDGHEVFLSLPARLGRGGILGVAEMDLTEAEAAQLRRSAKTLWENCQLLDL`
LDHA_HUMAN   .
ATLKDQLIYNLLKEEQTPQNKITVVGVGAVGMACAISILMKDLADELALVDVIEDKLKGE
MMDLQHGSLFLRTPKIVSGKDYNVTANSKLVIITAGARQQEGESRLNLVQRNVNIFKFII
PNVVKYSPNCKLLIVSNPVDILTYVAWKISGFPKNRVIGSGCNLDSARFRYLMGERLGVH
PLSCHGWVLGEHGDSSVPVWSGMNVAGVSLKTLHPDLGTDKDKEQWKEVHKQVVESAYEV
IKLKGYTSWAIGLSVADLAESIMKNLRRVHPVSTMIKGLYGIKDDVFLSVPCILGQNGIS
DLVKVTLTSEEEARLKKSADTLWGIQKELQF`
LDHA_LEPNU   .
STKEKLISHVMKEEPVGSRSKVTVVGVGMVGMASAISILLKDLCDELAMVDVMEDKLKGE
VMDLQHGSLFLKTKIVGDKDYSVTANSKVVVVTAGARQQEGESRLNLVQRNVNIFKFIIP
NIVKYSPNCILMVVSNPVDILTYVAWKLSGFPRHRVIGSGTNLDSARFRHLIGEKLHLHP
SSCHAWIVGEHGDSSVPVWSGVNVAGVSLQGLNPQMGTEDDGENWKAIHKEVVDGAYEVI
KLKGYTSWAIGMSVADLVESIIKNMHKVHPVSTLVQGMHGVKDEVFLSVPCVLGNSGLTD
VIHMTLKAEEEKQLQKSAETLWGVQKELTL`
LDHA_MACFA   .
ATLKDQLIHNLLKEEQTPQNKITVVGVGAVGMACAISILMKDLADELALVDVIEDKLKGE
MMDLQHGSFFLRTPKIVSGKDYSVTANSKLVIITAGARQQEGESRLNLVQRNVNIFKFIV
PNVVKYSPNCKLLIVSNPVDILTYVAWKISGFPKNRVIGSGCNLDSARFRYLMGERLGVH
PLSCHGWVLGEHGDSSVPVWSGMNVAGVSLKTLHPDLGTDKDKEQWKEVHKQVVESAYEV
IKLKGYTSWAIGLSVADLAESIMKNLRRVHPVSTMIKGLYGIKDDVFLSVPCILGQNGIS
DLVKVTLTPEEEARLKKSADTLWGIQKELQF`
LDHA_MONDO   .
GTVKDQLILNVLKEEQTPHNKITVVGVGAVGMACAISILMKDLADELALVDVIEDKLKGE
MMDLQHGSLFLKTPKIVSSKDYAVTANSKLVVITAGARQQEGESRLNLVQRNVNIFKFII
PNIVKYSPNCKLLVVSNPVDILTYVAWKLSGFPKNRVIGSGCNLDSARFRYLMGEKLGIH
SSSCHGWILGEHGDSSVPVWSGVNVAGVSLKSLHPALGTDSDSEQWKDVHKQVVESAYEV
IKLKGYTSWAIGLSVADLAESIMKNLRRVHPISTMIKGLYGINEDVFLSVPCILGQNGIS
DVVKVTLTTEEESRLKQSADTLWGIQKELQF`
LDHA_MOUSE   .
ATLKDQLIVNLLKEEQAPQNKITVVGVGAVGMACAISILMKDLADELALVDVMEDKLKGE
MMDLQHGSLFLKTPKIVSSKDYCVTANSKLVIITAGARQQEGESRLNLVQRNVNIFKFII
PNIVKYSPHCKLLIVSNPVDILTYVAWKISGFPKNRVIGSGCNLDSARFRYLMGERLGVH
ALSCHGWVLGEHGDSSVPVWSGVNVAGVSLKSLNPELGTDADKEQWKEVHKQVVDSAYEV
IKLKGYTSWAIGLSVADLAESIMKNLRRVHPISTMIKGLYGINEDVFLSVPCILGQNGIS
DVVKVTLTPEEEARLKKSADTLWGIQKELQF`
LDHA_NOTAN   .
STKEKLISHVMKEEPVGSRNKVTVVGVGMVGMASAISILLKDLCDELAMVDVMEDKLKGE
VMDLQHGSLFLKTKIVGDKDYSVTANSKVVVVTAGARQQEGESRLNLVQRNVNIFKFIIP
NIVKYSPNCILMVVSNPVDILTYVAWKLSGFPRHRVIGSGTNLDSARFRHLIGEKLHLHP
SSCHAWIVGEHGDSSVPVWSGVNVAGVSLQGLNPQMGTEGDGENWKAIHKEVVDGAYEVI
KLKGYTSWAIGMSVADLVESIIKNMHKVHPVSTLVQGMHGVKDEVFLSVPCVLGNSGLTD
VIHMTLKAEEEKQVQKSAETLWGVQKELTL`
LDHA_NOTCO   .
STKEKLISHVMKEEPVGSRNKVTVVGVGMVGMASAVSILLKDLCDELAMVDVMEDKLKGE
VMDLQHGSLFLKTKIVGDKDYSVTANSKVVVVTAGARQQEGESRLNLVQRNVNIFKFIIP
NIVKYSPNCILMVVSNPVDILTYVAWKLSGFPRHRVIGSGTNLDSARFRHLIGEKLHLHP
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SSCHAWIVGEHGDSSVPVWSGVNVAGVSLQGLNPQMGTEGDGENWKAIHKEVVDGAYEVI
KLKGYTSWAIGMSVADLVESIIKNMHKVHPVSTLVQGMHGVKDEVFLSVPCVLGNSGLTD
VIHMTLKAEEEKQVQKSAETLWGVQKELIL`
LDHA_PARCR   .
STKEKLISHVMKEEPVGSRNKVTVVGVGMVGMASAISILLKDLCDELAMVDVMEDKLKGE
VMDLQHGSLFLKTKIVGDKDYSVTANSKVVVVTAGARQQEGESRLNLVQRNVNIFKFIIP
NIVKYSPNCILMVVSNPVDILTYVAWKLSGFPRHRVIGSGTNLDSARFRHLIGEKLHLHP
SSCHAWIVGEHGDSSVPVWSGVNVAGVSLQDLNPQMGTEGDGENWKAIHKEVVDGAYEVI
KLKGYTSWAIGMSVADLVESIIKNMHKVHPVSTLVQGMHGVKDEVFLSVPCVLGNSGLTD
VIHMTLKADEEKQVQKSAETLWGVQKELTL`
LDHA_PARMG   .
STKEKLISHVMKEEPVGSGNKVTVVGVGMVGMASAISILLKDLCDELAMVDVMEDKLKGE
VMDLQHGSLFLKTKIVGDKDYSVTANSKVVVVTAGARQQEGESRLNLVQRNVNIFKFIIP
NIVKYSPNCILMVVSNPVDILTYVAWKLSGFPRHRVLGSGTNLDSARFRHLIGEKLHLHP
SSCHAWIVGEHGDSSVPVWSGVNVAGVSLQGLNPQMGTEGDGENWMAIHKEVVDGAYEVI
KLKGYTSWAIGMSVADLVESIIKNMHKVHPVSTLVQGMHGVKDEVFLSVPCVLGNSGLTD
VIHMALKAEEEKQVQKSAETLWGVQKELTL`
LDHA_PATTE   .
