REFERENCE GUIDE

TO THE

BASIC SCIENCE ASPECTS OF

THE MITOCHONDRIA

Daniel G. Clark, M.D.

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PREFACE

This Reference Guide to the Basic Science Aspects of the Mitochondria was compiled under the direction of the American Medical Research, LLC and its founder William S. Coury.

Until about twenty years ago, the mitochondria were the redheaded stepchildren of biology and biology based scientific research. Three discoveries: 1) that mitochondria are endosymbionts, with their own unique genome, and thus are an indispensable part of human genetics and 2) that these symbiotic organelles have a large number of functions until recently unsuspected, all relating to energy production and the handling of oxygen in human metabolism, have resulted in an explosion of research, and 3) the discovery that mitochondria and mutations of their genome are at the root of a large number of serious diseases, has led to an explosion of research.

That mitochondria are involved in the etiology and quite likely in the prevention and treatment of many diseases in which their involvement has been heretofore unsuspected is an evolving concept and one which is likely to be extremely important to physicians during the coming years.

In 1962, Luft R, Ikkos D, Palmeieri G, et al. *A case of severe hypermetabolism of non-thyroid origin with a defect in the maintenance of mitochondrial respiratory control: a correlated clinical, biochemical and morphological study.* J Clin Invest 1962;41:1776-804, was the first report that mitochondria are involved in human diseases. The development of that concept was chronicled by Luft R. *The development of mitochondrial medicine.* Biochem Biophys Acta 1995;1271:1-6. Until fairly recently, this was recognized in diseases involving the nervous system and muscles. Continuing research in that regard has led to the realization that mitochondrial dysfunction is involved in many other diseases in which this involvement is not so immediately apparent, since many of these other diseases make their appearance in middle age.

Since 1980, there have been in excess of 90,000 articles concerning mitochondria in the basic science literature. Only a few of these have been published in the clinical medical literature. The reporting in 1981 that mitochondria possess their own genome made these organelles an essential part of a study of genetics and the explosion of information about genetic influences on diseases, which is ongoing. One of the results of this is that recent college graduates who have had a course or two in biology are probably possessed of more of to day information about mitochondria than physicians who completed their education twenty-five years ago. The purpose of this guide line is to help physicians who are interested in integrating this newer knowledge into their practice catch up without having to read all 90,000 of the recently published references on mitochondria. This is not intended as a textbook, nor written with the rigor of textbooks. It is intended as a reference guide to help in self-education in an emerging

field of medicine, which is likely to be of extreme importance in the next few years.

We gratefully acknowledge the kindness of numerous scientists who furnished us with reprints of their published papers, in particularly the kindness of Dr. Douglas Wallace of Emory University in providing us with copies of the numerous papers published by his department, and the invaluable assistance of Maury Silverman in obtaining copies of hard to find references from the National Library of Medicine.

These references have been assembled and compiled by the joint efforts of William M. Cargile, D.C., L.Ac., Daniel G. Clark, M.D., Steve G. Haltiwanger, M.D., William H. Moore, Jr., M.D., J.D., and Marjorie Moore, with the invaluable assistance in research of David A. Steenblock, D.O. and the staff of the Institute of Quantum and Molecular Medicine.

Introduction

For years I have been involved in the clinical application of phytotherapy, nutritional supplements, amino acids, EDTA chelation therapy, hyperthermia using baths and saunas, and homeopathy for the treatment of chronic degenerative diseases. When I first began to study about an integrated approach to the treatment of these diseases, I recalled what I had learned at the Medical College of Georgia in my biochemistry class. My instructor was Ed Bresnick, PhD, who is now at Dartmouth Medical School. He made the biochemistry come alive by making application to clinical case studies. When we studied the cell, I was intrigued most by the powerhouses of the cell . . . the mitochondria. Things that he taught us in class kept taking me back to the mitochondria, which enumerated the importance of this important cellular organelle. As the battery of every cell, I learned that these units provide the energy that we need to run systems and functions of the body. I realized then that these were critical units and that if anything interfered with the Kreb's cycle or the oxidative system that the body would have a considerable amount of problems.

In 1978 I began to study the nutritional (i.e., vitamins, minerals, trace minerals, amino acids) application to the human body for the correction of diseases. I studied Dr. Royal Lee's writings from earlier years and was fascinated by what it revealed. I quickly went back to review Guyton's physiology, Leninger's biochemistry, and Dr. Bresnick's notes to see if there was a connection and there was!

I began practice in 1979 applying nutritional products to different disease conditions and I began to see improvements. After six months, the cancer patients began to call and at first I turned them away. I had been trained as a Gynecologist and had had three and a half years of training with our Gynecologic Oncologist. I knew the standard treatments well but I saw very poor results. One case in my residency stuck in my mind and I recalled this case as I began to get these calls from cancer patients.

I was in charge of a case of ovarian cancer. She had gone to the Contreras Clinic in Tijuana, Mexico five years before to get on an integrated program along with Laetrile. When I investigated her old records I discovered that she had had stage IV ovarian cancer and was told about the normal conventional protocol but very little hope was offered. Here she was five years later with a recurrence in the right pelvic area blocking her right ureter. I convinced her that surgery was the right approach so that we could remove the mass and save the right kidney.

At surgery, I discovered that there were no metastatic lesions as described by the previous post op dictation and that she had a mass in the right pelvis that was completely circular and approximately the size of a grapefruit. I simply lifted it out intact as there was no attachment to any organ. I told her after the surgery that she should continue her program because it had worked. When I discussed this with the chief of oncology, he simply rolled his eyes and stated

that it couldn't have been the nutritional supplements or the Laetrile.

I began to study the causes of cancer through books and materials of various physicians listed as follows: John Beard, PhD – "Enzyme Application to Cancer," William Fredrick Koch, MD, PhD – "Survival Factor in Viral and Neoplastic Diseases," Max Gerson, MD – "A Cancer Therapy – Fifty Cases," Harold Manner, PhD – "The Death of Cancer," Frank Shively, MD – "Multiple Proteolytic Enzyme Therapy in Cancer," Albert Szent-Gyorgi, PhD – Electronic Biology and Cancer," Otto Warburg – "The Prime Cause and Prevention of Cancer," and many more.

Through intensive study I began to realize that cancer and other chronic diseases were all due to blockage of the enzymes of oxidation. I began to search for ways of detoxification in order to free up these enzyme blockages. This search led me into the studies of herbology, homeopathy, nutritional medicine, environmental medicine, immunology, quantum physics, electrodermal screening, homotoxicology, EDTA chelation therapy, and matrix and matrix regulation. All of these areas of science and medicine helped me to formulate an approach to detoxification in which I saw reversal of cancer and other chronic diseases.

I began to lecture about 14 years ago about chronic degenerative diseases and cancer. One of my opening lectures I call "The Causes of Diseases" has been centered on oxygen, hydrogen and the mitochondria. I show how secondary causes interfere with oxygen utilization and block up the enzymes of the Kreb's cycle and oxidative phosphorylation. After revealing the factors that block the mitochondria, I proceed to describe the proper steps of detoxification.

A few years ago, Dr. Lewis Thomas, a physician and scientist at the Rockefeller Institute wrote a popular book entitled the *"Lives of a Cell: Notes of a Biology Watcher,"* in which he ruminated about the mitochondria:

> "It is a good thing for the entire enterprise that mitochondria and chloroplasts have remained small, conservative, and stable, since these two organelles are, in a fundamental sense, the most important living things on earth. Between them they produce the oxygen and arrange for its use. In effect, they run the place.
>
> My mitochondria comprise a very large proportion of me. I cannot do the calculation, but I suppose there is almost as much of them in sheer dry bulk as there is the rest of me. Looked at in this way, I could be taken for a very large, motile colony of respiring bacteria, operating a complex system of nuclei, microtubules, and neurons for the pleasure and sustenance of their families, and running, at the moment, a typewriter.
>
> I am intimately involved, and obliged to do a great deal of essential work for my mitochondria. My nuclei code out the outer membranes of each, and a good many of the enzymes attached to the cristae must be synthesized by me. Each of them, by all accounts, makes only enough of its own materials to get

along on, and the rest must come from me. And I am the one who has to do the worrying.

Now that I know about the situation, I can find all kinds of things to worry about. Viruses, for example. If my organelles are really symbiotic bacteria, colonizing me, what's to prevent them from catching a virus, or if they have such a thing as lysogeny, from conveying a phage to other organelles? Then there is the question of my estate. Do my mitochondria all die with me, or did my children get some of mine along with their mother's; this sort of thing should not worry me, I know, but it does."

From *The Lives of a Cell*
by LEWIS THOMAS

A few years ago I began to review information about aging and the causes of aging. I again began to look at the different theories and discovered the articles of Dr. Wallace from Emory University. He was revealing how mitochondrial senescence was one of the causes of aging.

I began to get intrigued by this concept because of earlier studies from Otto Warburg, PhD. I began to look at the current literature on mitochondria and discovered over 90,000 articles published since 1980. I talked with a few friends about this and, together, we decided that the information about mitochondrial function and dysfunction emerging from the avalanche of published research probably offered the first systematic scientific basis for the empirical therapeutics we had all known for years to be effective. This gave an explanation of how nutrition and energetic therapies achieved their results in treating and curing degenerative diseases! Together we hurriedly compiled this beginning Reference Guide to bridge the gap from research to the clinical setting.

This Reference Guide for the Basic Science Aspects of the Mitochondria is a preliminary effort to cull out of the vast literature on the mitochondria, some guideposts for understanding how the mitochondria are not only involved, but pivotal in the etiology, function and reversal of these degenerative diseases.

Daniel G. Clark, M.D.

BASIC SCIENCE ASPECTS OF THE MITOCHONDRIA
SECTION I

OVERVIEW OF MITOCHONDRIAL STRUCTURE AND FUNCTION

Mitochondria are organelles found in virtually all eukaryotic cells, animal and vegetable, where they perform similar, if not absolutely identical, functions in the cell and the organism.

They are believed by many scientists to be independent living organisms, which live in symbiosis with the cells they occupy. They have their own genome and reproduce independently of the cells' reproductive cycle. The functions they perform will be discussed below, after the structure of the organelle is discussed. While their size and shape may vary greatly, all mitochondria are organelles consisting of two membranes enclosing two spaces.

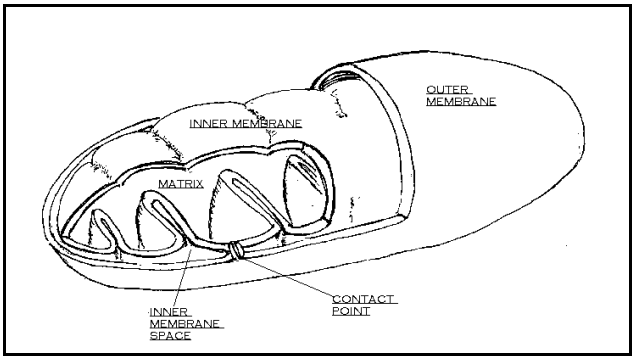

Figure 1

The outer membrane is a bi-lipid membrane quite similar to other cellular membranes. Contained within their outer membrane is the enfolded inner membrane which is quite different from the outer membrane and other cellular membranes. This inner membrane and the enzymes embedded or floating in it, surround the matrix which is a dense liquid containing many enzymes and the genome of the organelle.

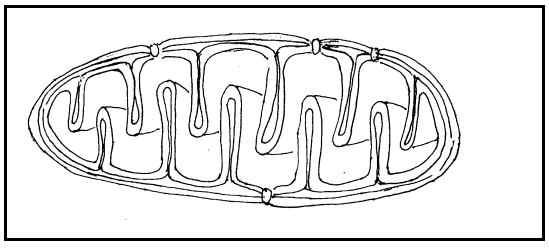

Figure 2a

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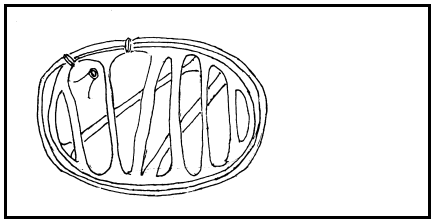

Figure 3a

The outer membrane is quite permeable, whereas the inner membrane is largely impermeable; three quarters of its volume is made up of proteins. It is here and in the matrix that the work of the mitochondria is carried out.

The inter membrane space between the outer and inner membranes is generally about like the cytosol due to the permeability of the outer membrane, and the two membranes are joined at several anchor pores called contact points which anchors the inner membrane. These points are also pores for the entry of nuclear encoded proteins and pre-proteins into the matrix of the organelle.

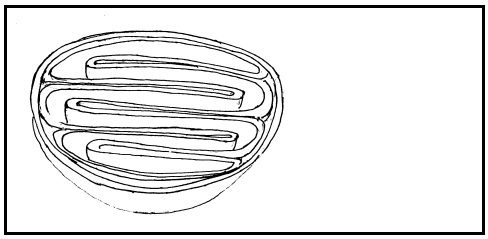

Figure 3b

The infolding of the inner membrane forms the cristae, which assume varied shapes, the original descriptions of these were of the classic baffle type. More advanced types of microscopic imaging have shown that in some mitochondria, the cristae are

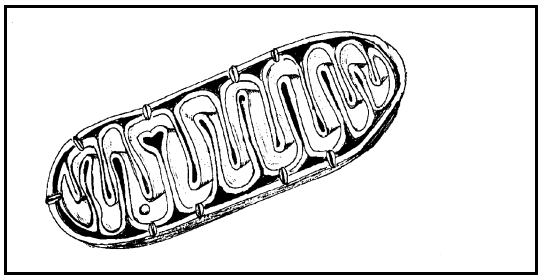

Figure 2b

tubular and in some cases the in-foldings are longitudinal rather than lateral.

None of these differences have much effect on their functions which will be discussed below.

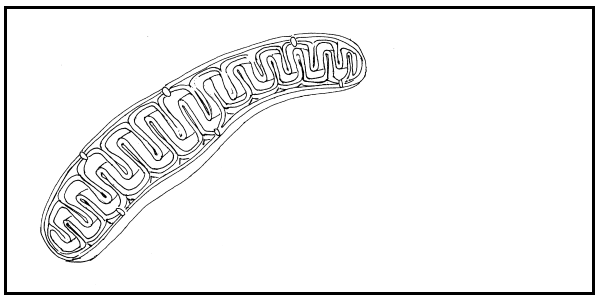

Figure 3d

In general, mitochondrial structure is quite similar to that of a bacteria, leading most scientists to the belief that mitochondria are bacteria which were engulfed or invaded eukaryotic cells in the past and established a mutually beneficial symbiotic relationship with these cells.

Mitochondria possesses a genome, several copies of which are contained in the matrix, and this genome is relatively small. Many scientists believe that at some time in the past, around 80% of the genes of the original bacterial genome were transferred to the nucleus of the cells, and only around 20% remains in the organelle. Many of the proteins used by the mitochondria are now encoded in the nuclear DNA and are transferred to the mitochondria from there through the pores at the contact points, after which they are incorporated into the structure. Most mitochondrial structure is a combination of a few proteins encoded in the mitochondrial genome and several encoded in the nuclear genome.

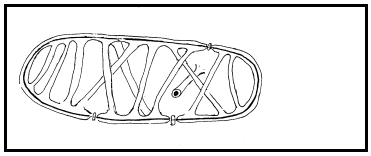

Figure 3c

Those encoded in the mitochondrial genome are maternally inherited, while those in the nuclear genome are subject to mendelian inheritance. This will be discussed in detail in a separate chapter.

Mitochondria reproduce by fission, and apparently are able to fuse and separate freely.

GENERAL FUNCTIONS

Mitochondria have several important functions in the cell, the most well known being that of the production of Adenosine Triphosphate by the process of oxidative phosphorylation. Around 95% of the ATP produced in the body is produced in the mitochondria. For this reason, they are often referred to as the power house of the cell, although they have several other equally important functions as well.

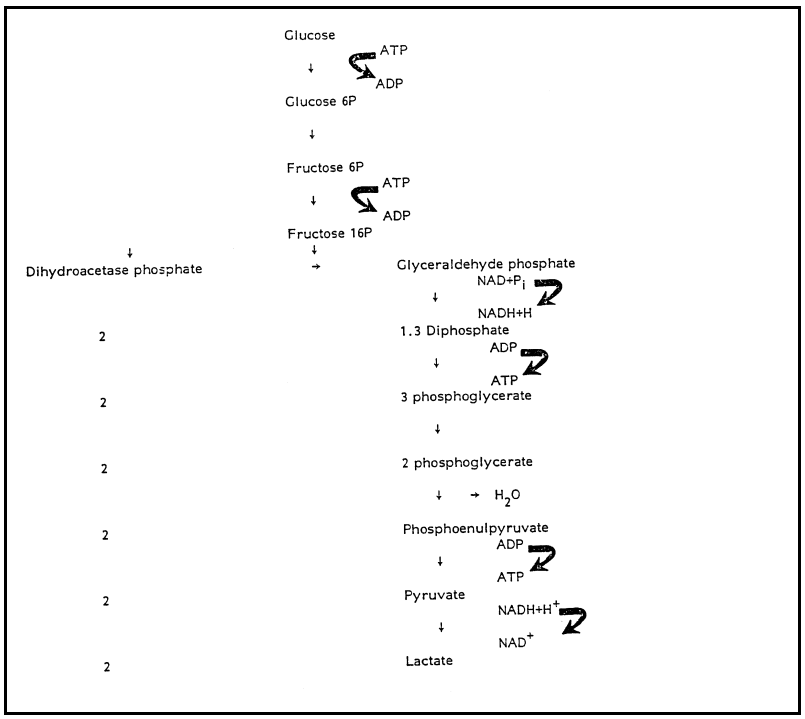

Figure 4

All molecules contain energy, stored in the molecular structure itself. A portion of that energy can be used to do work. This is called free energy. Oxidation of a molecule results in the release of free energy. Complete combustion (burning) of organic molecules, for example, releases all of the available free energy as heat. Reduction of a molecule requires an input of energy.

Energy can be transferred from one molecule to another by enzymes. The molecules that are converted by enzymes, that is, the reactants, are called substrates.

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Nutrients are organic molecules that are ultimately derived from food sources. They start off as fats, carbohydrates, and proteins. Enzymes involved in intermediate metabolism oxidize nutrient molecules to a form that can be converted to energy by mitochondria. Fats, carbohydrates and proteins are broken down to individual fatty acids, simple sugars and amino acids.

Cells utilize glucose, and its breakdown products, as well as fatty and amino acids as fuel. These are broken down in the process of digestion and transported to the cells via the circulatory system where they diffuse from the capillaries through the intracellular space and into the cytosol where the first step, that of anaerobic glycolysis occurs.

This is the method by which some anaerobic organisms produce ATP for their energy needs.

The end product of anaerobic glycolysis is pyruvate, which is broken down to two carriers, Nicotinamide Adenine Dinucleotide (NAD) and Flavin Adenine Dinucleotide (FAD), which must be transported across the virtually impenetrable mitochondrial inner membrane to the matrix.

This is accomplished by two shuttle systems, the malate aspartate and glycero-phosphate shuttles, which deliver these co-enzymes to the matrix in the oxidized state.

In the matrix, these are processed in the Krebs or Citric Acid Cycle where they are reduced, that is to say, where they have additional hydrogen ions attached to them, and after this reduction, they are carried to the inner membrane for further processing by the electron transport chain, which is made up of five complexes and two carriers.

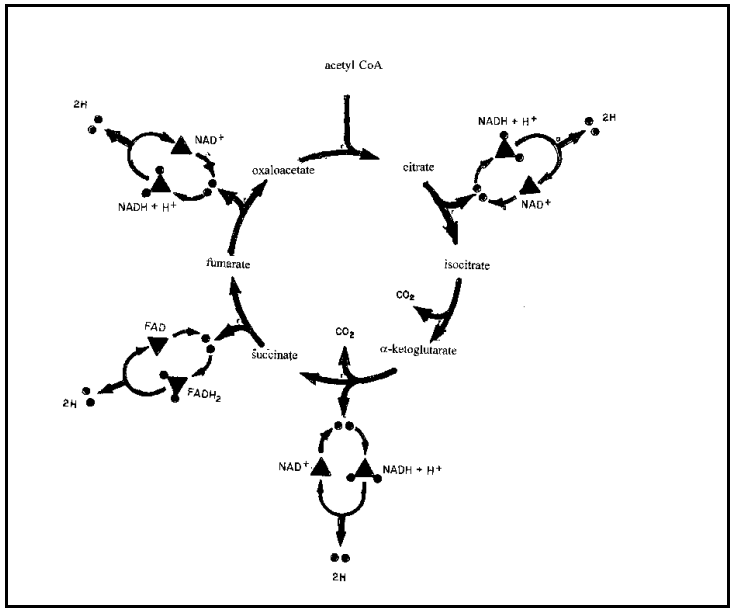

Figure 5

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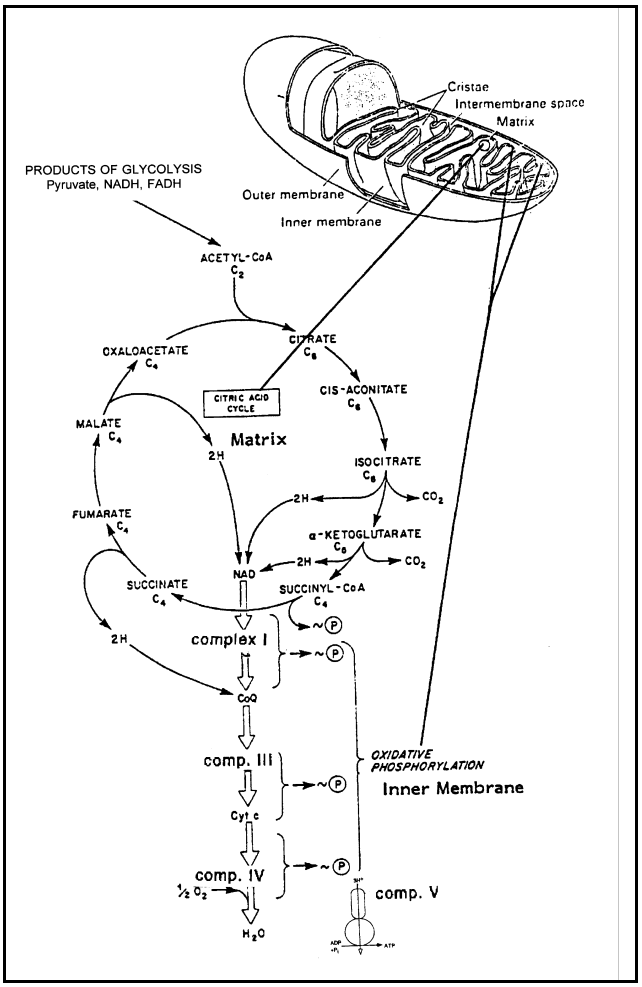

Figure 6

To understand this process, we should know the basic structure of the inner membrane, a bi-lipid membrane in which are embedded the complexes which carry out the electron transport function.

INNER MEMBRANE

The inner membrane is much larger and more extensive than the outer membrane and is enfolded within the inner membrane, the enfoldings forming a cristae. The inner membrane in its mass consists of approximately 3/4 proteins, which are various transport mechanisms and the five complexes of the electron transport chain and while these are said to be embedded in the inner membrane, they actually float in the inner membrane. Textbook illustrations tend to show them in a straight line so one can understand how electrons are transported from one to the other; actually, they are not embedded in an orderly linear fashion, and are rather scattered. (Figures 8 & 9). Also embedded in the inner membrane are enzyme carriers such as co-enzyme Q10 and cytochrome c which accept electrons from one complex and transport them to another. These enzymes and complexes are extremely numerous and are in very close proximity to each other. Figures 7, 8 and 9 show a fairly accurate depictation of their

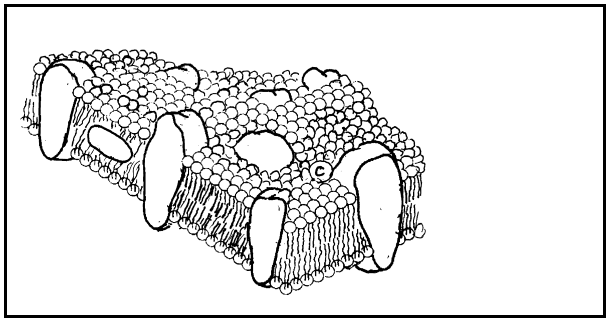

Figure 7

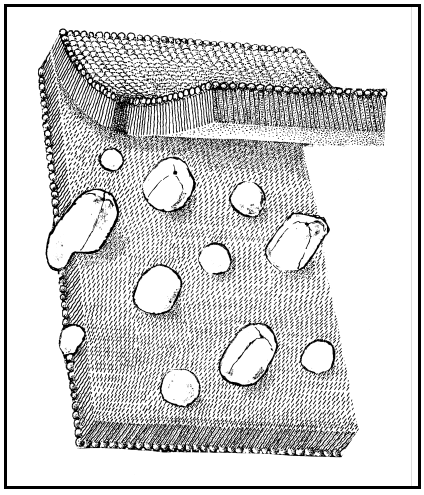

Figure 8

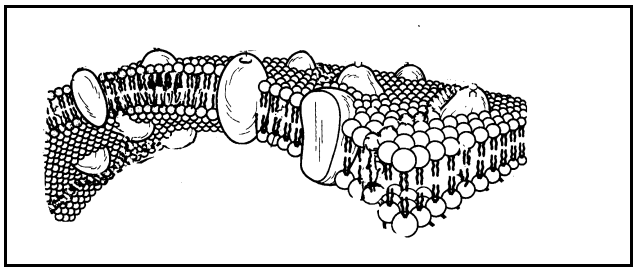

Figure 9

number and proximity to each other. Figure 10 is a more stylistic depiction showing the complexes and the various transport mechanisms involving moving substances in and out of the otherwise impermeable inner membrane. The inner membrane is permeable to gasses but without an import mechanism is impermeable to most other molecules. For adenosine diphosphate to be processed in oxidative phosphorylation, much of it must be moved across the inner membrane after Adenosine Triphosphate is created it must be moved back out across the membrane to be used in other parts of the cell. The electron carrier enzymes NAD and FAD as mentioned above must be transported into the matrix by shuttles. (Figure 10)

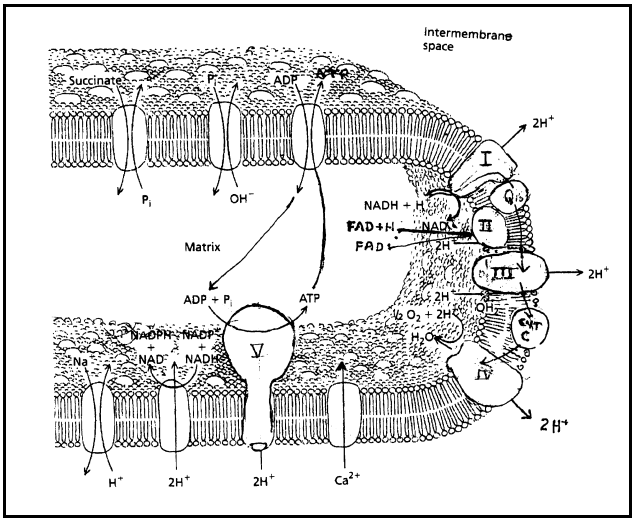

Figure 10

Complex I accepts fuel from the citric acid cycle in the form of NADH, which donates electrons to the chain. Part of the energy of these electrons are used

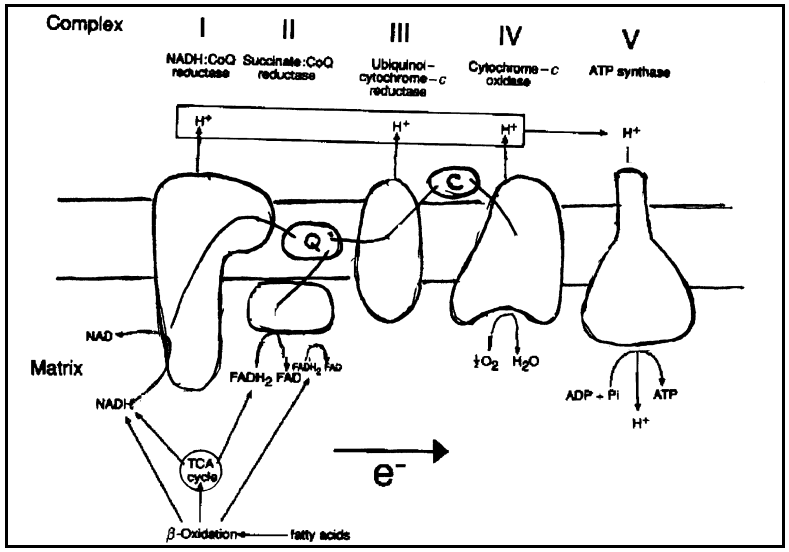

Figure 11

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to pump a proton across the inner membrane, after which the electrons are passed to Complex III via coenzyme Q. Complex II accepts electrons from FADH2 and also passes them to Complex III via coenzyme Q. Complex III uses another part of the energy of these electrons to pump another proton across the inner mitochondrial membrane. The electrons are then passed to Complex IV via cytochrome C where the remaining energy is used to pump the third proton across the membrane. The de-energized electron is then transferred to oxygen to generate water.

The relative excess of protons in the intermembrane space creates a pH and redox gradient across the inner mitochondrial membrane. The energy of this gradient, which is known as the proton motive force, is used by Complex V to convert adenosine diphosphate (ADP) to adenosine triphosphate (ATP), the chemical energy "currency" of the cell. ATP can then be transported to where work needs to be done. In this fashion, electron transport to oxygen in Complex IV is said to be coupled to oxidative phosphorylation in Complex V. If for any reason the electron transport system is diverted or does not create sufficient proton motive force to operate Complex V, then the electron transport chain and oxidative phosphorylation are said to be

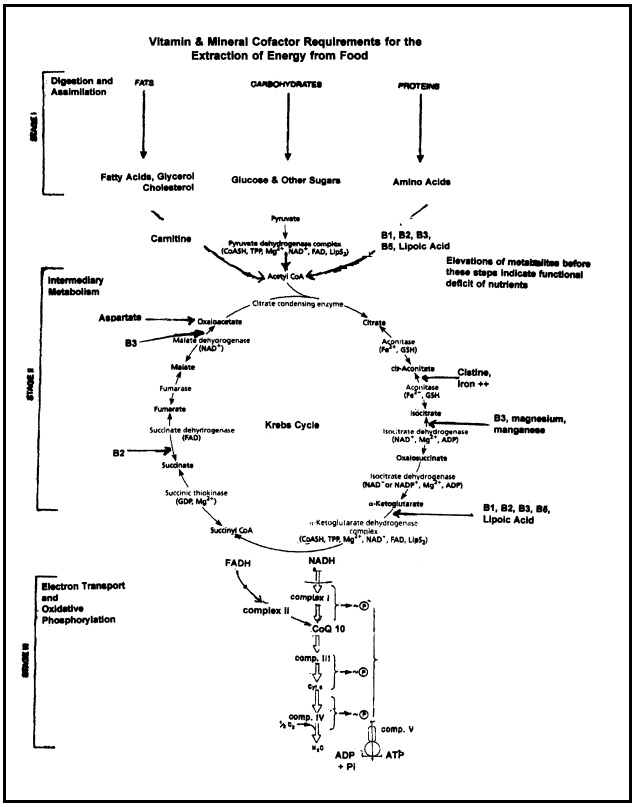

Figure 12

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uncoupled. The coupling and uncoupling of electron transport and oxidative phosphorylation will be discussed in a later chapter in detail. The coupling of electron transport to oxidative phosphorylation is essential for normal cell function and, ultimately, for the life of the cell.

Several nutrients and enzymes are involved in Krebs Cycle and electron transport chain functions. Deficiencies of these, may lead to dysfunctions. (Figure 12)

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BASIC SCIENCE ASPECTS OF THE MITOCHONDRIA
SECTION II

MEMBRANE TRANSPORT MECHANISMS IN MITOCHONDRIAL FUNCTION

Cells as well as subcellular organelles are generally surrounded by membranes which are more or less impermeable and which protect the cell or the organelle from adverse influences in their environment. Despite this, in order to reproduce and grow and carry out their normal functions, cells and organelles must constantly import and export substances in exchange with the environment. This is accomplished by means of a variety of membrane transport mechanisms.

A few solutes such as carbon dioxide and oxygen can simply diffuse across the membrane. The vast majority of solutes entering and leaving cannot diffuse across the membrane. Their transfer depends on membrane transport proteins which span the membrane and provide passage across it to specific substances. The particular membrane transport proteins in the plasma membrane of the cell or in the membranes of the intracellular organelle, determine exactly what solutes can pass into or out of that cell or organelle. Two major classes of membrane transport proteins can be distinguished: 1) carrier proteins which bind the solute on one side of the membrane and deliver it to the other side through a change in the conformation of the carrier protein. Solutes transported in this way may be either small organic molecules or inorganic ions. 2) By contrast, channel proteins form tiny hydrophilic pores in the membrane to which solutes can pass by diffusion. Most channel proteins let through inorganic ions only and are usually referred to as "ion channels". In some cases, where the substance to be transported is an oxidized or reduced molecule, it cannot pass through such carrier proteins or ion channels and must be disassembled on one side of the membrane and reassembled on the other side. This is referred to as a shuttle

mechanism. Two such shuttle mechanisms, the malate-aspartate and glycerol phosphate shuttles, exist to transport the reduced pyridines resulting from glycolysis in the cell into the matrix of the mitochondria where these molecules contribute their electrons and protons to drive the electron transport chain and the oxidative phosphorylation mechanism which produces ATP. Without these shuttles, mitochondria cannot produce energy and eventually the mitochondria and the cell it inhabits will undergo destruction. For proper function of electron transport and oxidative phosphorylation, mitochondria must import not only the reduced pyridines, NADH and FADH, but it must also import ADP, calcium and phosphate, which are imported through protein carriers. Once ATP is formed, this must be exported through a carrier. Failure of the shuttles or of the import and export protein carriers will result in or lead to destruction of the mitochondria and the cell it inhabits. The rate of the function of these mitochondria is controlled by the availability of the various substances required for this function. By way of example, the rate of the production of ATP by oxidative phosphorylation being dependent on the presence of phosphate and ADP cannot proceed at a greater rate than that permitted by a constant supply of phosphate and ADP. The rate of electron transport is dependent upon the availability of oxygen at Complex IV and its rate is controlled both by the influx of NADH and FADH through the shuttle mechanisms and the availability of oxygen.

Carrier proteins are required for the transport of almost all organic molecules across cell membranes with the exception of fat-soluble molecules and small uncharged molecules that can pass directly through the lipid bi-layer by simple diffusion. Each carrier protein is highly selective, oftentimes transporting only one type of molecule. To guide and propel this complex traffic of

small molecules into and out of the cell and between the cytosol and different membrane-bound organelles, each cell membrane must contain a set of different carrier proteins appropriate to that particular membrane. In the plasma membrane there are carriers to import nutrients such as sugar, amino acids and nucleotides. In the inner membrane of mitochondria there are carriers for importing pyruvate, ADP and for exporting ATP. An important feature of any transport process is what causes it to go in one direction rather than another. Movements of molecules downhill from a region of high concentration to a region of low concentration occurs spontaneously provided the pathway exists. Such movements are called "passive" because they need no other driving force. If a solute is present at a high concentration outside the cell and a low concentration inside the cell and an appropriate channel or carrier protein is present in the membrane, the solute will move spontaneously across the membrane and into the cell by passive transport without expenditure of energy by the transport protein. On the other hand, to move a solute against its concentration gradient, a transport protein has to do work. It has to drive the uphill flow by coupling it with some other process that provides energy. Transmembrane solute movement driven in this fashion is called "active transport" and can be carried out only by carrier proteins which can harness some energy source to the transport process.

Transport mechanisms

It is customary to distinguish between ‘passive transport’ which may occur by physical diffusion or by enzymatic mechanisms (‘carrier-mediated transport’), and ‘active transport’. Passive transport is down a chemical or electrochemical gradient, and active transport occurs against such a gradient. Since active transport is thermodynamically uphill it can only proceed if

energy in the form of ATP is available. In general, the term 'translocation' (instead of transport) may be used to denote the catalyzed movement of electrons, chemical groups, ions, and molecules from one place to another. In terms of the translocation of ions and molecules, one can distinguish between three systems of transport: uniport, symport, and antiport. A uniport reaction is a non-coupled solute translocation, one which allows a single solute to equilibrate across a membrane; in short, 'facilitated diffusion' which is responsible for e.g. glucose transport across the blood-brain or the cell membrane barriers. A symport reaction (symcoupled solute translocation) is one in which two solutes are transported together ('co-transport') in such a way that the translocation of one solute is coupled to the movement of another, such as the co-transport of amino acids and amines during the (passive) influx of Na+ along its electrochemical gradient. An antiport reaction, finally, implies counter- transport, the translocation of one solute is coupled to the translocation of another solute in the opposite direction. An example of antiport reaction is sodium-potassium -dependent ATPase. Since this enzyme catalyses 'active' translocation of sodium and potassium in opposite directions (and passive 'exchange diffusion' of sodium when there is an unsurmountable energy barrier) it constitutes an antiporter'.

Sodium-Potassium ATPase is an example of an energy-yielding metabolic system which, by being membrane bound and properly oriented in the membrane, can achieve vectorial translocation of ions between the cytoplasm and extracellular fluid. The complexes of the respiratory chain are so oriented in the mitochondrial membrane that the passage of electrons along the chain allows translocation of protons to the outside. This would then be another example of metabolic energy driving ion transport. The respiratory-linked separation of protons across the mitochondrial

membrane, by creating an electrochemical gradient for protons, reverses the mitochondrial ATPase reaction to form ATP. Electron transport is accompanied by extrusion of protons from the mitochondria which has two effects: it tends to maintain an alkaline pH inside the mitochondria, and it gives the mitochondrial matrix a negative potential with respect to the cytosol.

For electrically charged molecules, whether small organic ions or inorganic ions, an additional force comes into consideration. Most cell membranes have a voltage across them, a difference in the electrical potential on each side of the membrane which is called the membrane potential This membrane potential exerts a force on any molecule that carries an electrical charge. The cytoplasmic side of the cell membrane is usually at a negative potential relative to the outside, which tends to pull positively charge solutes into the cell and drive negatively charged ones out. While a charged solute also tends to move down its concentration gradient, the force driving a charged solute across a membrane is a combination of two forces, one due to its concentration gradient, the other due to the voltage across the membrane. This combined driving force is called "the electrochemical gradient" for any given solute. This gradient determines the direction of passive transport across the membrane. For many ions voltage and concentration gradient work in the same direction creating a relatively steep electrochemical gradient. This is the case, for example, with sodium, which is both positively charged and at a higher concentration gradient outside the cells than inside. Sodium, therefore, tends to enter the cell quite easily. On the other hand, potassium, a positively charged ion which is present at a high concentration inside the cell has a small electrochemical gradient across the membrane despite its large concentration gradient, with the result that there is little potassium movement across the membrane. Active transport of solutes

against their electrochemical gradient is thus necessary to maintain the intracellular ionic composition of cells and to import solutes that are at lower concentrations outside the cell than inside. Cells carry out active transport in two fashions. Coupled transporters couple the uphill transport of one solute across the membrane to the downhill transport of another. ATP driven pumps couple uphill driven transport into the hydrolysis of ATP. In the plasma membrane an ATP driven pump transports sodium out of the cell against its electrochemical gradient and the sodium then goes back in down its electrochemical gradient so the influx of sodium can also drive the active movement of other substances into the cell against their electrochemical gradient. If the sodium pump (sodium-potassium ATPase) ceased operating, the sodium gradient would soon slow down and transport through the sodium coupled transporters would come to a halt. For this reason, the ATP driven sodium pump has a central role in membrane transport since the ATP drive sodium pump is not only a carrier protein but also an enzyme in ATPase. It couples the outward transport of sodium to an inward transport of potassium. This sodium-potassium pump typically accounts for 30% or more of the cells total ATP consumption. Much like the bilge pump in a leaky ship, it operates continuously to expel the sodium that's constantly entering through other carrier proteins and ion channels and keeps the sodium concentration in the cell's interior around 30 times lower than in the extracellular fluid and potassium concentration about 30 times higher. The positive ions tend to be pulled into the cell, because the inward electrochemical driving force of sodium is large, being a combination of a driving force of its concentration gradient and a voltage gradient in the same direction.

Like sodium, calcium is kept at a low concentration at the interior of the cell compared with

its concentration in the extracellular fluid. The movements of calcium across cell membranes are crucially important because calcium can combine tightly with any other molecules in the cell altering their activities. The influx of calcium into the cell through calcium channels often serves as a signal to trigger other intracellular events such as secretion of signalling molecules or the contraction of muscle cells. The lower the concentration of free calcium in the cytosol, the more sensitive the cell is to an increase in cytosolic calcium. Cells maintain very low concentrations of free calcium in their cytosol in the face of much higher extracellular calcium by means of ATP driven calcium pumps in the plasma membrane and the endoplasmic reticular membrane which actively pump calcium out of the cytosol. Like the sodium potassium pump, the calcium pump is an ATPase pump.