STKEKLISHVMKEEPVGSRSKVTVVGVGMVGMASAISILLKDLCDELAMVDVMEDKLKGE
VMDLQHGSLFLKTKIVGDKDYSVTANSKVVVVTAGARQQEGESRLNLVQRNVNIFKFIIP
NIVKYSPNCILMVVSNPVDILTYVAWKLSGFPRHRVIGSGTNLDSARFRHLIGEKLHLHP
SSCHAWIVGEHGDSSVPVWSGVNVAGVSLQGLNPQMGTEGDGENWKAIHKEVVDGAYEVI
KLKGYTSWAIGMSVADLVESIIKNMHKVHPVSTLVQGMHGVKDEVFLSVPCVLGNSGLTD
VIHMTLKAEEEKQVQKSAETLWGVQKELTL`
LDHA_PELSJ   .
SVKELLIQNVHKEEHSHAHNKITVVGVGAVGMACAISILMKDLADELALVDVIEDKLRGE
MLDLQHGSLFLRTPKIVSGKDYSVTAHSKLVIITAGARQQEGESRLNLVQRNVNIFKFII
PNVVKYSPDCMLLVVSNPVDILTYVAWKISGFPKHRVIGSGCNLDSARFRYLMGEKLGIH
SLSCHGWIIGEHGDSSVPVWSGVNVAGVSLKALYPDLGTDADKEHWKEVHKQVVDSAYEV
IKLKGYTSWAIGLSVADLAETVMKNLRRVHPISTMVKGMYGVSSDVFLSVPCVLGYAGIT
DVVKMTLKSEEEEKLRKSADTLWGIQKELQF`
LDHA_PIG   .
ATLKDQLIHNLLKEEHVPHNKITVVGVGAVGMACAISILMKELADEIALVDVMEDKLKGE
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LDHA_PYTRG   .
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LDHA_RABIT   .
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LDHA_RAT   .
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LDHA_RHIDE   .
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LDHA_SCEUN   .
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LDHA_SPHAG   .
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LDHA_SPHID   .
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LDHA_SQUAC   .
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LDHA_TRASC   .
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LDHA_XENLA   .
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LDHB_ALLMI .
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LDHB_BRARE .
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LDHB_CAICA .
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LDHB_CHICK .
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LDHB_COLLI .
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LDHB_FUNHE .
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LDHB_HORVU .
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LDHB_HUMAN .
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LDHB_MONDO .
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LDHB_MOUSE .
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LDHB_PELSJ .
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LDHB_PIG .
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LDHB_RABIT .
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LDHB_RAT .
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LDHB_RHIOR .
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LDHB_SCEUN .
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LDHB_SCEWO   .
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LDHB_SQUAC   .
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LDHB_TRASC   .
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LDHB_XENLA   .
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LDHC_HUMAN   .
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LDHC_MOUSE   .
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LDHC_PIG   .
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TMDLQHGSLFFNTSKIVSGKDYSVSANSKIVIVTAGARQQEGESRLALVQRNVNIMKSII
PTIVQHSPDCKMLIVSNPVDILTYVAWKLSGLPATRVIGSGCNLDSARFRYLIGKKLGVH
PTSCHGWIIGEHGDSSVPLWSGVNVAGVALKTLDPKLGTDSDKDQWKNIHKQVIGSAYEI
IKLKGYTSWAIGLSVTDLVGSILKNLRRVHPVSTMVKGLYGIKEEIFLSIPCVLGRNGVS
DIVKVNLNAEEEALFKKSANTLWNVQKDLTF`
LDHC_RAT   .
STVKEQLIQNLAPDEKQSRCKITVVGVGNVGMACAISILLKGLADELALVDADENKLKGE
ALDLLHGSLFLSTPKIVFGKDYSVSANSKLVIITAGARMVSGESRLALLQRNVTSMKAIV
PGVIQNSPDCKIMIVTNPVDILTYVVWKISGLPVSSVIGSGCNLDSARFRYLIGEKLGVN
PSSCHGWVLGEHGDSSVPIWSGVNIAGVTLKSLNPAIGSDSDKEQWKTVHKQVVDGGYEV
LNLKGYTSWAIALSVTDIAASILKNLKRVHAVTTLVKGLYGIKEEIFLSIPCVLGQSGIT
DLVKVNMNTEEEALFKKSCDILWNIQKDLQL`
LDHC_VULVU   .
STVKEKLIENLIEEDKISQRKITIVGTGAVGMACAICILLKDLADELALVDVAVDKLKGE
MMDLQHGSLFFNTSKITSGKDYSVSANSKLVIVTAGARQQEGESRLALVQRNVNIMKSII
PAVVQHSPDCKMLIVSNPVDILTYVVWKLSGLPATRVFGSGCNLDSARFRYLIGEKLGVH
PTSCHGWIIGEHGDSSVPLWSGVNVAGVALKTLDPKLGTDADKDQWKNIHKQVVESAYEI
IKLKGYTSWAIGLSVTDLVGSVLKNLRRVHPVSTMVKGLYGIKEEIFLSIPCVLGQNGVS
DIVKINLNSDEEALFKKSADTLWNVQKELVF"
LDHC_XENLA   .
SSVQENLITNVCQDKAAKPTNKITIVGVGQVGMACAVSVLLKELADELALVDILEDKLKG
EVMDLQHGSLFLKTPTIVADKDYSVTANSRIVVVTGGVRQQEGESALNLVQRNVNVFKFI
IPQVVKYSPDCIIIVVSNPVDILTYVTWKLSGLPQHRIIGSGTNLDSARFRHLISEKLGV
HPSSCHGFILGEHGDTSVAVWSGVNVAGVSLQSLKPEIGTDQDSCNWKEVHKKVVDSAYE
VIKLKGYTNWAIGFSVAEIVESITKNLGRVHPVSTMVKGMYGIETEVFLSLPCVLNGNGL
TSVISQKLKDDEVGQLQKSSETLWGIQKDLQVL`
```

Appendix III

a) File generated by spectrometer every 20 second
b) Example of optical density data regarded experimental unit with LDH exposed to 745nm.

Data files recorded from the spectrometer: LDH was exposed at 835 nm for 15 min. The spectra was measure 20 sec after LDH was mixed with the solution of NADH and pyruvate. Only count at 340.27 was of the interest for the monitoring concentration of LDH

```
OOIBase32 Version 2.0.1.4 Data File
++++++++++++++++++++++++++++++++++++
Date: 11-23-2005, 13:46:35
User: Valued Ocean Optics Customer
Spectrometer Serial Number: USB2G3844
Spectrometer Channel: Master
Integration Time (msec): 20
Spectra Averaged: 3
Boxcar Smoothing: 9
Correct for Electrical Dark: Enabled
Time Normalized: Disabled
Dual-beam Reference: Disabled
Reference Channel: Master
Temperature: 27.0
Spectrometer Type: S2000
ADC Type: USB2000
Number of Pixels in File: 2048
Graph Title:


λ      count          λ     count        λ     count          λ     count        λ count

>>>>>Begin Spectral Data<<<<<
```

177.85	0.000	208.10	125.070	238.18	128.491	268.07	132.754	297.77	940.807
178.23	112.000	208.48	125.193	238.56	128.667	268.45	133.123	298.15	957.474
178.62	113.000	208.87	125.456	238.94	128.719	268.83	133.439	298.52	971.877
179.00	114.667	209.25	125.509	239.32	128.596	269.20	133.895	298.90	986.754
179.38	113.667	209.63	125.579	239.70	128.684	269.58	134.368	299.27	999.579
179.77	117.667	210.01	125.737	240.08	128.719	269.96	134.719	299.64	1010.211
180.15	116.000	210.39	125.719	240.46	128.596	270.33	135.088	300.02	1020.316
180.54	119.333	210.78	125.702	240.84	128.439	270.71	135.509	300.39	1030.351
180.92	118.667	211.16	125.684	241.22	128.386	271.09	136.018	300.77	1038.983
181.30	117.333	211.54	125.719	241.60	128.421	271.47	136.298	301.14	1046.263
181.69	116.333	211.92	125.754	241.97	128.439	271.84	136.649	301.52	1053.053
182.07	118.667	212.30	125.649	242.35	128.509	272.22	137.158	301.89	1058.561
182.46	117.333	212.68	125.737	242.73	128.526	272.60	137.754	302.27	1063.421
182.84	118.000	213.06	125.754	243.11	128.509	272.97	138.263	302.64	1067.246
183.22	119.667	213.45	125.719	243.49	128.544	273.35	138.947	303.01	1070.491
183.61	121.333	213.83	125.702	243.87	128.561	273.73	139.737	303.39	1073.035
183.99	122.667	214.21	125.825	244.25	128.491	274.10	140.439	303.76	1074.316
184.37	121.000	214.59	125.807	244.63	128.386	274.48	141.105	304.14	1074.509
184.76	119.333	214.97	125.807	245.01	128.333	274.86	141.842	304.51	1073.526
185.14	119.333	215.35	125.825	245.39	128.298	275.23	142.544	304.88	1072.597
185.52	120.333	215.73	125.789	245.77	128.158	275.61	143.246	305.26	1070.386
185.91	120.000	216.12	125.825	246.14	128.105	275.99	144.211	305.63	1068.474
186.29	120.333	216.50	126.018	246.52	128.246	276.36	145.263	306.01	1064.789
186.67	120.000	216.88	126.088	246.90	128.246	276.74	146.421	306.38	1060.316
187.06	118.667	217.26	126.070	247.28	128.298	277.12	147.579	306.75	1057.018
187.44	121.737	217.64	126.070	247.66	128.386	277.49	148.930	307.13	1052.754
187.82	121.737	218.02	126.228	248.04	128.421	277.87	150.509	307.50	1046.351
188.21	121.912	218.40	126.439	248.42	128.386	278.24	152.105	307.88	1040.035
188.59	122.123	218.78	126.561	248.80	128.421	278.62	154.105	308.25	1034.228
188.97	122.333	219.17	126.667	249.18	128.404	279.00	156.105	308.62	1025.737
189.36	122.632	219.55	126.877	249.55	128.404	279.37	158.281	309.00	1018.491
189.74	122.895	219.93	126.842	249.93	128.526	279.75	160.526	309.37	1010.509
190.12	123.018	220.31	126.930	250.31	128.474	280.13	163.053	309.74	1003.421
190.51	123.140	220.69	127.070	250.69	128.368	280.50	165.561	310.12	995.825
190.89	123.404	221.07	127.193	251.07	128.263	280.88	168.316	310.49	986.877
191.27	123.526	221.45	127.175	251.45	128.351	281.25	171.439	310.87	979.070
191.66	123.702	221.83	127.246	251.83	128.211	281.63	174.754	311.24	970.947
192.04	123.702	222.21	127.263	252.20	128.246	282.01	178.333	311.61	962.404
192.42	123.807	222.59	127.316	252.58	128.474	282.38	182.228	311.99	953.983
192.81	123.807	222.97	127.474	252.96	128.561	282.76	186.667	312.36	945.860
193.19	123.947	223.35	127.579	253.34	128.737	283.13	191.474	312.73	936.035
193.57	124.018	223.74	127.596	253.72	128.754	283.51	196.579	313.11	927.140
193.95	124.053	224.12	127.544	254.10	128.842	283.89	202.456	313.48	918.246
194.34	124.140	224.50	127.439	254.47	128.912	284.26	208.789	313.85	908.631
194.72	124.228	224.88	127.579	254.85	129.018	284.64	215.877	314.23	899.439
195.10	124.351	225.26	127.456	255.23	129.053	285.01	223.474	314.60	890.579
195.49	124.421	225.64	127.421	255.61	129.105	285.39	232.018	314.97	881.316
195.87	124.509	226.02	127.474	255.99	129.175	285.76	241.105	315.35	871.789
196.25	124.561	226.40	127.491	256.37	129.281	286.14	250.895	315.72	863.105
196.63	124.561	226.78	127.421	256.74	129.333	286.52	261.807	316.09	853.772
197.02	124.544	227.16	127.421	257.12	129.368	286.89	273.596	316.46	845.193
197.40	124.596	227.54	127.421	257.50	129.386	287.27	286.298	316.84	835.263
197.78	124.719	227.92	127.456	257.88	129.526	287.64	300.509	317.21	825.544
198.17	124.789	228.30	127.439	258.26	129.614	288.02	315.930	317.58	815.614
198.55	124.719	228.68	127.544	258.63	129.509	288.39	332.140	317.96	804.842
198.93	124.544	229.06	127.544	259.01	129.772	288.77	349.912	318.33	794.158
199.31	124.491	229.44	127.632	259.39	129.842	289.15	369.070	318.70	783.175
199.70	124.491	229.82	127.596	259.77	129.754	289.52	390.140	319.08	772.228
200.08	124.526	230.20	127.596	260.14	129.825	289.90	411.544	319.45	761.140
200.46	124.596	230.58	127.561	260.52	129.737	290.27	434.386	319.82	750.947
200.84	124.649	230.96	127.561	260.90	129.807	290.65	459.421	320.19	739.649
201.22	124.667	231.34	127.509	261.28	129.825	291.02	484.596	320.57	728.263
201.61	124.474	231.72	127.544	261.66	129.912	291.40	511.614	320.94	717.316
201.99	124.404	232.10	127.561	262.03	129.825	291.77	538.228	321.31	706.281
202.37	124.439	232.48	127.614	262.41	129.860	292.15	566.316	321.68	695.825
202.75	124.474	232.86	127.614	262.79	129.895	292.52	594.702	322.06	686.193
203.14	124.544	233.24	127.596	263.17	129.912	292.90	622.684	322.43	677.368