Channel proteins form small channels which allow water soluble molecules to cross from one side of the membrane to the other. An example of such protein channels are the porins that form channels in the outer membrane of the mitochondria. Most of the channel proteins have narrow highly-selective pores. Ion channels, on the other hand, as distinguished from simple channel proteins, have ion selectivity permitting some inorganic ions to pass but others not to pass. They have charged amino acids in their linings which are usually negative and deter negative ions from entering because of the electrostatic repulsion between like charges. These ion channels are said to be gated and they switch between an open and a closed state. Their advantage over membrane pumps is that the passage of ions through them when they are open is much faster than the passage through pump mechanisms. When a channel opens, ions rush through it and this rush of ions amounts to a pulse of electrical charge delivered into the cell or out of the cell. This ion

flow can change the voltage across the membrane - the membrane potential. More than 100 types of ion channels have been discovered so-far. New ones are still being found. They differ from one another with regard to ion selectivity, (or the types of ions they allow to pass) and gating, (the conditions that influence their opening and closing).

All of these mechanisms are operative in the mitochondria, particularly the aspartate-malate and glycerol-phosphate shuttles which are made necessary by the uniquely impermeable nature of the inner membrane. These mechanisms can be interfered with by environmental pollutants and toxins, as well as oxidative damage to the mitochondrial genome. It is quite apparent that a number of essential life processes in the body are dependent on the proper function of these transport mechanisms. It has recently been reported by way of example, that non-insulin dependent diabetes is the result of failure or interference with the shuttle systems in mitochondrial beta cells leading to improper function of these cells in the production of insulin and other hormones secreted by the islets of Langerhans, all of which are heavily dependent on proper membrane transport function to these cells.

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BASIC SCIENCE ASPECTS OF THE MITOCHONDRIA
SECTION III

MITOCHONDRIAL FUNCTION IN OXIDATIVE PHOSPHORYLATION

Since most of the scientific discoveries concerning mitochondrial function, dysfunction and pathophysiology have been made in the past 10 years, much of this was unknown at the time most physicians currently in practice were being trained.

To enable them to catch up with the Basic Science Aspects of Mitochondrial Disease upon which clinical practice must be based, the following summary of the developing knowledge is presented before discussing the clinical aspects of these diseases:

Mitochondria are composed of two membranes that create two compartments: the matrix, inner and outer membranes, and the intermembrane space, each harboring unique activities. The matrix contains hundreds of types of enzymes including those involved in the fatty acid and pyruvate oxidation cycles that yield acetyl CoA, the essential initial substrate of the citric acid cycle. The matrix also contains 5—10 copies of the mitochondrial genome composed of about 16 500 nucleotides and 37 endogenous genes. Thirteen of these genes encode for subunits of cytochrome oxidase, NADH dehydrogenase, cytochrome C, and ATP synthase that are essential components of the energy production system. There are 22 tRNA genes that are of prokaryotic origin and, thus, differ from the nuclear tRNA genes. In addition, there are two rRNA genes encoding for the 12S and 16S components of the mitochondrial prokaryotic-type ribosomes. The essential processes of DNA replication, transcription, and translation take place in the matrix and depend on imported proteins from the cytoplasm (e.g., DNA polymerase, RNA polymerase, and all the ribosomal proteins).

The inner membrane forms folds, which considerably increase its surface area. These can be maintained only if the mitochondrion is under intact osmotic regulation. The inner membrane is the site of the respiratory chain, the ATP-generating ATP synthase complex, and the function of the proteins that regulate metabolite transport into the matrix. Because of the dependence of ATP synthase on the electrochemical proton gradient that is established across the membrane by the respiratory chain, this membrane must be impermeable to small ions. Any transport problems can disrupt the respiratory chain and have far-reaching effects on the generation of ATP. Incoming cytoplasm-generated proteins carry out most of the transport functions. The electron transfer chain inside this membrane, which uses oxygen as an acceptor, is not always complete and a relatively small yield of superoxide radical is generated constantly in the mitochondrion. If not averted, this may cause serious damage to mitochondrial components. The intermembrane space contains enzymes that use ATP which is specifically transported out of the matrix to phosphorylate other nucleotides imported into the mitochondrion and essential for intramitochondrial nucleic acid synthesis and GTPase activities. The outer membrane is like a sieve because its large pores let molecules of up to 5, 000 Daltons enter freely into the intermembrane space.

The inner membrane is responsible for the fine-tuning of the transport of small molecules into and out of the organelle. The outer membrane contains receptors of mitochondrial proteins, which are produced in the cytoplasm and translocated into the mitochondria with the aid of chaperones. The incorporation of the hundreds of proteins into the organelle, starts with the preprotein translocases in this membrane which also contain enzymes involved in mitochondrial lipid synthesis from precursors imported from the cytoplasm.

There are between a few hundreds to 20,000 mitochondria per cell, depending on the cell type and its energy requirements at various physiological states. The organs with the higest number of mitochondria are heart, brain, liver, GI tract, adrenal and kidneys. In cell division, there is more-active mitochondrial division, but mitochondrial division is not synchronous nor strictly linked with mitosis. Mitochondria are polyploid, each one containing 5—10 copies of its small circular genome. Mitochondria are essentially prokaryotic with regard to DNA replication, transcription, and the translational apparatus. Mitochondria are vulnerable to oxidative stress because of the high production of superoxide radical as a byproduct of the electron transport chain. Such damage is encountered in the DNA, lipid, and protein components. Mitochondria generate most of the ATP in the cell and rely on ATP for its function. ATP is obligatory for mitochondrial DNA replication, transcription, and translation and for uptake of phosphate (for ATP synthesis from ADP) and the ion transport system. The mitochondria must import more of their proteins and lipid precursors from the cytoplasm (with the exception of the 13 proteins encoded by its own genome). This process requires ATP and intact membrane receptors. The correct folding of the imported proteins in the matrix requires internal chaperones and ATP. Mitochondria that are damaged beyond their self-repair capacity, and cannot function sufficiently are removed by phagocytes.

While respiration and ATP synthesis are the most important functions of the mitochondria, they also perform other important functions, such as synthesis of heme, lipids, amino acids, and nucleotides.

Mitochondria constitute the greatest source of reactive oxidants. The electron transport system consumes approximately 85—90% of the oxygen utilized by a cell, and, in the process,

generate pro-oxidants. The mitochondria also contain heavy metal ions such as iron and copper, a situation that favors formation of reactive oxygen species (ROS). The most damaging of ROS produced by mitochondria is the nonspecifically oxidizing hydroxyl radical which can cause oxidative damage to mitochondrial lipids, proteins, and DNA.

Potential loss of the mitochondrial energy-generating function can lead to depletion of cellular ATP, and this is the primary cause of cell death. Normal aging is considered a reliable risk factor for the onset of neurodegenerative diseases. Substantial evidence gathered over the years indicates that mitochondrial function deteriorates with the aging process. Twenty-five years ago, Harman proposed that the maximal life-span of a particular mammalian species is mainly an expression of the genetic control over the rate of oxygen utilization. Further, he argued that the rate of mitochondrial damage produced by free radical reactions increases with increasing oxygen consumption, which ultimately causes cell death.

Many cells require high levels of energy in the form of ATP for their functional activity. The electron transport chain that generates ATP by oxidative phosphorylation is composed of five enzyme complexes. mtDNA encodes 13 of the proteins while the nuclear DNA encodes the approximately 60 other mitochondrial proteins. Complexes I—IV are involved in the oxidation of NADH and succinate, electron transport, and the generation of an electrochemical gradient created by pumping protons across the inner mitochondrial membrane. This proton motive force is utilized by complex V to help phosphorylate ADP to produce ATP.

The generation of oxyradicals is a minor toxic by-product originating during oxygen metabolism in the mitochondria. These oxyradical reactions in biological systems are known to

increase during aging, and the consequent damage is found to accumulate with age. Several studies in the past two decades have shown that the main source of oxyradicals in mammalian systems is the mitochondria.

It has been established that the ubisemiquinone-cytochrome b region of the mitochondrial electron transport chain is the major source of O_2 and H_2O_2 generation. Generation of these ROS occur primarily under state 4 conditions when no exogenous ADP, but an excess amount of respiratory chain substrate, is present. Respiratory stimulation with NADH, which delivers reducing equivalents to complex I, or succinate, a complex II substrate, results in sequential reduction of the lipid-soluble electron shuttle coenzyme Q to the semiubiquinone and then to the dihydroubiquinone. Succinate dehydrogenase while converting succinate to fumarate results in transfer of generated reducing equivalents to FAD to form the reduced nucleotide ($FADH_2$). This reduced component, which is covalently bound to the dehydrogenase, cycles between its reduced and oxidized states while transferring electrons to coenzyme Q (ubiquinone). Protons, a component of the proton motive force, are also generated. Under normal conditions, the completely reduced electron shuttle coenzyme Q transfers its electrons to ubiquinol cytochrome c reductase (complex III). The reduced complex III delivers its electrons to cytochrome c oxidase (complex IV) via a water-soluble electron transfer protein, cytochrome c (C), and finally to the ultimate electron acceptor of the respiratory chain—oxygen. Oxy-radical production in mitochondria occurs mainly due to the semireduced coenzyme Q donating its electron to oxygen which is abundant in the mitochondria. This leads to the generation of superoxide (O_2). A subsequent one-electron reduction of superoxide by another semireduced coenzyme Q results in the production of hydrogen

peroxide.

The generation of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide and their interaction with the mitochondrial pool of nonheme iron in classical Fenton chemistry leads to the production of hydroxyl radicals. A high content of brain iron may be essential, particularly during development, but its presence means that injury to brain cells may release iron ions that can stimulate the production of free radicals.

There is also a high concentration of ascorbic acid in the gray and white matter of the central nervous system. Ascorbate, an antioxidant in the absence of transition metal ions, can generate free radicals in the presence of iron or copper. If any level of catalytic iron were generated in the brain due to injury or disease, ascorbic acid might stimulate hydroxyl radical generation within the brain and CSF. The hydroxyl radical can initiate lipid peroxidation in membranes of the mitochondria and other organelles of cells, mtDNA oxidation, and mitochondrial protein oxidation resulting in their dysfunction.

Over the years, researchers have focused on the mitochondrial ROS-generating capacity and its role in the aging process. Mechanisms for intrinsic mitochondrial aging leading to cellular senescence have also been explored. It has been proposed that mitochondria of fixed postmitotic cells may be the site of intrinsic aging due to attack by free radicals and lipid peroxides. The origin of these damaging species has been considered to be due to reduction of oxygen during respiration in the mitochondria.

Some investigators proposed that the mitochondrial DNA (mtDNA) was the site of irreversible injury and resulted in the inability of mitochondria to divide, which leads to an

age-related decline in the number of functional mitochondria, and a decreased cellular production of ATP. Wallace et al. established that oxidative damage to mtDNA isolated from various human brain regions is at least 10-fold higher than that of nuclear DNA. This correlates well with the earlier finding that there is a 17-fold higher mutation rate in mtDNA as compared to nuclear DNA. Some of the factors that make mtDNA more susceptible to oxidative attack than nuclear DNA are (1) location of DNA near the inner mitochondrial membrane, a site for maximal ROS formation; (2) lack of histones, a group of protective molecules that surrounds nuclear DNA and shields them from oxidative damage; and (3) minimal DNA repair activity by the mitochondria. Consistent with the increased risk of damage for mitochondrial DNA, Ames and coworkers reported that, a two- to threefold higher level of 8-Oxo-2'-deoxy- guanosine (oxo^8 dG) was detected in liver mitochondria isolated from a 24-month old rat as compared to a 4-month old rat.

The efficient operation of the oxidative phosphorylation (OXPHOS) machinery in the mitochondrion to sustain the constant energy demands of a cell require several proteins. The OXPHOS genetics is complex in nature, since the genes encoding the component peptides of the OXPHOS proteins are distributed in both the nuclear DNA and the mtDNA. Oxidative lesions to any of the mtDNA coding the OXPHOS enzymes result in dysfunction of the respiratory chain activity. Wallace and coworkers suggest that one's OXPHOS capability at birth is lost with advancing age. Many OXPHOS-related diseases are caused by the high mutation rates of OXPHOS enzyme-coding mtDNA resulting in the OXPHOS energy-generating capacity falling below the energetic threshold of a particular organ. Many degenerative diseases are associated with OXPHOS defects.

The role of mitochondrial dysfunction in Parkinson's diseases (PD) has been studied by several research groups who all found a selective decrease in complex I activity of the substantia nigra. Nondemented PD patients show decreased cortical and subcortical glucose metabolism indicative of impaired oxidative phosphorylation. In a recent study, an increase in lactate concentrations in the basal ganglia was found in PD patients, compared to controls. This is indicative of impaired energy metabolism in PD. An increase in the frequency of a small number of point mutations have also been detected in patients with PD pathology. Reduced cytochrome oxidase activity has been detected in the cerebral cortex of AD patients. Beal and coworkers have found significant decreases in cytochrome oxidase in four separate regions of the cerebral cortex. Studies using highly purified mitochondria from AD brains show a 50% decrease in complex IV activity. The hippocampus is one of the brain regions in AD patients in which cytochrome oxidase activity is compromised. These important studies provide evidence of mitochondrial dysfunction and oxidative damage in AD.

Many studies have found that the fluidity of cellular membrane decreases with age. Such a change has been attributed partially to the oxidation of plasma and mitochondrial membrane lipid components. One reason for this propensity toward oxidation with age is the change in composition of membrane lipids. In liver microsomal and mitochondrial membrane fractions isolated from rodents, there appears to be progressive decline in the amount of 18:2 linoleic acid. This change is paralleled by an increase in the amount of long-chain polyunsaturated fatty acids (22:4 and 22:5), a subclass of lipids that exhibit a higher degree of unsaturation and are more susceptible to oxidation reactions than linoleic acid. Most of the 18:2 to 22:4 and 22:5 fatty acid

substitutions are found to occur in the fatty acid composition of cardiolipin, a diphosphatidyl glycerol derivative found principally in mitochondria and is important in mitochondrial membrane structure and function. The enzymatic activities of several proteins of the inner mitochondrial membrane are maintained due to their interaction with cardiolipin. This pivotal lipid is found to decrease with age in a number of tissues and is thought to be associated with age-related decrease in state 3/state 4 respiratory control ratio and other mitochondrial functions. Cardiolipin contains a higher ratio of unsaturated to saturated fatty acid residues compared with the other phospholipids of the inner mitochondrial membrane. This characteristic increases its sensitivity to oxidation.

Conditions leading to oxidative stress, like episodes of ischemia-reperfusion, generate oxidants that decrease cardiolipin levels, presumably by causing lipid peroxidation of unsaturated fatty acyl side chains. The destruction of cardiolipin has been shown to lead to inactivation of cytochrome oxidase and other mitochondrial enzymes and also an increase in the permeability of the inner mitochondrial membrane. The targeted insertion of nuclear-encoded mitochondrial proteins into the inner mitochondria in the form of a precursor protein relies upon cardiolipin availability. Thus, oxidative modifications in the fatty acyl side chain of cardiolipin could adversely affect the transport of the nucleus-encoded precursor protein into the mitochondria leading to functional deficits of this organelle. The brain is particularly vulnerable to oxidative damage, since it contains relatively high concentrations of easily peroxidizable polyunsaturated fatty acids, and the brain is not highly enriched with protective antioxidant enzymes or small molecule-antioxidants.

The likelihood of oxidative insult to normal neurons relative to other cells depends on

catalytically active metals (i.e., iron and copper) present in the brain, particular ROS-generating enzymes (nitric oxide synthase, xanthine oxidase, etc.), abnormal processing of the brain amyloid precursor protein resulting in production of β3-amyloid (Aβ), aldehydic lipid-peroxidation products like 4-hydroxynonenal (HNE) and malondialdehyde (MDA) generated due to peroxidative reactions in the brain. Another factor that results in greater oxidative insult to brain, relative to other tissues, is that this organ consumes one-fourth of the total O_2 intake, and, consequently, generates more oxyradicals than most other organs in the body based on weight. Finally, due to the postmitotic neuronal cellularity of the brain, further differentiation and/or cellular repletion does not occur. As a consequence, the brain's organelles are more likely to accumulate more oxidatively damaged biomolecules than do cells that undergo mitosis, resulting in a loss of function.

The brain neurons and their organelles must survive for longer periods with oxidatively damaged dysfunctional organelles, which occurs as a function of age and long-term metabolic stress. A functional consequence of membrane lipid oxidation is membrane rigidization. This rigidization can lead to a decline in receptor-mediated signal transduction mechanisms partially explaining the decreased responsiveness of the 13-adrenergic, dopaminergic, and muscarinic receptor systems to agonist stimulation. There is substantial evidence of membrane deterioration in various regions of the brains of AD patients as shown by magnetic resonance spectroscopy and lipid composition studies. Evidence for a membrane defect has been demonstrated in the midtemporal cortex of an AD brain as compared to a normal elderly subject. Cerebellum, an area of the brain that is relatively unaffected by the disease, did not show any membrane destabilization in both elderly normal and AD patients.

Thus, various lines of evidence show that lipid peroxidative events do occur in age-related degenerative diseases like AD. It is conceivable that an endless loop of other peroxidative events occur, resulting in a continual damage to neuronal mitochondria. This can eventually lead to the inability of the mitochondria to generate energy and translocate high-energy phosphates to the cytosolic compartment, resulting in altered ion homeostasis and neuronal death.

The accumulation of oxidatively damaged proteins within and among tissues increases with age. Sohal and coworkers have shown that with mtDNA, mitochondrial protein undergoes age-related oxidative damage as estimated by carbonyl levels. The view that one of the fundamental feature of aging involves alteration of mitochondrial function is further enhanced by evidence of oxidative inactivation of the components of the electron transport chain. Some of the ROS, like superoxide (O_2) and hydrogen peroxide (H_2O_2), may diffuse to interact with remote cellular components, reactions of hydroxyl radical (OH) are diffusion controlled. It is conceivable that OH can oxidatively damage the proteins of the electron transport chain. Cytochrome oxidase (complex IV) is the enzyme located at the terminal step in the respiratory chain. This enzyme transfers electrons from cytochrome c into oxygen to produce water.

The accumulation of oxidatively damaged dysfunctional protein in the mitochondria can conceivably lead to loss of biochemical and physiological function in the mitochondria

Several defense mechanisms exist to counter the flux of harmful oxidants generated within a cell. Among these antioxidant mechanisms are enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx). SOD catalytically dismutes superoxide radical anion to hydrogen peroxide, while catalase and GPx render hydrogen peroxide generated in the

mitochondria harmless by converting it into water and oxygen. GPx utilizes GSH to destroy hydrogen peroxide and other lipid hydro peroxides. These enzymes remove ROS like superoxide and hydrogen peroxide before it can interact with metal ions to increase the production of hydroxyl radicals. There is a large body of evidence showing that cells respond to oxidative stress by inducing these enzymes as a protective mechanism.

The small molecule antioxidants that are present in the various organelles of a cell include glutathione, vitamins C, and vitamin E. Vitamin E (also called α-tocopherol), which is a highly lipid-soluble antioxidant, has generated a great deal of interest in its ability to act as a chain-breaking antioxidant. This molecule inhibits the chain reaction of lipid peroxidation by scavenging intermediate peroxyl radicals. This renders the peroxyl radicals harmless and incapable of attacking fatty acid side chains or membrane proteins. A deficiency of vitamin E has been shown to produce severe neurological derangements. Vitamin E is one of the most important chain-breaking antioxidant inhibitor of lipid peroxidation in humans. Recent evidence has shown that vitamin E present in brain mitochondria is more susceptible to oxidation than the pool available in synaptosomes. Even though ascorbate levels are quite low within mitochondria, this nutrient may play a major role as a first line of defense against oxidative stress. The relative ease of oxidation of mitochondrial tocopherol in these studies suggests a general vulnerability of the mitochondrial membranes to oxidation.

The antioxidant protective role of vitamin E may be crucial for the maintenance of tissues, such as brain, whose function is critically dependent on the availability of high-energy phosphates. Recent ischemia-reperfusion studies in animal models show a reasonable degree of protection with

the use of vitamin E against damage to mitochondria in the brain. It is well established that the role of vitamin C in the brain is a double-edged sword with regard to causing free radical damage. Gray and white matter of the brain contain substantial amounts of ascorbic acid. Apart from its proxidant ability to interact with metal pools in the brain, vitamin C also functions as an antioxidant in recycling the vitamin E radical back to vitamin E. Thus, vitamin C can act as an antioxidant or a prooxidant in the brain depending on its relative concentration and the availability of other constituents available for interaction in the cellular mileu. Two endogenous compounds that are being evaluated for their antioxidant properties in the brain are ubiquinone derivatives and melatonin. Ubiquinone (CoQ_{10}), is already known as a critical small molecule for transporting electrons in the mitochondria for the generation of ATP. The completely reduced form of ubiquinone is ubiquinol. Ubiquinols have been shown to possess much greater antioxidant activity than the oxidized ubiquinone forms and cause greater inhibition of lipid peroxidation.

The indole neurohormone, melatonin, a constituent of the pineal gland, has also been shown to possess free radical scavenging properties. This molecule readily passes through the blood-brain barrier and enters neurons and glial cells. A unique feature of melatonin is that upon exogenous administration, this molecule rapidly enters the brain. Due to its lipid and water solubility, it distributes readily into all subcellular compartments. This lack of compartmentalization makes melatonin a more effective antioxidant, as compared to vitamins E and C, which are restricted to either the lipid cell membranes or the aqueous environment of the cytosol, respectively. Preliminary evidence indicates that melatonin may restrict lipid peroxidation by prevention of initiating events as well as interfering with the chain reaction.

Mitochondrial energetic and oxidative dysfunctions which are associated with cytoplasmically inherited mutations and/or mutations in the mitochondrial genome caused by free radical production from electron transfer are emerging as pathological factors in late- onset neurodegenerative diseases. Electron paramagnetic resonance (EPR) studies indicate that exposure of isolated cerebellar and cerebral mitochondria to elevated Ca^{2+} and ADP induces generation of extremely reactive hydroxyl radicals (·OH). Mitochondrial Ca^{2+} influx is via a uniporter and efflux via Na+ and H+ exchange mechanisms with both influx and efflux dependent on the transmembrane electrochemical proton gradient ($\Delta\Psi$). When Ca2+ exceeds a critical level the inner membrane undergoes a permeability transition that collapses the electrochemical proton gradient $\Delta\Psi$.

Such depolarization prevents oxidative phosphorylation and increases free radical production from electron transfer complex I and cytochrome b_{566} in complex II. Mitochondria expressing congenital or oxidatively induced mutations show increased radical production and/or decreased potential for oxidative phosphorylation.

The study of degenerative diseases is undergoing a fundamental paradigm shift. The fields of neurodegenerative diseases, free radical pathophysiology, excitotoxin-mediated neuronal death, and mitochondrial physiology have begun to converge as evidence accumulates that excitatory amino acids and oxidative pathology figure in neuronal apoptosis and the etiology of chronic degenerative diseases. A growing body of evidence surveyed in this book implicates mitochondrial energetic and oxidative dysfunction due to congenital genetic defects, and perhaps to radical-induced mutations and oxidative enzyme impairment, in numerous degenerative disorders.

I have confined my perspective to only a few processes that are capable of precipitating mitochondrial dysfunction, and that are likely to contribute to degenerative disease. The reader is encouraged to consult the extensive and thoroughly documented literature of mitochondrial Ca2+ metabolism, of free radical pathophysiology, and of oxidative pathology in age-related changes in mitochondrial function and oxidative equilibria, and of the role oxidative and energetic pathology may play in the etiology of degenerative disease.

Although the scope of much of the literature is mitochondrial dysfunction in degenerative disease, there are benefits from a broader comparative perspective that encompasses cellular and mitochondrial pathology associated with ischemia and reoxygenation of aerobically poised tissues such as myocardium, as well as excitotoxicity of glutamate and its dicarboxylic analogue NMDA. These pathologies share in common several functional parallels with mitochondrial dysfunction in neurodegenerative disease. There are reductions in ATP/ADP ratios, that can result when ATP utilization outpaces production during the repeated or prolonged neuronal depolarizations that are characteristic of excitotoxin stimulation, when availability of O_2 as the terminal electron acceptor in the mitochondrial electron transfer system is inadequate due to hypoxia, or when genetic defects in electron transfer components impair the ability to generate ATP.

Although the causes of ATP depletion are diverse, the functional responses of the mitochondria to accelerate oxidative phosphorylation are similar. It is evident that increases in free cytosolic Ca2+ precede neuronal death following exposure to dicarboxylic excitotoxins, and after transient hypoxia in both neurons and cardiac myocytes. Such increases in free Ca2+ result in a host of responses, including activation of phospholipase A2 with consequent arachidonate metabolism,

activation of proteases, and alterations in numerous metabolic pathways. Such increases in free Ca2+ can also perturb oxidative phosphorylation or even completely destroy mitochondrial function. Cellular oxidative status results from an equilibrium between radical production and the sum total of the cellular (and extracellular) oxidative defenses, including enzymes such as superoxide dismutase (both cytosolic Cu2+—Zn2+ and the mitochondrial Mn2+ morphs.), catalase, and glutathione perioxidase-reductase, as well as antioxidants such as α-tocopherol, ascorbate, and urate. For example, between approximately 1 % and 6% of the O_2 consumed by normally respiring mammalian mitochondria is univalently reduced to superoxide radical $O_2\cdot^-$ by the electron transfer system instead of tetravalently to water by the terminal cytochrome oxidase.

Although such low-level, chronic production of oxygen radicals reasonably contributes both to aging and to increased mutation rates in mitochondrial DNA, this radical production can hardly be regarded as a potentially lethal oxidative threat unless the antioxidative defenses are impaired to the point where even such mild radical production exceeds defenses, and then oxidative damage will accrue. The antioxidant defenses are not absolutely efficient, and oxidative damage gradually accumulates as a function of age. A wealth of evidence indicates that mitochondrial dysfunction, with corresponding impairment of ATP status and increased radical production, figures prominently in excitotoxicity and neurodegenerative disease despite normal, or even increased, levels of cellular antioxidant potential.

At physiologically relevant pH, the conjugate peroxynitrous acid spontaneously isomerizes into the less stable trans configuration, whose decomposition results in the production of highly reactive hydroxyl radical and nitrogen dioxide. Thus, activities of antioxidant enzymes, such as

SOD, although obviously indicative of antioxidant defenses, imperfectly describe the oxidative environment in vivo where availability of other radicals, such as NO, will shift equilibria and correspondingly alter radical production. Clearly, lethal cellular damage, such as plasma membrane lipid peroxidation and consequent loss of integrity, can result from intense radical exposure or loss of oxidative defenses, but cellular radical production only slightly exceeding total antioxidant defenses can also yield lethal long-term consequences. For example, several key metabolic regulatory enzymes including glutamine synthase, phosphofructokinase (PFK), creatine phosphokinase, and lactate dehydrogenase are unusually susceptible to oxidative inactivation, showing significant losses of activity after $O_2\cdot^-$ and ·OH exposures three orders of magnitude less than those first used to study enzyme inactivation by oxygen radicals. Pyruvate dehydrogenase activity is reduced in canine cerebral cortex following transient hypoxia in vivo, and both this inactivation and protein oxidation are partially ameliorated by treatment with the antioxidant acetyl-L-carnitine.

There is evidence that increases in free cytosolic Ca2+ precede neuronal death following exposure to dicarboxylic excitatory neurotransmitters, such as glutamate, and numerous xenobiotic excitotoxins such as kainate, ibotenate, and NMDA. Stimulation of postsynaptic NMDA-type glutamate receptors contributes to neuronal death due to hypoxia, hypoglycemia, and seizures and to chronic neurodegenerative disorders such as Parkinson's, Alzheimer's, and Huntington's diseases as well. Depending on which receptor is stimulated, increases in neuronal free Ca2+ occur via ligand- and voltage-gated mechanisms as well as via metabotropic responses, and these result in a plethora of potentially damaging effects. In light of simultaneous Ca2+ increases and ATP

depletion, it was proposed some years ago that the metabolic pathology responsible for excitotoxin-induced neurodegeneration was analogous to the tissue damage that is observed in aerobically poised tissues, such as myocardium, following transient hypoxia.

According to this proposal, Ca2+-induced activation of a cytosolic serine protease causes the conversion of xanthine dehydrogenase into xanthine oxidase, a form that generates H_2O_2 and superoxide radicals $O_2\cdot^-$. Although $O_2\cdot^-$ is reactive, the proximate mediator of oxyradical toxicity is likely not $O_2\cdot^-$ but rather an ensuing flux of hydroxyl radicals (·OH) formed by either Fenton reactions that involve homolytic H_2O_2 cleavage catalyzed by Fe2+ and other transition metals, or by decomposition of the peroxynitrite that is formed in reactions between $O_2\cdot^-$ and nitric oxide (NO). Hydroxyl radicals are the most oxidizing radical species found in biological systems.

Considering the ubiquity of both transition metals and NO synthetase in tissues including brain, OH is likely to be responsible for most oxyradical toxicity. Despite much evidence for radical involvement in excitotoxicity, only very low xanthine dehydrogenase/oxidase activities have been detected in neurons, thus raising doubts that xanthine oxidase is an important source of radicals under conditions of ATP depletion and Ca2+ mobilization.

In light of the extremely low xanthine dehydrogenase/oxidase activities in neurons, radical production from mitochondrial electron transfer would seem to be a more likely candidate for oxidative stress during excitotoxicity and hypoxia, especially given the Ca2+ sensitivity of mitochondrial function and the large potential for radical production in aerobically poised tissues like brain and myocardium.

It has long been recognized that free radicals are produced by the mitochondrial electron

transfer system in direct proportion to ambient P_{O2} and to the rate of O_2 utilization Such radical production is independent of radical efflux from mitochondrial adrenodoxin-dependent P-450s within the matrix, and it is associated not only with Ca2+ loading and/or transient ischemia in the intact myocardium and isolated cardiac myocytes but also with the cytotoxicity of tumor necrosis factor-α.

All of the mitochondrial electron transfer components, all of which have lower reduction potentials than molecular O_2 are capable of reducing O_2 to $O_2\cdot^-$ (Eo' for electron transfer components range from —0.32 V for NADH— CoQ reductase to +0.56 V for cytochrome c oxidase, versus +0.82 V for O_2 reduction). Although most redox reactions within the normally functioning electron transfer system occur via paired electron transfers, orbital spin restrictions dictate that O_2 reduction must proceed univalently, the first product of which is $O_2\cdot^-$. Most studies of mitochondrial $O_2\cdot^-$ and H_2O_2 production assess the effect of various inhibitors of electron transfer based on the reasoning that "downstream" blockade will increase the number of "upstream" carriers in the reduced state, and correspondingly increase the probability of their undergoing autoxidation and redox cycling. For example, using rotenone and antimycin A, inhibitors of electron transfer that block electron transfer between ubiquinone (coenzyme Q) and cytochrome b, and between cytochrome b and c 1, the early data indicated that NADH-CoQ reductase (complex I) and ubiquinone were the two most likely sources for radical production.

The univalently reduced radical form of coenzyme Q, ubisemiquinone, is an intermediate step in both ubiquinone formation and reversible electron transfers between NADH dehydrogenase, succinate dehydrogenase, oL-glycerophosphate dehydrogenase, and cytochrome b—c_1. Studies

with electron transfer inhibitors by Loschen (1971) and by Trumpower and Simmons (1979) indicated the source of radicals was between succinate dehydrogenase and cytochrome b— c_1, and attention focused on the univalently reduced ubisemiquinone as a likely source of electrons for O_2 reduction. Persuasive evidence also indicates that reduced Coenzyme Q serves as an antioxidant, facilitating $O_2\cdot^-$ dismutation rather than producing $O_2\cdot^-$, except under pathological conditions such as ischemia or when intramitochondrial Ca2+ is elevated.

The Fe—S centers in complexes I, II, and III have been proposed as the real sources of electrons fueling $O_2\cdot^-$ formation. Based on the hydrophobic nature of coenzyme Q and the milieu of its intramembrane location, it has been argued that neither coenzyme Q nor its radical form ubisemiquinone is able to interact with molecular O_2 in the surrounding aqueous environment, thereby eliminating it as a primary source of radical production. In mitochondria suspended in acetonitrile media, the EPR spectrum of membrane-bound ubisemiquinone in intact mitochondria remains stable despite the presence of molecular O_2. Upon addition of water to the media, the ubisemiquinone signal dissipates as carbon-centered, $\cdot OH$, and O_2, EPR signals increase, indicating that addition of protons permits transfer of electrons between the semiquinone and O_2. Comparable EPR data from isolated myocardial mitochondria during hypoxia-reoxygenation also show loss of the semiquinoine signal coincident with the appearance of radical signals, suggesting that even if the interaction is indirect, the net result is oxyradical formation fueled by semiquinone oxidation. More recent EPR studies further suggest that such radical production is likely due to spatial alterations within the membrane that serve to relocate ubisemiquinone closer to protons in the aqueous phase, and consequent increased permeability of the inner membrane to protons.

Sci-mito-I

Electron double nuclear resonance (ENDOR) studies of mitochondrial ubisemiquinoine in situ reveal not only the protons that stabilize the interaction of Coenzyme Q with membrane proteins which modulate the thermodynamic responses of ubiquinone but also protons with weak hyperfine coupling, suggesting that they are exchangeable. Such exchange would provide ubisemiquinone indirect access to the aqueous solvent that is normally minimized by the impermeability of the inner mitochondrial membrane to protons and by the fully embedded sequestration of the quinone. Regardless of whether the mechanism at the molecular level entails direct redox coupling or an indirect transfer of electrons via unidentified intermediate(s), the data indicate that during normal respiration under physiologically relevant conditions, electron transfer to O_2 occurs from the span in the mitochondrial electron transfer system between ubisemiquinone and cytochrome c_1 in complex III. Production of oxyradicals from this region of the respiratory system is exacerbated by numerous pathologically relevant perturbations, such as transient hypoxia, exposure to $O_2\cdot^-$ or ·OH, and changes in cytosolic Ca2+ that correspondingly increase mitochondrial Ca2+.

Use of selective electron transfer inhibitors has also identified complex I (NADH—Coenzyme Q reductase) as another site of electron leakage to molecular O_2. Complex consisting of at least 27 different protein subunits (only 7 of which are encoded in the mitochondrial genome), at least six—and perhaps eight—FE—S centers, plus noncovalently bound FMN, and two species of bound ubiquinone. Rates of H_2O_2 and $O_2\cdot^-$ production increase significantly when electron transfer from the NADH-dehydrogenase to coenzyme Q is blocked with antimycin A, suggesting a site capable of oxidation within the complex that is accessible to molecular O_2. Electron flow through

complex III is via two catalytic sites; the Q_o and Q_1 sites (for proton output and input) that oxidize dihydroquinone and divalently reduce ubiquinone. By inhibiting reduction of ubiquinone at the Q_1 site, antimycin increases the overall reduction state of complex I and the Co—Q pool, correspondingly increasing the probability of autoxidation and radical formation.

A flavin radical signal is detected using either EPR upon complete reduction of complex I with NADH (being buffered via an enzymatic generating system). Such a signal can only arise from single or, less likely, odd numbered polyvalent reductions normally in competent complex I, despite the obligatory initial 2-electron redox nature of NADH.

Addition of NADH or succinate to rat liver submitochondrial particles not only induces production of EPR-detectable hydroxyl and carbon-centered radicals when electron flow from complex I is impeded using antimycin A but also induces formation of organic radicals by direct reduction in the absence of such blockade. Complex I activity is significantly elevated in familial ALS patients who carry a point mutation that reduces cytosolic SOD activity, yet it is diminished in Parkinson's patients, perhaps as a consequence of oxidative inactivation.

When exposed to elevated Ca2+ concentrations typical of those occurring in the cytosol during excitotoxin receptor stimulation, isolated mitochondria from adult rat cerebral cortex and cerebellum produce a variety of radicals, including ·OH and carbon-centered species that are detectable with EPR spin-trapping techniques. In the absence of exogenous Ca2+ and Na+, no EPR radical signals are detected in mitochondria isolated from cerebellum and suspended in 100 mM KC1, 50 mM KH2PO4, 10 mM succinate, and 10 mM ADP. However, radical signals are detected from these same mitochondria when Ca2+ and Na+ are increased to 2.5 μ and 14 mM,

respectively. The hyperfine splitting characteristics of the spin trap (α-pyridyl 1-oxide N-*tert*-butyl nitrone; POBN) indicate that these signals are likely to be due to a mixture of hydroxyl and lipid peroxyl radical adducts, an interpretation that is supported by the observation that both ·OH and carbon-centered radicals are detected under identical conditions using the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). Because of the extreme reactivity of ·OH and the inner membrane location of electron transfer components, it is unlikely that ·OH is diffusing across one, and probably two, mitochondrial membranes before reacting with the spin trap. The observed ·OH signal is more reasonably ascribed to diffusion of less reactive H_2O_2 out of the mitochondria and subsequent Fenton catalysis, a realistic reflection of events likely to occur in vivo given the paucity of catalase activity in brain. These mitochondrial ·OH signals persist in the presence of excess catalase (1800 U/ml), an observation that argues against the notion that ·OH arises from Fenton catalysis of effluxed H_2O_2. Such persistence of ·OH signals despite exogenous catalase suggests that catalase has less access to effluxed H_2O_2 than the spin trap.

Assuming that NO is constitutively present, minor increases in mitochondrial $O_2^{\cdot -}$ production would correspondingly increase peroxynitrite formation even without invoking the increase in NO production induced by stimulation of the glutamate-NMDA receptor. Inhibition of NO synthase moderates Ca2+ release and consequent toxicity in cultured rat cerebellar granule cells following NMDA exposure, and protects cortical neurons in vitro from glutamate exposure, clearly implicating NO in excitotoxicity. NO synthase inhibition moderates only slightly the radical efflux detected from intact cerebellar granule cells following NMDA stimulation, suggesting that peroxynitrite is not the primary source of the extracellular OH detected with EPR.

Sci-mito-I

Regardless of the underlying mechanism(s), it is clear that radical signals are observed from isolated neuronal mitochondria only after Ca2+ and Na+ are increased. Hypoxia increases radical production from mitochondria isolated from cardiac myocytes, probably because the proportion of the electron transfer components in the reduced state increases as availability of O_2 declines. The radical production observed from cerebellar and cortical mitochondria could also be due to hypoxia because oxygen in the closed EPR cuvette was depleted by the respiring mitochondria well before completion of EPR data collection. Several lines of evidence argue against this possibility: no radical signals are seen under identical circumstances from equally hypoxic mitochondria in the absence of elevated Ca2+ and Na+, indicating that excess Ca2+ and Na+ are responsible for the observed radical production, not hypoxia per Se; in the presence of excess reducing potential, buffer P_{o2} would have to decline to ca. 0.3 μ M , the K_m for the terminal oxidase for O_2, before reduction of upstream carriers would be substantially augmented, yet the experiment ended well before P_{o2} declined to such a low level.

To eliminate hypoxia as a confounding variable, sample P_{o2} was regulated by passing O_2 over the mitochondrial sample in an O_2-permeable Teflon tube within the resonance cavity. No EPR signals are detected from well-oxygenated mitochondria until Ca2+ and Na+ are increased as above, indicating that the observed radical production is induced by elevated Ca2+ and Na+, not hypoxia. This radical production requires sustained Ca2+ exposure and does not occur when Ca2+ is increased only briefly. Morbidity of hippocampal neurons following glutamate exposure *in vitro* is also precipitated by a gradual sustained increase in cytosolic Ca2+ that occurs hours after an initial transient increase. When Ca2+ alone is increased, EPR signals from isolated neuronal

mitochondria are fully 80% of those obtained when both Ca2+ and Na+ are increased.

The evidence indicates that a continuous slow cycling of Ca2+ and Na+ occurs across the mitochondrial inner membrane and that alterations of intramitochondrial Ca^{2+} are associated with metabolic regulation. In cardiac myocytes, fluctuating levels of mitochondrial free Ca2+ between 0.2 and 2 μ M are responsible for increases in oxidative metabolism in response to increased activity. This is primarily due to the allosteric regulation of several dehydrogenases and glycerol-3-phosphate dehydrogenase, a component of the glycerophosphate shuttle.

The phosphorylase that activates pyruvate dehydrogenase (PDH) is activated by Ca^{2+} concentrations of ca. 3 x $10^{—6}$ to $l0^{-7}$ M, within the range typically detected after physiologically relevant extracellular Ca^{2+} pulses. In response to increased activity in electrically paced cardiac myocytes, the proportion of PDH in the active form increases threefold, from less than 20% to almost 70% when intramitochondrial Ca2+ increases from 2.7 to 4.1 nmol/mg protein. Both isocitrate dehydrogenase (IDH) and α-ketoglutarate dehydrogenase show increased substrate affinities when Ca2+ is increased. Moderate increases in mitochondrial Ca2+ are also reported to increase activity of F_0F_1-ATPase, adenine nucleotide translocase, and electron transfer *per se*. Cytosolic Ca2+ concentration is typically 50— 100 nM, depending on cell type. When Ca2+ levels reach 200—300 nM, mitochondria begin to accumulate Ca2+ as a function of the equilibrium between influx via a Ca2+ uniporter versus efflux via both Na+-dependent and Na+-independent carriers. Ca2+ influx via the uniporter is fast, passive, and second order for Ca2+, indicating initial potentiation of influx by Ca2+. A number of other cationic metals and some antibiotics can substitute for Ca2+ and activate the uniporter. Ca2+ influx is completely dependent on the negative

transmembrane electrochemical potential ($\Delta\Psi$) established by electron transfer, and influx fails to occur in the absence of $\Delta\Psi$ even when an eightfold Ca2+ concentration gradient is imposed.