203.52	124.614	233.62	127.684	263.54	129.982	293.27	651.088	322.80	669.719
203.90	124.526	234.00	127.877	263.92	130.088	293.65	679.439	323.17	662.386
204.28	124.544	234.38	127.965	264.30	130.263	294.02	707.439	323.55	655.614
204.67	124.632	234.76	128.035	264.68	130.561	294.40	735.544	323.92	649.842
205.05	124.719	235.14	127.930	265.05	130.754	294.77	762.561	324.29	644.509
205.43	124.754	235.52	127.895	265.43	130.860	295.15	788.509	324.66	640.035
205.81	124.807	235.90	127.965	265.81	131.193	295.52	813.860	325.04	635.035
206.19	125.000	236.28	128.053	266.19	131.333	295.90	837.719	325.41	629.175
206.58	125.193	236.66	128.228	266.56	131.456	296.27	860.895	325.78	623.632
206.96	125.088	237.04	128.439	266.94	131.702	296.65	882.351	326.15	617.246
207.34	125.105	237.42	128.474	267.32	132.053	297.02	903.649	326.52	611.316
207.72	125.035	237.80	128.509	267.70	132.386	297.40	923.737	326.90	605.456

327.27	600.596	356.56	616.140	385.63	1620.211	414.48	1800.035	443.09	1660.000
327.64	596.070	356.93	622.877	386.00	1631.035	414.84	1798.667	443.45	1659.509
328.01	590.947	357.30	630.912	386.36	1641.246	415.20	1797.246	443.81	1658.386
328.39	586.754	357.67	638.561	386.73	1650.333	415.57	1795.912	444.17	1657.631
328.76	582.596	358.04	646.386	387.10	1657.930	415.93	1793.123	444.53	1659.474
329.13	577.123	358.40	654.421	387.46	1665.983	416.29	1790.825	444.89	1660.263
329.50	571.491	358.77	663.123	387.83	1673.509	416.66	1788.526	445.25	1657.930
329.87	565.544	359.14	671.474	388.20	1683.386	417.02	1782.597	445.61	1652.807
330.24	559.281	359.51	680.439	388.56	1691.983	417.39	1779.018	445.98	1649.877
330.62	551.965	359.88	689.649	388.93	1701.298	417.75	1779.754	446.34	1647.018
330.99	544.842	360.25	699.719	389.29	1712.509	418.11	1780.667	446.70	1644.755
331.36	537.509	360.62	709.860	389.66	1726.070	418.48	1783.368	447.06	1640.228
331.73	530.649	360.99	719.877	390.03	1737.755	418.84	1789.456	447.42	1633.369
332.10	524.246	361.36	730.158	390.39	1747.754	419.20	1793.561	447.78	1627.895
332.47	519.053	361.73	741.158	390.76	1753.368	419.56	1794.772	448.14	1627.035
332.85	514.754	362.09	752.193	391.12	1761.456	419.93	1795.158	448.50	1622.509
333.22	511.684	362.46	763.667	391.49	1767.000	420.29	1795.579	448.86	1619.737
333.59	508.351	362.83	774.789	391.86	1772.807	420.65	1794.807	449.22	1616.140
333.96	505.018	363.20	786.316	392.22	1775.860	421.02	1793.825	449.58	1610.088
334.33	502.158	363.57	798.298	392.59	1780.983	421.38	1797.386	449.94	1601.053
334.70	499.333	363.94	811.175	392.95	1784.702	421.74	1797.228	450.30	1592.842
335.08	496.965	364.31	823.035	393.32	1787.983	422.11	1797.070	450.66	1585.509
335.45	494.246	364.68	835.982	393.69	1790.579	422.47	1798.544	451.02	1579.193
335.82	491.544	365.04	849.281	394.05	1795.789	422.83	1797.579	451.38	1568.228
336.19	489.368	365.41	862.561	394.42	1802.719	423.19	1794.667	451.74	1560.070
336.56	487.158	365.78	874.860	394.78	1812.456	423.56	1791.035	452.10	1556.333
336.93	485.070	366.15	888.772	395.15	1820.719	423.92	1788.667	452.46	1549.579
337.30	483.368	366.52	902.000	395.51	1831.158	424.28	1784.351	452.82	1541.544
337.67	482.316	366.89	914.895	395.88	1839.316	424.65	1777.895	453.18	1533.807
338.05	480.526	367.26	926.842	396.25	1845.246	425.01	1772.351	453.53	1528.912
338.42	479.193	367.62	940.316	396.61	1847.614	425.37	1768.140	453.89	1521.597
338.79	478.158	367.99	954.491	396.98	1848.474	425.73	1760.438	454.25	1515.316
339.16	477.140	368.36	967.719	397.34	1851.421	426.10	1756.842	454.61	1510.474
339.53	476.807	368.73	981.404	397.71	1855.000	426.46	1756.053	454.97	1501.333
339.90	476.140	369.10	994.965	398.07	1859.404	426.82	1759.333	455.33	1494.649
340.27	***475.544***	369.47	1008.526	398.44	1861.351	427.18	1762.912	455.69	1487.281
340.64	475.474	369.83	1022.245	398.80	1864.667	427.55	1767.386	456.05	1483.000
341.01	475.561	370.20	1035.561	399.17	1868.719	427.91	1770.193	456.41	1477.509
341.38	475.351	370.57	1050.088	399.53	1871.298	428.27	1766.263	456.77	1473.877
341.75	475.439	370.94	1063.579	399.90	1872.421	428.63	1766.316	457.13	1474.825
342.13	476.070	371.31	1078.000	400.26	1874.228	428.99	1764.105	457.49	1474.246
342.50	476.632	371.67	1092.210	400.63	1877.491	429.36	1759.351	457.85	1473.614
342.87	477.316	372.04	1106.438	400.99	1879.702	429.72	1759.053	458.21	1477.070
343.24	478.404	372.41	1120.719	401.36	1879.263	430.08	1758.368	458.57	1475.386
343.61	479.456	372.78	1137.105	401.72	1878.702	430.44	1759.018	458.92	1472.298
343.98	480.667	373.15	1151.193	402.09	1876.263	430.81	1759.140	459.28	1470.842
344.35	482.105	373.51	1165.667	402.45	1873.175	431.17	1757.298	459.64	1468.140
344.72	483.737	373.88	1181.246	402.82	1870.807	431.53	1756.368	460.00	1465.421
345.09	485.509	374.25	1197.088	403.18	1866.649	431.89	1754.158	460.36	1463.544
345.46	487.772	374.62	1211.842	403.55	1863.140	432.25	1749.860	460.72	1462.281
345.83	489.421	374.98	1227.334	403.91	1860.281	432.61	1749.965	461.08	1463.807
346.20	491.404	375.35	1244.158	404.28	1855.438	432.98	1748.105	461.44	1464.123
346.57	493.140	375.72	1261.825	404.64	1853.614	433.34	1745.456	461.79	1467.333
346.94	495.281	376.09	1279.561	405.01	1852.702	433.70	1741.895	462.15	1472.123
347.31	497.579	376.45	1296.684	405.37	1854.824	434.06	1735.719	462.51	1480.755
347.68	500.000	376.82	1314.052	405.74	1855.421	434.42	1729.860	462.87	1488.737
348.05	502.807	377.19	1331.245	406.10	1854.614	434.78	1722.632	463.23	1496.193
348.42	505.895	377.56	1347.000	406.47	1854.930	435.15	1721.667	463.59	1501.017
348.79	509.053	377.92	1364.649	406.83	1856.140	435.51	1716.141	463.95	1501.263
349.16	511.895	378.29	1380.088	407.20	1855.035	435.87	1713.509	464.30	1502.386
349.53	514.877	378.66	1395.579	407.56	1852.088	436.23	1716.123	464.66	1503.123
349.90	518.684	379.03	1409.825	407.92	1850.193	436.59	1713.825	465.02	1503.667
350.27	522.386	379.39	1425.421	408.29	1847.017	436.95	1711.316	465.38	1507.421
350.64	526.404	379.76	1439.298	408.65	1841.070	437.31	1707.737	465.74	1510.386
351.01	530.702	380.13	1454.702	409.02	1837.596	437.68	1704.263	466.10	1515.298
351.38	534.825	380.49	1469.439	409.38	1832.193	438.04	1702.702	466.45	1521.649
351.75	539.070	380.86	1482.386	409.75	1828.017	438.40	1698.825	466.81	1527.386
352.12	543.807	381.23	1495.246	410.11	1827.895	438.76	1697.912	467.17	1533.614
352.49	548.579	381.60	1509.246	410.47	1828.035	439.12	1698.333	467.53	1538.368
352.86	553.965	381.96	1521.263	410.84	1828.614	439.48	1694.719	467.89	1538.561
353.23	559.632	382.33	1532.246	411.20	1829.860	439.84	1687.088	468.24	1540.070
353.60	565.333	382.70	1541.368	411.57	1828.579	440.20	1683.561	468.60	1538.737
353.97	571.053	383.06	1550.561	411.93	1823.438	440.57	1679.298	468.96	1536.842
354.34	577.140	383.43	1560.456	412.29	1818.404	440.93	1676.719	469.32	1537.035
354.71	582.561	383.80	1570.912	412.66	1814.123	441.29	1673.000	469.68	1534.474
355.08	588.737	384.16	1579.421	413.02	1811.403	441.65	1672.140	470.03	1535.105
355.45	595.053	384.53	1590.386	413.39	1805.596	442.01	1665.948	470.39	1541.298
355.82	601.719	384.90	1599.298	413.75	1801.614	442.37	1663.018	470.75	1546.018
356.19	608.912	385.26	1610.562	414.11	1800.368	442.73	1662.403	471.11	1555.982

471.46	1568.053	499.59	2089.403	527.46	1940.351	555.06	2012.351	582.39	2818.438
471.82	1578.649	499.94	2079.123	527.81	1940.404	555.41	2007.930	582.73	2825.561
472.18	1592.491	500.30	2069.824	528.16	1940.544	555.75	2000.141	583.08	2831.772
472.54	1606.631	500.65	2061.088	528.51	1940.772	556.10	1992.281	583.42	2834.456
472.89	1621.316	501.01	2053.719	528.86	1940.333	556.45	1985.123	583.77	2837.193
473.25	1633.456	501.36	2050.474	529.21	1940.193	556.80	1980.158	584.11	2839.877
473.61	1647.052	501.71	2047.562	529.56	1940.158	557.14	1977.193	584.45	2829.842
473.97	1661.316	502.07	2044.982	529.91	1941.790	557.49	1976.842	584.80	2809.789
474.32	1675.614	502.42	2041.737	530.26	1945.123	557.84	1979.105	585.14	2780.561
474.68	1691.877	502.78	2037.088	530.61	1947.912	558.19	1982.456	585.49	2750.070
475.04	1710.052	503.13	2033.632	530.97	1952.035	558.53	1985.825	585.83	2723.965
475.40	1727.789	503.48	2028.351	531.32	1956.508	558.88	1985.702	586.17	2698.666
475.75	1743.912	503.84	2025.930	531.67	1960.895	559.23	1985.438	586.52	2682.316
476.11	1756.684	504.19	2021.772	532.02	1963.175	559.58	1986.351	586.86	2672.368
476.47	1771.596	504.55	2017.702	532.37	1964.614	559.92	1988.368	587.20	2665.579
476.82	1787.825	504.90	2010.965	532.72	1965.053	560.27	1993.000	587.55	2655.842
477.18	1802.211	505.25	2004.614	533.07	1964.649	560.62	1999.123	587.89	2641.351
477.54	1819.158	505.61	1997.368	533.42	1963.544	560.96	2004.246	588.23	2620.140
477.89	1830.579	505.96	1990.263	533.77	1962.754	561.31	2009.719	588.58	2592.614
478.25	1845.386	506.31	1983.123	534.12	1963.421	561.66	2015.562	588.92	2567.105
478.61	1861.404	506.67	1977.035	534.47	1964.544	562.00	2019.667	589.26	2549.474
478.97	1873.017	507.02	1971.245	534.82	1967.842	562.35	2024.456	589.61	2541.825
479.32	1886.509	507.38	1965.158	535.17	1971.228	562.70	2028.070	589.95	2546.737
479.68	1897.719	507.73	1959.737	535.52	1976.017	563.05	2030.877	590.29	2555.649
480.04	1911.702	508.08	1953.281	535.87	1981.017	563.39	2030.526	590.64	2558.737
480.39	1926.158	508.44	1948.123	536.22	1986.158	563.74	2028.877	590.98	2555.579
480.75	1937.807	508.79	1941.807	536.57	1988.684	564.08	2025.421	591.32	2547.351
481.10	1952.175	509.14	1937.246	536.92	1989.965	564.43	2021.860	591.67	2538.386
481.46	1969.316	509.50	1933.368	537.27	1989.825	564.78	2018.509	592.01	2531.842
481.82	1985.386	509.85	1929.965	537.62	1987.403	565.12	2017.456	592.35	2528.140
482.17	2006.649	510.20	1928.298	537.97	1986.824	565.47	2019.684	592.70	2524.842
482.53	2033.614	510.56	1925.807	538.32	1984.614	565.82	2023.368	593.04	2517.684
482.89	2058.667	510.91	1924.000	538.67	1983.649	566.16	2030.421	593.38	2511.895
483.24	2083.105	511.26	1922.017	539.02	1981.649	566.51	2040.000	593.72	2508.035
483.60	2104.597	511.61	1919.649	539.37	1981.176	566.86	2050.263	594.07	2511.333
483.96	2123.439	511.97	1916.825	539.72	1981.632	567.20	2062.965	594.41	2518.614
484.31	2141.930	512.32	1913.702	540.07	1982.404	567.55	2080.018	594.75	2527.842
484.67	2159.632	512.67	1911.947	540.42	1983.369	567.89	2102.158	595.09	2539.421
485.02	2167.562	513.03	1911.193	540.77	1985.105	568.24	2127.579	595.44	2552.614
485.38	2173.579	513.38	1911.158	541.12	1985.912	568.59	2157.491	595.78	2566.509
485.74	2182.456	513.73	1911.105	541.47	1987.193	568.93	2187.807	596.12	2578.597
486.09	2187.000	514.08	1910.877	541.82	1987.772	569.28	2217.123	596.46	2586.193
486.45	2192.473	514.44	1909.597	542.17	1988.280	569.62	2245.473	596.81	2590.544
486.80	2196.456	514.79	1908.281	542.51	1986.895	569.97	2276.263	597.15	2597.527
487.16	2199.596	515.14	1907.456	542.86	1984.404	570.32	2308.473	597.49	2606.702
487.52	2203.281	515.49	1908.193	543.21	1982.842	570.66	2341.842	597.83	2615.737
487.87	2204.912	515.85	1908.754	543.56	1983.140	571.01	2375.193	598.18	2623.719
488.23	2203.930	516.20	1911.491	543.91	1985.000	571.35	2409.930	598.52	2626.772
488.58	2202.210	516.55	1912.035	544.26	1990.719	571.70	2443.351	598.86	2626.509
488.94	2195.579	516.90	1912.754	544.61	1996.140	572.04	2473.930	599.20	2626.737
489.29	2182.281	517.26	1913.140	544.96	2001.579	572.39	2501.088	599.54	2627.912
489.65	2169.982	517.61	1913.351	545.31	2005.895	572.73	2522.070	599.89	2630.649
490.01	2158.562	517.96	1914.070	545.66	2010.298	573.08	2539.035	600.23	2634.649
490.36	2148.632	518.31	1916.000	546.00	2012.123	573.43	2558.386	600.57	2639.000
490.72	2138.930	518.67	1917.404	546.35	2013.456	573.77	2574.719	600.91	2640.684
491.07	2130.175	519.02	1921.474	546.70	2014.088	574.12	2595.035	601.25	2641.053
491.43	2124.035	519.37	1923.877	547.05	2013.930	574.46	2619.158	601.59	2637.860
491.78	2121.544	519.72	1924.158	547.40	2011.386	574.81	2646.245	601.94	2630.491
492.14	2120.596	520.07	1923.860	547.75	2008.719	575.15	2672.456	602.28	2620.597