A significant consequence of such dependence on $\Delta\Psi$ is that mitochondria release Ca2+ via this uniporter when the membrane potential is disputed, as occurs with uncouplers like 2,4-dinitrophenol and carbonyl cyanide-*p*-trifluoro-methoxyphenylhydrazone (FCCP). The uniporter also transfers a host of other divalent cations including Fe2+. Because $O_2^{\cdot -}$ can increase free Fe2+ availability by releasing it from protein carriers, oxidative stress could potentially increase mitochondrial Fe2+ uptake, thereby exacerbating intramitochondrial Fenton reactions. The uniporter is inhibited by Mg2+.

Although initially considered passive ion exchangers, both efflux mechanisms (Na+-independent Cu2+ efflux, and the Na+-dependent efflux) pump Ca2+ out of mitochondria against an electrochemical gradient using energy derived from opposing favorable gradients for Na+, in the case of the Na+-dependent mechanism, and H+ for the Na+-independent mechanism.

Unlike many transmembrane ion pumps, of which Na+/K+-ATPase is the archetype, mitochondrial Ca2+ efflux mechanisms do not entail direct hydrolysis of ATP, relying rather on Na+ and H+ gradients across the inner membrane for energy. Eefflux is still considered active in that use of the proton motive gradient to fuel Ca2+ efflux represents a loss of phosphorylation potential because the exchanged H+ bypasses F_0F_1-ATPase. Activity of the inner membrane Na+—H+ exchanger that maintains the favorable mitochondrial Na+ gradient needed for Ca2+ efflux is not associated with ATP hydrolysis, yet by using the H+ gradient for purposes other than phosphorylation of ADP via F_0F_1 -ATPase, it represents an energy sink and is considered active.

Sci-mito-I

To the extent that mitochondrial Ca2+ loading exceeds normal levels required for metabolic regulation, the net result of using Na+ and H+ (and other cation) gradients to regulate intramitochondrial Ca2 + is that energy derived from the electron transfer system is not reflected in ATP production. When cytosolic Ca2+ levels increase abnormally— whether due to opening of voltage-gated NMDA channels, failure of plasma membrane ion-dependent ATPases from lack of ATP, or oxidative damage to the plasma membrane— the energy required to pump Ca2+ out of the mitochondria is lost to the adenylate pool in direct proportion to the Ca2+ load, and this can become a significant energetic drain. As cells age and the potential for ATP production declines, Ca2+ loading that would have previously been of minor significance may become increasingly difficult to compensate for, and the resulting increases in intramitochondrial Ca2+ can have lethal consequences because of irreversible mitochondrial collapse.

Although intramitochondrial Ca2+ sequestration and removal during pathological overloading of cytosolic Ca2+ results in futile energy dissipation, sharply increased intramitochondrial Ca2+ levels (ca. 25 μM) induce a corresponding sudden change in the inner membrane permeability, and a sharp increase in mitochondrial radical production, that presages complete loss of mitochondrial function and cell death. Calculations indicate that in respiring mitochondria, an intramitochondrial Ca2+ concentration of 25 μM would be established when external Ca2+ is ca. 1 μM, and use of Ca2+-activated fluorescent dyes such as fura 2 indicates that under unstressed conditions, intramitochondrial Ca2+ is between two- and fivefold higher than cytosolic [Ca2+]. This permeability transition (PT) entails opening of a nonselective proteinaceous pore in the inner membrane, likely associated with adenylate translocase, that not only collapses the

mitochondrial membrane potential but also results in mitochondrial swelling due to colloid osmotic pressure. The ability of Ca2+ to open the permeability transition pore is dramatically synergized by a variety of physiologically pertinent "inducing agents," such as elevated inorganic phosphate, oxaloacetate, lysophospholipids from phospholipase A_2 activation, and most notably in the present context, hydroperoxides and free radicals. Antioxidants and augmentation of mitochondrial glutathione protect against Ca2+- induced PT.

Depletion of mitochondrial glutathione precipitates the permeability transition and is correspondingly lethal to hepatocytes. In liver and myocardial mitochondria, the PT pore is selectively inhibited by the immunosuppressive agent cyclosporin A. When the electrochemical gradient and membrane potential collapses, mitochondria release Ca2+ which increases local [Ca2+] and the Ca2+ load on nearby mitochondria thereby setting up a chain reaction. Independent of the many pathological sequelae of PT collapse—including increased radical production from uncoupled electron transfer—the ensuing loss of ATP *per se* is also directly lethal to most aerobically poised cells, including a glial cell line. Aside from the obvious problems caused by a loss of cellular ATP, lack of ATP in the mitochondria exacerbates PT collapse. Permeability transition collapse of the mitochondrial potential, the consequent uncoupling of electron transfer and increase in radical production, plus the loss of phosphorylation potential—all results of perturbations in Ca2+ regulation—will carry dire consequences for any cell.

Severe radical exposures and Ca2+ loading induce a collapse of mitochondrial function in terms of inability to generate ATP and retain Ca2+ due to permeability transition. Less severe mitochondrial Ca2+ loading and free radical exposures are not without deleterious consequences to

both electron transfer and subsequent mitochondrial free radical efflux. For example, Fe—S centers in the electron transfer complexes are exquisitely susceptible to radical mediated inactivation, but so also are many enzymes including cerebral cortex pyruvate dehydrogenase, which shows inactivation following ischemia-reoxygenation in canine cortex. Mitochondrial transcription is extremely sensitive to oxidative inactivation by a variety of pro-oxidants.

Bovine heart mitochondrial complex I, NADH oxidase, succinate dehydrogenase, succinate oxidase, and F_0F_1 -ATPase are all exquisitely susceptible to inactivation by ·OH, while complex I, NADH oxidase, and ATPase are also substantially inactivated by $O_2\cdot^-$ despite its being a less reactive radical than ·OH. The adenine nucleotide translocase of the inner membrane is susceptible to oxidative fragmentation and degradation under oxidative conditions that cause only mild peroxidation of inner membrane lipids.

Exposure of isolated liver mitochondria to $O_2\cdot^-$ or ·OH significantly impairs respiration fueled by glutamate/malate yet does not impede succinate-fueled respiration. State 3 respiration (induced by adding ADP to stimulate respiration) in these liver mitochondria is inhibited by $O_2\cdot^-$ or ·OH, but state 4 is relatively unaltered, with the result that respiratory control ratios significantly decline. Cytochrome c oxidase activity is highly resistant to inactivation following oxidative exposures sufficient to inactivate complex I, succinate dehydrogenase, and others.

Mitochondrial membrane lipid peroxidation, loss of structural integrity, and functional impairment are detected in rat hepatocytes exposed to $O_2\cdot^-$ and ·OH (xanthine plus xanthine oxidase without removal or pentaorbital chelation of transition metals), whereas transient ischemia-reoxygenation induces differential hydrolysis of mitochondrial membrane phospholipids

in rat brain mitochondria. Cyanide treatment in vivo induces lipid hydroperoxides (conjugated dienes) in brain but not in liver or heart, indicating not only that peroxidation is a real consequence of ATP depletion, but also that there are real tissue differences in susceptibility to oxidative stress. Removal of exogenous Ca2+, or pretreatment of cortical slices with the Ca2+ channel blocker diltiazem, significantly diminishes CN-induced lipid peroxidation, suggesting that perturbations in Ca2+ regulation contribute to the observed oxidative toxicity. In addition to issues of permeability (especially important for maintaining mitochondrial $\Delta\Psi$), peroxidation and consequent alterations in membrane fluidity could well alter diffusion coefficients of the electron transfer components and thus potentially disrupt coordinated electron flow that is partially dependent on fixed rates of lateral diffusion within the membrane.

Oxidative susceptibility of complex I and repression of state 3 respiration have also been reported for isolated rat brain mitochondria exposed to the toxin 1-methyl-4-phenylpyridinium (MPP+) or H_20_2 plus Cu2+ (tantamount to $\cdot OH$). Such MPP+-induced complex I inhibition is blocked by antioxidants such as glutathione, catalase, and ascorbate, further implicating an oxidative mechanism. In vivo, impairment of oxidative phosphorylation by MPP+ induces ATP depletion and compensatory lactate accumulation, and generates lesions in the rat striatum which are prevented by MK-801 blockade of NMDA-receptors, indicating that Ca2+ perturbations and oxidative mitochondrial dysfunction contribute to neuronal death under these circumstances.

Hydroxyl radical exposure not only restricts electron entry via complex I but, when succinate is provided as substrate, it significantly increases respiration of neuronal mitochondria by 27% ($P < 0.04$, paired *t*-test) and increases radical production almost ninefold. Similar results have

also been reported for isolated renal mitochondria where exposure to ·OH plus 30 μM Ca2+ significantly impairs complex I activity and uncouples oxidative phosphorylation, while having little effect on electron entry via complex II.

In a rat strain with congenitally elevated mitochondrial radical production, state 3 respiration, uncoupled respiration, respiratory control ratios, and mitochondrial membrane potential are all lower. It is apparent not only that respiration proceeds at a high rate following combined Ca2+ and radical exposure when complex II substrates are provided, but also that such treatment increases subsequent free radical generation from mitochondrial isolated from neurons as well as other cell types.

Complex I dysfunction comparable to that induced by ·OH exposure is observed in mitochondria from substantia nigra and systemic tissues of Parkinson's disease patients, from platelets of Huntington's disease patients, and from neocortex of Alzheimer's patients. Complexes II and IV are reportedly reduced in basal ganglia of Huntington's patients. Complex IV is reportedly reduced in platelets and in postmortem cortex from Alzheimer's patients compared to age-matched controls. Mitochondria isolated from skin fibroblasts of Alzheimer's patients sequester less Ca2+ than mitochondria from age-matched normal controls. Cytochrome c oxidase activities are reportedly reduced in postmortem Alzheimer's brain tissue, whereas oxygen utilization by mitochondria isolated from cerebral biopsies from Alzheimer's patients is elevated under conditions of low ADP availability compared to mitochondria from normal cortex.

Some data suggest that electron transfer is partially uncoupled in Alzheimer's disease, but such polarographic assays do not resolve how O_2 is being reduced. Increased reliance on

substrate-level phosphorylation from both glycolysis and the Kreb's cycle in response to impaired oxidative phosphorylation offers a plausible explanation for the increased $^{14}CO_2$ production reported for neocortex biopsies from Alzheimer's patients and for elevated brain lactate levels detected using MRI in Huntington's disease patients.

Consistent observations of mitochondrial defects in systemic tissues of patients with neurodegenerative diseases raises the possibility that such diseases are systemic with the CNS as the primary site of frank pathology. The brain, rich in unsaturated lipids susceptible to peroxidation, is responsible for 20% of total O_2 consumption, yet represents only 2% of the total biomass.

Such elevated aerobic respiration exerts an increased oxidative load, yet brain antioxidants such as superoxide dismutase and catalase are lower than in other, less aerobically active tissues. Most of the brain glutathione and SOD are located not in neurons but rather in glia which are resistant to oxidative insult.

Neuronal mitochondria are more like myocardial mitochondria in that both show rapid fluctuations in [Ca2+] in response to changes in cytosolic Ca2+, primarily because of an increased proportion of the faster Na+-dependent Ca2+ efflux mechanism. Liver mitochondria, on the other hand, respond more slowly to changes in cytosolic Ca2+. Such differences in response to prolonged Ca2+ exposure may account for differences between brain and liver mitochondrial radical production.

The oxidative pathophysiology of the brain is different from other tissues. In addition to the study showing tissue differences in cyanide toxicity, in rats fed 36% of their daily caloric intake as

ethanol, state 3 respiration has been shown to decline and $O_2^{\cdot -}$ efflux to increase with succinate as substrate in mitochondria isolated from brain but not heart or liver. Monoamine oxidase activity and monoamine autoxidation are often construed as indigenous sources of oxidative stress that put the brain at higher oxidative risk. Such reactivity is a function of neuronal redox state; under some conditions, norepinephrine, dopamine, homovanillate, serotonin, and t-hydroxyindole acetic acid all function as antioxidants and protect rat brain mitochondria from lipid peroxidation induced by Fe2+ exposure. Evidence indicates that oxidative load from mitochondria increases with age, and the brain is the first organ to show oxidative degradation in aging.

Neurons may well be at higher risk of oxidative injury because of a paucity of antioxidant defenses and because their mitochondria are more prone to generate free radicals when Ca2+ and ADP are elevated

Increases in mitochondrial radical generation with age correspondingly increase oxidative damage to nuclear and mitochondrial DNA; mutation rates in the mitochondrial genome are about tenfold higher than in nuclear DNA, because of close proximity to the inner membrane site of radical production, lack of histones, poor DNA repair mechanisms in the mitochondria, oxidative susceptibility of the mitochondrial transcription machine, and the absence of nonencoding regions so that any oxidatively induced mutations are unavoidably transcribed. Mitochondria contain multiple DNA copies, accumulation of oxidatively induced mutations increases with age until a point when declining potential for ATP production, combined with increased oxidative load, puts the neuron at risk.

Congenital defects in electron transfer components become increasingly prominent as the

cell ages. These genetic defects are not present in all copies of DNA in all mitochondria but rather are dispersed among sub-populations of the maternally inherited mitochondria. After segregation into tissues during embryogenesis, the resulting heteroplasmy would remain stable in the absence of selective pressures within the cell. In most tissues (except striated muscle), the number of mitochondria per cell is relatively stable. Mitochondrial populations are not static, and highly coordinated communication between the nucleus and mitochondria results in mitochondrial reproduction via fission, with an organelle's half-life of approximately five days in normal hepatocytes.

Although the exact cue(s) that initiates mitochondrial reproduction remains unknown, it is likely to be a marker of mitochondrial senescence, probably related to reduced potential for oxidative phosphorylation or increased oxidative damage. Compared to normal mitochondria, those expressing genetic mutations are more likely to show decreased potential for oxidative phosphorylation and/or increased radical production and are therefore more likely to reproduce. If mitochondria expressing mutations are replaced via reproductive fission more often than normal organelles over time, an initial heteroplasmic mix of healthy and defective organelles will inexorably drift toward homoplasmy of defective mitochondria until a critical threshold is achieved where the cell is energetically or oxidatively imperiled. The rapidity with which this threshold will be obtained is dictated by the nature of the mutations. This gradual dominance of the mitochondrial pool by energetically and/or oxidatively dysfunctional organelles offers an explanation not only for the late onset of these diseases, but also for the pronounced susceptibility of long-lived, postmitotic cells, such as terminally differentiated neurons.

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BASIC SCIENCE ASPECTS OF THE MITOCHONDRIA
SECTION IV

MITOCHONDRIAL FUNCTION IN ADDITION TO OXIDATIVE DE-PHOSPHORYLATION

Under physiological conditions, approximately 98% of molecular oxygen is consumed by the mitochondria at the cytochrome c oxidase complex; the terminal ATP oxidase of the mitochondrial electron transport chain. At this complex, molecular oxygen is reduced by four electrons to two molecules of water. The free energy obtained during electron transport towards the final electron acceptor, molecular oxygen, is used to promote phosphorylation of ADP to ATP at complex V of the mitochondrial inner membrane. The 1 — 2% of O_2 which is not consumed at the mitochondrial cytochrome c oxidase is mono- or divalently reduced to $O_2\cdot^-$ and H_2O_2 at mitochondrial and extra-mitochondrial sites.

There is a continuous slow cycling of Ca2+ and Na+ across the mitochondrial inner membrane and alterations of intramitochondrial Ca2+ are associated with metabolic regulation. In cardiac myocytes, fluctuating levels of mitochondrial free Ca2+ between 0.2 and 2 μM are responsible for increases in oxidative metabolism in response to increased activity. This is primarily due to the allosteric regulation of several dehydrogenases and glycerol-3-phosphate dehydrogenase, a component of the glycerophosphate shuttle. The phosphorylase that activates pyruvate dehydrogenase (PDH) is activated by Ca2+ concentrations of ca. $3 \times 10^{—6}$ to 10^{-7} M, within the range typically detected after physiologically relevant extracellular Ca2+ pulses. In response to increased activity in electrically paced cardiac myocytes, the proportion of PDH in the active form increases threefold, from less than 20% to almost 70% when intramitochondrial Ca2+

increases from 2.7 to 4.1 nmol/mg protein. Similarly, both isocitrate dehydrogenase (IDH) and α-ketoglutarate dehydrogenase show increased substrate affinities when Ca2+ is increased, although the amount of Ca2+ required for the K_m shift of IDH (>30 μM) would appear to be too high for physiological regulation under normal conditions. Nevertheless, moderate increases in mitochondrial Ca2+ are also reported to increase activity of F_0F_1-ATPase, adenine nucleotide translocase, and electron transfer *per se*.

Cytosolic Ca2+ concentration is typically 50—100 nM, depending on cell type. When Ca2+ levels reach 200—300 nM, mitochondria begin to accumulate Ca2+ as a function of the equilibrium between influx via a Ca2+ uniporter versus efflux via both Na+-dependent and Na+-independent carriers. Ca2+ influx via the uniporter is fast, passive, and second order for Ca2+, indicating initial potentiation of influx by Ca2+. A number of other cationic metals and some antibiotics can substitute for Ca2+ and activate the uniporter. Importantly, Ca2+ influx is completely dependent on the negative transmembrane electrochemical potential ($\Delta\Psi$) established by electron transfer, and influx fails to occur in the absence of $\Delta\Psi$ even when an eightfold Ca2+ concentration gradient is imposed.

Although the K_m of the uniporter for Ca2+, ca. 1—100 μm, is substantially higher than that for typical cytosolic Ca2+, evidence from studies using several tissues and different assay techniques indicates that despite such a low affinity, intramitochondrial Ca2+ does fluctuate in parallel with changes in cytosolic Ca2+. Indeed, it has been argued that such a low affinity for so rapid a uniport mechanism indicates that its primary function is to lower cytosolic Ca2+ when it is pathologically elevated due to ATP depletion and/or abnormal influx across the plasma membrane.

Given the huge potential for rapid Ca2+ influx shown by the uniporter, regulation of intramitochondrial [Ca2+] falls to the two efflux mechanisms, both of which are active but substantially slower than the uniporter. The V_{max} of the Ca2+ uniporter is three orders of magnitude higher than the Na+-independent Ca2+ efflux mechanism (uniporter V_{max} = ~1750 nmol x mg protein^{-1} x(min^{-1} vs. 1 nmol x mg protein^{-1} x min^{-1} for Na+-independent efflux in liver) and some 100-fold faster than the Na+-dependent mechanism (V_{max} = ~18 nmol x mg protein^{-1} x min^{-1} in heart, but only ~3 nmol x mg protein^{-1} x min^{-1} in liver). Most cell types contain both the Na+-independent and Na+-dependent carriers, but the proportion of the faster Na+-dependent protein increases in cells that normally experience rapid changes in cytosolic Ca2+ such as neurons and cardiac myocytes. Although initially considered passive ion exchangers, both efflux mechanisms pump Ca2+ out of mitochondria against an electrochemical gradient using energy derived from opposing favorable gradients for Na+, in the case of the Na+-dependent mechanism, and H+ for the Na+-independent mechanism.

Unlike many transmembrane ion pumps, of which Na+/K+-ATPase is the archetype, mitochondrial Ca2+ efflux mechanisms do not entail direct hydrolysis of ATP, relying rather on Na+ and H+ gradients across the inner membrane for energy. Although ATP hydrolysis may not be required to pump Ca2+ out of the mitochondrion, efflux is still considered active in that use of the proton motive gradient to fuel Ca2+ efflux represents a loss of phosphorylation potential because the exchanged H+ bypasses F_0F_1 -ATPase. Likewise, activity of the inner membrane Na+ —H+ exchanger that maintains the favorable mitochondrial Na+ gradient needed for Ca2+ efflux is not associated with ATP hydrolysis, yet by using the H+ gradient for purposes other than

phosphorylation of ADP via F_0F_1 -ATPase, it represents an energy sink and is considered active.

To the extent that mitochondrial Ca2+ loading exceeds normal levels required for metabolic regulation, the net result of using Na+ and H+ (and other cation) gradients to regulate intramitochondrial Ca2+ is that energy derived from the electron transfer system is not reflected in ATP production.

Just as signaling by cell-surface receptors is a highly compartmentalized process involving arrays of signalling units, intracellular signaling pathways also depend on tightly regulated and highly ordered arrays of proteins. Calcium plays a prime role in signaling, and its specificity is related to the local Ca2+ concentration.

Mitochondria can sequester and release large amounts of Ca2+, which import and export of Ca2+ helps to adjust energy production to cellular needs, and these fluxes play a major role in normal Ca2+ signaling.

Studies on isolated mitochondria have demonstrated their extraordinary ability to take up Ca2+ added to the bathing solution which, due to electrophoretic entry through a Ca2+-selective channel — the mitochondrial Ca2+ uniporter — driven by the large inside-negative membrane potential of energized mitochondria. Uptake can be blocked by ruthenium red and stops if membrane potential is discharged by application of protonophores such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or carbonyl cyanine *p*-trifluoromethoxyphenylhydrazone (FCCP). Export of Ca2+ from isolated mitochondria is typically Na+-dependent and mediated by a Na+/Ca2+ exchanger, also Ca2+ can be liberated more catastrophically through opening of a high-conductance permeability transition pore.

Mitochondrial Ca2+ transport can have a significant impact on the time course of cytoplasmic Ca2+ transients in living neurons. Mitochondria Ca2+ uptake is fast compared with other cellular Ca2+-clearance mechanisms, so mitochondria are a significant buffer for cytoplasmic Ca2+. The subsequent gradual export of Ca2+ from mitochondria can prolong the final return to rest. This affects Ca2+-dependent phenomena such as secretion and synaptic transmission.

The outer mitochondrial membrane is readily permeable to small molecules, so the machinery for Ca2+ transport lies primarily at the inner membrane.

Uptake of Ca2+ by isolated mitochondria is through an ion channel. Estimated flux through the uniporter exceeds 10,000 ions per second, somewhat greater than that of the fastest known pumps and exchangers. The Ca2+ import depends directly upon membrane potential, and the flux is downhill. A steep dependence of Ca2+-uptake rates upon bathing [Ca2+] suggests that the channel opens only if cytoplasmic Ca2+ rises. Cytosolic components including ATP, Pi, and Mg2+ can prevent or modulate its opening. Export of Ca2+ is usually uphill, driven by entry of more than two Na+ ions per Ca2+, and is blockable by the inhibitor CGP-37157 or the less selective but more readily available clonazepam. In a 'cytosolic' medium containing ATP, brain mitochondria remove external Ca2+ to a Na+-dependent 'set-point' of ~300 nM that represents kinetic equilibrium between residual Ca2+ uptake through the uniporter and Ca2+ export by the Na+/Ca2+ exchanger.

Electrogenic entry of Ca2+ via the uniporter directly decreases membrane potential and briefly interrupts ATP synthesis. Sufficient elevation of mitochondrial free [Ca2+] activates dehydrogenases of the tricarboxylic acid cycle thereby increasing electron transport chain fluxes

and ATP synthesis. Mitochondrial energy production anticipates periods of energy demand.

The transition pore seems to open when the mitochondrial Ca2+ load exceeds some limit that is sensitive to pro-oxidants, inhibitors of the adenine nucleotide translocator, and other agents. The cyclophilin ligand cyclosporin A (CsA) or its more selective 4MeVal-analog prevents or delays the permeability transition. When open, the pore allows passage of ions and small molecules (1.5 kDa), suggesting a pore size of ~3 nm. Under some circumstances the pore may open transiently in a 'low-conductance mode' to allow more selective ionic fluxes. Possible molecular correlates of the transition pore are a multiconductance 'megachannel' detected in patches of mitochondrial inner membrane and a high relative molecular weight (>400 kDa) complex found in extracts of brain mitochondria.

Textbook descriptions of cellular Ca2+ metabolism focus on transporters and ion channels of the plasma membrane and the reticular Ca2+ stores. Mitochondria have recently came to the fore in studies on isolated neurons in which cytoplasmic Ca2+ was monitored optically as cells were depolarized by KCl, by electric currents or by neurotransmitters to induce Ca2+ entry. Typically, cytoplasmic Ca2+ rose to much higher levels during Ca2+ entry if mitochondrial Ca2+ uptake was inhibited in a variety of ways, but the final stages of recovery of cytoplasmic Ca2+ were often hastened. Application of carbonyl cyanide *m*-chlorophenylhydrazone or carbonyl cyanine *p*-trifluoromethoxyphenylhydrazone some seconds after Ca2+ loading released a bolus of Ca2+ into the cytoplasm from the mitochondria. Lowering the cytosolic [Na+] or pharmacological inhibition of the mitochondrial Na+/Ca2+ exchanger had little effect on early cytoplasmic Ca2+ clearance but hastened its late recovery. Similar results have been obtained with smooth muscle,

chromaffin, and anterior pituitary cells. This work implies that mitochondria can be a major sink for Ca2+ clearance when cytoplasmic Ca2+ rises into the low micromolar levels, and that Ca2+ is subsequently slowly extruded from mitochondria via the Na+/Ca2+ exchanger so that mitochondria speed the initial fall of cytoplasmic Ca2+ from high values (>500 nM) by Ca2+ uptake and then prolong the final phase of recovery by continuing to feed out accumulated Ca2+ into the cytoplasm.

These ideas have now been tested in several ways and are being accepted. An important initial control was to ensure that Ca2+ clearance was not being slowed by CCCP and FCCP simply because the cell had run out of ATP. Three kinds of evidence argue against that explanation. First, experiments and solution changes can be done rapidly, and the effects of the protonophores are evident within <5 s of their application. Second, early clearance of cytoplasmic Ca2+ is still delayed greatly by treatment with CCCP when oligomycin is present to minimize mitochondrial consumption of cytosolic ATP. Finally, the experiments can be done using whole-cell pipettes containing millimolar levels of ATP to provide a continuous supply of energy.

Mitochondria are significant for cellular Ca2+ handling since they can transport Ca2+ at rates comparable to or exceeding those of other Ca2+-transporting devices in the same cell. In chromaffin cells, mitochondrial Ca2+ uptake at cytoplasmic Ca2+ = 1 μM is four times faster than the sum of transports by the Na+/Ca2+ exchanger of the plasma membrane and by the Ca2+-ATPases of the plasma membrane and reticular Ca2+ stores. Thus, it is clearly dominant even during relatively modest physiological cytoplasmic Ca2+ elevations so that 80% of the initial Ca2+ load is cleared first into the mitochondria. Other types of cells may be different to some

extent.

A Ca2+ signal is conveyed from cytoplasm to mitochondria and exerts positive control on NADPH production in hepatocytes and cardiac muscle.

Ca_m in living cells has been monitored directly with optical indicators, which showed that after selective quenching of cytosolic indo-1 fluorescence with Mn2+, a residual compartmentalized signal reported a rise of Ca_m of single cardiomyocytes in response to electrical stimulation of the cell. Others have relied on imaging to focus on the signals produced from mitochondrial regions that were identified by other means. Ideally one might use some physiological property of mitochondria to target Ca2+-selective reporters directly to that compartment. Rizzuto et al. used a mitochondrial import sequence to develop a targeted version of the photoprotein aequorin that monitors Ca_m in fields of transfected cells. Their probe reported that Ca_m rises transiently upon mobilization of reticular Ca2+ stores and upon induced Ca2+ entry. Another approach takes advantage of the tendency for membrane-permanent cations to accumulate in the very negative mitochondrial matrix compartment. In particular, the cationic acetoxymethyl ester of the Ca2+ probe rhod-2 accumulates in mitochondria of living cells (like its parent chromophore rhodamine 123) and there generates a trapped Ca_m indicator. The rhod-2 signal reports that Ca_m rises in a variety of cell types when cytoplasmic Ca2+ is elevated by Ca2+ entry or by mobilization of reticular stores.

Results have been most complete with chromaffin cells. These cells can be loaded with rhod-2 in the mitochondria and Calcium Green in the cytoplasm and then subjected to voltage-clamp steps of membrane potential to inject controlled Ca2+ loads into the cytoplasm. A

0.5 s depolarization makes an immediate elevation in cytoplasmic Ca2+, which decays in about 3 s as much of the Ca2+ is cleared into mitochondria. Calcium also rises quickly in the mitochondria but takes about 60 s to be extruded. A subsequent exposure to CCCP causes loss of residual Ca2+ from mitochondria and a gain in the cytoplasm. With CCCP still present, a second cytoplasmic Ca2+ load is not well taken up into mitochondria and takes much longer than 3 s for its initial clearance from the cytoplasm via other routes. In other experiments, the export of accumulated mitochondrial Ca2+ can be slowed considerably by inhibitors of the mitochondrial Na+/Ca2+ exchanger CGP-37157 and clonazepam.

Ca2+ enters mitochondria and will limit the spread of micromolar elevations of cytoplasmic Ca2+ within the cell, maintaining the strong local character of Ca2+ signaling, and cutting off the spread of exocytosis and of Ca2+-dependent inactivation of various Ca2+ entry channels. Slow export from mitochondria extends the period during which cytoplasmic Ca2+ remains modestly elevated (to 150–500 nM) after a bout of Ca2+ signaling, prolonging the period of activation of Ca2+-dependent enzymes and of processes dependent on residual Ca2+ such as gene expression and post-tetanic potentiation of synaptic transmission. In addition, elevation of Cam raises the rate of energy production in the mitochondria. Mitochondria occupy special positions near sources or sinks of Ca2+, allowing preferential exchanges of Ca2+ between organelles. Indeed, mitochondrial Ca2+ uptake is important for Ca2+ oscillations and propagation of Ca2+ waves. Like reticular Ca2+ stores, mitochondria may generate signals that reflect their filling state. For example, they are sources of messengers, such as the pyridine nucleotide metabolites cyclic ADP-ribose (cADPR) or nicotinic acid adenine dinucleotide phosphate (NAADP), that affect other Ca2+ signaling

systems.

A hallmark of the permeability transition pore studied in isolated mitochondria is the complex modulation of its sensitivity to the Ca2+ content of these organelles. The possibility is now being considered that the threshold Ca2+ content required for opening of the pore is reached during normal cellular responses. Even more attention is being focused on possible opening of the pore or more selective alterations in mitochondrial ionic permeability as key events in ischemia/reperfusion injury, in neurodegenerative disease and in the commitment to apoptosis.

Apoptosis or programmed cell death is required for normal neural development and provides a line of defense against invasive or malignant challenge. It may also underlie some neurodegenerative diseases and other pathologies. Interest in apoptosis increased upon recognition that at least two of its mediators, the 'caspases' and Bcl-family proteins, are evolutionarily found in nematode to man. Mitochondrial involvement in the commitment to apoptosis was suggested by the preferential localization of Bcl-2 to these organelles, by the early changes in $\Delta\Psi_m$ that are elicited by many apoptotic stimuli, and by a release of cytochrome c from the mitochondrial intermembrane compartment, which may evoke activation of caspases.

In several systems apoptotic responses are altered by CsA or other agents that target the transition pore. These observations favor a model in which opening of the pore causes swelling of the mitochondrial matrix, rupture of the outer membrane and nonselective release of cytochrome c. Overexpression of Bcl-2 or Bcl-XL protects against various apoptotic stimuli and mitochondria from such cells have greatly increased Ca2+ uptake capacity, suggesting that Bcl-2 inhibits opening of the permeability transition pore. It remains possible that cytochrome c is released selectively

through a macromolecular assembly of Bcl-family proteins; Bcl-XL resembles the bacterial pore-forming proteins diphtheria toxin and colicins in structure. Recombinant anti-apoptotic Bcl-2 and pro-apoptotic Bax both form channels in lipid bilayers or vesicles, and apoptotic stimuli evoke translocation of Bax to mitochondria. However, other recent evidence indicates that Bcl-family proteins evoke mitochondrial swelling and rupture by controlling ion-selective permeability. Regardless of the outcome of such debates, continued study of the involvement of mitochondria in apoptosis will almost certainly increase knowledge of their roles in normal cellular Ca2+ signaling and gene expression.

Patterned neural activity modifies central synapses during development and the physiological properties of skeletal muscle by selectively repressing or stimulating transcription of distinct genes. The effects of neural activity are mostly mediated by calcium. Of particular interest are the cellular mechanisms that may be used to sense and convert changes in calcium into specific alterations in gene expression. Recent studies have addressed the importance of spatial heterogeneity or of temporal changes in calcium levels for the regulation of gene expression.

The functional properties of neurons and skeletal muscle are plastic during development and in the adult, and they are modulated by afferent innervation. The ability of the nervous system to be remodeled with experience, and of skeletal muscles to adapt to different environmental demands, results, at least in part, from the regulation of gene expression in response to patterned electrical activity. For this reason, it is important to understand how distinct patterns of stimuli are coupled to specific changes in gene expression. Electrical activity regulates different transcription factors, which, in turn, regulate the expression of neurotransmitter receptors, ion channels,

neurotrophic factors, cell adhesion molecules, cytoskeletal proteins, contractile proteins, and metabolic enzymes.

Immediate early genes (IEGs) have been the focus of numerous studies in the nervous system regarding the coupling of electrical activity to transcription, but little is known of how the regulation of these factors results in changes in the expression of the structural proteins that modify neural and muscle function. In order to understand how activity contributes to the formation and phenotypic differentiation of the nervous system, it is important to know how patterns of depolarization are sensed, decoded, and transduced into changes in the levels of transcription factors and other regulatory proteins that control the expression of genes encoding structural proteins. It is generally accepted that calcium is a major signaling molecule that transduces the activity into changes in cellular properties. But how the different frequencies of depolarization and the calcium currents elicited are then decoded and then translated into distinct signals is not completely understood. Different routes of calcium entry into the cell may be distinct signaling pathways that respond differentially to patterned activity. Perhaps the temporal and quantitative accumulation of calcium in different subcellular compartments activates distinct transduction pathways. Transcriptional specificity may be achieved in response to depolarization patterns.

Adult skeletal muscle is plastic. In response to changing extrinsic demands, muscle has the capacity to adapt by modifying its contractile and metabolic properties in response to different patterns of motoneuron activity. Skeletal muscle historically has served as an excellent model to study activity-dependent plasticity and the modulation of gene expression in response to neural activity, because peripheral nerves are accessible for experimental manipulation and the resulting

changes in muscle physiology can be easily quantified (e.g. force generation, twitch time). The importance of motoneuron innervation was first described by Eccles and colleagues, by demonstrating that fast-twitch muscles adopt slow-twitch properties when they are re-innervated ectopically by a nerve that normally innervates a slow muscle, and vice-versa. Activity, and not myotrophic factors released by the nerve, were shown to be instructive to muscle because electrical stimulation of motor nerves or denervated muscles with patterns of stimuli that mimic natural motoneuron activity are sufficient to modify its contractile properties to the same extent as cross-innervation.

The transition of muscle properties evoked by patterned stimulation results from changes in the expression of numerous genes encoding specific fast and slow protein isoforms that determine the contractile and metabolic properties of muscles. For example, depolarization of fast-twitch muscles with tonic, slow frequency (10 Hz) stimulation induces a sequential change in the synthesis of myosin heavy chain isoforms, as well as changes in the calcium handling mechanisms of the cell. The latter include changes in the sarcoplasmic reticulum calcium-ATPase, dihydropyradine, ryanodine-sensitive calcium channels, the calcium-sequestering proteins phospholamban and parvalbumin.

The restricted expression of contractile proteins in specific muscle types and their regulation in response to neural activity is mostly controlled at the level of transcription. Despite the fact that a large repertoire of muscle-specific genes regulated by patterned activity has been identified, little is known of the signaling pathways and transcription factors that couple the temporal changes in stimuli to specific changes in transcription. Direct proof of a role for calcium has not been formally

established, but is emerging from research. These type of studies have been hindered because the full extent of muscle-type diversification does not occur in vitro and thus requires analysis in vivo. One approach for mapping the pathways that lead from activity to fiber-type-specific transcription has been to use transgenic mice and somatic gene transfer (by the intra-muscular injection of DNA constructs into adult muscles) to map DNA regulatory sequences conferring fiber-type specificity. The fiber-type-specific expression of the troponin I slow and fast isogenes, which requires motoneuron innervation and is differentially regulated by slow (10Hz/10s) and fast (100Hz/1s) bursts of activity, is conferred by enhancers of 128 and 148 base pairs, respectively. Interestingly, both enhancers have conserved DNA motifs that bind to the transcription factors MyoD, MEF-2 and Sp1/CACC and that are required for transcription; these regulatory elements are also found in other muscle-specific genes. However, mutational analysis and the generation of chimeric enhancers — where the conserved motifs from the slow and fast enhancers have been swapped and tested in transgenic mice — demonstrate that although these sites are required for the enhancers to be active, they fail to restrict transcription to either slow or fast muscle fibers. Novel sequences that reside adjacent to the conserved motifs are necessary to direct transcriptional specificity of the troponin I genes. Sequences required for the activity-dependent transcription of two slow myosin light chain genes were mapped by somatic gene transfer. Both light-chain promoters share three of the elements found in the troponin I genes, but a sequence responsible for conferring the ability to respond to specific patterns of activity has not yet been found. The identification of these sites will be invaluable for determining how elements in the transcription regulatory complex interact to respond to patterned activity in a tissue-specific fashion.

Neural impulse activity can regulate a number of functional processes in the central and peripheral nervous systems, including neuronal phenotype, neurite outgrowth, axon fasciculation, synaptogenesis and remodeling, and activity-dependent changes in synaptic strength. The genes involved in these forms of activity-dependent plasticity are largely unknown, but there are numerous examples of neuronal genes that can be regulated by impulse activity, including immediate-early genes and genes that encode proteases, neurotrophins and neurotrophic factors, neurotransmitter receptors, cell adhesion molecules, cell surface molecules, and novel membrane and cytoskeletal molecules. As in muscle, the frequency or pattern of electrical impulses in neurons can be an important factor in activity-dependent gene regulation. High-frequency stimulation induces transcription of c-fos, c-jun and junB in hippocampal neurons, but stimulus patterns inducing long-term potentiation (LTP) selectively trigger induction of zif268. Frequency-specific regulation of neuronal genes has also been described for some structural genes. Expression of the cell adhesion molecule L1 is downregulated by 0.1 Hz stimulation in dorsal root ganglion (DRG) neurons, but 1 Hz stimulation is without effect. In contrast, N-cadherin mRNA is downregulated by both 0.1 Hz and 1 Hz stimulation in DRG neurons, whereas NCAM-180 expression is not altered measurably. Together, these results suggest that the pattern of neural impulse activity a neuron experiences can have significant functional effects that are dependent on the activation of the appropriate genes. Three examples illustrate this at the behavioral, cellular, and synaptic levels of organization, respectively. First, the conversion of short-term memory into long-term memory, which requires CREB-dependent gene expression, only takes place when training sessions are repeated at appropriate intervals. The same number of trials presented in one session are ineffective

both in altering gene expression and in allowing memory. A second example, myelination of axons, involves changes in expression of a large number of genes controlling this highly regulated interaction between neurons and glia. Recent work shows that induction of myelination by Schwann cells is influenced by the specific temporal pattern of impulse activity in the axon, through changes in axonal expression of the cell adhesion molecule L1. A third example of how activity can contribute to the synaptic level of organization comes from CA1 neurons of the hippocampus: brief synaptic activation at high frequency (100 Hz), or short high-frequency bursts repeated at 5 Hz intervals, induce LTP. In contrast, prolonged low-frequency stimulation (15 min at 1 Hz) depress the strength of the same synapses, a phenomenon referred to as long-term depression (LTD). Maintenance of LTP requires gene transcription that is associated with MAPK and CREB phosphorylation.

How specificity between stimulus and response is maintained within a broadly interactive network of intracellular signaling reactions and transcriptional regulatory processes is a general problem in cell biology. In excitable cells, however, the problem is compounded because membrane depolarization stimulates signaling pathways primarily through changes in intracellular free calcium levels, rather than through discrete receptors. How can different patterns of calcium flux activate different signaling pathways to the nucleus? The signaling mechanisms that confer stimulus–response specificity in cells could be divided into three general categories: discrete pathways, spatial heterogeneity, and temporal specificity.

Activation of specific receptors by appropriate ligands can stimulate discrete signaling pathways to regulate expression of target genes, but it is conceivable that the multiple points of

convergence and divergence among signaling pathways, and the interactions among DNA-binding proteins, would undermine this specificity. For example, the transcription factor CREB mediates responses to both cAMP and calcium in some cells. Down-stream from calcium, signaling cascades can propagate from multiple calcium-sensitive kinases, including calmodulin (CaM) kinase II, CaM kinase IV and MAPK, all of which are capable of phosphorylating CREB. Within the nucleus, the transcriptional apparatus also shows a high degree of interaction that would appear to further entangle signaling pathways from distinct stimuli to specific response genes. For example, the c-fos promoter contains two different regulatory sequences, the serum response element (SRE) and the cAMP response element (CRE), that were initially associated with trophic factor and cAMP/calcium responses, respectively. However, potential cross-talk between these two pathways and the combinatorial interactions among the DNA-binding proteins and transcriptional apparatus complicate the separation of signaling pathways from different stimuli.

Such interactions between signaling pathways activated by either growth factors or membrane depolarization could degrade response specificity. However, such interactions could also increase stimulus–response specificity by integrating multimodal stimuli within cells. A recent example is the regulation of the NR2C subtype of NMDA receptors, which requires both the activation of ErbB tyrosine kinase receptors by neuregulin and the activation of NMDA receptors by glutamate. Both of these stimuli can be provided by the mossy fiber inputs that innervate granule cells during development and that upregulate NR2C expression. The requirement for conjoint activation of Trk tyrosine kinase and NMDA receptors has also been observed in the regulation of dendritic growth, but the genes mediating this response have not been identified. The

fact that transcriptional enhancers function in a combinatorial fashion, and that the cooperative interaction of distinct DNA regulatory elements are often required to achieve transcriptional activation, is consistent with the idea that the temporal summation of calcium or synergy by co-activation of distinct pathways during synaptic transmission could be important for stimulus-dependent transcription.

Different frequencies or patterns of impulse activity can be encoded in the concentration of calcium produced in the cell. Higher levels of calcium could activate different intracellular signaling pathways than lower levels, and thereby activate different cellular substrates or genes. The balance between calcium-regulated kinase and phosphatase activity also could be shifted by the level of calcium. Transgenic mice overexpressing a constitutively active form of calcineurin, a calcium-dependent phosphatase, in the hippocampus support the idea of a temporal, activity-dependent balance between phosphorylation and dephosphorylation that regulates the transition from early-phase LTP to late-phase LTP. These calcineurin-overexpressing mice manifest deficits in spatial and visual recognition tasks requiring long-term memory when the number of training trials is low but perform as well as wild-type mice when the number of trials is increased, suggesting that the amount of activity is important for the transition from early- to late-phase LTP.

The calcineurin-dependent pathway, acting through the transcription factor NF-AT (nuclear factor of activated T cells), has also been proposed to regulate the transcription of contractile genes expressed specifically in slow-twitch skeletal muscle in response to patterned activity. However, the NF-AT-binding site located in the enhancer of the slow troponin I gene — proposed in these studies to be the site conferring transcription specifically in slow muscles — has recently been

shown not to be necessary for the expression of the troponin I enhancer in the slow-twitch muscles of transgenic mice. Further experiments will be needed to determine how the calcineurin/NF-AT pathway may regulate the fiber-type-specific expression of slow contractile genes, or their levels, in response to patterned activity.

Spatial heterogeneity can provide specificity between a stimulus and response when either cell surface receptors, signaling enzymes, or transcription factors are localized in distinct subcellular compartments. Indeed, the subcellular localization of transcription factors, such as NF-B and NF-AT, is regulated by calcium. Whether calcium influx controls transcription of c-fos through SRE or CRE can depend on whether calcium enters through NMDA channels or L-type calcium channels, because the signaling pathways associated with each mode of calcium entry are distinct and are distributed in different parts of the neuron. Recently, two groups have reported on the differential regulation of immediate early gene expression and CREB phosphorylation with respect to the spatially distinct subcellular localization of calcium in the cytoplasm versus nucleus, and the nucleus versus the submembranous compartment. Microinjection of an immobilized calcium chelator into the nucleus of AtT20 pituitary cells was used to demonstrate that distinct cis-acting elements in the c-fos promoter are differentially responsive to increases in cytoplasmic versus nuclear calcium after activation of L-type voltage-gated calcium channels. Whereas increases in cytoplasmic calcium signal via the c-fos SRE, nuclear calcium signals through the CRE. Interestingly, phosphorylation of CREB at Ser133 is necessary but not sufficient to mediate transcription via the CRE. Nuclear calcium and CaM kinase IV are required for the recruitment and activation of the CREB-binding protein (CBP), and the activation of CRE-mediated transcription.

There is little evidence, however, that the nucleus represents a diffusion barrier to calcium or that differences in action potential firing patterns result in disproportionate increases in cytoplasmic versus nuclear calcium in neurons.

By contrast, the extent of CREB phosphorylation at Ser133 in dissociated hippocampal neurons differs with stimulus frequency and does not require nuclear calcium. Deisseroth et al. proposed the existence of a submembranous calcium sensor after observing that the amount of activity-dependent CREB phosphorylation varies when neurons are preloaded with either EGTA or BAPTA, which are predicted to differentially remove calcium based on their different rates of chelation. Calmodulin was proposed as a candidate for the calcium sensor located near the site of calcium entry. Activation of NMDA receptors and L-type voltage-sensitive channels leads to the rapid translocation of calmodulin to the nucleus and CREB phosphorylation at Ser133.

Together, these results indicate that signaling from the plasma membrane to the nucleus can be highly dependent on the subcellular compartmentalization of calcium and proteins in the signaling cascade. The recent demonstration of direct interactions between channels and receptors with other signaling molecules sequestered by PDZ-domain proteins emphasizes the importance of specialized microdomains that could sense localized changes of calcium and connect these to intracellular signal transduction pathways. Indeed, a close association between the Drosophila photoreceptor TRP (transient receptor potential) calcium channel and a signaling complex that includes calmodulin, rhodopsin and phospholipase C is mediated via the PDZ-domain-containing protein INAD. On the basis of these findings, the possibility exists that PDZ-containing proteins at hippocampal synapses could closely associate L-type voltage-gated channels to calmodulin-or

calmodulin-binding proteins to couple local calcium transients to pathways that signal to the nucleus.

The spatial distribution of cytoplasmic calcium in dendrites could be important for signal processing and gene expression in response to sensory stimulation. Changes in free calcium can be localized to subregions of the dendritic shaft or confined to individual dendritic spines and active sodium or calcium conductances can lead to global increases in dendritic calcium. The influence of back-propagating action potentials on dendritic calcium transients varies with dendritic branching, pattern of neuronal activity, and physiological conditions. Measurements using two-photon microscopy under normal physiological conditions have shown that the amplitude of dendritic calcium transients is proportional to the number of sodium action potentials induced by vibrissae stimulation, and that the concentration of calcium declines steeply with increasing distance from the soma. Thus, some patterns of synaptic activity or action potentials can be converted into spatial differences in calcium, and could activate different signaling pathways in distinct subcellular compartments.

Another mechanism for signaling specificity would be provided if temporal patterns of calcium entry could be 'decoded' to selectively regulate gene expression. Measurements of intracellular calcium dynamics in mouse DRG neurons in response to action potentials have shown that some growth cone responses are correlated with the rate of calcium increase rather than the peak concentration of calcium. In addition, in DRG neurons, c-fos mRNA levels are correlated with the interval of time between calcium influx induced by bursts of action potentials, not by the concentration of calcium. The latter experiments revealed three unexpected results: first,

transcription of the c-fos gene did not require a sustained increase in cytoplasmic calcium; second, large increases in intracellular calcium are less effective in stimulating c-fos expression than small increases presented at shorter interburst intervals; and finally, c-fos expression is increased by stimuli that produced minimal, nearly undetectable changes in cytoplasmic calcium, provided the stimulus was repeated at appropriate temporal intervals. Similar results have been obtained from studying regulation of potassium channel maturation and neurite outgrowth in frog spinal cord neurons. These functions correlate with the frequency of calcium spikes and waves (2–15/h), not with the amplitude of the increase in cytoplasmic calcium.

Gene regulation in response to action potentials of very low frequencies have also been documented, where only minimal transient changes in cytoplasmic calcium would be generated. One action potential every 10 s is sufficient to stimulate c-fos expression in DRG neurons. The same low-frequency stimulation lowers expression of the cell adhesion molecule L1, but higher-frequency stimulation or chronic depolarization with potassium-chloride are without effect. These results illustrate the importance of the temporal dynamics of calcium entry, rather than calcium concentration, in regulating gene expression in response to action potentials in neurons.

One hypothesis for how action potential patterns regulate different genes is that intracellular signaling reactions will propagate temporally varying stimuli differently depending upon the kinetic responses of the pathway. For example, the kinetics of MAPK and CaM kinase activation and inactivation differ markedly, and should therefore respond preferentially to stimuli with temporal dynamics that favorably match the dynamics of each pathway. It has been shown that different intracellular pathways regulating transcription of the c-fos gene can be activated selectively by

patterns of action potentials that are favorably matched to the temporal dynamics of the signaling pathway. In these neurons, phosphorylation of CREB is rapid and sustained for several minutes, but phosphorylation of MAPK is more transient. Pulsed stimuli that were repeated at intervals that were too long (e.g. 3–5 min) failed to allow levels of phosphorylated MAPK to accumulate, but did activate CREB at maximal levels. Consistent with the combinatorial requirement for multiple DNA-binding proteins and the basal transcriptional complex, maximal c-fos expression was obtained in response to pulsed patterns of action potentials that coordinately activated both the MAPK and CREB signaling pathways. In response to action potential stimulation that produces either a large or a prolonged increase in intracellular calcium, the serine/threonine CaM kinase II and the phosphatase calcineurin undergo changes in reaction kinetics. This behavior may permit them to act as spike-frequency or stimulus-duration 'switches or detectors' that could activate specific signaling pathways regulating gene transcription in response to appropriate temporal features of impulse activity in neurons and muscles. Autophosphorylation of CaM kinase II changes the calcium sensitivity and reaction kinetics (i.e. rate of calmodulin dissociation from CaM kinase II) to transmit signals from high-frequency stimuli (i.e. those producing higher levels of intracellular calcium) through the CaM kinase II pathway. The importance of CaM kinase II autophosphorylation activity was recently shown in vivo using mice harboring a mutation in threonine-286 of CaM kinase II, which blocks the autocatalytic activity of the enzyme. Mutant mice show impaired NMDA-dependent LTP and spatial learning deficits on the Morris water maze.

Experiments performed with purified CaM kinase II immobilized to a rapid perfusion chamber have shown that different frequencies of calcium oscillations result in different levels of

autonomous kinase activity. At low frequencies, the autonomy of the enzyme due to autophosphorylation is lower but increases sharply when stimuli are delivered at higher frequencies. The levels of kinase activity are also regulated by the subunit composition of the enzyme, which have different affinities for calmodulin. Such frequency-dependent regulation of CaM kinase II autophosphorylation needs to be demonstrated in vivo using natural stimuli because alternative models have been proposed.

The activity-dependent regulation of phosphatases can also modify the kinetics of signaling in response to calcium. A mechanism for detecting the duration of action potential bursts in dissociated hippocampal neurons has been proposed. Longer duration bursts (e.g. 180 s versus 18 s at 5Hz) resulted in inactivation of calcineurin and prolonged the period that phospho-CREB remained at elevated levels in the nucleus. This lead to elevated levels of c-fos and somatostatin gene expression, which are regulated via the CRE. Phosphatases (e.g. PP-1, PP-2B/calcineurin) also appear to play an important role in sustaining high levels of phospho-CREB in response to L-type calcium channel activity and in inducing c-Fos in developing striatal slice preparations.

Temporal segregation of intracellular signaling is not unique to neurons or muscle. Modulating intracellular calcium periodically by exposure to potassium-chloride or a calcium ionophore is more effective than sustained calcium influx in regulating prolactin gene expression in pituitary cells. Two recent papers have addressed how calcium oscillations optimize the efficiency and specificity of gene expression in non-neuronal cells; it is important to emphasize that the frequencies of intracellular calcium oscillations in these systems are considerably lower than the action potential frequencies observed in mature neurons and muscles. A calcium-clamp technique

was used to regulate the frequency and amplitude of intracellular calcium in populations of T lymphocytes, which were treated with thapsigargin to deplete internal calcium stores, to analyze how distinct oscillation patterns regulate the expression of transcription factors that module interleukin-2 and interleukin-8 transcription. NF-B was activated by low-frequency oscillations, whereas high frequencies were necessary to recruit NF-AT, Oct and NF-B. In turn, the promoters for the interleukin-8 and interleukin-2 genes demonstrated distinct preferences for the frequencies of intracellular calcium that correspond to their preferential regulation by NF-B (low frequency) and NF-AT and Oct (higher frequency), respectively. The regulation of NF-AT by oscillation frequency was also observed by RY Tsien and colleagues, who used a cell-permeant caged inositol 1, 4, 5-triphosphate (InsP3) activated by ultraviolet light to induce oscillations in intracellular calcium in a variety of cell lines by releasing calcium from internal stores. NF-AT-driven gene expression was found to be more effective if the changes of InsP3 and cytoplasmic calcium changed in waves, instead of being maintained at high steady-state levels. The time intervals between the peaks of cytoplasmic calcium levels were important for maximum NF-AT function. These studies emphasize the importance of the temporal changes of calcium, rather than changes in amount.

Regulation of gene expression by specific patterns of impulse activity is essential for nervous system and skeletal muscle function. As experiments on transcriptional regulation in excitable cells moves from identifying the components of the system to observing how the components operate as a system in a dynamic state, the field is entering into an exciting new phase. Many properties that are essential for stimulus–transcription coupling may not be apparent from

analysis of the system in a static state. Studies under dynamic conditions will bring us closer to understanding how the nervous system responds and adapts to changes in the environment and in its operation over time. A future challenge will be to identify the structural genes that are regulated by patterned activity that modifies neuronal plasticity, and to elucidate their mode of regulation. The integration of regulatory factors activated by activity patterns, and their interaction with transcription factors confined to specific cell types by lineage or development, provides further refinement for stimulus–transcription coupling.

These latter studies, while so far inconclusive, may offer an explanation for why fluctuating electromagnetic fields such as those used in the Demitri Sodi Pallares device are capable of bringing about tissue regeneration, as noted in section six below.

Mitochondria synthesize the Heme molecule, as well as cardiolipin, coenzyme Q and the lipids which move up the mitochondrial inner membrane. The urea cycle takes place partially in the mitochondrial matrix, and the Krebs cycle takes place entirely in the mitochondrial matrix. All of these functions are necessary for the mitochondria to function, produce energy, and handle oxygen efficiently.

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BASIC SCIENCE ASPECTS OF THE MITOCHONDRIA
SECTION V

MITOCHONDRIA, FREE RADICALS AND CHRONIC DEGENERATIVE DISEASE

During the 1940's and 1950's extensive research identified mitochondria as cellular centers of energy metabolism. By 1960 similar studies on mitochondria had classified their gross structure and the bioenergetics of the respiratory chains which produces most of the energy in cells.

By 1975 scientists had developed the information that mitochondria have their own DNA separate and distinct from that of the cells they inhabit and by 1981 the gross genetic structure of human mitochondrial DNA was mapped.

This was the beginning of scientific understanding of the mitochondria as an endosymbiant, and of the pivotal role of mitochondria in human health and diseases.

In 1959, Dr. Rolf Luft of Koralinska Hospital of Stockholm Sweden diagnosed the first mitochondrial disease, which is now known as Luft's Disease. The disease and its discovery set into motion a new medical discipline which Dr. Luft calls mitochondrial medicine. With the increasing scientific studies of the organelle and its genome, mitochondrial medicine has expanded dramatically in several directions. By 1988, some 120 mitochondrial diseases had been identified and several basic principles of mitochondrial pathophysiology have been established.

In 1963, two scientists NAS and NASS demonstrated that mitochondria have their own separate DNA genome. Unlike nuclear DNA, there are thousands of copies of mitochondrial DNA in every nucleated cell - several in each mitochondrion. These undergo mutations 5-10 times faster than the nuclear DNA.

By now quite a lot has been determined about mitochondrial DNA and its effect on energy

production diseases and aging.

It is now well established that mitochondrial DNA is maternally transmitted in the ovum and that the sperm contributes no mitochondria or mitochondrial DNA to the fertilized egg. Therefore, mitochondrial diseases are entirely inherited from the mother and never from the father. Mitochondrion and their DNA are passed along just as they were in the mother at the time the ovum was created. If that DNA contained mutations, they are passed along to the child.

Some mitochondrial DNA mutations cause serious disorders like Luft's Disease, others do not immediately cause problems and will not cause problems so long as the cell in which they are housed is able to produce adequate energy for its functions.

If during the life of the individual who has inherited mitochondrial DNA which is defective and has point deletions, the mutated DNA inherited from the mother will begin to manifest itself as a mitochondrial disease. In the individual who inherited defective mitochondrial DNA new mutations or aging gradually causes a loss of the capacity to produce sufficient energy.

Damage to mitochondrial DNA and the capacity of the mitochondria to continue to produce ATP for the cells energy needs can occur due to damage from free radicals produced in the mitochondria in the process of producing ATP for fuel.

While the body and particularly its mitochondria have a built-in free radical defense system, this can become inefficient for its purposes due to the presence of chemicals or nutritional deficiency. The free radicals produced normally in the oxidative system begin to damage the mitochondria, its membranes and structures to the extent that the mitochondria can no longer produce ATP efficiently. If this happens to enough of the mitochondria in a cell, the cell will not be

able to function and will degenerate.

The mitochondria require fuel, oxygen and component proteins produced by the nuclear DNA for their functions. If the supply of these is interrupted, the cell will undergo energy failure and loss of function which, if a sufficient number of such cells are affected, will produce the symptoms of one or more chronic degenerative diseases.

What this means simply stated is that all of the chronic degenerative diseases, i.e., arteriosclerosis - heart diseases, strokes, cancer, adult onset diabetes, arthritis, cataracts, Franconi's anemia, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, essential hypertension, amyloidosis, and Lannec's cirrhosis are truly mitochondrial diseases. The symptoms appear as we get older, when the individual cells and the mitochondria begin to lose that capacity to produce sufficient ATP to maintain their health and function.

This also means that these diseases are both preventable and treatable by treatments which restore the capacity of the mitochondria to mount an antioxidant defense and to efficiently process fuel to produce ATP.

This is perhaps a startling concept to physicians who were trained before 1992 and it should come as no surprise that most busy physicians do not follow each and every detail of the scientific literature on research about the structure, function and particular genetics of mitochondria. Until a few short years ago, mitochondria were not considered to be involved in disease processes. For this reason, we will set forth briefly the research which supports this rather startling conclusion that all chronic degenerative diseases are indeed diseases of mitochondrial structure and function. The equally startling concept arises from the fact that many of the treatments which have been used for

treating chronic diseases over the past several years are not only ineffective, but may actually be contributing to the disease they were designed to treat.

There are several excellent reviews of the scientific research which have been published during the past five years in journals which publish basic research.

Mitochondria are the most important intracellular source of reactive oxygen species, although alternative sources in other organelles and the cytoplasm are present in cells. Based upon a large body of experimental evidence, reactive oxygen species participate in aging and aging-associated pathology. In both the mitochondrial and the cytosol, enzymatic and nonenzymatic systems are available, which serve to decrease the reactive oxygen species steady-state concentration. Despite this, DNA, proteins, and lipids are subject to oxidative damage that increases with age and leads to the chronic degenerative diseases associated with aging.

Accumulation of various mutations in the mitochondrial genome is now established as an important contributor to both aging and chronic degenerative diseases. The frequency of nucleotide substitutions in the striatum of Parkinson's disease patients is significantly higher than that in control tissues, but at the same time increased protein modification by 4-hydroxy-2-nonenal is observed. These results have been taken to mean that a primary mitochondrial mutation induces a mitochondrial respiratory defect, which increases leakage of reactive oxygen species that trigger accumulation of secondary mtDNA mutations. Such a mechanism is supported by the data showing that mitochondrial complex I deficiency leads to increased production of superoxide radicals (O_2^-) and induction of superoxide dismutase (SOD). However, an increase in oxygen radical production when complex I activity is diminished would not actually occur, due to the

presence of sufficient SOD and of inducibility of this enzyme. The data in fact show that, due to mitochondrial superoxide dismutase (Mn-SOD) induction, the actual level of O_2^- production may decrease, sometimes below that seen in control fibroblast mitochondria.

A number of observations support the hypothesis that mtDNA damage does accumulate with age and indicate that respiratory stress greatly elevates mitochondrial damage. In the brain cortex, the deleted: total mtDNA ratio ranged from 0.00023 to 0.012 in 67 to 77-year-old brains and was up to 0.034 in subjects over 80 years of age. In the putamen, the deletion level ranged from 0.0016 to 0.010 in 67 to 77 year olds up to 0.12 in individuals over the age of 80 years. The cerebellum remained devoid of mtDNA deletions. It is puzzling that a general radical injury spares certain tissues, which remain totally devoid of mutations. It is also unclear how such a low level of mutation can be the cause of age-dependent devastating processes.

One more intriguing question is the apparent lack of inheritance of the actual mother's age (if age is a function of the number of mitochondrial mutations) by the offspring. As in somatic cells, oocytes from older women contain more deletions of mtDNA. It has also been found that oocytes harbor measurable levels (up to 0.1 %) of the so-called common deletion, an mtDNA molecule containing a 4977 bp rearrangement that is present in high amounts in many patients with "sporadic" Kearns-Sayre syndrome (KSS) and progressive external ophthalmoplegia (PEO). Why these and other mitochondrial defects are maternally inherited, and yet the mutations considered at the basis of the aging process are not, remains to be clarified. Possibly the answer will come from understanding the relationships between mtDNA damage, nDNA damage, protein and lipid damage, and the aging process. Obviously, environmentally and non-genetically determined events

must be involved as well.

Much recent experimentation support the views that the genetic, biochemical and bioenergetic functions of mitochondria deteriorate during normal aging. Deletion mutations of the mitochondrial genome accumulate with age in nerve and muscle tissue of humans and several other species. In muscle, a tissue that undergoes age-related atrophy in humans, there is an exponential rise in the number of cytochrome-oxidase-deficient fibers which is first detectable in the fourth decade of age. Most biochemical studies of animal mitochondrial activity indicate a decline in electron transport activity, as well as decreased bioenergetic capacity with age, as measured by mitochondrial membrane potential. Mitochondrial mutations may be both the result of mitochondrial oxidative stress and cells bearing pure populations of pathogenic mitochondrial mutations. Oxidant stress to mitochondria is known to induce the mitochondrial permeability transition, which is involved in the release of cytochrome c and the initiation of apoptosis. Several lines of evidence support a contribution of mitochondrial dysfunction to the phenotypic changes associated with aging.

While sustained damage inflicted by endogenously produced oxidants is likely one of the causes of the age-related deficits in mitochondrial function, this decline is associated with a generalized physiological decline that is common to all aging organisms. In human's, oxidation is a major contributor to cellular aging and the degenerative diseases that accompany aging such as cancer, cardiovascular disease, immune-system decline, brain dysfunction, and cataracts.

Oxidants produced continuously at a high rate as a by-product of aerobic metabolism include superoxide (O_2._), H_20_2, and hydroxyl radicals (HO.) (the same oxidants produced by

radiation) and possibly singlet oxygen ($1O_2$). All of these can damage cellular macromolecules, including DNA, protein, and lipid. Accumulation of such damage likely contributes to aging and age-associated degenerative diseases.

The continuous threat of oxidant damage to the cell, tissue, and organism as a whole, is countered by the existence of an impressive array of cellular. There is evidence that dietary antioxidants, such as ascorbate, tocopherol, and carotenoids, the main source of which are fruits and vegetables, protect against these degenerative diseases. However, these defenses are not perfect and consequently, cellular macromolecules become oxidatively damaged. The accumulation of these damaged macromolecules contribute significantly to aging, and the incidence of diseases.

Mitochondria constitute the chief source of oxidants because 1) The mitochondrial electron transport system consumes approximately 90% of the oxygen utilized by the cell. 2) Compared with other oxidant producing systems of the cell (cytochrome P-450, various cytosolic oxidases, ß-oxidation of fatty acids in peroxisomes, etc.), mitochondria are required for the production of ATP and are present in relatively high numbers in essentially all cells of the body. Cellular energy deficits caused by declines in mitochondrial function can impair normal cellular activities and compromise the cell's ability to adapt to various physiological stresses. This oxidative damage, and in particular oxidative damage to mitochondria, is a major factor in aging.

Levels of oxidative damage to mtDNA isolated from rat liver or various human brain regions are at least 10-fold higher than those of nuclear DNA. This increase correlates with the 17-fold higher mutation rate in mtDNA compared to nuclear DNA. These higher levels of oxidative damage and mutation in mtDNA have been ascribed to location of the DNA near the

inner mitochondrial membrane sites where oxidants are formed, lack of protective histones, and lack of DNA repair activity. Oxidative lesions in mtDNA accumulate as a function of age in human diaphragm muscle, human brain, and rat liver. The age-associated accumulation of oxidative damage to mtDNA correlates with the level of mtDNA deletions seen in a number of tissues composed of post-mitotic cells and this damage leads to mutations that results in dysfunctional mitochondria. Oxidative damage to brain mtDNA may contribute to the age-dependent increase in the incidence of neurodegenerative diseases.

The accumulation of oxidatively damaged proteins, varying within tissues, increases with age, as does age-associated increase in oxidative damage to mitochondrial protein. The accumulation of oxidized dysfunctional protein with reactive carbonyl groups leads to inter- and intramolecular cross-links with protein amino groups and causes loss of biochemical and physiological function in mitochondria. Thus the age related accumulation of protein oxidation products in mitochondria leads to loss of energy production and increased production of oxidants.

The fluidity of cellular membranes decreases with age, a change due in part to oxidation of plasma and mitochondrial membrane lipid components. Part of this increased sensitivity to oxidants is due to changes in membrane lipid composition. For example, in the liver microsomal and mitochondrial membrane fractions isolated from rodents, there appears to be a progressive decline in the amount of linoleic acid. This change is roughly paralleled by an increase in the amount of long-chain polyunsaturated fatty acids, a subclass of lipids that exhibit a higher degree of unsaturation and are therefore more sensitive to oxidation reactions than linoleic acid. Most of these substitutions appear to occur in the fatty acid composition of cardiolipin. Because cardiolipin

plays a pivotal role in facilitating the activities of mitochondrial inner membrane enzymes, changes that increase its susceptibility to oxidative damage are deleterious to normal mitochondrial function.

The age-dependent accumulation of lipids more prone to peroxidation tends, following peroxidation, to decrease the fluidity of cell membranes. Mitochondria account for essentially all the net loss of water that occurs with age in liver and heart, which is consistent with the age-associated increase in membrane rigidity. Similarly, decreases in lateral diffusion of plasma membrane proteins (e.g., receptors) is associated with a general decline in signal transduction that is commonly observed in aging organisms.

Phospholipase A2 is involved in repair of oxidatively damaged lipids. Phospholipase A2 activity in the inner mitochondrial membrane increases in response to conditions associated with increased oxidant production, such as bacterial endotoxin treatments. Increases in inner mitochondrial phospholipase A2 activity is also seen in mitochondria isolated from fish oil-fed and vitamin E-deficient rats, dietary treatments associated with increased lipid peroxidation. Efficient membrane antioxidants such as ubiquinol and its synthetic derivatives inhibit release of fatty acids catalyzed by phospholipase A2, probably by inhibiting oxidation of lipids. Physiological conditions such as which are associated with reduced mitochondrial oxygen consumption, are associated with a marked decline in phospholipase A2 activity. These observations support the suggestion that phospholipase A2 is a repair enzyme that catalyzes the removal of oxidized lipids in membranes. Without such a repair activity peroxidized lipids accumulate, the consequence of which is increased membrane permeability and loss of mitochondrial respiratory control.

The components of the electron transport chain, which catalyze the phosphorylation of ADP

to ATP, work as an integrated system comprised of a total of 5 protein complexes. mtDNA encodes 13 of the proteins and nuclear DNA encodes approximately 60. Complexes I-IV are involved in the oxidation of NADH, electron transport, and the generation of an electrochemical gradient. This electrochemical gradient, which is created by pumping protons across the inner mitochondrial membrane, is utilized by ATP synthase (complex V) as a source of energy. Relevant to mitochondrial function is the efficiency of electron movement through the electron transport chain and its coupling to oxidative phosphorylation to produce ATP. The coupling efficiency can be measured experimentally by determining the ratio of ATP production to molecular oxygen consumed (ADP/O), and whether the mitochondria are in State 3 or State 4.

State 3 represents a condition where oxidative phosphorylation is not rate limited by ADP concentration. State 4, a condition where the level of ADP limits oxidative phosphorylation, is associated with a reduced respiratory chain activity leading to increased formation of O2.- byproduct.

Temporary or sustained loss of mitochondrial function and ATP production can have a major impact on the fidelity of cellular defenses and repair processes. This results in increased mutational load, increased accumulation of dysfunctional cellular macromolecules, and a decreased capacity to mount an appropriate stress response when challenged. Probable age associated loss of function in mitochondria is suggested by the evidence of the following: 1.) increased mtDNA deletions and point mutations, 2.) increased oxidative damage to mtDNA, 3.) increased levels of aberrant forms of mtDNA, 4.) formation of mtDNA-protein crosslinks, 5.) increased production of mitochondrially-derived oxidants, 6.) decreased State 3/State 4 ratio, 7.) decline in activities of

complexes I, II and IV, and 8.) age-related decreases of mitochondrial cytochrome oxidase in post-mitotic tissues. Marked changes in mitochondria with age have been observed histologically, including enlargement, matrix vacuolization, shortened cristae and loss of dense granules. As only about half of these enlarged mitochondria can be recovered from old animals, it is quite possible that differences in the function of mitochondria isolated from old versus young animals are underestimated by this selective loss and may be one reason for the apparent lack of age-associated biochemical changes in this organelle. Along with the histological changes cited above, the potential for lipid peroxidation in the inner mitochondrial membrane increases, making the mitochondria more susceptible to damage by oxidants. Furthermore, the decreased content of 18:2-containing lipids, which are optimal for cardiolipin interactions with proteins of the inner mitochondrial membrane, accounts for the decreased State 3/State 4 ratio, and increased O_2.- and H_2O_2 formation that has been observed in some tissues with age. These changes, in turn, contribute to increased loss of efficiency and lead to mitochondrial dysfunction. Some of these defects are genetically inherited and have been shown in some instances to be associated with an extensive amount of mtDNA deletions (30-80% of all mtDNA) or point mutations resulting in energy deficits and compromised tissue function. mtDNA deletions, many of which are produced because of illegitimate recombinational events at direct repeat sequences are particularly prevalent in post-mitotic tissues. Associated with these deletions are myopathies and increased susceptibility to neurodegenerative disorders.

The same type of deletions and point mutations in mtDNA that cause inherited myopathies are those observed to increase with age. The age-associated increase in the level of any of the

common deletions (e.g. mtDNA 4977, mtDNA 7436, mtDNA 10,422) produced spontaneously is low (<0.1% versus 30-80% for inherited cases). While the effect of this low level of deletions may not be significant, it is postulated that these deletions represent only a small portion, "the tip of the iceberg", of the multitude of deletions and point mutations that might exist and accumulate with age. It is plausible that the accumulation of all mtDNA defects could account for the age-related deficits in mitochondrial bioenergetic capacity and function.

The role of oxidants in the formation of mtDNA deletions is supported by the observation that doxorubicin, a compound that stimulates mitochondrial oxidant production, creates a marked elevation in mtDNA deletions in cardiac tissue; this effect is blocked by ubiquinone (Coenzyme Q10), a key component of the mitochondrial electron transport system whose reduced form, ubiquinol, exhibits antioxidant properties. The age-associated accumulation of the common deletion mtDNA 4977 also appears to correlate with oxygen consumption as well as functional workload. This and other mtDNA deletions are likely to be responsible for the degeneration of neurological function, cardiovascular function, and muscle movement that are common in older individuals.

Studies that have examined the content of cytochrome oxidase in mitochondria show a progressive and random loss in this enzyme activity which correlates well with the age-associated decline in mtRNA synthesis. A study of human diaphragm muscle indicates that cytochrome c oxidase decreases markedly beyond the seventh decade of life. Examination of various muscle tissues (extraocular muscles, human diaphragm, skeletal muscles), brain, liver, heart, and lung indicate age-associated increases in mtDNA deletions. These deletions create tissue bioenergy

mosaics that account for losses in bioenergetic capacity. This has been shown to occur with age in skeletal muscle and in liver.

The loss of functional mitochondria with age appears to be compensated in part by the increased workload of the remaining intact population of mitochondria. The increase in senescent tissue of mtDNA copy number supports the idea of an adaptive mechanism designed to restore mitochondrial function. These changes may account for the apparent lack of effect of aging on the level of adenine nucleotide levels observed in cells of aged organisms.

Thus, the increases in either mtDNA copy number or increases in the expression of nuclear encoded proteins for oxidative phosphorylation may be feedback mechanisms that compensate for mitochondria harboring defective proteins or mtDNA. The result of such a mechanism is to allow a cell to adapt to a localized loss of mitochondrial function.

Compensatory mechanisms in the fully functional cells mask the inefficiencies of their dysfunctional neighbors but in doing so increase their workload, their energy expenditures, and the probability of incurring damage and loss of function.

Analogous compensatory effects are proposed to account for the age-dependent loss of dopaminergic neurons. Functional deficits in dopaminergic neurons are not observed until approximately 80% of these cells are lost. This implies that with the attrition of these nerve cells, the remaining viable neurons increase their workload to the point of adequately compensating for this loss.

Damage to inner membrane proteins comprising the electron transport chain can alter the efficiency of electron transport. Imbalances in the stoichiometry of functional electron transport

proteins is proposed to lead to a leakage in the flow of electrons to the terminal electron acceptor, cytochrome oxidase. The decreased age-related expression of cytochrome oxidase in tissues such as the heart, liver, and brain are of particular relevance. Furthermore, alteration in protein conformation due to direct oxidative damage or through DNA mutation may cause inefficient transfer of electrons through the electron transport chain. This would increase the likelihood of superoxide formation. Treatment of submitochondrial particles with glutaraldehyde, increases O2.- and H_2O_2 production, presumably by inducing crosslinks between proteins and lipids of the inner mitochondrial membrane. Crosslinks of inner mitochondrial membrane proteins by oxidants, or reactive aldehydes generated from lipid peroxidation, may also result in increased O_2.- and H_2O_2 production, thus further increasing the damage that can lead to mitochondrial dysfunction.

Mutations in the many nuclear DNA encoded proteins of the mitochondria can also lead to mitochondrial dysfunction. Such mutations, which are likely to be produced in part by endogenous oxidative damage, may result, for example, in lowered efficiencies of electron transport components, lowered efficiencies of substrate and phosphate transporters, and lowered rates of ATP synthesis. Nuclear genes encoding mitochondrial proteins are transcribed continuously and are therefore expected to be at an increased risk of mutation compared with other regions of the genome that are transcribed at a lower rate, if at all. Although nuclear DNA is considerably less susceptible to mutation than mtDNA, once formed, the products of such mutation would affect the bioenergetics of all of the mitochondria in the cell. Mutations to nuclear DNA that encode proteins of the mitochondrial electron transport chain will include lethal mutations that are cytotoxic and dominant mutations that cause mitochondrial dysfunction, but still allow the cell to remain viable.

Cardiolipin, a diphosphatidyl glycerol derivative found principally in mitochondria, plays an important role in mitochondrial membrane structure and function. The decrease of cardiolipin with age is associated with a decrease in State 3/State 4 ratio. Cardiolipin interacts with various proteins of the inner mitochondrial membrane and plays a pivotal role in maintaining their activities. In addition, cardiolipin appears to play an important role in controlling the permeability of the inner mitochondrial membrane to small molecules as well as in establishing mitochondrial proton gradients.

Mitochondrial cardiolipin content has been reported to decrease with age in a number of tissues including heart, liver, and nonsynaptic brain mitochondria. This loss, which has been proposed to be due to a decline in mitochondrial cytidine triphosphate (CTP): phosphotidate cytidyltransferase activity, may play a critically important role in the age-related decrements in mitochondrial function. The change in mitochondrial cardiolipin is paralleled in mitochondria by a decrease of the inner membrane surface area, a smaller, sparser cristae, and increased fragility.

The functional changes in mitochondrial enzyme activities that accompany the modifications in cardiolipin composition include a decrease in the activity of cytochrome oxidase that is involved in the age-related increases in the production of mitochondrially-derived oxidants. Other proteins of the inner mitochondrial membrane that also require interaction with cardiolipin for optimal catalytic activity include the ADP/ATP translocator, phosphate translocator, mitochondrial ATP synthase, mitochondrial substrate transporters, as well as the palmitoyl carnitine transferase and carnitine translocase systems. Under certain experimental conditions that strip cardiolipin off protein, denaturation and complete loss of activity of many of these proteins are

observed. Cardiolipin appears to be essential for the activity of the proteins it interacts with, because substitution with other mitochondrial phosopholipids (e.g., phosphatidylcholine and phosphatidylethanolamine) has little or no effect in reconstituting activity. The age-related decrease in heart mitochondrial cardiolipin is correlated with an increased cholesterol:phospholipid ratio, a change that is associated with increased membrane rigidity. Acetyl-L-carnitine (ALCAR) fed to old rats increases the amount of cardiolipin to levels similar to that of young rats, suggesting that ALCAR administration may improve cellular bioenergetics in the aged rat.

Cardiolipin contains a higher ratio of unsaturated to saturated fatty acid residues compared with the other phospholipids of the inner mitochondrial membrane, a characteristic that increases its sensitivity to oxidation. The sensitivity of cardiolipin to peroxidation increases with age in rodents, an effect that appears to be attributable in large part to the substitution of 18:2 acyl side chains with more readily peroxidizable 22:4 and 22:5 acyl side chains. The mechanism underlying the age-related change in the composition of the acyl side chains of cardiolipin is not known. Interestingly, calorie restriction, a dietary regimen that extends lifespan in rodents, maintains the level of 18:2 acyl side chains and inhibits the cardiolipin composition change to the 22:4 and 22:5 class of lipids. Calorie restriction was shown not to have a marked effect on cardiolipin levels.

Oxidative stress conditions decrease cardiolipin levels by inducing oxidation of its unsaturated fatty acyl side chains. After episodes of ischemia-reperfusion, cardiolipin appears to be destroyed selectively by oxidants. The extensive lipid peroxidation that occurs at the inner mitochondrial membrane as a result of this challenge appears to be catastrophic to the integrity of cardiolipin. This in turn leads to the inactivation of cytochrome c and other mitochondrial

enzymes and increases the permeability of the inner mitochondrial membrane. Because cardiolipin is important in precursor protein import into mitochondria, changes in its level or acyl side chain composition could adversely affect targeted insertion of nuclear encoded mitochondrial proteins.

Exposure of HeLa cells to 80% O_2 for two days inhibits mitochondrial respiration and is associated with growth inhibition and loss of mitogen responsiveness. This treatment leads to the inactivation of the thiol-containing mitochondrial enzymes NADH dehydrogenase, succinate dehydrogenase and [[alpha]]-ketoglutarate dehydrogenase. Studies in mammalian cell culture show that oxidative stress can adversely affect the activity of key mitochondrial enzymes that subsequently leads to a decline in ATP production. The important lesions that lead to decline in mitochondrial enzyme activities are not known but could be derived from mutations to mitochondrial or nuclear genes. Epigenetic effects such as direct protein damage (e.g. oxidation of vicinal and mono-thiol containing enzymes of the mitochondrial inner membrane) may also create a condition that indirectly leads to genetic damage of these key genetic loci. For example, oxidant-induced damage to inner mitochondrial membrane proteins can lead to increased leakage of O_2.- and H_2O_2 that then may cause mtDNA mutations. Oxidative inactivation of mitochondrial proteins that leads to decreased mitochondrial efficiency may be related to the age-associated depression of mitochondrial proton motive force observed in human fibroblasts and murine lymphocytes. A decline in mitochondrial membrane potential can lead to lower ATP production and decrease the efficiency of energy dependent processes such as signal transduction. The loss of mitogen responsiveness in lymphocytes isolated from elderly individuals has been attributed in part to the decrease in mitochondrial and plasma membrane potentials.

The ß-oxidation of fatty acids serves as a key source of energy for many tissues. For these tissues the activity of carnitine-acylcarnitine exchange across the inner mitochondrial membrane is of great importance. Investigations of heart mitochondria indicate that the activity of this exchange reaction, which is mediated by a thiol containing and mersalyl-sensitive carrier protein, is decreased significantly with age. It has been suggested that the lower intramitochondrial pool of carnitine is in part responsible for this age effect.