492.49	2118.491	520.43	1922.544	548.10	2004.053	575.50	2692.123	602.62	2607.632
492.85	2123.842	520.78	1921.789	548.45	2002.000	575.84	2707.368	602.96	2592.053
493.20	2130.228	521.13	1923.368	548.79	2000.386	576.19	2719.439	603.30	2579.403
493.56	2136.228	521.48	1924.562	549.14	2002.017	576.53	2728.053	603.64	2568.631
493.91	2141.544	521.83	1925.649	549.49	2004.965	576.88	2733.562	603.98	2562.948
494.27	2144.772	522.19	1925.895	549.84	2008.298	577.22	2735.877	604.33	2563.088
494.62	2145.140	522.54	1925.983	550.19	2010.105	577.57	2738.947	604.67	2566.930
494.98	2143.649	522.89	1924.474	550.54	2011.509	577.91	2752.210	605.01	2572.614
495.33	2141.579	523.24	1924.404	550.88	2009.982	578.26	2774.070	605.35	2576.842
495.69	2139.491	523.59	1923.245	551.23	2006.860	578.60	2803.351	605.69	2582.070
496.04	2138.158	523.94	1923.632	551.58	2003.965	578.95	2832.140	606.03	2584.562
496.40	2135.912	524.29	1924.877	551.93	2002.228	579.29	2856.421	606.37	2583.948
496.75	2132.597	524.65	1924.421	552.28	2003.421	579.63	2876.070	606.71	2579.983
497.11	2126.421	525.00	1925.140	552.62	2007.000	579.98	2883.684	607.05	2570.860
497.46	2121.527	525.35	1927.649	552.97	2009.824	580.32	2883.210	607.40	2557.877
497.82	2115.053	525.70	1929.579	553.32	2012.947	580.67	2871.631	607.74	2543.772
498.17	2111.140	526.05	1932.298	553.67	2014.632	581.01	2852.386	608.08	2528.351
498.53	2107.421	526.40	1934.614	554.02	2015.509	581.36	2831.614	608.42	2512.175
498.88	2103.070	526.75	1936.421	554.36	2016.772	581.70	2815.175	608.76	2492.929
499.23	2097.719	527.11	1938.579	554.71	2016.053	582.05	2812.947	609.10	2470.947

609.44	2449.316	636.20	2281.439	662.67	2246.649	688.83	2253.070	714.67	2128.719
609.78	2425.789	636.54	2282.544	663.00	2245.070	689.15	2248.562	715.00	2128.386
610.12	2400.947	636.87	2283.229	663.33	2244.772	689.48	2244.719	715.32	2128.561
610.46	2375.807	637.21	2283.035	663.66	2243.509	689.81	2241.912	715.65	2129.351
610.80	2349.772	637.55	2283.105	664.00	2242.386	690.14	2238.772	715.97	2129.614
611.14	2327.386	637.88	2282.807	664.33	2242.158	690.47	2236.404	716.30	2128.176
611.48	2311.948	638.22	2282.684	664.66	2241.438	690.80	2234.438	716.62	2124.667
611.82	2307.053	638.56	2282.333	665.00	2239.912	691.13	2231.895	716.95	2121.754
612.16	2305.667	638.89	2280.965	665.33	2239.351	691.46	2229.053	717.27	2117.895
612.50	2307.281	639.23	2277.140	665.66	2237.947	691.79	2226.351	717.60	2113.930
612.84	2306.702	639.57	2271.210	665.99	2238.245	692.11	2223.298	717.92	2108.755
613.18	2305.474	639.90	2266.070	666.33	2239.088	692.44	2222.158	718.25	2105.228
613.52	2303.088	640.24	2261.000	666.66	2239.948	692.77	2219.965	718.57	2100.579
613.86	2301.930	640.58	2259.596	666.99	2239.562	693.10	2217.877	718.90	2095.719
614.20	2298.649	640.91	2258.684	667.32	2240.895	693.43	2214.842	719.22	2092.930
614.54	2294.491	641.25	2256.737	667.66	2239.439	693.76	2215.035	719.55	2091.333
614.88	2290.316	641.58	2255.228	667.99	2239.930	694.09	2217.105	719.87	2089.930
615.22	2284.965	641.92	2254.649	668.32	2240.456	694.41	2217.754	720.19	2089.175
615.56	2281.316	642.26	2254.193	668.65	2244.263	694.74	2219.438	720.52	2088.210
615.90	2275.123	642.59	2253.562	668.99	2251.035	695.07	2219.947	720.84	2086.860
616.24	2267.649	642.93	2251.211	669.32	2256.737	695.40	2218.614	721.17	2084.000
616.58	2261.632	643.27	2249.351	669.65	2261.772	695.73	2216.263	721.49	2080.105
616.92	2256.246	643.60	2246.368	669.98	2266.596	696.06	2213.403	721.81	2075.509
617.26	2251.158	643.94	2243.754	670.31	2270.754	696.38	2211.018	722.14	2070.474
617.60	2242.018	644.27	2241.719	670.65	2274.825	696.71	2207.544	722.46	2066.930
617.94	2229.456	644.61	2240.965	670.98	2278.404	697.04	2203.702	722.79	2064.807
618.28	2212.579	644.94	2242.333	671.31	2280.544	697.37	2198.965	723.11	2062.614
618.62	2198.930	645.28	2245.421	671.64	2281.930	697.70	2195.737	723.43	2061.790
618.96	2189.105	645.62	2251.228	671.97	2283.597	698.02	2192.930	723.76	2059.772
619.30	2186.649	645.95	2259.491	672.30	2284.877	698.35	2189.702	724.08	2059.141
619.64	2186.140	646.29	2265.509	672.64	2285.105	698.68	2186.351	724.41	2057.053
619.98	2188.421	646.62	2272.263	672.97	2285.509	699.01	2182.772	724.73	2055.544
620.31	2191.263	646.96	2277.877	673.30	2286.702	699.33	2180.579	725.05	2054.772
620.65	2198.596	647.29	2280.877	673.63	2286.982	699.66	2180.491	725.38	2053.824
620.99	2208.105	647.63	2283.544	673.96	2287.772	699.99	2178.228	725.70	2053.228
621.33	2219.649	647.96	2286.175	674.29	2288.035	700.32	2173.421	726.02	2052.526
621.67	2229.965	648.30	2288.070	674.62	2286.386	700.64	2168.070	726.35	2052.509
622.01	2238.632	648.63	2290.825	674.96	2283.895	700.97	2159.895	726.67	2052.509
622.35	2247.965	648.97	2293.351	675.29	2279.772	701.30	2152.052	726.99	2051.667
622.69	2258.667	649.30	2297.053	675.62	2275.105	701.63	2146.281	727.32	2051.824
623.03	2267.000	649.64	2301.070	675.95	2271.176	701.95	2141.702	727.64	2054.527
623.36	2274.790	649.97	2306.386	676.28	2269.877	702.28	2137.246	727.96	2057.491
623.70	2284.351	650.31	2311.842	676.61	2270.018	702.61	2132.930	728.29	2062.527
624.04	2296.035	650.64	2316.614	676.94	2270.491	702.93	2128.860	728.61	2066.597
624.38	2308.439	650.98	2319.000	677.27	2271.649	703.26	2125.316	728.93	2069.228
624.72	2319.790	651.31	2318.877	677.60	2273.737	703.59	2125.035	729.25	2071.649
625.06	2326.438	651.65	2315.982	677.94	2275.158	703.91	2126.825	729.58	2072.035
625.40	2331.316	651.98	2312.158	678.27	2276.491	704.24	2129.895	729.90	2073.316
625.73	2332.754	652.32	2320.649	678.60	2278.087	704.57	2136.912	730.22	2075.579
626.07	2335.210	652.65	2359.842	678.93	2280.175	704.89	2142.667	730.54	2078.053
626.41	2338.316	652.99	2422.263	679.26	2281.509	705.22	2148.228	730.87	2079.649
626.75	2342.561	653.32	2491.965	679.59	2281.456	705.55	2151.790	731.19	2082.614
627.09	2344.649	653.65	2554.070	679.92	2278.614	705.87	2153.228	731.51	2084.017
627.43	2345.544	653.99	2595.315	680.25	2275.860	706.20	2153.175	731.83	2086.368