A rapidly growing body of evidence suggests that the apparent age-related deficits in mitochondrial function can be slowed or reversed by ALCAR, a normal component of the inner mitochondrial membrane that serves as a precursor for acetylCoA as well as the neurotransmitter acetylcholine. Once deacetylated, L-carnitine, which remains in the inner mitochondrial membrane, can be reacylated and further serve to shuttle lipid substrates into mitochondria for ß-oxidation. ALCAR has been shown to reverse the age-related decrease in the levels of mitochondrial membrane phospholipid cardiolipin and the activity of the phosphate carrier in rat heart mitochondria. Furthermore, the age-associated decrease in mtDNA transcription is reversed rapidly by ALCAR. Chronic administration of this compound to rats is associated with a reduction in the accumulation of lipofuscin in Purkinje neurons and pyramidal neurons of the prefrontal cortex and hippocampus. It has also been shown to attenuate the age-related decrements in active avoidance learning. ALCAR's effect on mitochondrial function in the aging brain is supported by its ability to create a shift in ATP production from glycolytic pathways to mitochondria. In vitro studies indicate that ALCAR increases the number of N-methyl-D-aspartate (NMDA) receptors of cultured cerebellar granule cells, prevents age-associated reduction of nerve growth factor binding

to PC12 cells, and attenuates the rate of mortality in rat dorsal root ganglia neurons. In aged mice treated with ALCAR for 3 months, dopamine release is enhanced compared with untreated control animals. The age-associated loss of the D1 subclass of striatal dopamine receptors is also attenuated by this treatment. In addition, ALCAR reverses the age-associated decrease in mitogen-induced lymphocyte proliferation and protects lymphocytes isolated from old donors from cytotoxicity following a challenge with oxidants. ALCAR appears to completely protect canine frontal cortex proteins from oxidation after cardiac arrest and restoration of circulation. The multiplicative effects of ALCAR in reversing the age-related decline in various physiological parameters associated with mitochondrial function may be attributable to its ability to deliver acetylCoA equivalents to the tricarboxylic acid cycle and to facilitate the mitochondrial ß-oxidation of fatty acids, thereby increasing the production of ATP. It is plausible that ALCAR can increase the metabolic efficiency of compromised subpopulations of mitochondria, and cause a redistribution of the metabolic workload, resulting in increased cellular efficiency and possibly a decrease in the rate at which mitochondria-derived oxidants are produced.

Physical fitness in animals is increased by the delay of reproductive function during periods of low food availability; the saved resources are invested in maintenance of the body until food resources are available for successful reproduction. For example, during the winter months when food is scarce, Syrian hamsters undergo a process of gonadal regression that is accompanied by patterns of daily torpor, a metabolically depressed state. These two important physiological adaptations minimize consumption of metabolic fuel by diverting resources away from reproductive function and by transiently lowering metabolic rate. When animals that had already undergone

gonadal regression are treated with testosterone, daily torpor is not observed. This suggests a potentially important link between neuroendocrine modulation of reproductive function and whole body aerobic metabolism. Another example of the dramatic life-extending effects of lowered oxygen consumption rates is the unusually long 8-year lifespan of the pocket mouse Perognathus longimembris, a rodent with a low waking metabolic rate and the ability to undergo daily torpor.

During torpor or hibernation, a condition of extended torpor, mitochondrial respiration of liver drops markedly. This decrease in cellular respiration is associated with increased microviscosity and decreased permeability of the inner mitochondrial membrane, a change that leads to decreased transport of substrates for intramitochondrial energy production. Phospholipase A2 activity also decreases, indicating that oxidation of lipids of the inner mitochondrial membrane is reduced. Specifically, electron transfer through the ubiquinol:cytochrome c1-segment of the respiratory chain of liver mitochondria isolated from hibernating ground squirrels (Citellus undulatus) is inhibited by 70-80% when compared with mitochondria isolated from non-hibernating control animals.

The physiological mechanisms that control the conservation of energy during periods of low food availability, as observed experimentally with calorie restriction, may temporarily but profoundly affect the metabolic rate of organisms capable of entering this state. Therefore, daily torpor, by lowering the metabolic rate and oxidant production that can exert pro-aging effects, is likely to be responsible for at least some of the life prolonging effects of calorie restriction. These findings are not necessarily inconsistent with the observation of total lean body O_2 consumption being unchanged when the effects of calorie restriction and ad libutum feeding are compared.

While whole body metabolic rate may remain constant, significant shifts in the proportion of oxygen consumed by the different organs will occur, as would be predicted based on principles of physiological adaptation. When food consumption is low (i.e. calories are restricted), workload is reduced in organs (stomach, intestines, colon, liver, and kidney) that participate in food absorption and digestion.

Oxidants and mitochondrial deficits may lead to increased neuronal loss through excitotoxic mechanisms. Because central nervous system function is critical in homeostasis, the attrition of a sufficient number of neurons can lead to age-associated disability and loss of various physiological functions such as receptor-mediated signal transduction.

The loss of sensitivity in central neuronal receptors to agonist stimulation is a hallmark of the aging process. This appears to be particularly true in central (hippocampal, striatal) muscarinic cholinergic systems and in the striatal dopamine systems. Decreased receptor numbers and less efficient signal transduction seem to be responsible for a marked decline in cognitive and motor functions. Oxidation has been implicated in membrane and mitochondrial damage in neurons. The central nervous system is enriched in both unsaturated lipids and nonheme iron, two ingredients that could cooperate to produce oxidant damage and cell death. A functional consequence of oxidant damage is increased membrane rigidity that can lead to a decline in receptor mediated signaling, possibly explaining the age-associated decline in responsiveness of the ß-adrenergic, dopaminergic and muscarinic receptor systems to agonist stimulation. The effective age-related loss in signal transduction can be mimicked in younger animals by treatments such as kainic acid or ionizing radiation that produce oxidative damage to these receptors.

The decline in mitochondrial function that is proposed to occur with age in terminally differentiated neurons appears to increase their sensitivity to cell death by excitatory factors. It has been shown in vitro and in vivo that deficits in mitochondrial function can lead to neuronal degeneration and death by sensitizing neurons to the excitotoxic effects of endogenous glutamate, a neurotransmitter that binds to the NMDA receptor and under normal physiological conditions is excitatory. The neurotoxic effects of excitatory amino acids have been implicated in a number of neurodegenerative processes such as Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis, Alzheimer's disease, and the AIDS dementia complex and may also be involved in a number of neurological disorders including stroke, epilepsy, trauma, and neuropathic pain. It is suggested that voltage-dependent NMDA receptor ion channels will become increasingly sensitized to the neurotoxic effects of glutamate when cell membranes become partially depolarized because of decreased mitochondrial energy production. The decline in the plasma membrane potential would thus permit the release of the voltage dependent blockade of the NMDA receptor channel by Mg2+. This release allows persistent and uncontrolled receptor activation that leads to calcium mobilization from intracellular stores, oxidant production, neuronal damage, and cell death. Such a mechanism could account for the progressive age-associated loss of these neuronal populations.

The prooxidant effect of glutamate receptor activation, which appears to be amplified markedly when mitochondrial function is compromised, is revealed in studies that show increases in the formation of lipid peroxidation products and lipofuscin by the NMDA receptor agonists NMDA and kainic acid. Studies on the effect of NMDA-induced neuronal death suggests reactive byproducts of nitric oxide as being one of the principal cytotoxic species. NMDA receptor

activation triggers a rapid influx of calcium into the cell that stimulates calmodulin-dependent nitric oxide synthase activity, creating a marked elevation in nitric oxide production and subsequent cellular damage. This effect of nitric oxide appears to be mediated by peroxynitrite, a powerful oxidant formed from nitric oxide and superoxide. Under physiological conditions peroxynitrite rapidly decomposes to generate oxidants with a reactivity similar to that of hydroxyl radical. Inhibitors of nitric oxide synthase effectively protect neuronal cells in culture from the cytotoxic effects of NMDA and glutamate. Antioxidant treatments with 21-aminosteroids appear to be somewhat effective in attenuating the neurotoxic effects of these excitatory agents in vitro. In addition, both GSH and GSSG are protective against the acute neuronal toxicity, but act by distinct pathways; GSH appears to act as an antioxidant, while GSSG binds to the vicinal thiol residues of the NMDA receptor, thus blocking receptor activation. The prophylactic effects of antioxidant treatments on age-associated, glutamate-dependent neuronal losses have yet to be tested. Long term treatment of rats with ALCAR also attenuates the age-associated reduction in the density of hippocampal NMDA receptors. Ubiquinone also protects neuronal cells in culture from glutamate toxicity. It is not clear, however, whether the protection afforded by ubiquinone in this cell culture model is due to its role as a component of mitochondrial electron transport or as an antioxidant. Coadministration of nicotinamide (a precursor of NADH) and ubiquinone are more neuroprotective to the neuronal injury caused by the mitochondrial toxin malonate than either compound alone; this treatment prevents depletion of ATP that is postulated to sensitize neurons to the cytotoxic effects of excitatory amino acids. The available evidence suggests that acute neurological damage or chronic neuronal degeneration that accompanies aging may be provoked in part by mitochondrial

dysfunction and the oxidants produced by the NMDA receptor-mediated pathway.

At this point, to put the information we have in perspective, it might be well to consider a number of questions raised a few years ago by Professor Imre ZS-Nazy, of the University Medical School at Debrecen, Hungary. In the Annals of the New York Academy of Sciences Conference on the Physiological process of ageing conference, published as Vol. 63 of the Annals of the New York Academy of Sciences, Professor ZS-Nagy in his "A Proposal for Reconsideration of the Role of Oxygen Free Radicals in Cell Differentiation and Aging" states:

> "Living systems exist as biological individuals and display various levels of complexity from unicellular organisms to humans. All molecular components of living systems can be isolated in pure form and put in test tubes; however, life itself is lost during such isolation and purification, since the living state is bound to a specific supramolecular organization and interaction of the compounds. On the other hand, it is clear from available physicochemical knowledge that the supramolecular organization can be created through certain intermolecular reactions that are based on the interactions of the external electron orbits of the macromolecules. Starting from these and many other considerations, Szent-Györgyi concluded that the living state is bound to particular functions of the electrons. Consequently, it is plausible to assume that the processes of biological maturation and aging are also related to alterations of the external electron orbits of the macromolecules. It is obvious that these thoughts and free radical biology have a number of common aspects.
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> Although oxygen free radicals have long since been implicated as causal factors of biological aging, their role in cell physiology and biochemistry has not yet been sufficiently elucidated. The free radical theory of aging assumes that free radicals are in general harmful byproducts of the aerobic life and as such represent the basic cause of aging and of numerous diseases. Although oxygen free radicals do occur in biological systems and represent a real danger for macromolecular conformation, and although they have been implicated in numerous biological phenomena such as cellular aging, mutagenesis, inflammation, and some other pathologies, at a deeper level of analysis one encounters a paradoxical situation that has to be explained. Namely, the aggressivity and chemical nature of oxygen free radicals do not change with age, yet it is a fact that young cells and organisms are able to grow and differentiate, while older cells progressively deteriorate in their structure and functional performance. In addition, it is a well-known fact that young individuals consume more oxygen per unit of mass and time than the old ones; that

is, there must be an even more intense radical formation in younger systems, as compared to older ones. In other words, the general statement that aging is caused by the oxygen free radicals remains shaky unless the above- mentioned discrepancy finds an explanation in the biological structure itself.

This paradox of the free radical theory of aging may be resolved by the membrane hypothesis of aging on the basis of the age-dependent increase of the physical density of living systems, which causes a lifelong increase of the damaging efficiency of the cross-linking effect of oxygen free radicals. In other words, even the lower level of oxygen free radicals in the old systems may represent a more serious danger, because at higher physical density even less radicals may produce more intermolecular cross-linking. Nevertheless, the concepts of theoretical gerontology, outlined in detail elsewhere, suggest further questions of general importance, relevant to the problem of oxygen free radicals as explained in this paper.

THE MAIN PROBLEMS TO BE RECONSIDERED

If we wish to understand the function of living systems and particularly such a complicated process as their aging, it is obviously necessary to start from a well-established theoretical basis that is in harmony with present knowledge of all branches of biomedical science. In other words, we need a synthesis of actual knowledge and interpretations. Unfortunately, however, such a synthetic approach is out of fashion nowadays, since at the existing level of overspecializations gerontologists and other scientists are too busy with details of living systems. Therefore, very few people are interested in the supramolecular organization of living material.

In this field one has to ask the following main question: what kind of forces bring about, maintain, and stabilize the supramolecular and cellular organization of macromolecules? This question is justified because the genetic code contains information only for the amino acid sequence of the protein chain; and although this sequence may largely determine the coiling of the chain and even the final conformation of the macromolecule, it is only scarcely known how the intermolecular bonds stabilizing the supramolecular organization of well-defined structural entities such as membranes, sarcomeres, and synapses come into being. The situation is similar to that in which we have all the components for constructing a house but do not know how to put together and strengthen the parts to make a stable house. One can speak of hydrogen bonds, hydrophobic interactions, and other “weak” interactions, for example, which are certainly involved in the construction of the cell structure; nevertheless, they are most probably not sufficient for this purpose. Therefore, one has to assume that more stable intermolecular bonds, such as covalent cross-links, should also play a role in the relatively high level of physiologically necessary stabilization of living structures. If this

assumption is accepted, oxygen free radicals of cross-linking effect, especially the $OH^{\cdot}$ free radicals, may be thought to be involved in this process. That is, we have to reconsider their role, which has so far been classified only as a damaging one. - - -

. . . One cannot exclude the existence of other important roles for $OH^{\cdot}$ radicals. For example, their formation and reactions are sufficiently fast to assume their involvement in brain memory formation. If we consider that the brain is practically devoid of catalase, it seems logical to assume that most of the hydrogen peroxide formed in the brain is directed toward $OH^{\cdot}$ radical production; and if this is true, these radicals may have roles other than the assumed damaging roles. Although this is speculation at present, it may be worth further exploration if we consider the almost complete lack of secure knowledge about brain memory mechanisms.

SUMMARY

The best-known factors able to cause molecular alterations in living systems are the oxygen free radicals, as has been suggested long ago. These radicals occur in biological systems and are implicated as causal factors in a great number of biological events, such as mutagenesis, aging, and some pathologies. The most widely accepted view is that these radicals are harmful by-products of the oxidative metabolism. The use of so-called antioxidants against them is thought to be a useful approach in any antiaging strategy. However, more and more observations indicate that the oxygen radicals in living systems are most probably necessary compounds in the maturation and differentiation processes of cellular structures, and perhaps also in brain memory functions. The complete elimination of these radicals would not only be impossible, but also harmful. The central idea of a possible new concept is that the supramolecular structure of cells is built up and kept relatively stable by using the cross-linking effect of the $OH^{\cdot}$ free radicals.. . . It should be stressed that the free radical theory of aging remains valid, but it becomes part of a more general concept: aging occurs when the ever- increasing physical density of differentiated cells contributes to an exponential increase in radical-induced intermolecular cross-linking in the cell membrane. "

Continued research in the 8 years since these observations by Professor ZS- Nazy have elucidated some of the functions of free radicals in cell physiology, particularly their roles in cell signalling which reinforce his consensus, that while consumption of adequate antioxidants is important in the treatment of degenerative diseases involving mitochondrial dysfunction, they are not a panacea and other modalities of treatment such as nutritional supplementation with

supplements such as Acetyl L-Carnitine, detoxification and electromagnetic treatments are going to be equally as necessary to reverse the manifestations of these diseases.

IRON, NITROGEN AND CELL SIGNALING

Iron is the most abundant metal in the earth and in the human body. Nitrogen is the most abundant gas in the atmosphere. It is no surprise that both iron and nitrogen should have become a part of the makeup of the bodys' cells and the mitochondria.

Over the past two decades, many biological functions of iron have been identified, especially its role in enzymatic processes. Iron is involved in many enzymatic processes in the body such as DNA, RNA and protein synthesis, many heme and non-heme enzymes as a co-factor, the formation of myelin, the function of the neuronal dendritic tree, defensive receptor function, interacts with other neurotransmitters such as GABA and serotonin, and plays a critical role in the heme molecule.

Within cells, there normally exists a pool of low molecular mass redox active iron which is essential for the synthesis of iron-requiring enzymes and proteins, and for the synthesis of DNA. This pool of iron is the target of iron chelators and is also a form of iron sensed by iron regulatory proteins. The amount, and nature, of the ligands attached to this iron, however, remain unknown. A recently introduced fluorescence assay based on calcein has enhanced our knowledge of intracellular iron pools. In contrast to the intracellular environment, extracellular compartments do not usually require, or contain, a low molecular mass iron pool. Iron-binding proteins such as transferrin and lactoferrin do not even remotely approach saturation in healthy subjects. They retain

a considerable iron-binding capacity and are able to remove mononuclear forms of iron that enter extracellular fluids.

The differences between intracellular and extracellular compartments and their requirements for low molecular mass iron deserves special comment, since it is iron in this form that is involved in signaling, and the most likely catalyst of biological free radical reactions. Inside the cell, low molecular mass iron need not pose a serious threat as a free radical catalyst, because the cell has specific enzymic defenses to safely and speedily remove all the $O_2^{\circ -}$ and H_2O_2 and organic peroxides (such as lipid peroxides) that could react with such iron. In the extracellular space, however, we see a different pattern of protection against free radical chemistry. Here, proteins bind, conserve, transport and recycle iron and keep it in non- or poorly-reactive form that does not react with H_2O_2 or organic peroxides. Proteins such as transferrin and lactoferrin bind mononuclear iron, whereas haptoglobins bind haemoglobin and haemopexin binds haem. In addition, plasma contains the ferrous ion oxidizing protein (ferroxidase) caeruloplasmin. By keeping iron in a poorly reactive state, molecules such as $O_2^{\circ -}$, H_2O_2, $^{\circ}NO$, HOCl, and lipid peroxides can survive long enough to perform important and useful functions as cell signaling and intercellular messenger molecules.

Nitrogen gas accounts for 78% by volume of the earth's atmosphere and, because of its inert properties, it is a major global antioxidant deterring combustion and other oxidative processes. Three simple oxides of nitrogen are of current biomedical interest; nitrous oxide (N_2O), a colorless gas with anesthetic properties (laughing gas), nitrogen dioxide ($^{\circ}NO_2$), a toxic brown colored paramagnetic gas (free radical), and nitric oxide ($^{\circ}NO$). Nitrogen dioxide is an environmental

pollutant and may be produced *in vivo* from reactions of $^{\circ}NO$, where it can act as an initiator of lipid peroxidation. Nitric oxide is a colorless gas and a weak reducing agent. Biological interest in $^{\circ}NO$ and other RNS has exploded since the recent observation that the vascular endothelium and other cells in the body produce small amounts of it from the amino acid L-arginine. Nitric oxide is poorly reactive with most molecules in the human body (non-radicals) but, as a free radical, it can react extremely rapidly with other free radicals such as superoxide, amino acid radicals, and certain transition metal ions. The reaction between nitric oxide and superoxide ($K_2 6.7 \times 10^9\ M^{-1}s^{-1}$) produces peroxynitrite ($ONOO^-$), which is, itself, a powerful oxidant that can decompose to yield further oxidants with the chemical reactivities of $^{\circ}NO_2$, °OH and NO_2+. The exact chemistry of damage by $ONOO^-$ is a matter of current debate. Activated phagocytes generate $O_2^{\circ-}$, H_2O_2, $^{\circ}NO$, (and, in the case of neutrophils, HOCl) as one of their many mechanisms for killing foreign organisms, and leave evidence of their activity on protein, lipid and DNA molecules. The interactions of RNS and ROS are also important for we see examples whereby H_2O_2 activates NF-$_K$B but $^{\circ}NO$ inhibits activation. In vascular endothelium $O_2^{\circ-}$ antagonizes the action of $^{\circ}NO$ and causes vasoconstriction; it has been suggested that this is a physiological mechanism for regulating vascular tone. Unfortunately, the rapid reaction of $^{\circ}NO$ with $O_2^{\circ-}$ generates $ONOO^-$, a species possibly responsible for several of the cytotoxic effects attributed to excess $^{\circ}NO$, such as destruction of Fe-S clusters in certain enzymes. Peroxynitrite heightens lipid peroxidation, but $^{\circ}NO$ reacts quickly with the peroxyl radicals that propagate this process. If the resulting ROONO species can be metabolized without the release of free radicals, then NO° effectively inhibits lipid peroxidation. Hydroxylation, nitration and nitrosylation, and chlorination of biological molecules, particularly

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when activated phagocytic cells are involved, is widely interpreted as 'damage accumulation'. However, it is also possible that modifications of proteins, lipids, and DNA in this way plays an important role in biological signalling.

Metals play a dual role in biological systems, serving as essential co-factors for a wide range of biochemical reactions yet these same metals may be extremely toxic to cells. To cope with the stress of increases in environmental metal concentrations, eukaryotic cells have sophisticated toxic metal sensing proteins which respond to elevations in metal concentrations. This signal is transmitted to stimulate the cellular transcriptional machinery to activate expression of metal detoxification and homeostasis genes. This review summarizes our current understanding of the biochemical and genetic mechanisms which underlie cellular responses to toxic metals via metalloregulatory transcription factors.

Redox (oxidation-reduction) reactions regulate signal transduction. Oxidants such as superoxide, hydrogen peroxide, hydroxyl radicals, and lipid hydroperoxides (i.e., reactive oxygen species) are now realized as signaling molecules under subtoxic conditions. Nitric oxide is also an example of a redox mediator. Reactive oxygen species induce various biological processes such as gene expression by stimulating signal transduction components such as Ca(2+)-signaling and protein phosphorylation. Various oxidants increase cytosolic Ca2+; however, the exact origin of Ca2+ is controversial. Ca2+ may be released from the endoplasmic reticulum, extracellular space, or mitochondria in response to oxidant-influence on Ca2+ pumps, channels, and transporters. Alternatively, oxidants may release Ca2+ from Ca2+ binding proteins. Various oxidants stimulate tyrosine as well as serine/threonine phosphorylation, and direct stimulation of protein kinases and

inhibition of protein phosphatases by oxidants have been proposed as mechanisms. The oxidant-stimulation of the effector molecules such as phospholipase A2 as well as the activation of oxidative stress-responsive transcription factors may also depend on the oxidant-mediated activation of Ca(2+)-signaling and/or protein phosphorylation. In addition to the stimulation of signal transduction by oxidants, the observations that ligand-receptor interactions produce reactive oxygen species and that antioxidants block receptor-mediated signal transduction led to a proposal that reactive oxygen species may be second messengers for transcription factor activation, apoptosis, bone resorption, cell growth, and chemotaxis. Physiological significance of the role of biological oxidants in the regulation of signal transduction as well as the mechanisms of the oxidant-stimulation of signal transduction are becoming more apparent with each month as new research is published.

There is increasing evidence that ROS, RNS and RIS are used as 'signal, messenger and trigger molecules'. We are seeing examples of redox regulation of gene expression; not only oxyR and NF-$_K$B but also the role of thioredoxin and of AP- 1.

In human studies, antioxidants can, in simple terms, be considered as dietary, constitutive, and inducible in origin. Knowledge concerning dietary antioxidants has been greatly stimulated by recent advances in the understanding of the molecular mechanisms leading to the development of atherosclerosis, and epidemiological links between diseases and the dietary intake of micronutrients. The adequate intake of dietary antioxidants is now recognized as essential for a healthy lifestyle.

Antioxidants derived from fruits, vegetables, and leaves, where they serve important

protective functions for the plant, appear to play long-term protective roles in preventing life-threatening diseases such as atherosclerosis and certain types of cancer. Evidence that dietary antioxidants alone can cure life-threatening diseases once they have occurred in man is still lacking.

If Reactive Oxygen Species, Reactive Nitrogen Species and Reactive Iron Species are intimately involved in the redox regulation of cell functions, then it is possible to understand why attempts to advantageously change antioxidant balance in disease and ageing experiments have not been completely successful. Cells normally function in a reducing environment but, as this is changed to a more oxidizing (or less reducing) state, cell functions and gene expressions also change. Different cells will respond in different ways to an oxidant challenge, but when resting cells in culture are subjected to increasing conditions of oxidation they can be triggered into proliferation or apoptosis. Following continuing severe oxidative (and also reductive) stress necrosis will eventually prevail.

Using redox control of cellular functions, and the use of antioxidants to control redox balances, makes it clearer why cells have an elaborate system to allow short-term changes in antioxidant intake to influence their cellular redox balance. The relationship between dietary, constitutive, and inducible antioxidants must, therefore, be complex and under genetic control. Understanding these relationships, and how to change them, remains a major challenge in the treatment of degenerative diseases. The role of synthetic anti-oxidants such as Stobadine and TPPB (triphenyl phosphonium bromide) remain to be elucidated.

The practical importance of this is that in the prevention and treatment of mitochondrial diseases, while the importance of dietary and supplemental antioxidants cannot be over-

emphasized, these alone cannot reverse the chronic degenerative diseases and other treatment modalities, such as detoxification, adequate oxygenation, herbs, homeopathic remedies, pulsating electromagnetic frequencies, acupuncture and manipulative therapies are going to be required to produce effective treatments along with the antioxidants. The role of environmental pollutants and the inhibiting and blocking pharmaceuticals must be elucidated and must be eliminated if these treatment modalities are to be successful.

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BASIC SCIENCE ASPECTS OF THE MITOCHONDRIA
SECTION VI

MITOCHONDRIA AND EVOLUTIONARY NUTRITION

Rapid changes in human diet, particularly the last 150 years, are potent promoters of chronic diseases such as atherosclerosis, essential hypertension, obesity, diabetes, and many cancers. In addition to diet, sedentary life styles and exposure to a vast host of noxious substances interact with genetically controlled biochemical processes to chronic degenerative diseases, which have increased exponentially during the past half century.

In the last decade it has been established that genetic factors, both nuclear and mitochondrial, determine susceptibility to disease. Environmental factors determine which genetically susceptible individuals will be affected and, when nutrition is an environmental factor of major importance. Major changes have taken place in the human diet since the beginning of the Agricultural Revolution, but human genes have not changed. Genetically speaking, humans today live in a nutritional environment that differs drastically from that of the genetic constitution of their great-grandparents 150 years ago.

For much of the recorded history of Western civilization, good nutrition meant getting enough calories to ward off hunger for many people, and this became a part of our genetic heritage.

The ability to utilize molecular oxygen in metabolism endowed man and animals with considerably greater efficiency in converting foods to energy. This heightened efficiency however has an inherent biological drawback: the potential toxicity of oxygen due to several unique properties of the oxygen molecule. The initial event in oxygen poisoning is the generation of toxic

free radicals. A number of highly reactive free radicals and related active species are generated from molecular oxygen in living systems, as an unavoidable consequence of aerobic metabolism. An antioxidant defense system is an intrinsic part of every cell to defend organelles, cells, and tissues against free radical-mediated oxidative stress.

The mammalian antioxidant defense system is highly sophisticated: it attempts to maintain redox balance homeostatically, through a variety of inter-regulated mechanisms. It utilizes non-enzymatic nutrient-derived antioxidants acting in concert with nutrient-modulated antioxidant enzymes, to detoxify oxygen radicals and other "activated" species derived from molecular oxygen. The antioxidant defense system is able to react adaptively in response to oxidative challenges, subject to the availability of nutrient-derived cofactors, cosubstrates, and other antioxidant factors.

The diet, before the advent of agriculture, was rich in plants which contained high levels of antioxidants which were utilized by the body's defense system for handling oxygen and the oxidative by-products of mitochondrial oxidative phosphorylation and electron transfer. In the beginning, man’s diet was rich in both antioxidants and omega 3 fatty acids, as well as minerals which abounded in the soil and the water.

The development of agriculture introduced seed grains, or grass seeds, into the human diet. After a few thousand years and extending up until the present time, wheat, corn and rice to a great extent replaced the intake of vegetables and fruits as the staple of the human diet. In the beginning humans ate an enormous variety of wild plants, whereas today about 17% of plant species provide 90% of the world’s food supply, with the greatest percentage contributed by cereal grains.

Three cereals, namely, wheat, maize, and rice together account for 75% of the world’s grain

production. The nutritional implications of such high grain consumption upon human health are enormous. Cereal grains are high in carbohydrates and ω-6 fatty acids, but low in ω-3 fatty acids and in antioxidants. Recent studies show that low fat/high carbohydrate diets increase insulin resistance and hyperinsulinemia, conditions that increase the risk for coronary heart disease, diabetes, and obesity. A number of nutritional, and genetic studies indicate that human's overall diet, including energy intake and energy expenditure, has changed over the past 6,000 years with major changes occurring during the past 150 years in the type and amount of fat.

Most modern food is calorically concentrated in comparison with wild game and the uncultivated fruits and vegetables. Today industrialized societies are characterized by (1) an increase in energy intake and decrease in energy expenditure; (2) an increase in saturated fat, ω-6 fatty acids and trans fatty acids, and a decrease in ω-3 fatty acid intake; (3) a decrease in complex carbohydrates and fiber; (4) an increase in cereal grains and a decrease in fruits and vegetables; and (5) a decrease in protein, antioxidants, and calcium intake.

The various roles of (1) essential fatty acids, including their influence on gene expression, and (2) antioxidants have been recognized as important factors in growth and development and in chronic diseases. The functions of essential fatty acids (EFA) and antioxidants are intertwined. Therefore, the dietary changes that have occurred in EFA and selected antioxidants through agriculture and industrialization are extremely important in terms of gene expression and health.

Studies on the aspects of diet indicate that major changes have taken place in the type and amount of essential fatty acids and in the antioxidant content. Advances in human biochemical genetics have produced data that suggest considerable biochemical variability within and between

human populations. Therefore, the relevance of this genetic information for human nutrition is considerable. Variation in nutritional requirements and the interaction of certain nutrients with genetically determined biochemical and metabolic factors suggest different requirements for individuals. Research is now defining the mechanisms by which genes influence nutrient absorption, metabolism and excretion, taste perception, and degree of satiation; and the mechanisms by which nutrients influence gene expression. Furthermore, advances in molecular and recombinant DNA technology have led to exquisite studies in the field of Genetics and the recognition in a much more specific way, through DNA sequencing, how unique each one of us is, and the extent to which genetic variation occurs in human beings. The importance of the effects of genetic variation has been extensively studied and applied in drug development and evaluation of their metabolism and adverse reactions. In the past two decades, physicians, geneticists, and nutritionists began to study the effects of genetic variation and gene-nutrient interactions in the management of chronic diseases.

Human populations represent vast resorvoirs of genetic variability. Advances in genetic studies have pinpointed significant variability in biochemical and immunologic characteristics for individuals that involve many enzymes, proteins, blood groups, human leukocyte antigen (HLA) systems, etc. Human variability has been demonstrated by use of linkage, other family studies, somatic cell genetic hybridization studies, and molecular genetic studies. Nuclear and mitochondrial genetics indicate that more extensive variability occurs at the DNA level of both these genomes.

Individuality is determined by genes - (both nuclear and mitochondrial genes),

constitutional factors (age, sex, developmental stage, parental factors) and environmental factors (time, geography, climate, exposure to herbicides, pesticides, industrial pollution, socioeconomic status, occupation, education, diet and other xenobiotic toxins). All sorts of interactions among these three sources of variation are possible. Genes do not provide an unalterable blueprint but merely a set of options, each more or less conditional, and to be taken up according to what is being experienced as well as what has been experienced. Thus, gene-environment (nutrient) interaction are variable.

The importance of advances in molecular biology and their application to genetic diseases has revolutionized our concepts and has provided the impetus to use the new techniques to identify those specifically at risk for chronic degenerative diseases. Thus, by the use of the tools of molecular genetics, which combine classical family genetic studies with the newest in recombinant DNA techniques, it is now possible to develop diagnostic tests for specific inherited diseases. These studies were first used for the diagnosis of single gene defects. It is now possible to use this approach to detect an individual's susceptibility to diseases caused by multiple factors. Research advances have developed data pertaining to the diagnosis of genetic predisposition to four major multifactorial polygenic diseases: atherosclerosis, hypertension, diabetes, and cancer.

The increased consumption of ω-6 fatty acids in the last 100 years is due to the development of technology at the turn of the century that marked the beginning of the modern vegetable-oil industry and to modern agriculture with emphasis on grain feeds for domestic livestock (grains are rich in ω-6 fatty acids). The invention of the continuous screw press, named Expeller® by V. D. Anderson, and the steam-vacuum deodorization process by D. Wesson made possible the industrial

production of cottonseed oil and other vegetable oils for cooking. Solvent extraction of oilseeds came into increased use after World War I as the large-scale production of vegetable oils became more efficient and more economical. Subsequently, hydrogenation was applied to oils to solidify them. The partial selective hydrogenation of soybean oil reduced the alpha-linolenic acid (LNA) content of the oil while leaving a high concentration of linoleic acid (LA). LNA content was reduced because LNA in soybean oil caused many organoleptic problems. It is now well known that the hydrogenation process and particularly the formation of trans fatty acids has led to increases in serum cholesterol concentrations, whereas LA in its regular state in oil is associated with a reduced serum cholesterol concentration. From the 1950s, until quite recently, research on the effects of ω-6 PUFAs in lowering serum cholesterol concentrations dominated research support on the role of PUFAs in lipid metabolism. The availability of methods for production of vegetable oils and the myth that their use in lowering serum cholesterol concentration was therapeutically justified led to an increase in both the fat content of the diet and the greater increase in vegetable oils rich in ω-6 fatty acids.

Agribusiness contributed further to the decrease in ω-3 fatty acids in animal carcasses. Wild animals and birds who feed on wild plants are very lean, with a carcass fat content of only 3.9% and contain about five times more PUFAs per gram than is found in domestic livestock. Most important, 4% of the fat of wild animals contains EPA. Domestic beef contains very small or undetectable amounts of LNA because cattle are fed grains rich in ω-6 fatty acids and poor in ω-3 fatty acids whereas deer that forage on ferns and mosses contain more ω-3 fatty acids (LNA) in their meat. Modern agriculture with its emphasis on production has decreased the ω- 3 fatty acid

content in many foods such as green leafy vegetables, animal meats, eggs, and even fish. Foods from edible wild plants contain a good balance of ω-6 and ω-3 fatty acids. Purslane compared to spinach, red leaf lettuce, buttercrunch lettuce, and mustard greens has 8 times more alpha-linolenic acid than the cultivated plants. Farmed fish generally contain less ω-3 and more ω-6 than their counterparts in the wild.

The fatty acid composition of egg yolk from free-ranging chicken compared to the standard U.S. Department of Agriculture (USDA) egg, shows the former has an ω-6/ω-3 ratio of 1.3 whereas the USDA egg has an ω-6/ω-3 ratio of 19.4. By enriching the chicken feed with fishmeal or flax, this ratio decreased to 6.6 and 1.6 respectively.

The use of artificial fertilizers to drive agriculture production in soils which have been depleted of essential minerals and other nutrients has also led to a serious decrease in the nutrient value of mass produced foods

It is evident that food technology and agribusiness provided the economic stimulus that dominated the changes in the food supply. From per capita quantities of foods available for consumption in the U.S. national food supply in 1985, the amount of EPA is reported to be about 50 and the amount of DHA is 80 mg/capita/day. The two main sources are fish and poultry. It has been estimated that the present western diet is 'deficient' in ω-3 fatty acids with a ratio of ω-6/ω-3 of 20 to 25:1, instead of 1:1 as is the case with wild animals and human beings. Before the 1940s, cod-liver oil was given to children as a source of vitamins A and D with the usual dose being a teaspoonful. Once these vitamins were synthesized, consumption of cod-liver oil was drastically decreased. Thus an absolute and relative change of ω-6/ω-3 in the food supply of Western societies

occurred over the last 100 years. Rapid dietary changes over short periods of time as have occurred over the past 100-150 years is a totally new phenomenon. A balance between the ω-6 and ω-3 fatty acids is a more physiologic state in terms of prostaglandin and leukotriene metabolism and interleukin-1 (IL-1) production. The current recommendation to substitute vegetable oils ω-6 for saturated fats leads to increases in IL-1, prostaglandins of the 2 series and leukotrienes of the 4 series, which are proinflammatory, prothrombotic, and vasoconstrictive.

Just as the ω-3 fatty acids were depleted from the food supply and led to an imbalance in the ω-6 and ω-3 ratio, the antioxidant intake has drastically decreased. Our forefathers ate meat, fish, and fresh fruits and vegetables whereas today most of the vegetables eaten are cooked and are limited in number. Prior to the Agricultural Revolution, humans typically used many species of wild plants for food, mostly green leafy vegetables. In addition, they consumed roots, beans, nuts, tubers, and fruit. These foods are rich sources of antioxidants.

Wild plants are rich in antioxidant vitamins E and C and other important antioxidants such as glutathione and carotenoids. The study of wild plants such as purslane provides information about the antioxidant intake of our ancestors. Purslane seeds found in caves date back to 16,400 years ago. This finding is of particular interest in terms of human evolution.

Studies of the fatty acid composition and antioxidant content of purslane show that it is particularly rich in α-tocopherol and glutathione. Glutathione is also a major component of the antioxidant system and has been suggested to play a role in the prevention and treatment of chronic diseases. Purslane contains higher levels of vitamin C but lower levels of β-carotene in comparison to spinach.

Purslane is one of the 8 most common plants dispersed on planet Earth and has been part of the diet of both humans and animals. The large native population encountered in the years 1790-1850 was nonagricultural and obtained their food by foraging and harvesting natural localized species of plants and animals. Recent studies show the vegetable food products of foraging economies of the Pacific Northwest were valuable sources of calcium, magnesium, iron, zinc, and ascorbic acid. One of the studies states:

> *"These members of the Lily, Purslane, Barberry, Currant, Rose, Parsley, Heath, Honeysuckle, Sunflower and Water-Plantain families are among those regularly collected by these foraging groups whose economic strategies were keyed to the use of multiple resources and the storage of large quantities of processed foods. Stored vegetable food along with dried fish provided ample and nutritious diets during seasonal periods of resource non-productivity... Analyses show that these native foods are superior to cultigens in necessary fiber, minerals and vitamins making substantial contributions to pre-contact diets.* "

A wide variety of foods were used to meet nutritional needs. Vegetable foods were systematically gathered and processed in quantity. The native preparation and preservation techniques were important factors in retaining nutrients and in maintaining a balanced diet during seasons of low productivity. Of the 1,300 known food plants, fewer than 20 are currently providing most of our food needs, making humans heavily dependent on a few crops to provide food. A number of these plants are rich sources of vitamins E and C. In addition, plants supply a wide variety of phytochemicals many of which have strong antioxidant activity.

The modern diet is rich in animal fat, which contains lower levels of fat-soluble antioxidants such as tocopherols than vegetable oils. In addition, the vegetable oils used in modern diets supply more ω-6 and less ω-3 than earlier diets. Oil seeds and nuts are good sources of

tocopherols and other antioxidants. The modern oil refining processes, however, remove the larger part of the tocopherols and other antioxidants.

The imbalance of ω-6 to ω-3 fatty acids induce inflammation, which increases oxidative stress. Phagocytes, for example, produce large amounts of free radicals. Saturated fatty acids, which are common in animal fats, appear to be atherogenic, a condition which involves phagocytes and inflammatory responses. In contrast, ω-3 fatty acids are antiinflammatory. Thus the change in the ratio ω-6:ω-3 may have contributed to conditions of oxidative stress. Other major changes in diet, such as higher intake of carbohydrates and especially refined sugars, produce hormonal and other metabolic changes which contribute to development of chronic diseases such as diabetes, heart disease and cancer, in which oxidative stress plays a major role in pathogenesis. Environmental factors such as air pollutants, UV radiation, and the intake of pharmaceuticals which block or inhibit enzymes at the cellular level, contribute to this pathogenesis.

Genetic variation is an important consideration in establishing dietary reference values for people. For this reason, general dietary recommendations are not appropriate for the prevention or management of chronic diseases and are not applicable to different populations.

Humans diets centuries ago were very different from current Western diets. They were lower in total fat, saturated fat, trans fatty acids, and ω-6 fatty acids, and were higher in ω-3 fatty acids, protein, ascorbic acid, and other antioxidants such as α-tocopherol and glutathione. These dietary changes came about as a result of the Agricultural Revolution and for the past 50 years by agribusiness, food technology, and the economic factors that provided the stimulus for the expansion of processed foods. The processing of foods has increased the incidence of chronic

disease, and unveiled a number of diseases that did not exist before, or were very rare in the general populace. Significant evidence suggests that the ratio of ω-6:ω-3 should be reduced to 4:1 or lower, and intake of foods rich in ascorbic acid, vitamin E, and folate should be increased by everyone.

Leading the parade of conditions which lead to chronic degenerative diseases is exogenous obesity which today effects sixty percent of Americans over 40 years of age. In animal husbandry, grass fed steers upon reaching maturity are taken to feed lots where they are fattened on grain. Seed grains are highly efficient fattening agents - for steers and for people.

Our food customs since the agricultural revolution incorporate grains into virtually every meal, sometimes to the exclusion of most other foods. It is likely, under these circumstances, that a pica mechanism leads to vastly overeating these nutrient-poor foods in an effort to attain levels of nutrients which are very low or are non-existent in them.

Individuals fed a nutritionally balanced diet almost invariably loose weight and achieve a fairly lean body configuration, provided they receive sufficient micronutrients and antioxidants. That the average American over 40 is obese and it is evident to anyone who goes shopping in the grocery store and sees people pushing over-laden shopping carts loaded with goodies, based primarily upon seed-grain constituents, such as breads, cakes, cookies, doughnuts, pies and delicious snack-crackers. These consumer products contain artificial flavors and are devoid of micronutrients or antioxidants. The entire nation would benefit from the consumption of far less such pseudo-foodstuffs and the eating of far more truly lean meat, fresh fruits and vegetables.

What particular nutritional supplements an individual needs are based on his or her peculiar genetic makeup and present state of functional health.

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BASIC SCIENCE ASPECTS OF THE MITOCHONDRIA
SECTION VII

MITOCHONDRIA AND ANTIOXIDANT FACTORS FOUND ONLY IN FOOD AND HERBS

With the exception of exogenous obesity, which will be discussed shortly, most of the chronic diseases may also be thought of as diseases of ageing. They occur most commonly with advancing age and with the changes associated with that. Increasingly diseases of ageing are seen at younger ages than was the case a century ago.

These diseases, some inherited and some acquired, invariably involve mitochondrial dysfunction. The mitochondrial dysfunction may be due to deletions and point mutations in mitochondrial DNA which are caused by environmental influences which can interfere with mitochondrial function.

The influence of nutrition on the appearance of ageing phenomenon and the related chronic degenerative diseases is profound.

There is not a perfect diet for the human race, and in a true sense given individual variation, one man's meat may well be another man's metabolic poison from the standpoint of ageing and the appearance of chronic degenerative diseases.

The "poison" nature of some classes of food is most apt to lie in its deficiencies in certain nutrients than its excesses. Some fats may be deleterious in excess largely because of failure of the diet to contain other fats which balance the ratio.

Excesses are largely in the gross intake of nutrients of any class, i.e., overeating of foods beyond the amount needed to provide energy.

This is a common practice in places where foods are plentiful and relatively inexpensive which characterizes the western nations, which are the affluent nations of the world.

The epidemic of exogenous obesity which is endemic in affluent nations is due to the over-consumption of foods based upon denatured wheat flours, breads, pasta, pies, cookies, cakes, crackers, flapjacks and canned foods, as well as the eating of meat which has been fattened by being fed grains before they are slaughtered.

Recent nutritional research reveals that foods based upon gluten-rich grains, such as wheat, may have an addictive quality, mediated by exodorphic substances which mimic endorphins, and chemically induce a sensation of well being.

The problem is made worse by the existence of a second human addiction of fairly recent origin, that of sweet tastes. Wheat-based foodstuffs are often laced with sugar or corn syrup to impart a sweet taste. Most breakfast cereals are sweetened, as are breads, pies, cakes, and donuts.

The problem is capped off by the 20th Century proclivity for drinking a large quantity of beverages based largely on sucrose and/or corn syrup, laced with a flavoring agent and usually carbonated.

All of this together sets the scene for metabolic disaster. There is a failure of the antioxidant defense system to be able to ward off damage due to absence of nutrient derived antioxidants which acts in concert with the nutrient-modulated antioxidant enzymes to detoxify oxygen radicals, as well as other radical species. The result is premature ageing and the early appearance of chronic degenerative diseases such as adult-onset diabetes, arteriosclerosis and cancer, which occur years before these conditions would make their appearance in adequately

nourished individuals.

Several years of research have shown that both animals and people whose caloric intake is restricted have extended life spans. This restriction had its beginnings over a century ago with the popularization of the myth that foods made from whole grains are "wholesome" and are all that is necessary for health. This was the platform upon which Dr. William Kellogg launched his nutritional programs built around whole grain foods - cereals and breads as all that was necessary for production of good health and a long and productive life. These are the perfect diet for the human race - a myth which continues to infect the thinking of nutritionists today to the detriment of the public's health.

The only way in the end to lose weight, i.e., to lose fat, is to eat less of the foods that are making the individual fat.

The chief cause of modern exogenous obesity is the overconsumption of foods made from seed grains, wheat, rice and corn, particularly those which are combined with sucrose, or corn syrup, all of which nutritionally can be viewed as empty calories, or calories not accompanied by nutrients.

A highly successful approach to this for thirty years has been the Adkins diet which limits the intake of carbohydrates to 15 grams daily, thereby effectively limiting or eliminating the intake of foods made from seed grains or containing sugar or corn syrup. This diet results in rapid weight loss and particularly loss of excessive fat. The diet permits the ingestion of green leafy vegetables as salads. The individual stays in a state of low-grade ketosis due to metabolism of fat stores.

The keto acidosis seems in individuals who stick to the diet to induce a sensation of well-

being and high energy which may replace the effects of glutean induced exodorphins.

The elimination of wheat, rice and corn based foods and their replacement with natural vegetables and fruits with a high antioxidant level and omega 3 fatty acids seems to restore balance.

The rest of the diet is high in protein, which should be from healthy animals, chickens, pigs and beef. Animals raised or fattened in feedlots or which are mass produced in captivity are not healthy. They are raised as an industrial product which results in the animal's suffering from the same sort of mitochondrial cytopathology which causes chronic degenerative diseases in humans. They are obese and completely out of nutritional balance. Despite the relatively young age at which they are slaughtered, they frequently suffer from cancer, and other degenerative diseases.

Flabby muscles make for tender meat. Beef is prized for human consumption by its tenderness and succulence, which means it contains a high fat content. Meat which is tender and well marbled with fat does not come from healthy steers.

Chicken and pork are also penned in, force-fed food that is not healthy nutrition, given excessive antibiotics, hormones and other pharmaceuticals until their muscles are fat and flabby which in the industry is called tender and well marbled.

The importance of meat in the human diet is emphasized by Eaton and Horner in their original article (New England J. Med. 312:293 (1985), thusly:

> "Paleolithic populations obtained their animal protein from wild game, especially gregarious ungulate herbivores, such as deer, bison, horses, and mammoths. The nutritional quality of such meat differs considerably from that of meat available in the modern American supermarket; the latter has much more fat -- in subcutaneous tissue, in fascial planes, and as marbling within the muscle itself. Domesticated animals have always been fatter than their wild ancestors because of their steady food supply and reduced physical activity, but recent breeding and feeding practices have further increased the proportion of fat to satisfy our desire for

tender meat. These efforts have succeeded: modern high-fat carcasses are 25 to 30 per cent fat or even more. In contrast, a survey of 15 different species of free-living African herbivores revealed a mean carcass fat content of only 3.9 per cent. Not only is there more fat in domesticated animals, its composition is different; wild game contains over five times more polyunsaturated fat per gram than is found in domestic livestock. Furthermore, the fat of wild animals contains an appreciable amount (approximately 4 per cent) of eicosapentaenoic acid (C20:5), a long-chain polyunsaturated, ω3 fatty acid currently under investigation because of its apparent antiatherosclerotic properties. Domestic beef contains almost undetectable amounts of this nutrient.

Meat from free-living animals has fewer calories and more protein per unit of weight than meat from domesticated animals, but the amino acid composition of muscle tissue from each source is similar. Since the cholesterol content of fat is roughly equivalent to that of lean tissue, the cholesterol content of game would not be expected to differ substantially from that of commercially available meat."

A diet using the leaner meats available from free ranging animals, which are now commercially available, can provide a protein and fat intake which are higher in content of the EPA or Omega-3 fatty acids. For such a diet to be healthy it must be augmented by the vegetables and fruits which contain the proper balance of fats as well as the high levels of antioxidants and antioxidant co-factors.

While the intake of antioxidants as supplements is uniformly recommended, such supplements have been universally disappointing as therapeutic agents unless accompanied by antioxidants contained in fruits and vegetables, which latter also contain the nutrient derived cofactors - cosubstrates and synergistic factors found in fruits and vegetables.

There are today at least 120 edible vegetables, legumes, and herbs which were routinely gathered and may be consumed by people as part of their diet.

The western vegetable diet is largely confined to 20 or so of these, which may not contain all of the co-factors, co-substrates and synergists which are essential to maintain good health.

Some of these continue to be used as spices throughout the world and others have been largely ignored because they aren't to be found neatly handled in the supermarket produce department. Much of the produce which is available from the supermarket is an odorless and tasteless imitation of that which grows in the wild because it has been force farmed on poor soil with the aid of synthetic fertilizers. The missing elements which make up the aroma and flavor left out of such force grown vegetables is the same micronutrients which make up the co-factors, co-substrates and synergists to the antioxidants which make them biologically effective as part of the diet.

Organically grown herbs, vegetables and fruits which are naturally fertilized and grown on a crop rotation basis are far more apt to provide essential nutrients than the pseudo-fruits and vegetables force grown by modern agribusiness.

Vitamins C and E are the workhorses of the cellular antioxidant defense system, and are obtainable only by nutrient intake. Neither of these is effective without their naturally occurring co-factors and synergists, which include the dietary Flavonoids, the carotenoids and Alpha-Lipoic Acid.

Many mammals are capable of synthesizing vitamin C. Primates, including humans, do not possess this ability. Thus, primates are totally dependent on dietary intake for normal function of their antioxidant defense system.

Vitamin C was first discovered and synthesized by Albert Szent-Gyorgi, who found it required co-factors to function properly. These co-factors are now known to be flavonoids.

An example of compounds which act as co-factors and co-substrates to the body's

antioxidant system is found in Flavonoids, carotenoids, and Alpha Lipoic Acid which are found in many fruits and vegetables.

Flavonoids are contained in most fruits that are red or purple, such as red grapes, cherries, or blackberries, apples and citrus fruits, broccoli, kale and onions.

More than 4,000 chemically unique flavonoids have been identified in fruits, vegetables, nuts, seeds and flowers, as well as in some beverages, such as tea and red wine. Flavonoids are also contained in several herbs such as Ginkgo Biloba, Pycogenol and Hypericum. These compounds play a crucial role in the body antioxidant defense, although none of them are synthesized in the body and must be obtained from the diet.

They are not listed in any published recommended daily allowances. Their crucial role in health has been appreciated for centuries by herbalists who prescribe foods and herbs which are rich in these substances for the treatment and prevention of a variety of chronic degenerative diseases.

The only way to ensure an adequate level of these compounds is the ingestion of the plants which contain them and their cofactors. Most seed grains are deficient in these compounds and their cofactors and cannot supply the levels needed.

More than 4000 chemically unique flavonoids have been identified in plants. These low molecular weight compounds are found in fruits, vegetables, nuts, seeds, and flowers, as well as in several beverages, and are important constituents of the human diet. They have important effects in plant biochemistry, acting as antioxidants, enzyme regulators, precursors of toxic substances, pigments, and light screens, to name a few. Selected flavonoids have been shown in numerous *in vitro* and *in vivo* experiments to have antiallergic, anti-inflammatory, antiviral, and antioxidant

activities. Some flavonoids have been shown to exert significant anticancer activity, including anticarcinogenic and prodifferentiative activities. Flavonoid intake has been shown to be inversely related to cardiovascular disease (CVD) risk in epidemiological studies conducted in the Netherlands and Finland. However, similar studies conducted in the United States and the United Kingdom have demonstrated either no association or a positive relationship. Nonetheless, a considerable body of evidence suggests that plant flavonoids may be health-promoting, disease-preventing dietary compounds.

The prominent flavonoids in foods are characterized by several subclasses, including anthocyanidins, flavanols, flavonones, flavones, flavonols, and their metabolic precursors, chalcones. The general structure of flavonoids is two benzene groups connected by a three-carbon (propane) bridge. With the exception of chalcones, all flavonoids found in foods have a pyran ring (oxygen-containing heterocyclic ring), which is formed by the addition of oxygen to position 2 of chalcones and subsequent cyclization of the three-carbon chain with the "A" ring. The various subclasses of flavonoids are derived from this basic structure by changing the oxidation state and substitution (primarily hydroxylation) of the propane portion of the molecule. Thus, flavanols are at the lowest oxidation state (saturated pyran ring) but are substituted with a hydroxyl group at position three (flavan-3-ols or catechins), whereas flavones and flavonols (3-OH flavones) are the most highly unsaturated with a 2,3 double bond and a keto group at carbon 4. All flavonoids have hydroxyl groups at positions 4', 5, and 7 and the corresponding positions of chalcones. Within each subclass, specific flavonoid molecules are identified based on additional substitutions. For example, quercetin is a flavonol with an additional hydroxyl substitution at the 3' position (3,5,7,3',4'

pentahydroxy flavone) and catechin is also a flavan-3-ol with an additional hydroxyl substitution at the 3' position (3,5,7,3',4'-pentahydroxy flavan). There are a limited number of flavonoids within each class that are prominent in plant foods commonly consumed by human beings. These include about three anthocyanidins (cyanadin, delphinidin, malvidin), three flavan3-ols (catechin, epicatechin, epigallocatechin), two flavanones (hesperetin, naringenin), two flavones (apigenin, luteolin), three flavonols (kaempferol, myricetin, quercetin), and three chalcones.

Carotenoids are pigments widespread in nature and more than 600 different compounds have been identified in various organisms. β-Carotene is the most prominent representative of this very lipophilic class of compounds. The basic structure of carotenoids consists of a tetraterpene skeleton, which may be cyclized at one end or both ends of the molecule. Cyclic end groups may be five- or six separate membered ring systems. Carotenoids, which are composed only from carbon and hydrogen atoms, are collectively assigned as carotenes, e.g., β-carotene, γ-carotene, and lycopene. However, most natural carotenoids contain at least one oxygen function such as keto (violerythrin), hydroxy (lutein), or epoxy groups (violaxanthin) referred to as xanthophylls or oxocarotenoids. A common structural element of carotenoids is the extended system of conjugated double bonds, which is responsible for their color and some of their biological functions, such as antioxidant activity.

Carotenoids are synthesized and stored in the photosynthetic apparatus of higher plants where they are involved in the light-harvesting system and in antioxidant defense against photooxidation. Animals and humans are not capable of synthesizing carotenoids but absorb them from the diet and make use of provitamin A carotenoids for the vitamin A supply. Important

dietary sources of carotenoids for the human are green leafy and orange to red vegetables as well as various fruits, including oranges, tangerines, or peaches. The provitamin A carotenoids β-carotene, α-carotene, and cryptoxanthin, as well as the nonprovitamin A compounds lutein, zeaxanthin, and lycopene are the major dietary carotenoids, which are mainly provided by plant-based food. More than 35 different carotenoids have been identified in human plasma, indicating that additional carotenoids are supplied with the diet. Although β-carotene and lutein are found in many different kinds of fruits and vegetables, only a few products contain important other carotenoids such as lycopene or zeaxanthin. About 90% of dietary lycopene in the United States derives from tomatoes and tomato products with more than 50% from processed food. The major source for zeaxanthin is corn. Carrots are an important source for β-carotene along with spinach, broccoli, or green and red peppers.

Fruits also contain considerable amounts of the provitamin A carotenoids β-carotene and β-cryptoxanthin, and it has been suggested that they represent the most important source for vitamin A in developing countries. Another major carotenoid found in fruits is lutein.

Hydroxylated carotenoids in fruits and vegetables may be present as either parent carotenols or esterified with various fatty acids. The carotenoid pattern of papaya and other fruits is dominated by carotenoid esters whereas only low amounts of free xanthophylls are found in some fruits. For example, tangerines contain high amounts of β-cryptoxanthin present mainly in esterified form. Major carotenoid esters in tangerines were identified as β-cryptoxanthin laurate, myristate, and palmitate. Additionally, small amounts of lutein and zeaxanthin esters are detectable.

Carotenoids are also found in various kinds of seafood, including lobster and salmon.

Commercially, astaxanthin is used widely as a feed additive to obtain the typical color of salmon flesh. Various carotenoids are also detected in algae, some of which are capable of storing high amounts of these compounds. Halotolerant algae such as *Dunaliella salina* produce several carotenoids, mainly different geometrical isomers of β-carotene, which amount to more than 10% of the dry weight.

Algae, fungi, bacteria, and other natural sources of carotenoids such as tomatoes or flowers are used to isolate carotenoids for the production of supplements, food, and feed additives. A concentrated source of lutein and lutein esters is found in marigold. Marigold petals contain 20 times higher concentrations of lutein than spinach. Large amounts of carotenoids are synthesized chemically for use in the food or feed industry.

There is increasing evidence that β-carotene and other carotenoids exhibit beneficial health effects in preventing the development of chronic diseases in humans. An increased consumption of a carotenoid-rich diet is associated with a diminished risk for some kinds of cancer and cardiovascular diseases.

It has been suggested that carotenoids may be helpful in preventing photoaging of the skin and sunburn reactions. β-Carotene supplements are widely used as oral sun protectants, and a daily intake over several weeks leads to increased carotenoid levels in human skin and plasma. Lutein and zeaxanthin are the predominant carotenoids in the human macula lutea; none of the major human carotenes are detectable in this tissue. Several lines of evidence indicate that carotenoids may protect from age-related macular degeneration (AMD). AMD is the leading cause of

irreversible blindness among Americans 65 years and older. This disease is the cause of partial vision loss for 1 of every 20 people in the United States. A genetic component for an increased AMD risk has been identified. A number of biological effects have been attributed to carotenoids, including antioxidant activity, influences on the immune system, control of cell growth and differentiation, and stimulatory effects on gap junctional communication. These effects are thought to be relevant with respect to their protective properties.

Research on the antioxidant activity of carotenoids was sparked by the initial description of their property as singlet oxygen ($^1O_2$) quenchers and their ability to trap peroxyl radicals. The discovery that carotenoids inactivate $^1O_2$ was an important advance in understanding the biological effects of β-carotene and other carotenoids. The mechanism by which carotenoids protect biological systems against $^1O_2$-mediated damage appears to depend largely on physical quenching. In this process, the energy of the excited oxygen is transferred to the carotenoid molecule. The energy is dissipated through rotational and vibrational interactions between the excited carotenoid and the surrounding solvent to yield the ground state carotenoid and thermal energy. In the process of physical quenching the carotenoid molecule is not destroyed. It may undergo further cycles of singlet oxygen quenching, thus acting like a catalyst. Isomerization of the carotenoid may occur in the quenching process, via the lowest triplet state of the molecule. The quenching of singlet oxygen by β-carotene and other biological carotenoids occurs with rate constants approaching diffusion control. Lycopene, the open-chain analog of β-carotene exhibited the highest rate constant. Thus, lycopene is the most efficient $^1O_2$ quencher among the biologically occurring carotenoids.

The quenching rate constants of several natural and synthetic carotenoids have been

investigated to elucidate structure—activity relationships. All of the investigated carotenoids proved to be efficient quenchers of singlet molecular oxygen. The quenching rate constant depends on the number of conjugated double bonds present in the molecule. The C-20-dialdehyde polyene showed the lowest quenching rate constant. The C-40-dialdehyde, dinor-canthaxanthin, and violerythrin exhibited the highest quenching rate constants but also retrodehydro-β-carotene, echinenone, 3-hydroxy-β-carotene, 4-hydroxy-β- carotene, canthaxanthin, the C-30-dialdehyde, capsorubin, and β-carotene itself effectively quenched $^1O_2$. The C-40-dialdehyde is among the most potent quenchers of $^1O_2$.

α-Lipoic acid, also known as thioctic acid, 1,2-dithiolane-3-pentanoic acid, 1,2-dithiolane-3-valeric, or 6,8-thioctic acid, is a naturally occurring potent antioxidant. It is present as lipoyllysine in various natural sources. In the plant material studied, the lipoyllysine content was the highest in spinach (3.15 μg/g dry weight; 92.51 μg/mg protein). When expressed as weight per dry weight of lyophilized vegetables, the abundance of naturally existing lipoate in spinach is over three- and fivefold higher than that in broccoli and tomato, respectively. A lower concentration of lipoyllysine is also detected in garden pea, Brussels sprouts, and rice bran. Lipoyllysine concentration has been found to be below detection limits in acetone powders of banana, orange peel, soybean, and horseradish. In animal tissues, the abundance of lipoyllysine in bovine acetone powders can be represented in the following order: kidney> heart> liver > spleen> brain > pancreas > lung. The concentration of lipoyllysine in bovine kidney and heart were 2.64 ± 1.23 and 1.51 ± 0.75 μg/g dry weight, respectively. Lipoic acid is also an integral component of the mammalian cell. It is present in trace amounts as lipoamide in at least five proteins where it is

covalently linked to a lysyl residue. Four of these proteins are found in α-keto acid dehydrogenase complexes, the pyruvate dehydrogenase complex, the branched chain keto acid dehydrogenase complex, and the α-ketoglutarate dehydrogenase complex. Three lipoamide-containing proteins are present in the E2 enzyme dihydrolipoyl acyltransferase, which is different in each of the complexes and specific for the substrate of the complex. One lipoyl residue is found in protein X, which is the same in each complex. The fifth lipoamide residue is present in the glycine cleavage system.

The mitochondrial E3 enzyme, dihydrolipoyl dehydrogenase, reduces lipoate to dihydrolipoate (DHLA) in the presence of NADH. This enzyme shows a marked preference for the naturally occurring R-enantiomer of lipoate. Lipoate is also a substrate for the NADPH-dependent enzyme glutathione reductase. Glutathione reductase shares a high degree of structural homology with lipoamide dehydrogenase. Both are homodimeric enzymes with 50-k Dalton subunits conserved between all species. In contrast to dihydrolipoyl dehydrogenase, however, glutathione reductase exhibits a preference for the S-enantiomer of lipoate. Although lipoate is recognized by glutathione reductase as a substrate for reduction, the rate of reduction to DHLA is much slower than that of the natural substrate glutathione disulfide. Whether lipoate would be reduced in a NADH or NADPH-dependent mechanism is largely tissue specific. Thioredoxin reductase catalyzes the NADPH-dependent reduction of oxidized thioredoxin. Thioredoxin reductase from calf thymus and liver, human placenta, and rat liver has been observed to efficiently reduce both lipoate and lipoamide in NADPH-dependent reactions. Under similar conditions at 20^0C and pH 8.0, mammalian thioredoxin reductase reduced lipoic acid 15 times more efficiently than lipoamide dehydrogenase. The relative contribution of the three different enzymes known to reduce lipoate in

mammalian cells is tissue and cell specific depending on the presence or absence of mitochondrial activity and of oxidized thioredoxin and GSSG.

A major property of lipoic acid is that it can serve as a proglutathione agent and enhance the cellular glutathione (GSH) level. Studies with human Jurkat T cells have shown that when added to the culture medium, lipoate readily enters the cell where it is reduced to its dithiol form, DHLA. DHLA, a potent reducing agent, accumulates in the cell pellet and, when monitored over a 2-hr interval, the dithiol is released to the culture medium. Following lipoate supplementation, extracellular DHLA reduces cystine outside the cell to cysteine. The cellular uptake mechanism for cysteine by the ASC system is approximately 10 times faster than that for cystine by the x_c^- system. Thus, DHLA markedly improves cysteine availability within the cell, resulting in accelerated GSH synthesis.

Both lipoate and its reduced form DHLA have remarkable reactive oxygen-detoxifying properties. Lipoate and DHLA scavenge several reactive species, including hydroxyl radicals, hydrogen peroxide, hypochlorous acid, and singlet oxygen. In addition, lipoate/DHLA have transition metal chelation properties by virtue of which it may avert the transformation of relatively weak oxidants such as the superoxide anions and hydrogen peroxide to the deleterious hydroxyl radical.

Antioxidant reactions are essentially oxidation—reduction reactions in which reactive forms of oxygen are reduced and thus scavenged by the antioxidants; in the process the antioxidant is oxidized to its functionally inert form. Effective functioning of redox antioxidants requires the recycling of the oxidized form of antioxidant to its potent reduced form. DHLA is a strong

reductant and is thus capable of recycling some of such oxidized antioxidants. DHLA can directly regenerate ascorbate and indirectly regenerate vitamin E from their respective oxidized radical forms.

α-Lipoic has been known to be an essential cofactor in oxidative metabolism for many years. It was first purified in 1951 after being recognized that this compound was responsible for a number of compounds displaying the same activity. The earliest of these was in 1937, when the term potato growth factor was coined to describe a substance necessary for the growth of bacteria. From these early studies it was noticeable that lipoic acid was a common constituent of normal animal and plant tissues and was in fact tentatively described as a vitamin after isolation. It is now known that both plants and animals can synthesize lipoic acid from octanoic acid, but the complete biosynthetic pathway is still unknown.

Lipoic acid is bound covalently to a lysine residue of five distinct mitochondrial proteins. Three of these are E2 subunits of the pyruvate dehydrogenase (PDC), the branched chain keto acid dehydrogenase, and the a-ketoglutarate dehydrogenase complexes. These complexes catalyze the oxidative decarboxylation of pyruvate into acetyl-CoA and α-ketoglutarate into succinyl-CoA, respectively. The PDC complex comprises a structural core of approximately 60 E_2 subunits to which multiple E_1 and E_3 components are attached. The E_1 component decarboxylates the substrate and transfers the acetyl group to E_2. The lipoate moiety of E_2 becomes acetylated and later transfers this group to coenzyme A, forming acetyl coenzyme A and dihydrolipoate. The E_3 component regenerates the oxidized form to continue the catalytic cycle. E_2 and E_3 are anchored together by protein X, which also contains the lipoyllysine moiety. The final lipoamide moiety is found as the

H-protein, part of the glycine cleavage system. In plants this is known as the glycine decarboxylase complex, where it catalyzes the conversion of glycine into CO_2, ammonium, and methylene tetrahydrofolate. Plants are also unique in that they contain chloroplastic PDC as well as mitochondrial PDC. Various amounts of each have been found to be species specific. Hence lipoate occupies a central position in metabolism as a regulator of carbon flow into the Kreb's cycle, resulting in the production of ATP.

In the last decade lipoic acid and its reduced form, dihydrolipoic acid (DHLA), have emerged as powerful antioxidants *in vitro*. The lipoate couple, having a potential of —0.32 V, is a strong reductant and as such has the ability of interacting with many cellular thiols, antioxidants, and reactive oxygen species. The antioxidant network is an important line of defense against potential free radical attack *in vivo.* It has been shown in various *in vitro* systems that DHLA can regenerate ascorbate, which in turn recycles vitamin E. Glutathione can also recycle ascorbate, but cannot recycle lipoic acid. DHLA, however, is able to reduce GSSG, and so lipoate can work at even higher levels of the antioxidant network, accepting electrons directly from NAD(P)H. Recycling, or protection, of vitamin E has also been shown in vivo in vitamin E-deficient mice. Lipoic acid supplementation prevents deficiency symptoms and sustains tissue vitamin E levels. Both LA and/or DHLA can scavenge a variety of reactive oxygen species, including hydroxyl, peroxyl, and superoxide radicals, hypochlorous acid, and singlet oxygen, as well as having the ability to chelate metal ions. Thus the lipoate/dihydrolipoate couple represents a powerful weapon in the fight against free radical attack.

This points to the uniqueness of some natural compounds found only in food and herbs

whose anti-oxidant properties cannot be imitated or substituted for by simple synthetic antioxidants.

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BASIC SCIENCE ASPECTS OF THE MITOCHONDRIA
SECTION VIII

DRUGS, CHEMICALS AND MITOCHONDRIA

Maternally inherited and acquired defects in mitochondrial function are now known to cause most of the common diseases of ageing, including Type II Diabetes Mellitus, Atherosclerotic Heart Disease, Stroke, Cancer, Alzheimer's Disease, and Parkinson's Disease. In all of these the function of mitochondria is markedly disturbed, and energy production declines to a critical level below which inherited or acquired mitochondrial errors express themselves.

In 1995, the entire program of the 25th Annual Meeting of the American Ageing Commission and the American College of Clinical Gerontology was directed to the role of mitochondria in the Chronic Diseases of Ageing.

Despite this, most physicians in America are not yet aware of the clear connection between these diseases and abnormalities in mitochondrial function, nor are they aware of the pivotal role their use of blocking and inhibiting drugs has had on creating the disturbances in mitochondrial function which leads to the appearance of these latter disorders.

The diseases of ageing outnumber mitochondrial diseases in children about 5,000 to 1 according to the directors of the Mitochondrial and Metabolic Disease Center at the University of California, San Diego. The chronic degenerative diseases of aging are due in part to the fact that most of the sufferers have been exposed for 20 to 30 years to a host of environmental and toxic pollutants which increase the risk for these diseases. Studying the mitochondrial diseases of children has shed some considerable light on the causes and may lead to the development of effective treatment and preventive strategies of the adult onset degenerative diseases.

The first convincing clue that environmental toxins may cause Parkinsonism came from some young drug users who developed neurological symptoms closely resembling Parkinson's disease. The physicians in the San Francisco Bay area were puzzled that all these patients with Parkinson's like symptoms were too young to have clinical symptoms usually seen in the elderly with Parkinson's disease. Investigating the causes of the neurological symptoms, led to the discovery that all the young patients were drug (heroin) addicts and that their neurological symptoms could be relieved by L-DOPA treatment. Postmortem examination of one of the patients who died of drug overdose showed that the agent which produced Parkinson-like symptoms was a contaminant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the drug preparation. Further studies showed that MPTP produced Parkinson-like symptoms in monkeys similar to those in humans, and from the demonstration that MPTP disrupts mitochondrial energy metabolism, damages the substantia nigra, and induces Parkinsonism in humans. The active and neurotoxic metabolite of MPTP is MPP+ which is formed in the body by the action of glial monoamine oxidaseB. MPP+ is then selectively transported via dopamine transporter into nigrostriatal dopaminergic neurons, where it inhibits mitochondrial complex I, depletes ATP and causes neurodegeneration by an unknown mechanism. Mitochondrial energy deficit is the primary cause of MPTP/MPP+ neurotoxicity. Some studies have shown that MPP+ interacts with mitochondrial complex I, produces free radicals, and causes an irreversible inactivation of complex I enzyme activity. Other studies have shown that nitric oxide mediates MPTP neurotoxicity which can be blocked by 7-nitroindazole, a potent inhibitor of neuronal nitric oxide synthase (NOS). It has also been demonstrated that mice lacking the NOS gene are refractory to MPTP neurotoxicity. A number of investigators have suggested that NMDA receptors play a crucial role in MPTP/MPP+

neurotoxicity, which can be blocked by NMDA receptor antagonists. Some investigators, however, failed to observe the protective effect of NMDA receptor antagonists against MPTP/MPP+ neurotoxicity.

3-Nitropropionate (3-NPA) is widely distributed in toxic plants such as *Astragalus* species and was identified in 1954 as the component of *Indigofera endecaphylla Jacq* responsible for its toxicity to domestic animals. 3-NPA is also produced by the fungus *Arthrinium spp* which was responsible for the development of an acute encephalopathy in Chinese children. Magnetic Resonance Imaging (MRI) of affected individuals showed a bilateral necrosis of the putamen with delayed dystonia in some patients. 3-NPA produces basal ganglia degeneration and extrapyramidal symptoms in humans and in experimental animals. Some investigators have reported age-dependent vulnerability of striatal neurons following intrastriatal, subacute, or chronic administration of 3-NPA in rats. Some investigators studied neurochemical and histologic changes following intrastriatal injection of 3-NPA, others investigated locomotor changes and striatal lesions in 3-NPA treated rats. One group of investigators observed axonal degeneration in the caudate-putamen region of rats treated with 3-NPA. Pretreatment with nerve growth factor, prior decortication, or treatment with glutamate antagonists was able to block the toxic effect of 3-NPA.

The chemical structure of 3-NPA is isoelectronic with succinate; 3-NPA acts as a suicide inhibitor of succinic dehydrogenase, an enzyme of citric acid cycle and a component of mitochondrial complex II. 3-NPA reduces energy supplies of cultured cortical explants and causes neuronal degeneration by an excitotoxic mechanisms. It has been demonstrated that noninvasive spectroscopic imaging can be used to detect neurochemical alterations induced by 3-NPA. Exposure of cultured striatal or cortical neurons to 3-NPA has shown that neuronal cell death

occurs by an apoptotic mechanism.

Studies have shown that 3-NPA decreases synaptosomal respiration in a concentration-dependent manner, and it was reported that the earliest sign of impairment of energy metabolism was a fall in the ratio of phosphocreatine/creatine. In the initial phase of intoxication, 3-NPA selectively inhibits tricarboxylic acid cycle (TCA) of GABAergic neurons; glial TCA cycle remained unaffected during this time. These studies explain why the caudate/ putamen neurons, which are GABAergic, are selectively damaged by 3-NPA. Other studies have suggested that an impairment of energy metabolism by 3-NPA may underlie neuronal death by an excitotoxic mechanisms in laboratory rats. They have provided in vivo evidence for the involvement of free radicals in excitotoxic death of neurons and shown that 3-NPA toxicity was significantly attenuated in copper/zinc superoxide dismutase transgenic mice. It was suggested that both bioenergetic and oxidative stress play an important role in neurodegenerative diseases.

Recent work has shown that chronic exposure to 3-NPA replicates the cognitive and motor deficits and behavioral pathology of Huntington's disease, in baboons and rats, respectively. It has been suggested that treatment of rodents and primates with 3-NPA provides a good animal model of HD. Treatment of animals with Q10 and nicotinamide, agents that improve oxidative phosphorylation and quench free radicals, ameiliorate striatal lesions. The combination of NMDA receptor antagonist, MK-801, with coenzyme Q10 was found to be a more effective treatment for protecting neurons.

Potassium cyanide is one of the most toxic occupational and environmental chemicals. Humans get exposed to toxic levels of cyanide from consumption of cyanophoric plants (e.g., cassava), from tobacco smoke, from alkyl-cyanides used as solvents, from cyanide salts used for

polishing and metal cleaning, and the antihypertensive drug sodium nitroprusside. The primary target organ of cyanide is the central nervous system. Cyanide rapidly inhibits COX activity, lowers energy supplies and causes neurological dysfunction within seconds. Cyanide exposure also causes neuronal degeneration in brain and produces progressive parkinsonism and dystonia. Magnetic resonance imaging (MRI) shows bilateral lesions of the basal ganglia. Positron emission tomography (PET) with 6-fluoro-L-dopa revealed marked dysfunction of dopaminergic transmission similar to that observed in parkinsonism. Chronic cyanide exposure has been associated with motor neuron disease. Cyanide depletes gamma aminobutyric acid and elevates glutamate concentrations in brain. Dopaminergic system of rodents is highly susceptible to cyanide neurotoxicity. Studies have suggested that cyanide selectively affects basal ganglia by an excitotoxic mechanism following disruption of energy metabolism. The role of COX inhibition as the primary biochemical lesion in cyanide toxicity is unresolved. It has been shown that cyanide rapidly depresses synaptic transmission without inhibiting COX activity.

Cyanide increases cytosolic free Ca2+ in energy-compromised neurons by the activation of NMDA receptors and initiates a series of intracellular cascades which culminate in cell death. In PC12 cells, cyanide activates phospholipase A2, stimulates generation of inositol triphosphate through an interaction with the glutamate/ metabotropic receptors and induces an apoptotic cell death. The toxic effect of cyanide can be blocked with NMDA receptor antagonists. Cyanide inhibits brain catalase, superoxide dismutase, and glutathione peroxidase and increases lipid peroxidation in the striatum. Studies suggest that oxidative stress plays an important role in the expression of CN neurotoxicity.

In parts of Africa, where cassava consumption is high and protein intake is low, cyanide is

etiologically implicated in causing neurodegenerative diseases, tropical ataxic neuropathy and konzo, a paralytic disorder characterized by spastic paraparesis. Populations subsisting on a low protein diet on a chronic basis, are good candidates for developing neurological diseases. Cassava harbors a cyanogenic glucoside, linamarin, which liberates cyanide, a potent inhibitor of COX activity. There are two defense mechanisms against cyanide toxicity. First, cyanide is rapidly, but reversibly, trapped by methemoglobin to form cyanomethemoglobin. Second, additional cyanide is detoxified to thiocyanate (SCN-) by the enzyme rhodanese. This mechanism requires sulfane sulfur substrates derived from dietary sulfur amino acids, cysteine and methionine. In protein-deficient individuals, where sulfur amino acid concentrations are low, detoxification of cyanide to SCN- may be impaired and cyanide may be metabolized to neurotoxic cyanate (OCN). Recent work has shown that OCN inhibits COX activity, uncouples oxidative phosphorylation, and blocks the activity of glutathione reductase and reduces glutathione in rodent brain.

Sodium azide is very reactive toxic chemical which is rapidly converted to volatile hydrazoic acid. Sodium azide is extensively used as a herbicide, fungicide, insecticide, and in inflatable “air bags” in automobiles and emergency escape chutes for aircraft. Sodium azide is a potent inhibitor of COX activity of the mitochondrial respiratory chain and may deplete energy supplies in certain brain regions. COX inhibition may lead to increased free radical (azidyl and hydroxyl) formation by the mitochondria. Chronic and continuous administration of sodium azide in rats impairs learning, and produces memory deficit. Evidence is accumulating that the toxic effects of NaN3 (such as convulsive seizures) may be due to its conversion to nitric oxide. Acute or chronic exposure with NaN3 produces pathological lesions in substantia nigra, a brain area commonly affected in parkinsonism. Demyelination, necrosis of the optic nerves, caudate nucleus,

and putamen are common in monkeys treated with repeated doses of NaN3. Recent work has shown that NaN3 causes striatal damage by an excitotoxic mechanism following energy depletion.

Carbon monoxide is a highly poisonous, odorless, colorless, and tasteless gas. It is an ubiquitous environmental pollutant produced by partial oxidation of hydrocarbons from natural gas or by the gasification of coal. Fuel combustion in areas of limited ventilation is a common cause of acute CO poisoning. CO combines with the hemoglobin of the blood to form carboxyhemoglobin and thereby blocks its oxygen binding/carrying properties. CO exposure blocks ATP generation,by inhibiting COX activity of the mitochondrial electron transport chain, and causes severe extrapyramidal degeneration. The toxic symptoms of CO poisoning may include dizziness, convulsions, coma, respiratory failure and death. Humans with CO poisoning develop parkinsonism six weeks postexposure. The pathological changes produced in the brain by CO are similar those seen in hypoxia-ischemia. Bilateral necrotic lesions of the globus pallidus are recognized as a hallmark of CO poisoning.

Manganese (Mn) is an essential element required for the maintenance of normal health, but it causes neurotoxicity in rodents, monkeys and humans. In mining workers, acute intoxication with Mn causes speech impairment, irritability and hallucinations. Human exposure to Mn occurs through use of potassium permanganate, a powerful oxidizing agent. Manganese is widely used as a fungicide in agriculture, where workers develop neurological signs of parkinsonism and dystonia. Neuropathological lesions in Mn poisoning are found in substantia nigra, globus pallidus, caudate nucleus, and putamen. Decreased dopamine levels are found in the striatum of humans, primates and rodents. Evidence has been provided that both divalent and trivalent manganese produce reactive oxygen species. Manganese preferentially accumulates in mitochondria, and causes

neuronal degeneration by an excitotoxic mechanism secondary to inhibition of cerebral oxidative energy metabolism.

Mercury occurs in the environment as an element and in inorganic and organic compounds. Many humans are continuously exposed to minute concentrations of inorganic and organic mercury through mercury-amalgam dental work. Since the outbreak of Minamata disease following mercury poisoning in Japan, extensive studies of the pathological and clinical changes in affected patients have been performed. Mercury and its compounds disrupt protein synthesis and energy transformation. Alkyl mercurials affect synaptosomal respiration and perturb citric acid cycle and mitochondrial electron transport chain. The predominant pathological changes occur in the cerebral cortex, but granule cells and basal ganglia are also affected. Mercury intoxicated subjects display parkinsonian features, rigidity, tremors, ataxia, impairment of speech, and memory deficit. Subcellular distribution studies have shown that Hg preferentially binds to the mitochondria and microsomes.

Despite a long history of lead poisoning, the precise mechanism of its neurotoxicity is unknown. Lead has neurotoxic effects on both central and peripheral tissue. Exposure to lead has greater toxic effects on the nervous systems of children than adults. In addition to neurotransmitter changes, lead affects energy metabolism before neuropathologic changes. Lead interacts with magnesium in the mitochondria and thereby affects oxidative phosphorylation. It has been reported that neonatal exposure of rats to low levels of Pb produces changes in phosphorylation activity in brain mitochondria. Relatively high concentrations of lead are required to inhibit mitochondrial respiration in the cerebellum.

In a significant proportion of patients with Acquired Immuno-deficiency Syndrome

myopathy has been observed in those receiving the anti-viral agent AZT (3'-Azido-3'-deoxythymidine), and this is often reversed if AZT treatment is suspended. Some of the reported clinical features observed in patients with AZT-induced myopathy include lactic acidosis, myalgia, muscle weakness and abnormal skeletal muscle mitochondria (i.e., ragged-red fibres). Such features are typical of mitochondrial myopathy, except that AZT-induced myopathic patients have elevated serum creatine kinase levels. Examination of mitochondrial respiratory complexes revealed reduced activities of succinate cytochrome-*c*-oxidase and cytochrome-*c*-oxidase. Mitochondrial DNA content was lower in patients with AZT-induced myopathy, presumably due to the inhibition of mitochondrial γ-DNA polymerase by AZT.