627.76	2344.246	654.32	2622.614	680.58	2273.158	706.53	2152.596	732.16	2087.386
628.10	2343.421	654.66	2642.649	680.91	2272.790	706.85	2152.193	732.48	2088.562
628.44	2343.000	654.99	2658.684	681.24	2271.474	707.18	2151.649	732.80	2088.579
628.78	2342.245	655.33	2671.228	681.57	2270.298	707.51	2151.544	733.12	2090.035
629.12	2339.701	655.66	2680.807	681.90	2272.298	707.83	2151.947	733.45	2090.000
629.45	2337.912	655.99	2685.473	682.23	2275.421	708.16	2153.035	733.77	2088.561
629.79	2334.561	656.33	2685.790	682.56	2277.965	708.49	2153.702	734.09	2086.737
630.13	2329.245	656.66	2683.649	682.89	2280.123	708.81	2154.474	734.41	2082.667
630.47	2323.193	657.00	2681.070	683.22	2280.930	709.14	2155.421	734.73	2078.509
630.80	2317.825	657.33	2679.421	683.55	2280.544	709.46	2156.562	735.06	2075.123
631.14	2313.228	657.66	2677.614	683.88	2280.158	709.79	2156.930	735.38	2070.105
631.48	2309.421	658.00	2675.807	684.21	2280.105	710.12	2156.737	735.70	2067.158
631.82	2304.825	658.33	2672.702	684.54	2279.228	710.44	2156.053	736.02	2064.912
632.15	2300.684	658.66	2657.772	684.87	2278.544	710.77	2152.070	736.34	2063.000
632.49	2296.947	659.00	2611.421	685.20	2275.965	711.09	2148.719	736.67	2061.614
632.83	2293.982	659.33	2543.193	685.53	2273.316	711.42	2145.754	736.99	2059.333
633.17	2290.895	659.67	2465.439	685.86	2271.281	711.74	2142.667	737.31	2053.333
633.50	2290.140	660.00	2397.825	686.19	2271.000	712.07	2140.632	737.63	2047.193
633.84	2289.105	660.33	2352.509	686.52	2271.965	712.40	2140.351	737.95	2039.000
634.18	2286.667	660.67	2322.105	686.85	2272.930	712.72	2140.930	738.27	2030.649
634.52	2284.912	661.00	2299.088	687.18	2271.228	713.05	2140.228	738.59	2022.228
634.85	2283.526	661.33	2281.947	687.51	2269.070	713.37	2138.228	738.92	2014.053
635.19	2283.035	661.67	2267.631	687.84	2265.526	713.70	2136.491	739.24	2005.000
635.53	2282.579	662.00	2257.403	688.17	2261.474	714.02	2133.140	739.56	1996.737
635.86	2281.772	662.33	2250.000	688.50	2257.719	714.35	2130.053	739.88	1989.263

740.20	1981.368	765.40	1965.158	790.26	1652.053	814.78	1310.912	838.95	1033.579
740.52	1972.807	765.72	1969.562	790.57	1648.719	815.09	1308.368	839.25	1029.421
740.84	1963.140	766.03	1973.176	790.89	1647.596	815.40	1306.386	839.55	1025.684
741.16	1952.983	766.35	1975.895	791.20	1646.526	815.70	1303.596	839.86	1022.123
741.48	1944.368	766.67	1977.983	791.51	1646.035	816.01	1300.965	840.16	1018.333
741.81	1934.351	766.98	1976.825	791.82	1645.614	816.32	1298.140	840.46	1013.754
742.13	1923.017	767.30	1974.474	792.14	1642.737	816.63	1293.614	840.77	1011.000
742.45	1910.053	767.62	1971.070	792.45	1640.491	816.94	1289.333	841.07	1007.544
742.77	1896.245	767.93	1966.404	792.76	1639.491	817.24	1284.386	841.37	1003.369
743.09	1882.895	768.25	1961.667	793.07	1637.509	817.55	1281.070	841.68	1000.263
743.41	1872.982	768.57	1956.280	793.38	1633.965	817.86	1278.491	841.98	997.439
743.73	1864.579	768.88	1951.947	793.70	1629.789	818.17	1276.614	842.28	993.877
744.05	1857.070	769.20	1947.667	794.01	1624.456	818.47	1274.263	842.59	990.789
744.37	1851.018	769.51	1944.562	794.32	1620.053	818.78	1272.456	842.89	987.246
744.69	1844.351	769.83	1942.474	794.63	1615.386	819.09	1269.772	843.19	984.000
745.01	1836.947	770.15	1940.877	794.94	1609.719	819.40	1265.614	843.49	980.596
745.33	1829.175	770.46	1938.754	795.26	1604.649	819.70	1262.614	843.80	975.719
745.65	1821.263	770.78	1935.737	795.57	1600.193	820.01	1258.842	844.10	970.842
745.97	1812.684	771.09	1931.614	795.88	1594.684	820.32	1255.070	844.40	966.930
746.29	1804.123	771.41	1925.649	796.19	1588.930	820.62	1251.877	844.70	963.018
746.61	1796.211	771.73	1917.965	796.50	1583.632	820.93	1247.474	845.01	959.596
746.93	1789.684	772.04	1909.579	796.81	1575.175	821.24	1243.947	845.31	955.070
747.25	1784.965	772.36	1901.369	797.12	1565.123	821.55	1240.351	845.61	950.737
747.57	1781.263	772.67	1894.579	797.44	1553.649	821.85	1237.562	845.91	946.825
747.89	1777.281	772.99	1888.807	797.75	1541.667	822.16	1234.298	846.22	943.596
748.21	1773.404	773.30	1882.351	798.06	1531.403	822.47	1232.754	846.52	939.176
748.53	1770.316	773.62	1876.421	798.37	1521.386	822.77	1230.860	846.82	935.456
748.85	1767.228	773.93	1870.281	798.68	1511.070	823.08	1228.737	847.12	931.912
749.17	1765.140	774.25	1862.579	798.99	1502.965	823.39	1225.474	847.43	927.614
749.49	1761.175	774.57	1854.947	799.30	1496.228	823.69	1221.561	847.73	923.000
749.81	1757.368	774.88	1846.070	799.61	1490.088	824.00	1218.316	848.03	919.070
750.13	1753.474	775.20	1839.000	799.92	1485.053	824.31	1214.965	848.33	914.526
750.45	1750.614	775.51	1831.983	800.24	1478.807	824.61	1212.807	848.63	910.754
750.77	1748.421	775.83	1825.439	800.55	1473.018	824.92	1211.123	848.93	906.667
751.09	1747.316	776.14	1818.597	800.86	1468.403	825.22	1210.825	849.24	902.421
751.41	1746.912	776.46	1813.211	801.17	1462.614	825.53	1209.386	849.54	898.579
751.72	1746.561	776.77	1808.947	801.48	1456.912	825.84	1206.807	849.84	895.333
752.04	1745.895	777.09	1803.684	801.79	1451.824	826.14	1204.474	850.14	890.912
752.36	1745.755	777.40	1798.842	802.10	1447.193	826.45	1201.123	850.44	886.877
752.68	1745.965	777.72	1793.737	802.41	1442.509	826.75	1199.193	850.74	881.754
753.00	1746.526	778.03	1790.018	802.72	1438.719	827.06	1196.544	851.05	878.158
753.32	1747.176	778.35	1787.842	803.03	1435.649	827.37	1194.386	851.35	873.895
753.64	1747.930	778.66	1785.947	803.34	1433.052	827.67	1190.930	851.65	870.175
753.96	1751.842	778.97	1784.053	803.65	1431.930	827.98	1187.719	851.95	865.877
754.28	1758.632	779.29	1783.404	803.96	1430.544	828.28	1183.211	852.25	861.947
754.60	1768.000	779.60	1781.842	804.27	1428.579	828.59	1178.421	852.55	857.526
754.91	1778.491	779.92	1780.000	804.58	1427.526	828.89	1174.316	852.85	853.105
755.23	1788.790	780.23	1778.281	804.89	1425.403	829.20	1169.579	853.15	849.526
755.55	1799.246	780.55	1776.895	805.20	1423.526	829.51	1164.421	853.45	844.965
755.87	1807.667	780.86	1776.088	805.51	1421.333	829.81	1159.105	853.75	840.561