When dosed at relatively high levels of AZT (50-100 mg/kg body weight per day) for extended periods (35-70 days), animals developed mitochondrial abnormalities in cardiac and skeletal muscle, weight loss, elevated serum creatine kinase and lactic acidosis. Histological studies revealed ragged-red fibres in skeletal muscle and mitochondrial proliferation of abnormal morphology in skeletal and cardiac muscle tissues in which the highest levels of AZT were found. Isolated skeletal muscle mitochondria from treated animals had impaired Complex I and II activities. Interestingly, brain mitochondria were unaffected, perhaps reflecting the biodistribution of AZT, where brain tissue contained less than 20% of the AZT content of skeletal muscle. It was concluded from these studies that AZT, as a consequence of its inhibition of mitochondrial γ-DNA polymerase, is a mitochondrial toxin affecting Complexes I and II.

In his chapter on Defects in mitochondrial function, in V. Darley-Usmar and A.H.V. Schapira (Eds), Chapel Hill and London, Portland Press (1994), "Mitochondria: DNA, Proteins

and Disease", David John Hayes lists the following drugs reported to cause mitochondrial dysfunction:

Agent	Use/Action	Abnormalities
DPI	Complex I Inhibitor	Reduced oxidative enzymes, muscle fatigue
Antimycin A.	Complex III Inhibitor	Swelling/disruption of mitochondria
Oligomycin	Complex V Inhibitor	Swelling/disruption of mitochondria
Fluoroacetate	Citric acid cycle inhibitor	Ultrastructural, reduced oxygen uptake
Crotoxin	Snake venom	Ragged-red fibres, ultrastructural
Prenylamine	Vasodilator	Reduced oxygen uptake, inhibition of calcium transport
Bayer K 8644	Calcium channel agonist	Ultrastructural
Germanium (GeO_2)	Trace element	Ragged-red fibres, ultrastructural
Zidovudine (AZT)	Antiviral	Ragged-red fibres, reduced oxygen uptake, ultrastructural
Clofibrate	Lipid lowering	Ultrastructural, CoQ deficiency?
Statines		
Lovastati		
Cyclosporin	Immune suppression	Ultrastructural
Emetine	Amoebiasis/vomiting induction used chronically by anorexics	Ultrastructural, reduced oxidative enzymes

Neurons have an absolute dependence on a continuous supply of ATP to support ion pumps in excitable and synaptic membranes, intracellular neuronal and axonal transport, neurotransmission, and the synthesis of energy requiring enzyme systems. A plethora of scientific evidence supports the notion that the rate and duration of energy deficit plays a major role in dictating the distribution and pattern of neurodegeneration. Neuronal peikarya with glutamatergic inputs, such as the striatum, hippocampus and substantia nigra, are especially vulnerable to an abrupt and severe toxin-induced decline in energy status through an excitotoxic mechanism. For example complex I inhibition by methyl phenyl pyridinium ion (MPP+), complex II inhibition by 3-NPA and malonate, complex IV (COX) inhibition by cyanide, causes selective neuronal degeneration in the brain. Sodium azide that inhibits COX activity, induces excitotoxic striatal

damage. Complex V inhibition by cyanate, which uncouples the strict relationship between electron transport and oxidative phosphorylation, inhibits brain COX activity both in vitro and in vivo, and causes striatal and motor neuron degeneration. Mild energy depression of chemical energy produced by the inhibition of the glycolysis-citric acid cycle or attenuation (rather than blockade) of mitochondria electron transport chain compromises the delivery of materials via axonal transport and causes primary distal axonal degeneration. Disruption of energy metabolism initiates a vicious cycle of biochemical events that culminates in neurodegeneration.

Similar processes, less immediately discernible than those in the nervous system take place in the cells, tissues, and organs of other systems which are subjected to chemicals which have the property of uncoupling oxidative phorphorylation or otherwise interfering with mitochondrial function in the heart, liver, kidneys, lungs, pancreas or muscles. Such chemicals may be environmental pollutants or may well be pharmaceuticals which have the property of blocking or inhibiting bodily processes.

Until the cultural gap between science and its assimilation into the medical education system closes, the facts of mitochondrial diseases and conditions will remain more of a mystery to the allopathic medical profession than the dark side of the moon and it will remain a part of the problem rather than the solution.

One of the bad mistakes we may have been making is the indiscriminate use of drugs to treat such diseases, because it is beginning to appear that many drugs also have the property of interfering with the ability of the mitochondria to make ATP although there is enough fuel and oxygen available for that purpose.

Although interference with mitochondrial function is the way in which many synthetic pharmaceuticals create subtle and long-term toxicity and unwanted side effects at this time, drugs are not tested nor are required to be tested to determine whether or not they have the property of interfering with the ability of mitochondria to produce ATP.

Many modern pharmaceuticals achieve their effect by blocking or inhibiting some biological process. For instance Prozac and Luvox are selective seratonin re-uptake inhibitors and antibiotics inhibit the metabolic processes of bacteria.

There is a trend in pharmacology to select drugs which block or inhibit some physiological

process or biochemical event. A few examples should suffice to illustrate this:

Selegiline (Eldepryl): Mechanism of Action: Potent monoamine oxidase (MAO) type-B inhibitor; may also increase dopaminergic activity by interfering with dopamine reuptake at the synapse.

Sertraline (Zoloft): Mechanism of Action: Selective inhibitory effect on presynaptic seratonin reuptake.

Sibutramine (Meridia): Mechanism of Action: Blocks the neuronal uptake of norepinephrine and serotonin and to a lesser extent, dopamine.

Simvastatin (Zocor): Mechanism of Action: competitively inhibiting the enzyme that catalyzes the reduction of HMB CoA reductase

Sparfloxacin (Zagam): Mechanism of Action: Inhibits DNA-gyrase; inhibits relaxation of supercoiled DNA and promotes breakage of double stranded DNA.

Tetracycline (Achromycin, achromycin V, Ala-Tet, Nor-Tet, Panmycin, Robitet, Sumycin, Teline, Tetracyn, Tetralan, Topicycline): Mechanism of Action: Inhibits bacterial protein synthesis by binding with the 30S and possibly the 50S ribosomal subunit(s) of susceptible bacteria; may also cause alterations in the cytoplasmic membrane.

This list might easily be made pages long, but these few examples should suffice to illustrate.

While mitochondria have their own DNA, they are also dependant to a large extent on the cells nuclear DNA to produce proteins, which they use.

While some mitochondrial disorders are due to intramitochondrial events, many others are dependent on both mitochondrial and nuclear DNA so the function of mitochondrion can be vulnerable to chemicals which inhibit either mtDNA, NDNA, or both.

Persons suffering from a Chronic Degenerative Disease should be thoroughly evaluated to determine what prescription drugs they ingest, what over-the-counter drugs they ingest and what recreational drugs they ingest as well as how much of their diet contains chemicals which are capable of interfering with the function of mitochondria and the production of ATP in sufficient quantities to maintain health. Unless such ingestion is stopped, there will be little possibility of reversing these diseases.

In antibiotic use, chemicals, which inhibit the life processes of bacteria, inhibit the life processes of all bacterium, including several which are essential to health such as the gut flora and also the mitochondria which are bacterial symbionts.

There are circumstances in which the administration of antibiotics are essential to save lives and such use is justified, but even then, these should be administered in such a way as to minimize their impact on non-pathogenic and essential bacteria.

It is common practice to administer antibiotics orally, in which case the bacterium, which make up the useful gut flora are harmed along with the pathogenic bacteria. The life saving therapy should be administered by injection rather than orally in order to avoid dysbiosis.

Much of the damage from the indiscriminate over-use of antibiotics has been done over the past 50 years has resulted in both the widespread development of antibiotic resistance, as well as the severe impairment of gut ecology in people who have received oral antibiotics for every conceivable indication. Antibiotics have no effect on viruses and are of no use in the treatment of viral disease, but they have been used to treat viral diseases routinely, because they were regarded as panaceas.

To the Allopathic mind, as well as to the regulatory agencies, toxicity to be appreciated, must be immediate; apparent within a day or week or it is ignored; long term toxicity is simply not considered or even thought of until months or years later, when it results in serious impairment or failure of some organ, frequently the liver.

Much the same is true of the thousands of synthetic compounds routinely added to processed foods to extend their shelf life.

In the race to develop new and patentable molecules and get them to market, long-term toxicity is ignored or information indicating its presence is suppressed and denied by the proponents of the clinical usefulness of the new molecule.

For the past two centuries, thoughtful people have been warning that the use of toxic substances as drugs is ultimately harmful. Samuel Hahnemann's polemics are well known. During the Civil War a Dr. R. T. Troll gave an address at the Smithsonian Institute, attended by President Lincoln and a number of Government officials in which he commented that the allopathic drug treatments then predominant and taught in medical schools were "active in philosophy, abysmal in

science, in opposition to nature and in direct conflict with every law of the vital organism, and that it's application to cure of diseases and the preservation of health is uncertain, dangerous and often fatal and, on the whole, vastly more injurious than useful, pointing out that in the treatment of sicknesses patients receiving no medicine did better than those who did." A Century later, the National Institute of Environmental Health Science in a large seminar on the Biological Relevance of Immune Suppression induced by Genetic, Therapeutic and Environmental Factors, had much the same message, which was also studiously ignored by the host of officials who attended. By 1998, adverse drug reactions had risen to the 4th leading cause of death in the United States and many people had begun, belatedly, to realize that much of drug therapy could be responsible for a literal epidemic of chronic degenerative diseases and organ dysfunction's which have occurred and are occurring during the 20th Century.

In Allopathic medicine, all diseases are classified as groups of signs and symptoms which usually have been recognized as a group or distinctive hallmark of a discrete illness. These are given names and from that point on, they are treated as an entity, known as the disease entity. In many of these, the cause of the disease is not known and treatment usually does not aim to remove or correct the cause or causes so much as to suppress the symptoms so the patient feels better.

Sometimes the causation of the disease is known or a cause is suspected and, in such cases, an effort is made to remove the cause. Many diseases are thought to be caused by bacteria and viruses; some of them are; in others, the bacteria appear after the tissue has been damaged by the disease and act as scavengers. Some diseases are caused by a lack of some one or more nutrients for which the body has a specific need and cannot make for itself. This lack leads to a breakdown in some essential process in the metabolism of a cell or group of cells which body stores of the nutrient are exhausted and not replaced by nutrition, either in the diet or by supplementation of the diet. Occasionally a disease might be caused by dietary excesses and intake of so much of a substance that the body cannot eliminate it or some metabolite of it – this happens, for instance, in gout. But whatever the mechanism, disease happens when the cells or some group of cells run out of energy and are no longer able to carry out their function or functions, and stay above the expression threshold of the inherited mitochondrial mutations.

Allopathic medicine has treated diseases by administering substances which have the effect

of masking symptoms, or attempting to do so. Increasingly, drugs which block some natural biological process were used to mask symptoms and were administered clinically for years to control symptoms of metabolic disorders, to lower blood sugar, to block hormone production, or to suppress enzyme production; these chronically administered drugs combined with chemicals in the food and water, as well as preservatives in milk, and meats also block life processes. Some of the processes blocked were in the mitochondria of cells, the organisms where energy is produced.

Mitochondria produce ATP in a complicated multi-step reaction, utilizing oxygen in its final step to combine with hydrogen, so that the waste product is water, a substance, which causes no damage to the structure of the cells. When this process is blocked or interrupted, energy production fails and free radicals are produced; these damage the structure. When cells are unable to produce energy they degenerate, become non-functional. They either become dormant or they become malignant and chronic degenerative disease is the result. If the process of energy production in the mitochondria can be restored, the cells and the organs they make can be restored to function – regenerated.

While many things are capable of interfering with mitochondrial function, pollution, extremely low frequency electromagnetic radiations, natural toxins, none does so more severely than chemicals, which are designed to interfere with biological function – pharmaceuticals, taken directly into the body at regular intervals for protracted periods of time.

The chemicals can be prescription drugs, over-the-counter drugs, street or illegal drugs, additives such as fluoride in the drinking water, aspartame used as an artificial sweetener or mercury leeched from a tooth filling – whatever the source, chemicals capable of disrupting mitochondrial function will hasten the expression of degeneration in cells if regularly ingested.

The first step towards regeneration is to stop ingesting the chemical which is producing the degeneration. We must assume that most synthetic chemicals are capable of having, causing or contributing to this degeneration.

The next step is to detoxify the body to remove all such chemicals and residues of such chemicals from the body. This is accomplished by a number of steps including the use of nutrition.

Then the oxygen levels of the tissues must be enhanced and finally, the appropriate electromagnetic vibrations are applied. Some of these are known, but further experimentation

based upon our present knowledge of which of these have been successful needs to be done.

Chemicals capable of interfering with mitochondrial function are quite ubiquitous in the environment of everyone living in the late 20th century. They are in the food, the water, the air and the dust which accumulates on every surface of homes and work places. The task is to avoid as many of these as possible. Since most people cannot move to a pristine environment, if there are any more of these, the best that can be done is to make our environment as pure as possible.

We can filter and treat the air we breathe indoors. We can filter and purify the water we drink and bathe in. We can treat the foods we eat to remove most of the contamination and we can avoid eating foods that have chemicals added to them in the processing. We can shield our houses and offices from some of the harmful Electro-magnetic radiations; we can use appliances and deices which do not produce these and avoid those that do. We can stop consuming synthetic pharmaceuticals as drugs and find more natural ways to achieve relief from symptoms.

Regeneration of cells, tissues and organs, which have become degenerated, is an ongoing and arduous task. No one can do it for us – there is no magic solution. If we are to accomplish this, we must devote a considerable portion of our energy, time and income to it. The government cannot do it for us; in fact, quite a lot of our present exposure to these chemicals can be laid at the door of governmental agencies which are supposed to regulate our environment but fail to do so adequately.

The FDA and Department of Agriculture allow the inclusion in many foods of chemicals which produce degeneration. Many industrial activities produce some of the chemicals as a by-product of their operations and where the government is slack on regulation, these are allowed to be dumped in the air we breathe and the water we drink and bathe in.

We can begin by sharply questioning the premises of the Allopathic School and its knee jerk prescription of synthetic drugs for every minor ache or pain. People should quit gulping pills with serious effects for the treatment of minor aches and pains. We can re-explore the rich heritage of the use of herbs over the millennia, as well as Homeopathy.

People should quit buying milk which has been laced with formaldehyde and other chemicals so it can be transported for thousands of miles and stored for months. In the process, it has been devitalized, degraded and turned into a slow poison rather than a healthy food. We should

refuse to buy or eat meats, which have residues of antibiotics, hormones and other harmful chemicals, which can and do harm their bodies. It is possible to find natural substitutes for milk in the diets. Real milk is a wholesome food, but it is not really essential for adult diets.

The main point of ingress of chemicals which cause degeneration is through the digestive tract and the liver and a healthy gut can prevent a lot of assimilation of many chemicals. Unfortunately, due to years of ingestion of oral antibiotics, many people suffer from dysbiosis, an imbalance of the helpful bacteria, which live in the gut and their replacement by other organisms. If the ingress of some toxins is to be controlled, it is necessary to restore healthy gut foundation, replace the harmful bacteria with those which should be there, and restoration of normal function.

A good liver detox, under the supervision of a health care provider who understands this procedure, should be done. A course of EDTA chelation to remove heavy metal contaminants from the body and blood is probably essential for anyone over 40 years of age and should be done if they feel healthy, in order to stay that way. If they are suffering from one or more degenerative diseases, then a more comprehensive program is essential to regain health and maintain it.

Every patient suffering from a chronic degenerative disease involving mitochondrial function should have a comprehensive detox program, including saunas, hot baths, liver flushes, coffee enemas, ozone baths, Rolfing, Chiropractic adjustment, Acupuncture and Homeopathics, all as outlined in Krohn, et al, "Natural Detoxification".

In addition, a comprehensive program for nutritional supplementation adjusted to the individual and the diagnosis, should be implemented after examination by a physician.

As can be seen from a review of the rapidly growing literature on Mitochondrial Diseases, quite a few diseases which are due to mDNA deletions are manifest in infancy and early childhood, as syndromes, some of which are rapidly fatal, some of which can be treated. These distinct syndromes can be and should be treated in centers where this is diagnosed and treated. There is a growing awareness of much later onset of mitochondrial diseases which are due to decline in mitochondrial function with age due to mutations or to toxic influences with mitochondrial processes. Deficiencies in oxygenation and repeated episodes of ischemia and reperfusion which

lead to generation of Reactive Oxygen Species and other free radicals can lead to mitochondrial damage and decline in mitochondrial function.

To distinguish these from the infant and early childhood diseases, we call these acquired mitochondrial diseases and disorders. These acquired mitochondrial dysfunctions which lead to degeneration of tissues and organs can be treated, reversed and prevented by regenerative therapies, most of which can be carried out on an outpatient basis.

The relatively slow onset of some of these disorders, due to a gradual diminution of tissue oxygenation rather than the abrupt onset of ischemic disorders, leads to damaging of tissues rather than the apoptosis seen after ischemia and reflow. The affected cells become dormant; their metabolic fires banked and slowly smoldering rather than amply oxygenated, and such dormant cells can be brought back to normal or near normal function by appropriate measures.

Examples of such dormant cells are found in macular degeneration which can be reversed by electro-magnetic therapies; dormant cells in the penumbra surrounding infarcts in strokes which can be restored to function by hyperbaric oxygen therapy which avoids the excitotoxin induced apoptosis of reflow by supplying oxygen to the tissues, and the reversal of cardiac myopathy by pulsed electromagnetic therapy discussed below.

Since mitochondria are similar to bacteria, it should come as no surprise that antibiotics which attack bacteria, arrest their growth, can have a profound and ofttimes adverse effect on the structure and function of some of the mitochondria of the cells. Oral antibiotics, which affect not only mitochondria but gut flora adversely, creates a dysfunctional gut ecology which is its turn allows toxins to reach the cells of various systems and interfere with mitochondria ecology.

Mitochondrial DNA encodes for the production of a number of proteins which are essential

for the carrying out of oxidative phosphorylation. The production of these proteins is being studied by genetic biochemists who routinely utilize common antibiotics to block such protein processing. Some of the antibiotics so used are chloramphenicol, tetracycline, and erythromycin. Other chemicals are capable of acting as uncouplers of phosphorylation, i.e., sodium fluoride, which in many places is routinely added to the drinking water.

There is no way of knowing what synthetic compounds used either as drugs or as herbicides, pesticides and fertilizers or resulting from industrial processes, and capable of interfering with or interrupting the life processes of mitochondria, are routinely dumped into the aquifer from which they find their way into the human digestive system and eventually reach the mitochondria to disrupt mitochondrial function.

Antibiotics are singled out here along with fluoride as examples but there are thousands of synthetic compounds used therapeutically and industrially which are capable of affecting the structure and function of mitochondria, interfering with the production of energy (ATP) as well as causing the generation of Reactive Oxygen Species and other free radicals.

Thus, a person swallowing two capsules of an oral antibiotic along with a glass of typical urban tap water laced with fluoride, as "treatment" for a common cold, may well end up suffering serious consequences to his or her mitochondrial energy production and ultimately suffer tissue and organ degeneration as a result.

Many food additives used to extend the shelf life of processed foods may be capable of producing mitochondrial damage as well.

Meats frequently contain the residues of antibiotics which are included in the feed of. Some of these antibiotics remain in the meat whether beef, pork or poultry, along with hormones used for

promoting growth can reach the human digestive system, and where faulty bowel ecology exists may allow these to reach the blood and then be carried to organs and to the mitochondria of the cells of organs.

The healthy cell is in what Szent-Gyorgi termed the β or oxidative resting state, producing abundant energy for its work through oxidative phosphorylation, and the electron transport chain of the mitochondria; when something interferes with this oxidative function to decrease energy production significantly, the cell begins to revert towards the α state, its original primordial or anaerobic state. When it does so, it loses its functions and is barely able to maintain its structure -it has become degenerated. It may revert completely to the primordial state and resume the incessant mitosis characteristic of malignancy, or it may not reach that state and simply become dormant - living but not functioning.

Along this path to regression, there is always the possibility that it can be returned to the healthy oxidative state which sometimes occurs spontaneously. The cells have the capacity to return to normal function, but ofttimes this capacity is not utilized.

The purpose of treatment is to assist this return to normal function. The only treatments which offer hope of return to normal function are those which remove the causes of the regression and restore the capacity to return to function. These appear to be:

(1) Detoxification

(2) Orthomolecular nutrition

(3) Oxidation

(4) Pulsed electromagnetic stimulation at an appropriate frequency

These measures have been highly successful in restoring healthy function by restoring the

function of the mitochondria. Such treatments may be a bit more complex than appear from this short list.

Detoxification, the essential first step, is a multiphase process which involves nutrition. Fortunately, these have recently been described with great precision and in detail by Josephine Krohn, M.D. and her co-authors in their book "Natural Detoxification," Point Roberts, Washington, Hartley & Marks Publishers (1996) which constitutes a definitive manual of detoxification procedures.

This manual includes measures for the restoration of normal bowel ecology. This is important since the bowel is the ingress route for virtually all the toxins capable of interfering with mitochondrial function. Much nutritional therapy is involved in detoxing and to those processes the consideration that human mitochondrial DNA occurs in eight distinct haplotypes, which probably coincide to the eight metabolic types should be taken into account. Orthomolecular nutrition, in this case, means a basic diet which is appropriate for the individual's metabolic type. The cancer therapy of William Donald Kelly, one of the most consistently successful alternative cancer therapies developed to date, is based largely on this concept. There are certain nutrients which support oxidative phosphorylation and certainly these and the known antioxidants should be supplied along with the basic diet appropriate to the individual's metabolic type. Appropriate nutrition means not only the intake of nutrients which are correct but also the avoidance of the substances which can adversely effect mitochondrial function. The diet, both food and drink must, of course, not contain any of the mitochondrial toxins. The water must be free of any traces of fluoride as well as the hundreds of chemicals which are routinely found in certain water supplies. The foods must not contain traces of herbicides, pesticides, inorganic fertilizers, food colors or

additives, including those approved by the Department of Agriculture and the FDA for use as food additives or as within allowable limits for use as food. The tolerance for such substances in the treatment of mitochondrial dysfunction is zero. The foods and beverages must contain no aspartame or Nutrasweet - this product is currently found in over 5,000 commercial foods and beverages. It is reported to cause a mosaic, a number of conditions, particularly Multiple Sclerosis and Systemic Lupus, both of which are mitochondrial disorders. Reportedly, when this product reaches a temperature exceeding 86 degrees F. it converts to Formaldehyde and Formic Acid - below that temperature, it metabolizes to methylalcohol. There is more than ample evidence to absolutely exclude this product from the diet of anyone who is suspected of mitochondrial dysfunction. Some of the metabolic type diets include meats, and these must be free of antibiotic residues; seafoods should be confined to those originating and living well away from coastal waters, particularly seafood consisting of Northern fish caught at least 50 miles off shore. Poultry should be of the free-range variety and not that raised in crowded cages and fed antibiotics and other chemicals. Fruits and vegetables, to be eaten raw, should be thoroughly ozonated at the point of consumption to eliminate all herbicide and pesticide residues, and to destroy pathogenic bacteria such as E. coli 0157-H7, Salmonella and similar organisms involved in Food-borne Diseases[1]. The toxins produced by such microorganisms can destroy mitochondrial function. Since toxins can be and are achieved transdermally, all bathing water should be purified by ozonation and not chlorination.

Another highly successful alternative cancer treatment, Essiac Tea, is an herbal

[1]See Fox, Nichols, SPOILED: The Dangerous Truth About Food Gone Haywire, New York, Basic Books (1997)

detoxification. The tea must be brewed from absolutely pure water. Green tea is also reported to be an extremely efficient preventive of cancer and it too must be brewed from absolutely pure water.

Oxygenation: To the methods of oxygenation discussed in Krohn, et al's Natural Detoxification should be utilized. This is extra pulmonary oxygen enhancement by transdermal diffusion. In this method, the individual is immersed up to the neck in a tub of water through which Ozone has been and is being bubbled. This leads to a rapid rise in tissue oxygenation which, reportedly, lasts longer than the increased tissue oxygenation achieved with hyperbaric oxygen and eliminates the drawbacks inherent in HBO. It is far less expensive than chambers, it causes no difficulty with the ears, it does not cause claustrophobia and, since it is extrapulmonary, does not entail oxygen toxicity which is a pulmonary event. It can be engaged in as often as necessary to maintain high levels of oxygen in the extracellular fluid where it is readily available to the cells, and it avoids problems such as oxy-hemoglobin disassociation shifts.

So far only one pharmaceutical, Dichloroacetate, has been reported to be of any benefit in the treatment of mitochondrial diseases; all other pharmaceuticals should be suspected of being mitochondrial toxins. Some may not be, but until that is reliably established, they should empirically be handled as if they are. All synthetic compounds, those not occurring in nature, and synthesized from petroleum should be assumed to be capable of interfering with mitochondrial function.

Dental amalgam restorations are certainly capable of producing a mitochondrial toxin in the form of methylated mercury. Most other heavy metals should be avoided and where present in fat stores, should be removed by detoxification.

In the regenerative treatment of mitochondrial dysfunctions, if at all possible,

pharmaceutical treatments should be replaced by herbal, nutritional, homeopathic or other natural therapies. Sometimes this may not be possible.

During and after treatment, the principles of Natural Hygiene should be followed so far as this is possible in an urban environment.

Air and water can be filtered, oxygenated and sometimes electrostatically treated to remove pollutants.

Geopathic stress can be avoided. Bedding, such as mattresses and springs, can be replaced by air mattresses, which should be placed on wooden platforms to avoid all coil springs and other metallic support which can generate or accumulate electromagnetic frequencies. Some household appliances which generated high gauss magnetic fields can be eliminated. Quartz watches should not be worn on the body. Areas of Geopathic stress should be avoided, particularly for work and sleeping areas.

Acupuncture by a skilled practitioner can support many systems and organs energetically and can be a valuable aid for maintaining good bowel function, which is represented along several acupuncture meridians.

Spinal manipulation by a skilled Chiropractor or Osteopath can be crucial where the nerve supply to organs arises from the spinal nerve as well as the autonomic nervous system, the ganglia of which originate from the spinal nerves. Body work, Rolfing and massage therapies are also valuable in this sort of treatment - which is discussed in the book by Krohn, et al, as a part of detoxification procedures.

Psychological and spiritual counselling are of inestimable importance in all treatment programs.

All psychopharmaceutical drugs should be completely avoided in persons being treated for mitochondrial dysfunction, particularly the Selective Serotonin Re-uptake Inhibitors and diazepam derivatives, most of which can be replaced by herbal treatments. Drugs which alter brain chemistry almost certainly are mitochondrial toxins.

Blood pressure medications can and should be replaced by herbal and nutritional programs where possible.

Non-steroidal anti-inflammatory drugs are absolutely contraindicated in people suffering from mitochondrial dysfunctions.

Carbonated beverages should be avoided, those which are sweetened artificially with Aspartame as well as those which are unsweetened. All carbonated beverages are buffered with phosphate which severely interferes with normal metabolism.

Alcohol should be avoided. If this is not done, the intake should be strictly limited to one ounce of alcohol dailysuch as red wines. Stimulant beverages such as coffee and tea should be avoided, although green tea may be beneficial in moderation.

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BASIC SCIENCE ASPECTS OF THE MITOCHONDRIA
SECTION IX

ARTERIOSCLEROSIS

Despite a recent improvement in the statistics cardiovascular disease remains the number one killer in the United States. Over one million Americans are expected to die from heart attack, stroke, hypertensive disease and other cardiovascular disorders in 2000. The cost of these diseases during 2000, in terms of medical care and lost productivity will amount to an estimated $65 billion.

In an attempt to discover the causes of heart disease, scientists have isolated a group of so-called "risk factors"; those parameters which are associated with an increased risk of cardiovascular mortality. Persons with elevated Homocysteine levels, total serum cholesterol or a deficiency of high density lipoprotein (HDL) cholesterol; those with hypertension, elevated uric acid or triglycerides; smokers, diabetics and significantly obese individuals all may have a greater than average chance of dying of a heart attack or an occlusive stroke. However none of these "risk factors" may be the culprit.

Much debate and hundreds of millions of dollars of research have focused on the question of whether risk can be reduced by modifying known cardiovascular risk factors. In the first place, as many as 40% of people with coronary heart disease (CHD) have no known risk factors whatsoever. In addition, statistical associations do not imply causation. Heart attacks may actually be caused by other factors which also happen to raise serum cholesterol, triglycerides, etc. If that is indeed the case, then modifying these risk factors by methods that do not eliminate the true cause of CHD is not worthwhile, and may also be harmful.

CONVENTIONAL RISK FACTOR THEORIES

The Multiple Risk Factor Intervention Trial dealt a lethal blow to the so-called "lipid hypothesis;" the theory that CHD is caused by eating too much meat, eggs, butter, whole milk, and other fatty foods. This hypothesis always was plagued by inconsistencies and unexplainable observations. However, it seemed so simple and, at least on the surface, so logical that it was difficult to reject. It is therefore important that we review this hypothesis; the data on which it is based and why it is not a good theory. Only then will we feel comfortable enough to move on and explore other, perhaps more fruitful, approaches to CHD.

Several studies have shown that conventional methods for lowering serum cholesterol are worthless and may be dangerous. In one long-term study (mean 9.6 years), hypercholesterolemic subjects treated with the cholesterol-lowering drug clofibrate had more deaths from ischemic heart disease (IHD) than placebo treated controls. In another study patients with IDH were treated either with a diet high in saturated fat and cholesterol (control group) or a low fat diet supplemented with about four tablespoons daily of corn oil. After two years the mean serum cholesterol in the corn oil group was lower than in the control group. However, during this time, nearly twice as many men fed corn oil (48%) suffered a major cardiac event as compared with those fed the allegedly atherogenic animal fats (25%).

The Multiple Risk Factor Intervention Trial (MR FIT) was the most recent, most expensive and probably the last, large scale study of whether risk factor modification reduces risk. Approximately 12,000 men at risk for coronary death were randomly allocated to two groups in this

six-year trial. The specific intervention (SI) group was counseled on standard dietary recommendations designed to lower blood pressure and serum cholesterol. Treatment with antihypertensive medication was also given when indicated. In addition, the men in the SI group were counseled individually on the importance of quitting smoking. The second group was assigned to usual care (UC) in the community and was given no specific advice.

Men in the SI group achieved a lowered serum cholesterol and blood pressure and a lowered percentage of smokers than those in the UC group. However, total death rates and mortality from CHD were nearly identical in the two groups. Thus, after six years and nearly $115 million in research, it has become clear that the battle against heart disease will not be won by merely cutting down on dietary cholesterol, saturated fat, salt, and cigarettes.

The lipid hypothesis is based on four major points.

1. that populations with high death rates from CHD consume large amounts of cholesterol and saturated fat;
2. that dietary cholesterol and saturated fat raise, while polyunsaturated fat lowers, serum cholesterol;
3. that atherosclerotic plaques contain cholesterol; and
4. that feeding large amounts of cholesterol to animals produces lipid deposits in the arteries.

From these four observations the following concept emerged - that a diet high in cholesterol and saturated fat causes an increase in serum cholesterol level which, in turn, leads to atherosclerotic changes and their complications (heart attacks and strokes). If all of the above information were correct, then the lipid hypothesis would, indeed, be quite compelling. However,

each of the four points has serious weaknesses.

Although an international survey did show a positive correlation between CHD incidence and national animal fat consumption, there are many other factors, both dietary and non-dietary, which are also associated with CHD. Why blame animal fats, rather than sucrose, margarine, deficiency of fiber, deficiency of proper nutrients or minerals, chlorinization of water or chemical pollution? There are a number of reports which contradict the lipid hypothesis. For example, the Masai tribesmen in Africa derive 60-65 percent of their calories from milk fat, which is both highly saturated and high in cholesterol. Yet, symptoms of occlusive vascular disease occur in less than 1 percent in Masai males aged 45-64, although atherosclerotic plaque buildup is just as prevalent in Masai tribesmen as it is in American males. The incidence of CHD among Yemenite Jews increased greatly after they immigrated to Israel. However, the amount of animal fats in their diet was no different in Yemen than it was in Israel. What did increase considerably in the diet of these Yemenite Jews was the amount of sugar, margarine and vegetable oil. In addition to the above studies of specific cultural groups, most surveys have failed to find a difference in dietary cholesterol and saturated fat between coronary and non-coronary cases within any given population. Furthermore, those with high cholesterol and triglycerides do not eat any more of these animal fats than do others in the same society.

While consumption of animal fats does affect serum cholesterol level in some people, the level of these dietary constituents accounts for only a small part of the variation in serum cholesterol in the general population. There are a number of other foods, such as yogurt, carrots, oat bran, soy beans and garlic with hypocholesterolemic activity that cannot be explained by their

fat and cholesterol content.

The fact that atherosclerotic plaques contain cholesterol does not necessarily support the lipid hypothesis either. Recent work has shown that much of the cholesterol in plaques is not derived from the blood, but is synthesized de novo by arterial cells. Thus the concept that cholesterol travels from the food to the blood and then sticks to the arteries like chewing gum is not borne out by our current understanding of the pathogenesis of atherosclerosis.

The final observation on which the lipid hypothesis is based, that cholesterol feeding causes arterial lipid deposits in animals, is also apparently in error. When pure cholesterol is exposed to room air, the cholesterol undergoes spontaneous oxidation to form angiotoxic derivatives. These angiotoxins, when fed even in minute quantities produced atherosclerotic lesions in animals. Pure, unoxidized cholesterol was not atherogenic, even when administered in large amounts. Thus it is not cholesterol per se, but its oxidative byproducts, that are atherogenic. While some cholesterol might certainly become oxidized during normal food preparation, the level of angiotoxins is probably far below that which would occur in feeding experiments where massive amounts of pure cholesterol sit in feed cups for long periods of time.

From the original lipid theory that the cholesterol in food had a direct effect on arteriosclerosis, we have advanced to the High Density Lipoprotein-Low Density Lipoprotein theory.

Fats in the diet are broken down and fed, two carbon atoms at a time, into the same metabolic pathway that derives energy from breaking down sugars. Fats and related molecules also have a fundamental structural role in cells. They are the building blocks of membranes—cell

membranes, mitochondrial membranes, and many others. All these molecules are collectively termed lipids.

Most of the structural molecules in membranes are modified in a generic way. A fat molecule is a triglyceride: a molecule of glycerol with a many-carbon organic acid attached to each of its three carbons. When a fat molecule is broken down, the acids are stripped off and fed into the tricarboxylic acid cycle. When a fat molecule is to be used in a membrane, only one of the three chains is removed and, rather than being replaced by a hydrogen atom, it is replaced by a group centered around a phosphorus atom. The resulting molecule is a phospholipid. Two phospholipids can thus differ in the nature of the phosphorus-centered group (the head group) and/or in the two fatty-acid side chains that are retained. Neither makes an enormous difference to the chemistry of the molecule, but differences in the head groups have more effect on that than differences in the side chains, so the nomenclature of phospholipids is based on the head groups. What the side chains do affect is the physical properties of a membrane made of phospholipids: if one of the chains is polyunsaturated (contains two or more C=C double bonds) then the molecule takes up more room. A membrane with large amounts of that type of phospholipid is more fluid than otherwise, which is a property that membranes need in order not to rupture.

Mitochondria have a lot of a diphospholipid called cardiolipin (so named because it was first discovered in the heart). Cardiolipin is formed by joining two copies of the simplest phospholipid, phosphatidate, at their phosphates. Cardiolipin is the only anionic lipid present in significant amounts in the inner mitochondrial membrane, and its concentration decreases with age. This change has been shown to cause reduced performance of certain membrane proteins. A

possible mechanism for this effect is that loss of cardiolipin causes an increase in the pH of the water right next to the membrane, which may markedly affect mitochondrial function. All membranes also have a component which is rather more distantly related to fats. It is classed as a lipid, but unlike phospholipids it is not derived from fats but instead is built from scratch in the liver, as well as being extracted from food by epithelial cells in the gut. It is cholesterol. Cholesterol is a steroid, a molecule only about half the length of the average phospholipid, and its presence in membranes increases their fluidity, which is necessary to keep them intact. Cells in culture that have been genetically modified to lack cholesterol are very prone to suffer membrane rupture, which is fatal to the cell; the same effect has been shown in red blood cells.

Most of our cells, despite needing cholesterol so vitally, do not make it themselves—at least, not in the quantity they need. They are also rather ineffective at destroying it (or packaging it away) when they transiently need less of it, though they do package it to some extent. They can afford this because a few types of cell, particularly ones in the liver and in the gut, have a very high capacity for cholesterol synthesis and degradation (for liver) or absorption and release (for gut), and can therefore buffer the less capable cells elsewhere. But in order to achieve this buffering, the liver and gut cells must somehow exchange cholesterol with all other cells. They do this via the blood stream. Cholesterol is secreted by the liver and gut and imported by other cells in a particle called a low-density lipoprotein, or LDL; and it is transported the other way in a similar (but easily distinguishable biochemically) particle called a high-density lipoprotein (HDL). Both of these transport processes are highly regulated by the cells that are doing the import and export. The particles themselves are also highly structured, being organized around a protein scaffolding (just

one big polypeptide in the case of LDL and being wrapped in a coat of phospholipid, which is necessary in order to allow the particle to move freely in the blood, since it makes the particle more water-soluble.

A similar situation exists for fatty acids. Most cells are capable of building the phospholipids (and related molecules) that they need, but not from absolute scratch: they need the component fatty acids to be supplied in the blood. Fatty acids are present in LDL, but the amount of LDL imported (or HDL exported) is fixed by the cell's cholesterol requirements and is thus not (necessarily) adequate for the cell's fatty acid requirements. Most fatty acid is acquired by a different mechanism: it circulates in the blood bound to albumin, which mediates its uptake by cells. In contrast to cholesterol, however, there is no "reverse transport" system to rid the cell of excess fatty acid: this is not necessary, because it can be used as fuel for the TCA cycle. Homeostasis is achieved by regulating the uptake and storage of the TCA cycle's other main fuel, glucose.

The blood stream is used as a transporter not only of cholesterol but of all nutrients. LDL and HDL particles can suffer chemical changes during their journey which can render them toxic to the cells that are their intended destination. The main modification is oxidation. In the case of HDL, the destination cells (in the liver) are purpose-built to deal with all manner of toxins. The situation with LDL is much more serious. We know that LDL particles can undergo modifications that make them toxic to most cells. The intracellular machinery that imports LDL specifically rejects LDL that has been significantly oxidized. A certain type of white blood cell called a monocyte has the potential to turn into a scavenger for oxidized LDL, by attaching itself to the

artery wall and expressing a different type of surface protein that imports oxidized LDL even more assiduously than unoxidized. A highly deleterious side-effect of this process is atherosclerosis. The blood contains high levels of various chemicals that reduce the rate at which LDL oxidation occurs in the first place; these are the antioxidants.

There is a major paradox concerning the presence of oxidized LDL in the blood stream. It was shown in 1984 that the oxidation of LDL by cultured cells is totally abolished by the addition to the culture medium of antioxidants such as vitamin E. The same is true of the arterial intima. Thus, LDL oxidation should not be happening in the body at all! That it does may very well be due to the failure of the immune system to control chronic bacterial and viral infections in the arteries.

The inside of the entire cardiovascular system is coated with a single-cell layer of specialized cells called endothelial cells. These cells have a protective function. One aspect of that function is the removal from the blood of oxidized fats. They do not do this directly, but rather by changing their surface in such a way that another type of cell, which is normally free in the blood, attaches to them. This is a type of white blood cell called a monocyte, and once fixed to the artery wall it changes its character sufficiently that it is given a different name. It is called a macrophage. Macrophages have a multi-faceted defensive role, but the one that concerns us here is that they absorb LDL particles which have undergone peroxidation. This is in contrast to most cells, which import LDL particles (mainly for cholesterol supply) that are unoxidized, but which are not receptive to them once they have become significantly oxidized. Thus this is a defence mechanism that prevents the build-up of oxidized LDL in the blood. Initially, and ideally, macrophages ingest oxidized LDL and rapidly break it down.

However, especially in people with a high-fat diet, this mechanism is saturable. Macrophages eventually become unable to cope with the rate of incoming LDL and cease to function properly. Macrophages that have reached this point are called foam cells. Once foam cells begin to accumulate in a region of arterial wall, things go from bad to worse: a fibrous cap forms over the foam cells, probably in an attempt to contain it and stop it from injuring the epithelial cells nearby. Moreover, a low-cholesterol diet does not have a greatly inhibitory effect on the rate of progress of atherosclerosis, because plasma LDL levels are not much altered; presumably the liver makes up the deficit from the gut. This is no surprise, because ultimately we need to supply our cells with cholesterol, a vital component of membranes which most cells do not make in sufficient quantity for their needs. Only the liver and gut can supply it fast enough; it is thus necessary to have it (as LDL) in the blood in order to make it available elsewhere. In line with this, the genetic and environmental risk factors which cause a wide variation in different people's susceptibility to atherosclerosis are largely related to the later, hyperplastic stages of lesion formation: foam cells are apparent at about the same (early) age in all individuals.