756.19	1815.965	781.17	1773.439	805.82	1419.123	830.12	1154.438	854.06	836.526
756.51	1823.632	781.49	1770.667	806.13	1416.614	830.42	1149.333	854.36	832.140
756.82	1832.070	781.80	1767.035	806.44	1413.491	830.73	1143.070	854.66	827.351
757.14	1839.825	782.12	1763.368	806.75	1409.930	831.03	1137.018	854.96	823.123
757.46	1849.140	782.43	1759.439	807.06	1407.456	831.34	1131.281	855.26	818.439
757.78	1858.737	782.74	1754.737	807.37	1404.211	831.64	1126.789	855.56	814.140
758.10	1870.105	783.06	1751.368	807.68	1401.052	831.95	1121.614	855.86	810.053
758.42	1881.544	783.37	1747.754	807.99	1398.474	832.25	1117.105	856.16	804.965
758.73	1891.614	783.69	1744.930	808.30	1394.474	832.56	1112.105	856.46	800.491
759.05	1901.088	784.00	1740.965	808.61	1390.877	832.86	1107.544	856.76	795.439
759.37	1908.228	784.31	1734.000	808.92	1387.333	833.17	1102.684	857.06	790.825
759.69	1914.105	784.63	1726.965	809.22	1382.807	833.47	1099.298	857.36	785.860
760.00	1918.579	784.94	1720.368	809.53	1376.667	833.78	1095.649	857.66	780.719
760.32	1921.561	785.25	1714.667	809.84	1370.719	834.08	1092.825	857.96	775.632
760.64	1921.474	785.57	1710.737	810.15	1364.895	834.39	1089.632	858.26	770.158
760.96	1922.158	785.88	1706.772	810.46	1358.842	834.69	1086.386	858.56	765.140
761.28	1923.737	786.19	1704.140	810.77	1353.842	834.99	1082.509	858.86	760.123
761.59	1926.579	786.51	1700.140	811.08	1348.983	835.30	1079.298	859.16	755.298
761.91	1932.632	786.82	1695.211	811.39	1345.211	835.60	1076.614	859.46	750.965
762.23	1940.596	787.13	1690.491	811.70	1341.386	835.91	1072.368	859.76	745.737
762.55	1948.930	787.45	1686.351	812.00	1338.438	836.21	1068.211	860.06	740.509
762.86	1955.316	787.76	1682.614	812.31	1334.614	836.52	1064.158	860.36	735.912
763.18	1961.211	788.07	1678.860	812.62	1331.263	836.82	1059.456	860.66	730.754
763.50	1963.702	788.39	1674.333	812.93	1326.807	837.12	1055.456	860.96	726.491
763.81	1964.877	788.70	1671.211	813.24	1323.965	837.43	1051.316	861.26	721.263
764.13	1964.053	789.01	1667.474	813.55	1321.544	837.73	1047.369	861.56	716.298
764.45	1962.649	789.32	1663.561	813.86	1317.983	838.03	1044.228	861.85	711.684
764.77	1961.544	789.64	1658.930	814.16	1315.930	838.34	1041.193	862.15	706.842
765.08	1962.017	789.95	1655.053	814.47	1313.614	838.64	1037.281	862.45	702.158

862.75	697.404
863.05	692.158
863.35	687.789
863.65	682.912
863.95	679.018
864.25	674.351
864.55	669.614
864.84	664.965
865.14	659.491
865.44	655.053
865.74	650.386
866.04	644.895
866.34	639.825
866.63	634.509
866.93	629.456
867.23	624.421
867.53	619.754
867.83	615.175
868.12	610.368
868.42	605.210
868.72	600.737
869.02	596.316
869.32	591.842
869.61	586.719
869.91	581.684
870.21	576.491
870.51	571.614
870.80	567.474
871.10	562.772
871.40	557.842
871.70	553.351
871.99	548.719
872.29	543.825
872.59	539.404
872.88	535.140
873.18	530.404
873.48	526.070
873.78	521.421
874.07	517.298
874.37	513.140
874.67	508.509
874.96	504.035
875.26	499.684
875.56	495.702
875.85	491.368
876.15	487.035
876.44	482.825
876.74	478.596
877.04	474.632
877.33	470.789
877.63	467.035
877.93	463.684
878.22	459.491
878.52	455.474
878.81	451.825
879.11	447.544
879.40	443.772
879.70	439.842
880.00	435.912
880.29	431.965
880.59	428.070
880.88	424.123
881.18	419.983
881.47	416.316
881.77	408.000
882.06	406.333
882.36	406.333
882.65	405.333
882.95	399.000
883.24	392.333
883.54	386.333
883.83	384.000
884.13	384.000

>>>>>End Spectral Data<<<<<

For each frequency of EMR used in experiment the optical densityes at 340 nm are stored in the following format. Given example Is for experimental unit irradiated at 745 nm.

appendix	Exaple of data collected for the optical density measured at 340nm after exposure at 745nm.									
recording step in sec	No of exp.	745-1	745-2	745-3	745-4	745-5	745-6	745-7	745-8	
20		1.0355	1.1266	1.0677	1.0576	1.0641	1.0268	1.0280	1.0320	
40		0.8750	0.9720	0.9067	0.9143	0.9046	0.8783	0.9046	0.9485	
60		0.8489	0.9484	0.8809	0.8780	0.8748	0.8519	0.8178	0.9201	
80		0.8240	0.9245	0.8534	0.8337	0.8420	0.8269	0.7938	0.8930	
100		0.7966	0.8994	0.8268	0.8076	0.8224	0.8032	0.7711	0.8674	
120		0.7714	0.8722	0.8015	0.7843	0.7902	0.7809	0.7497	0.8434	
140		0.7438	0.8507	0.7755	0.7591	0.7662	0.7567	0.7264	0.8172	
160		0.7172	0.8233	0.7484	0.7323	0.7410	0.7354	0.7060	0.7942	
180		0.6890	0.7973	0.7227	0.7086	0.7161	0.7113	0.6828	0.7682	
200		0.6644	0.7756	0.6951	0.6816	0.6896	0.6843	0.6570	0.7391	
220		0.6369	0.7481	0.6688	0.6563	0.6637	0.6625	0.6360	0.7155	
240		0.6112	0.7220	0.6407	0.6303	0.6376	0.6330	0.6077	0.6837	
260		0.5845	0.6968	0.6167	0.6047	0.6101	0.6105	0.5861	0.6594	
280		0.5589	0.6754	0.5895	0.5786	0.5858	0.5874	0.5639	0.6344	
300		0.5348	0.6521	0.5645	0.5547	0.5612	0.5623	0.5398	0.6073	
320		0.5076	0.6255	0.5389	0.5301	0.5484	0.5368	0.5154	0.5798	
340		0.4832	0.6032	0.5138	0.5061	0.5126	0.5151	0.4945	0.5563	
360		0.4578	0.5798	0.4901	0.4832	0.4998	0.4896	0.4700	0.5288	
380		0.4338	0.5585	0.4662	0.4593	0.4892	0.4684	0.4497	0.5059	
400		0.4101	0.5362	0.4439	0.4374	0.4721	0.4484	0.4305	0.4843	
420		0.3941	0.5131	0.4198	0.4151	0.4504	0.4294	0.4123	0.4638	
440		0.3625	0.4928	0.3978	0.3914	0.4127	0.4052	0.3890	0.4377	
460		0.3552	0.4704	0.3760	0.3706	0.3953	0.3847	0.3693	0.4155	
480		0.3223	0.4502	0.3559	0.3543	0.3680	0.3630	0.3485	0.3921	
500		0.3023	0.4297	0.3346	0.3368	0.3490	0.3444	0.3306	0.3720	
520		0.2838	0.4096	0.3153	0.3132	0.3294	0.3234	0.3105	0.3493	
540		0.2664	0.3921	0.2977	0.2944	0.3116	0.3049	0.2927	0.3293	
560		0.2500	0.3748	0.2793	0.2776	0.2895	0.2898	0.2782	0.3130	
580		0.2362	0.3563	0.2644	0.2628	0.2751	0.2736	0.2626	0.2955	
600		0.2221	0.3395	0.2492	0.2545	0.2601	0.2577	0.2474	0.2784	
620		0.2101	0.3238	0.2361	0.2411	0.2448	0.2423	0.2326	0.2616	
rate of change		-0.0230	-0.0224	-0.0231	-0.0221	-0.0225	-0.0218	-0.0209	-0.0236	MEAN -0.0224
										St. dev 0.0008

Example of the optical density of NADH versus time data measured for after exposure of LDHB tho the EMR at 745nm.