Atherosclerosis is a multifactorial disease based on the action of various risk factors that become effective on an appropriate genetic background. The disease is characterized by the appearance of mononuclear cells (MNC) in the vessel wall at certain predilection sites, such as arterial branching points, which are known to be subject to altered hemodynamic (turbulent) stress. With progression of lesions, smooth muscle cells (SMCs) from the media immigrate into the intima, where they proliferate and lead to the deposition of extracellular matrix (ECM) proteins, notably collagen fibers. This sequence of events leads to thickening and hardening of arteries

(arteriosclerosis). Atherosclerosis is characterized by the additional formation of foam cells, for example, macrophages and SMCs that have taken up chemically modified, that is, oxidized low-density lipoproteins (ox-LDL) by nonsaturable scavenger receptors, leading to overloading of these cells with lipids and eventual deposition of extracellular cholesterol crystals. According to a conventional, classic view of atherogenesis, whitish, cushion-like lesions, so-called fatty streaks, with a predominance of foam cells, constitute the precursors of more severe, rupture-prone, often exulcerated and even calcified lesions, that is, atherosclerotic plaques. The exact natural history, macroscopic and microscopic appearance, distribution, and functional characteristics of various stages of atherosclerotic lesions are discussed below.

The cholesterol theory of atherogenesis did not ascribe a major significance to inflammatory-immunologic processes as possible primary pathogenetic factors. The "response to injury hypothesis" originally postulated an alteration of the endothelium and the intima (mechanical injury, toxins, oxygen radicals, and so forth) as the initiating event leading to endothelial dysfunction. This was followed by increased permeability, expression of adhesion molecules, and release of growth factors and chemotactic factors. As a consequence, platelet aggregation and monocyte adhesion and activation take place, the latter being attracted into the subendothelial space of the intima, where they meet with SMCs immigrating from the media, followed by foam cell formation. As the lesion progresses, a fibrotic "cap" and a rupture-prone "shoulder" region characterized by ECM deposition are formed. The "altered lipoprotein hypothesis" postulates an initiating role of chemically altered lipoproteins, notably ox-LDL, which lead to the primary formation of foam cells in the intima. This hypothesis has recently been modified since it has been

shown that only native rather than ox-LDL is found in the circulation. The native LDL transported into the intima through the endothelium is modified (oxidized) and retained there, where it acts as a chemoattractant for monocytes and SMCs and is later taken up by these cells, resulting in foam cell formation ("retention of modified LDL hypothesis").

The endothelial cells which line the circulatory system are the tissues in the body which are most constantly in contact with blood. In most instances, they are the only tissues which are in direct contact with blood and all of the constituents of blood, the formed elements, the plasma and all substances dissolved or suspended in blood. In the arteries, the endothelial cells are constantly in contact with blood under pressure.

Depending on circumstances of exposure, the blood contains at any time a bewildering number of compounds, some quite toxic in nature, being transported suspended in blood from the point of ingress to the point where they are detoxified or eliminated by the kidneys and liver.

During the past century, human beings are constantly being bombarded with heavy metal toxins, such as lead, mercury, cadmium, arsenic, chromium, antimony, beryllium, thallium and others.

Blood flow through arteries is ordinarily laminar. However, laminar flow is disrupted at places where arteries branch. At these points laminar flow is disrupted and turbulence occurs in the flow.

Areas of turbulence due to changing diameter and branching off of arterial trunks constitute the areas which are most frequently the site of arteriosclerosis in humans.

It is in these areas where turbulent flow brings the contents of blood into direct contact with

the endothelium repeatedly and constantly bringing the toxic metals and chemicals into contact with the endothelial cells which avoid contact where laminar flow occurs.

Toxins can damage endothelial cells, their membranes and subcellular organelles, and bring about death of such cells which results in injury to the intima, the initiating event in arteriosclerosis. Such damage is likely free radical damage.

The process of arteriosclerosis begins with injury to the endothelial cells which line the arteries, this injury is probably toxic but it could conceivably be bacterial or viral.

The injury usually occurs at or near the bifurcation of arteries or in locations where for some anatomical reason, the laminar flow of blood becomes disrupted and turbulence occurs. This brings the epithelial cells into direct contact with all of the matter suspended in blood, which does not occur in areas where the movement of the blood is laminar.

The injurious agent may be an environmental toxin, or a chemical injected as a drug or food additive. All that is necessary is that it be present in the blood either repeatedly or for a prolonged length of time to attack the endothelial cells and injure them.

The next step is an inflammatory response to the injury.

The increase in myocardial infarction and strokes due to arteriosclerosis began in the latter quarter of the 19th Century. This was the beginning of industrial development in the United States which brought about an exponential increase in the levels of environmental toxins as the use of toxins in industrial processes, agriculture and petroleum products for transportation increased. As the air, water and food supply became more contaminated with toxins and the use of synthetic chemical compounds replaced the use of herbally derived compounds in medicine, the exposure of

the endothelial cells increased to levels which had never occurred before. Endothelial injury increased and the aftermath of endothelial injury, inflammation and the response to injury increased, a rare disease became commonplace.

The response-to-injury hypothesis of atherosclerosis is that the protective, inflammatory response followed by the formation of a fibroproliferative response begins as a protective mechanism that with time and continuing insult may become excessive. In its excess, both the inflammation and the fibrous connective tissue proliferation become, in themselves, the disease process. This is the essence of the process of atherogenesis.

After the initial injury, the earliest cellular events that occur during atherogenesis is a specialized type of chronic inflammatory response that precedes migration and proliferation of arterial smooth muscle cells. The first events are increased accumulation of lipid and lipoprotein particles beneath the endothelium, presumably from increased transport and/or permeability of the lining endothelial cells. This is rapidly followed by attachment, adherence, and spreading of peripheral blood monocytes and T-lymphocytes at sites throughout the arterial tree, particularly at branches and bifurcations. These cells adhere from the formation of adhesive cell-surface glycoproteins by the endothelium and the leukocytes, which interact in a ligand-receptor manner. Thus one of the earliest changes induced by hypercholesterolemia and hypertension is altered endothelial permeability, together with the adherence of leukocytes, representing the first phase of an inflammatory response.

The leukocytes migrate across the surface of the endothelium, probe between the junctions of the endothelial cells, and are chemotactically attracted into the subendothelial space where they

begin to accumulate within the intima. In the presence of oxidized low-density lipoprotein, the monocytes become converted to activated macrophages and, through their scavenger receptors, take up the modified lipoprotein particles and become foam cells. The formation of foam cells and their continued accumulation in the intima lead to the first ubiquitous lesion of atherosclerosis, the fatty streak. If the offending agent, such as hypercholesterolemia or another risk factor, continues, then the inflammatory response will also continue. What began as a protective, inflammatory response can become deleterious to the cells of the artery wall.

This condition may lead to an expanded, intermediate (or fibrofatty) lesion that may contain multiple layers of smooth muscle, connective tissue, macrophages, and T-lymphocytes. Eventually, if the conditions that induce the response continue long enough, remodeling of the lesion may occur with the formation of a fibrous cap. The cap consists of numerous smooth muscle cells surrounded by collagen, elastic fibers, and proteoglycans that cover the numerous proliferated smooth muscle cells, macrophages, and T-lymphocytes, together with varying amounts of necrotic cell debris, intracellular and extracellular lipid, and potentially massive amounts of new connective tissue. The advanced lesion, or fibrous plaque, can then intrude into the artery wall. Changes in the surface characteristics of the fibrous cap, such as ulceration or rupture, can lead to formation of a thrombus that can cause sudden death or organize itself and lead to further lesion progression and compromise the flow at the local site.

Thus the advanced lesions of atherosclerosis represent the culmination of cellular and molecular events in which there is replication of both smooth muscle cells and macrophages that had previously entered the artery wall. The interactions among these cells and the overlying

endothelium and T-lymphocytes may lead to a massive fibroproliferative response. Terminal events, such as myocardial or cerebral infarction, are usually derived from secondary changes within the fibrous plaque that lead to the formation of an occlusive thrombus. These changes include fissuring, cracking, or ulceration in the surface of the lesion, which can become subjected to altered rheologic forces caused by the encroachment on the lumen by the lesion.

Plaque disruption with superimposed thrombosis is the main cause of the acute coronary syndromes of unstable angina, myocardial infarction, and sudden coronary death. Atherosclerosis would be a much more benign disease if plaque disruption and thrombosis could be prevented. Therefore the focus should be on plaque composition and vulnerability to rupture rather than on plaque size and stenosis severity.

The risk of plaque disruption depends more on plaque type than on plaque size; lipid-rich and soft plaques are more vulnerable and prone to rupture than are collagen- rich and hard plaques. Furthermore, they are highly thrombogenic after disruption because of high content of tissue factor. Pathoanatomic studies have identified 3 major determinants of a plaque's vulnerability to rupture: (1) the size of the lipid-rich core; (2) inflammation with plaque degradation; and (3) lack of smooth muscle cells (SMC) with impaired healing.

Lipid accumulation, macrophage infiltration, and lack of SMCs destabilize plaques, making them vulnerable to rupture. In contrast, SMC-mediated healing and repair processes stabilize plaques, protecting them against disruption. Plaque size or stenosis severity tell nothing about a plaque's vulnerability. Many vulnerable plaques are invisible angiographically because of their small size and compensatory vascular remodeling. The atheromatous core of these plaques is

avascular, hypocellular, lipid-rich, soft like gruel, and totally devoid of supporting collagen. The size of such a soft core is critical for the stability of the plaque. At autopsy, many observers found much larger atheromatous cores in coronary plaques with disrupted (versus intact) surfaces, and a strong relation between core size and plaque rupture in aorta. Recent studies have identified macrophage-specific antigens and apoptotic nuclear fragments within the gruel, indicating that lipid and other cell constituents released from dead macrophage foam cells could contribute significantly to the formation and growth of the atheromatous core. It also has been referred to as the "graveyard of dead macrophages," emphasizing the inflammatory origin of this destabilizing core.

Inflammation and immune responses play an important role in atherogenesis. It is associated with increased endothelial permeability, increased transcytosis and intimal retention of lipoproteins, and endothelial activation with focal expression of vascular cell adhesion molecule-1 (VCAM-1), leading to monocyte and T-lymphocyte recruitment. Within the intima, the monocyte-derived macrophages engulf the blood-derived low-density lipoprotein (LDL), probably through their scavenger receptor after oxidative modification, and become lipid-filled foam cells. These inflammatory cells constitute by far the major part of the early fatty streak lesion, with a ratio of approximately 1:10 to 1:50 between T cells and macrophages. They probably play a significant role in the progression of fatty streaks to mature atherosclerotic plaques. The presence of activated macrophages and T cells strongly suggests that an immunologic reaction has taken place in the atherosclerotic plaque. The antigens that elicit this response are both autoantigens (eg, against oxidized LDL) and microorganisms (eg, Chlamydia pneumoniae, Cytomegalo Virus and Herpes Virus.)

Sci-mito-7

Disrupted fibrous caps are usually heavily infiltrated by macrophage foam cells, and recent observations have shown that such rupture-related macrophages are activated, indicating ongoing inflammation at the site of plaque disruption. Superficial macrophage infiltration in plaques beneath coronary thrombi are seen. Whether or not the underlying plaque was disrupted or just eroded, recent studies of coronary thrombi responsible for sudden coronary death could not confirm that observation. Evaluation with immunohistochemical techniques shows that macrophages and adjacent T lymphocytes (SMCs were usually lacking at rupture sites) were activated, indicating ongoing disease activity. A study of atherectomy specimens showing an inverse relation between the extent of inflammatory activity in plaque tissues of culprit lesions and the clinical stability of the ischemic syndrome. However, there was considerable overlap between groups, indicating that not all patients with clinically stable angina have histologically stable plaques. These observations confirmed a previous study of atherectomy specimens from culprit lesions responsible for stable angina, unstable rest angina, or non-Q-wave infarction. Culprit lesions responsible for the acute coronary syndromes contained significantly more macrophages than did lesions responsible for stable angina pectoris (14% vs 3% of plaque tissue occupied by macrophages).

Macrophages are capable of degrading extracellular matrix by phagocytosis or by secreting proteolytic enzymes such as plasminogen activators which may weaken the fibrous cap, predisposing it to rupture. Collagen confers stability to plaques, and human monocyte-derived macrophages grown in culture are indeed capable of degrading the old and mature collagen present in advanced aortic plaques. Simultaneously, they express MMP-1 (interstitial collagenase) and induce MMP-2 (gelatinolytic) activity in the culture medium. Besides macrophages, a wide variety

of cells may produce MMPs. Activated mast cells may secrete powerful proteolytic enzymes such as tryptase and chymase that can activate pro-MMPs secreted by other cells (eg, macrophages). Mast cells are actually present in shoulder regions of mature plaques and at sites of disruption although at very low density. Neutrophils are also capable of destroying tissue by secreting proteolytic enzymes, but they are rare in intact plaques.

Several infectious agents play an active role in the development of cardiovascular diseases, particularly *C pneumoniae* but also herpes- viruses (including cytomegalovirus) and *Helicobacter pylori.* Chlamydia has been identified in atherosclerotic plaques; it contains lipopolysaccharide and heat shock protein 60, which are well-known strong inducers of many enzymes, among others MMPs.

Recently, nonspecific but sensitive blood markers of inflammation (acute phase reactants such as C-reactive protein and serum amyloid A) have been identified as strong risk factors for future cardiovascular events in apparently healthy men and women, in patients with stable and unstable angina, and after myocardial infarction.

Obviously, the thickness and collagen content of the fibrous cap is very important for its strength and stability; the thinner the cap is, the weaker it is and the more vulnerable is the plaque to rupture. Ruptured aortic caps contain fewer SMCs and less collagen than intact caps, and SMCs are usually missing at the actual site of disruption.

Collagen is responsible for the mechanical strength of the fibrous cap, and it is synthesized by intimal SMCs. It is important to realize that SMC proliferation and matrix synthesis may be good in protecting plaques against disruption. Local loss of SMC or impaired SMC function may

be bad, leading to gradual plaque destabilization due to impaired healing and repair. It is unknown why SMCs are lacking at sites of disruption, but apoptotic cell death could play an important role.

Rupture of vulnerable plaques occurs frequently. Autopsy data indicate that 9% of healthy persons harbor disrupted plaques (without superimposed thrombosis) in their coronary arteries, which increases to 22% in persons with diabetes or hypertension. In fatal coronary artery disease, more than one disrupted plaque, with or without superimposed thrombosis, usually is present in the coronary arteries.

Disruption of the plaque surface occurs most often where the cap is thinnest and most heavily infiltrated by macrophages.

Approximately 75% of thrombi responsible for acute coronary syndromes are precipitated by plaque disruption, whereby the highly thrombogenic gruel is exposed to the flowing blood. In the remaining 25%, superficial plaque erosion without frank disruption (ie, no deep injury) is usually present. Most disrupted plaques are resealed by a small mural thrombus, and only sometimes does a major luminal thrombus evolve. There are 3 major determinants of the thrombotic response to plaque disruption/erosion: (1) the local thrombogenic substrate; (2) the local flow disturbances; and (3) the systemic thrombotic propensity. Inflammatory cells might also play an important role in the thrombotic response to plaque disruption/erosion through tissue factor expressed locally in plaque macrophages and systemically in blood monocytes. The thrombotic response to plaque disruption is dynamic; thrombosis and thrombolysis occur simultaneously in many patients with acute coronary syndromes, with or without concomitant vasospasm, causing intermittent flow obstruction. The initial flow obstruction is usually caused by platelet aggregation,

but fibrin is important for the subsequent stabilization of the early and fragile platelet thrombus. Therefore both platelets and fibrin are involved in the evolution of a persisting coronary thrombus.

Plaque disruption itself is asymptomatic, and the associated rapid plaque growth is usually clinically silent. However, rupture-related hemorrhage into the plaque, luminal thrombosis, and/or vasospasm may cause sudden flow obstruction, giving rise to an acute coronary syndrome. The culprit lesion is frequently "dynamic," causing intermittent flow obstruction, and the clinical presentation and the outcome depend on the severity and duration of myocardial ischemia. A nonocclusive or transiently occlusive thrombus most frequently underlies primary unstable angina with pain at rest and non-Q-wave infarction, whereas a more stable and occlusive thrombus is most frequently seen in Q-wave infarction—overall modified by vascular tone and collateral flow. The lesion responsible for out-of-hospital cardiac arrest or sudden coronary death is often similar to that of unstable angina: a disrupted plaque with superimposed nonocclusive thrombosis.

The injury to endothelial cells which triggers the inflammatory process culminating in plaque formation is a mitochondrial event - apoptosis, due to free radical damage, involving antioxidant breakdown. Recent research shows that inadequate vitamin C levels may be responsible.

VITAMIN C AND THE RESPONSE TO INJURY CASCADE IN ARTERIOSCLEROSIS

In the January 18, 2000 issue (Vol. 97; 841-846) issue of Proceedings of National Academy of Science, Nobuyo Maeda, Hiroyuki Hagihara, Yukiko Nakata, Sylvia Hiller, Jennifer Wilder, of the Department of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, NC and Robert Reddick of the Department of Pathology, University of Texas Health Science

Center, San Antonio, TX, report the occurrence of Aortic wall damage in mice unable to synthesize ascorbic acid.

In this breakthrough study, the authors inactivated the gene for L-gulono-γ-lactone oxidase, a key enzyme in ascorbic acid synthesis, thus generating mice that, like humans beings, must depend on dietary vitamin C.

These authors report:

> "By inactivating the gene for L-gulono-γ-lactone oxidase, a key enzyme in ascorbic acid synthesis, we have generated mice that, like humans, depend on dietary vitamin C. Regular chow, containing about 110 mg/kg of vitamin C, is unable to support the growth of the mutant mice, which require L-ascorbic acid supplemented in their drinking water (330 mg/liter). Upon withdrawal of supplementation, plasma and tissue ascorbic acid levels decreased to 10-15% of normal within 2 weeks, and after 5 weeks the mutants became anemic, began to lose weight, and die. Plasma total antioxidative capacities were approximately 37% normal in homozygotes after feeding the unsupplemented diet for 3-5 weeks. As plasma ascorbic acid decreased, small, but significant, increases in total cholesterol and decreases in high density lipoprotein cholesterol were observed. The most striking effects of the marginal dietary vitamin C were alterations in the wall of aorta, evidenced by the disruption of elastic laminae, smooth muscle cell proliferation, and focal endothelial desquamation of the luminal surface. Thus, marginal vitamin C deficiency affects the vascular integrity of mice unable to synthesize ascorbic acid, with potentially profound effects on the pathogenesis of vascular diseases. - - -
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> Taken together, these data demonstrate that a suboptimal ascorbic acid has a small, but significant, effect on the plasma cholesterol levels and suggests that it increases the levels of atherogenic lipoprotein particles. However, these effects are small, and the Gulo / mice are not overtly hypercholesterolemic.
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> Rupture of Elastic Lamina, Smooth Muscle Cell Proliferation, and Injury of the Luminal Surface in the Thoracic Aorta. Ascorbic acid is an important cofactor for the hydroxylation of proline and lysine necessary for the crosslinking of collagen and elastin, important structural components of vessel wall. Inspection under both light microscopy and TEM of cross sections of the thoracic aorta from animals with low levels of plasma and tissue ascorbic acid revealed marked alterations in the pattern and integrity of the elastic laminae. Prominent breaks and fragmentation of the elastica were located in both the superficial and deep media. The endothelial cells overlying the areas of internal elastic lamia disruption were attenuated as a

result of the accumulation of basement membrane material, collagen/elastic tissue, and aggregates of smooth muscle cells. Some smooth muscle cells had an altered morphology, were devoid of cytoplasmic filaments, and contained myelin figures indicative of cellular degeneration. Small clusters of smooth muscle cells, ranging from a few to a diffuse collections of several cells, were present within the subintima below the basement membrane and above the superficial elastic lamina. Most of the smooth muscle cells present within the intima were mildly activated as judged by a slight increase in their rough endoplasmic reticulum. Small collections of elastic fibers were located between smooth muscle cells both in the intima and in the deep media, suggesting that reorganization of the elastic lamina is a dynamic process in these mice.

On scanning microscopy, the aortic arches from the vitamin C-deficient mice contained focal areas where the normal endothelial cell pattern was absent. The foci with loss of normal endothelial architecture were oriented in the direction of blood flow and most often were located in the aortic wall near the takeoff of the carotid arteries. The surface was rough and irregular but devoid of fibrin deposition and attached blood cells, with the exception of occasional red blood cells. Cytoplasmic extensions of adjacent endothelial cells were in contact with the edges of the defects, and remnants of endothelial cells were present within the altered areas. Although aortic segments from all 10 Gulo/ mice evaluated had at least one of such lesions, sections of the aortic arch in none of the wild-type mice showed abnormalities in the arrangements and integrity of the endothelial cells."

Vitamin C is, of course, a powerful antioxidant and free radical scavenger; it also regenerates other small molecule antioxidants such as alpha tocoperol, Glutathione, urate and β-carotene from their respective radical species. It has additional properties, such as the regulation of collagen biosynthesis and protects vascular smooth muscle against apoptosis induced by oxidized Low Density Lipoproteins and protects against plaque instability.

These and other functions of Vitamin C alone or acting in concert with other antioxidant nutrients play an important role in preventing and reversing arteriosclerotic plaque, in mice and in men. It also has an important role in the function of the immune system.

It is clear from the above data that we must look beyond the lipid hypothesis if major advances against cardiovascular disease are to be made. This review advances the concept that

nutritional deficiency is a major etiologic factor in cardiovascular disease. This idea has not received a great deal of attention. However, based on available research, it is not unreasonable to believe that correction of micronutrient and essential fatty acid deficiencies will greatly reduce cardiovascular mortality by addressing the enhancement of the immune functions and regulation of the metabolic process involved.

It has been postulated that the major role of elevated homocysteine levels in the etiology of arteriosclerosis is to act as an irritant or molecular abrasive which scrapes and injures the endothelial cells damaging them and setting up the inflammatory cascade, making them vulnerable to bacterial and viral infection. Elevated homocysteine levels are also evidence of a block in the metabolic process which produces melatonin and taurine as downstream metabolites of methionine. Either way, it is clear that the reduction of homocysteine levels by nutritional means stop whatever deleterious role it plays in arteriosclerosis.

The ultimate answer will not be the study or use of single nutrients to prevent, treat or reverse the lesion but a combined nutritional approach utilizing a balance of nutrients, antioxidants and factors which work together to inhibit and reverse the arteriosclerotic process.

Homocysteine is an intermediate compound in the conversion of the amino acid methionine to cysteine. If a person is relatively deficient in folic acid, vitamin B6, or vitamin B12, there will be an increase in the level of homocysteine. This compound has been implicated in a variety of conditions, including atherosclerosis. Homocysteine is thought to promote atherosclerosis by directly damaging the artery and by reducing the integrity of the vessel wall, as well as by preventing the oxidation of low density lipoproteins.

Elevated homocysteine levels are an accurate predictor of heart attack, stroke, or peripheral vascular disease. Elevations in homocysteine are found in approximately twenty to forty percent of patients with heart disease. It is estimated that folic acid supplementation (400mgc daily) alone would reduce the number of heart attacks suffered by Americans each year by ten percent. However, given the importance of vitamin B12, B6, folic acid and trimethyl glycine to proper homocysteine metabolism, it simply makes more sense to use all five together.

In one study, the frequency of suboptimal levels of these nutrients in men with elevated homocysteine levels was found to be 56.8 percent for folic acid, 59.1 percent for vitamin B12, and 25 percent for vitamin B6. These results suggest that folic acid supplementation alone would not lower homocysteine levels in many cases since homocysteine levels would still be elevated in men with either B12 or B6 deficiency. In other words, folic acid supplementation will only lower homocysteine levels if there are adequate levels of vitamin B12 and B6. Because of the interconnectedness of these three B-vitamins, the best approach to lowering homocysteine levels is to supplement all three.

There are three biochemical pathways used by the body to reduce homocysteine. In one pathway, Trimethylglyciene (TMG) donates a methyl group which detoxifies homocysteine. In this reaction TMG is turned into dimethylglycine (DMG) - yes, that familiar product sold as a supplement for its energizing effects. In other routes, folic acid, B12 and B6 convert homocysteine into non-toxic substances.

Some people can't utilize one or another of these pathways. That is why a combination of all the supernutrients is the only way to guarantee that homocysteine will be lowered. Nearly all

cases of elevated homocysteine can be corrected with a combination of TMG, folic acid, vitamin B12, and vitamin B6. A note about vitamin B6 (pyridoxine): B6 can be toxic in high doses. Some people who take more than 250mg/day have developed tingling and numbness in their hands and feet, and balance problems. The symptoms of vitamin B6 toxicity will subside when the dose of the vitamin is reduced.

Dr. Soo-Sang Kang of Rush Medical College in Chicago, reports that, along with genetic predisposition, elevated levels of homocysteine occur because of nutritional inadequacies. Vitamin B6, B12 and folate deficiencies are common in people of all ages. Betaine deficiency has not yet been studied in the general population.

Although vitamin B6 is found in a variety of foods, processing, canning, and freezing quickly destroys it. Folic acid (originally found in spinach) is in most leafy vegetables, yet numerous studies indicate that people of all ages and cultures are deficient. In one study of healthy elderly patients, one or more elevated metabolites (indicating deficiency) were found in 63% of people tested. Using the metabolite method of measuring these vitamins reveals even greater deficiencies than suspected. In elderly hospitalized patients, 83% showed a deficiency in folic acid. In another study on elderly Spanish people, low folate levels correlated with an impaired mental state and diminished ability to carry out daily activities. The Nationwide Food Consumption Survey of 1980 showed that half of all Americans surveyed were getting less than the RDA of vitamin B6.

Vitamin B12 is found in anything that moves, but is reduced by antibiotics (which are used in most commercial animal farms). TMG (betaine) is found in most vegetables, and small fish and

shrimp. It requires great effort to get enough TMG through the diet to consistently lower homocysteine levels. Coffee may act as a inhibitor of betaine metabolism.

A quick look at the standard Western diet (where the french fry has made the potato the most commonly eaten vegetable) reveals the roots of heart disease. Homocysteine is a product of methionine, an amino acid found in meat protein. It promotes heart disease by promoting the oxidation of lipids. Homocysteine plays a role in the entire heart disease process. It causes platelets to stick together, enhances the binding of Lp(a) to fibrin; and promotes free radical damage to the inside of arteries. Autopsies of young men killed in Vietnam show that vascular degeneration begins very early in Western society - likely due to the American high protein/fat diet which is deficient in supernutrient-containing vegetables.

Dr. Nicholas Dudman and his group of the University of New South Wales studied 10 people with homocystinuria (genetically-elevated homocysteine levels) who took TMG plus supernutrients vitamin B6 and folic acid for almost 13 years. They found that the TMG supernutrient combination kept homocysteine levels to one-fourth their pre-TMG level, with no negative side effects.

According to the researchers, "it was necessary to continue taking TMG (betaine) regularly to maintain maximum effects." Homocysteine levels would rise within days if people neglected to take the combination; but quickly decreased once the TMG was resumed. The study is particularly striking in that homocysteine levels naturally rise with age, but in the patients taking TMG, levels actually fell substantially, even though the participants were getting older.

Elevated homocysteine can be reduced (or detoxified) in two ways. The most common

pathway is via the remethylation process, where "methyl groups" are donated to homocysteine to transform it into methionine and s-adenosylmethionine (SAMe).

A potent remethylation agent is TMG, which stands for tri-methyl-glycine. The "tri" means there are three "methyl" groups on each "glycine" molecule that can be transferred to homocysteine to transform (remethylate) it into methionine and SAMe. The remethylation (or detoxification) of homocysteine requires the following minimum factors: 1) Folic acid 2) Vitamin B12 3) Zinc 4) TMG.

Choline is another "methyl donor" that helps to lower elevated homocysteine levels and this conversion doesn't require co-factors. However, choline only enhances remethylation in the liver and kidney, which is why it is to important to take adequate amounts of remethylating factors such as folic acid and vitamin B12 to protect the brain and the heart. The published literature emphasizes that folic acid and vitamin B12 are critical nutrients in the remethylation (detoxification) pathway of homocysteine.

The other pathway that elevated homocysteine is reduced is via its conversion into cysteine and eventually glutathione via the "transsulfuration" pathway. This pathway is dependent on vitamin B6. The amount of vitamin B6 required to lower homocysteine has considerable individual variability. Methionine is the only amino acid that creates homocysteine. People who eat foods that are high in methionine such as red meat and chicken may need more vitamin B6. Elevated homocysteine can occur when there are insufficient vitamin co-factors (such as folate and vitamin B6) to detoxify the amount of methionine being ingested in the diet.

Elevated homocysteine can also be caused by a genetic defect that blocks the

transsulfuration pathway by inducing a deficiency of the B6-dependent enzyme cystathionine-B-synthase. In this case, high doses of vitamin B6 are required to suppress excessive homocysteine accumulation. Since one would not want to take excessive doses of vitamin B 6 (greater than 300-500 mg a day for a long time period), a homocysteine blood test can help determine whether enough B6 is being ingested to keep homocysteine levels in a safe range. There are some people who lack an enzyme to convert vitamin B6 into its biologically active form pyridoxal-5-phosphate. In this case, if low-cost vitamin B6 supplements do not sufficiently lower homocysteine levels, then a high-cost pyridoxal-5-phosphate supplement may be required.

For many people, the daily intake of 500 mg of TMG, 800mcg of folic acid, 1000 mcg of vitamin B12, 250mg of choline, 250mg of inositol, 30mg of zinc and 100mg of vitamin B6 will keep homocysteine levels in a safe range. But the only way to really know is to have your blood tested to make sure the homocysteine levels are under 7. If homocysteine levels are too high, then up to 6000mg of TMG may be needed along with higher amounts of other remethylation co-factors. Some people with cystathione-B synthase deficiencies will require 500 mg a day or more of vitamin B6 to reduce homocysteine to a safe level. For the prevention of cardiovascular disease, the homocysteine blood level should be under 7. For the prevention of aging, some people have suggested an even lower level is desirable, but more research needs to be done before any scientific conclusions can be made.

Elevated homocysteine can be a sign of a methylation deficiency throughout the body. Methylation is fundamental both to DNA repair and immune function. If DNA is not adequately repaired, mutations and strand breaks will result. This will lead to accelerated aging, as greater

amounts of faulty proteins are synthesized from the damaged DNA. The liver depends on methylation to perform the numerous enzymatic reactions required to detoxify every drug and foreign substance that the body is exposed to. Methylation is required for the growth of new cells. Without it, new cells cannot be made.

A study published in the journal Medical Hypothesis (1998, Vol. 51, Iss 3, pp-179-221) provides evidence that aging may be exclusively a result of cellular "demethylation", or said differently, the aging process is caused by the depletion of enzymatic "remethylation" activity that is required to maintain and repair cellular DNA. This study suggests that aging may be reversible if aged cells could be programmed to remethylate rather than demethylate.

Homocysteine induces cellular damage by interfering with the methylation process. Methylation will be compromised if homocysteine is elevated, and elevated homocysteine is a warning sign that the methylation cycle is not functioning properly. Homocysteine may also damage cells directly by promoting oxidation of low density lipoproteins.

There is a growing consensus that deficient methylation may be a major factor in the degenerative diseases of aging. The consumption of methylation-enhancing nutrients like TMG, choline, folic acid and vitamin B12 may be one of the most readily available and effective anti-aging therapies presently known. However, it is important to tailor the intake of methylation enhancing nutrients to the individual biochemistry. The best way of assessing the body's rate of methylation is to measure blood levels of homocysteine. Elevated serum homocysteine is the classic sign of a methylation deficiency (or demethylation) that is correctable with the proper intake of methylation enhancing nutrients such as TMG, folic acid, vitamin B12.

Sci-mito-7

ESTROGEN-ANDROGEN RELATIONS

The appearance of coronary artery disease and stroke late in life argues that the age-related decline in immune function and energy production is key to the symptomatic onset of a chronic condition which may have been present since adolescence. It does not manifest until the decline in physiological function associated with aging. A riddle yet to be answered is what role estrogens play in this process since arteriosclerosis is seldom seen in women before menopause, after which it appears in an accelerated fashion. Apparently this can be prevented by the administration of estrogen to post-menopausal women. However, it has now been reported that such estrogen administration to post-menopausal women greatly increases the risk of breast cancer. There are several phytoestrogens, found in soybeans and wild yams which do not have the property of increasing the risk of breast cancer.

Current clinical research suggests that sex hormones work in opposite ways in men and women to impact cardiovascular disease processes. While estrogen generally has a protective influence on cardiovascular health in women, high levels of estrogens in men are associated with an increased risk of angina, coronary artery disease and myocardial infarction. Conversely, though testosterone may exert a detrimental influence on cardiovascular dynamics in women, it produces strong beneficial effects on an amazingly wide array of CVD risk factors in men.

It has been demonstrated that higher levels of testosterone conferred a protective ratio against atherosclerotic coronary artery disease of greater than five-fold in men. There is an inverse correlation between free testosterone levels and both the degree of coronary artery disease and the various risk factors for myocardial infarction. Chronically low testosterone levels may actually

precede—and thus in part precipitate—coronary artery disease and thrombosis in men. A normal physiological level of testosterone may protect against the development of hyperlipidemia, hyperinsulinism, hypertension, thrombophilic tendency, obesity and increased waist-hip ratio," according to British cardiologists reports in the Quarterly Journal of Medicine. "The decline of testosterone with age may partly explain the greater risk of CAD with advancing years." They emphasize the importance of identifying young men with relative hypogonadism, who are at increased risk of premature CAD. These patients may only exhibit symptoms of fatigue and depression, while "the true diagnosis is unsuspected and undiagnosed. This recommendation is supported by a cross-sectional study of South African Indian men, which revealed lower testosterone levels in younger men with premature coronary artery disease. A variety of physiological mechanisms may explain the associations between testosterone imbalances and the pathogenesis of coronary disease, myocardial infarction, and stroke, in addition to testosterone's ability to modulate lipid and glucose factors. Low testosterone is linked to higher levels of fibrinogen and plasminogen activator inhibitor which play a crucial role in blood viscosity, plaque formation, and platelet aggregation. Experimental studies also show testosterone capable of triggering vasodilation of the arteries—a relaxant effect believed to have a beneficial impact on angina and other cardiovascular impairments.

Testosterone deficiency has been called the "primary event" initiating the synergistic process involving insulin resistance, diabetes, myocardial infarction and stroke. Testosterone levels independently predict the likelihood of developing diabetes, and restoring depleted levels has been shown to improve insulin resistance.

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While early studies examining the specific relationship between testosterone and cardiovascular factors in men sometimes produced inconclusive results, most investigators now believe that these inconsistencies stemmed from the limitations of measuring total testosterone levels in blood, rather than the more clinically significant bioavailable fraction of testosterone. The bioavailable form of testosterone is more readily available to target tissues and organs in the body, and has been shown to display a stronger negative correlation with coronary artery disease parameters than total testosterone levels.

BACTERIOSCLEROSIS: THE THEORY OF THE INFECTIOUS CAUSE OF ARTERIOSCLEROSIS

In the last two years a flood of new data points to a controversial but convincing hypothesis of the pathogenesis of atherosclerosis (arteriosclerosis). Atherosclerosis can apparently be the result of ultrachronic persistent infection by Chlamydia pneumoniae, Cytomegalo virus or both and not the result of different risk factors.

There are numerous studies that were able to find C. pneumoniae and Cytomegalo Virus infections as a contributing factor in atherosclerosis pathogenesis. Positive serology for C. pneumoniae was found in most studies in patients with atherosclerosis (IgG, IgA, IgM). Most studies were able to correlate an elevated IgA antibody titer rather than the IgG titer to the risk for atherosclerosis. C. pneumoniae and its components (DNA, antigens) were detected in atherosclerotic plaques using immunohistochemistry, PCR, electron microscopy and cell cultures. C. pneumoniae has been located in endothelium, smooth muscle cells and macrophages of arterial wall with atherosclerosis but not in normal arteries. Cellular models have shown that C.

pneumoniae is able to replicate in endothelium, macrophages and smooth muscle cells. A high C. pneumoniae antibody titer was found to correlate with high level of LDL and triglycerides and low level of HDL. C. pneumoniae infection increases platelet adhesion and adhesion molecules at the surface of endothelium. C. pneumoniae could be a cofactor for atherosclerosis combined with Cytomegalovirus. Strong cellular and humoral immunity have been found in men with atherosclerosis and positive C. pneumoniae titers. This organism could be found in diverse arteries with atherosclerosis. One particular C. pneumoniae strain (AR 39) appears to be more frequently involved in atherosclerosis. Antibiotic treatment with azithromycin appears to be protective against atherosclerosis complications. Two large randomized clinical trials are currently underway evaluating azithromycin treatment in patients with atherosclerosis which will hopefully give us answers about the role of antibiotic treatment, but these may be misleading since it appears that there may be a viral component.

The Koch-Henle postulates for the proof of the etiology are largely fulfilled--even if there are doubts about the validity of these criteria in chronic local infections. A number of unexplainable aspect of atherosclerosis can be viewed in a new light. The higher incidence of coronary heart disease in young males has a parallel in the remarkable androtropism of many bacterial diseases (pneumococcal pneumonia, tuberculosis). The rise in the incidence of atherosclerotic diseases in the United States since 1965 can be explained by the much higher exposure to the organisms due to the sexual revolution and other life-style changes.

The increase of inflammatory parameters (C-reactive protein, fibrinogen, leucocytes) before acute coronary infarction may not be risk factors but signs of an active chronic infection. The

interpretation is possible, that atherogenic changes in lipids like increase of LDL and decrease of HDL are the consequences of chronic arterial infection by chlamydia and/or viruses. Vascular infection can be related to the age of the patient at the primary infection. With low hygiene, intestinal primary infections in early childhood can be possible. Arterial infection would be thus a result of a primary infection in adolescence. There are strong arguments for the thesis that C. pneumoniae and viruses are the primary cause of atherosclerosis and not a secondary invader.

The answer lies not in putting antibiotics in the water supply like fluoride but in non-toxic treatments which enhance, rather than further decrease, mitochondrial function. The rationale for dietary supplementation and herbal therapies which are capable of sustaining immune function during later life and assisting the body in ridding itself of chronic infection should be considered. The entire issue of the journal *Nutrition*, July-August 1998, Vol. 14:7-8, is a special issue on the subject of immunonutrition, which contains a wealth of data and information on the role of nutrition in human immune function. Most of this information is relevant to understanding the role of nutrition in arteriosclerosis.

THE TIMOTHY JOHNSON-ABC NEWS THEORY OF HIDDEN ARTERIOSCLEROTIC PLAQUE

Six weeks after the publication in the American Heart Journal of the 1st International Symposium on Infection and Atherosclerosis, November 30, 1999, on January 20, 2000 with some considerable pre-broadcast fanfare and advertisement Timothy Johnson, MD, medical reporter for American Broadcasting Corporation announced a new theory of atherosclerosis, named by him "hidden plaque." He postulated that space-occupying atherosclerotic plaque customarily seen in angiogram and sonogram is seldom the cause of heart attack or coronary occlusion. It is rather the

plaque lurking in the media of the artery which from time to time ruptures, spilling its contents into the lumen, produces the vast majority of coronary thrombosis and stroke. This event may be prevented by the ingestion of one of the six Statin drugs recommending that these should be consumed by everyone at risk, whether or not they have elevated cholesterol levels, decreased high density lipoprotein, increased low density lipoprotein or any of the conventionally accepted risk factors. He stated that while he had no risk factors, after learning of this "new type" of heretofore unsuspected "hidden plaque" he has personally elected to begin chronic ingestion of Statin drugs and recommends that anyone over 50 years of age should do likewise. ABC runs frequent commercials for some brands Statin drugs. Dr. Johnson postulated that other treatments would soon be developed, such as ultrasonic catheters and hyperthermia tips for catheters which could locate and treat these concealed pockets of plaque but reiterated the primary treatment and prevention would be with Statin drugs. He characterized this as the most important news report he had ever made. This is a innovative procedure for bypassing customary publication of papers announcing such discoveries in peer reviewed medical or scientific journals, by announcing it directly to the public on television newscasts by networks that carry frequent commercials for the drugs highly recommended in the newscast. The report gave no clue as to where an interested physician might go in the peer reviewed literature to learn the technical details not covered in the broadcast. It is not known whether such papers will appear in the near future. It is believed that interested scientists and physicians may be able to purchase the video tape of the January 20, 2000 20/20 broadcast from ABC if they wish to learn more details concerning this theory.

Dr. Johnson's belief that the Statin drugs might be a successful therapeutic strategy for

treatment and prevention of this newly discovered form of arteriosclerosis was not set forth in the broadcast. However, by implication, it would appear that Dr. Johnson and his sponsors reject the notion that the primary cause of arteriosclerosis is nutritional, bacterial and viral in nature or at least feel that their newly discovered secret plaques are caused by something different from the plaques that are called "unhidden plaque". Perhaps in the forthcoming months, ABC will enlighten the scientific and medical community by further video publication of their theory.

There is no question that the quantities of vitamins, minerals and other nutrients in the typical Western diet have fallen considerably during the past 150 years. This reduction in dietary micronutrients has resulted both from changes in food consumption patterns and from a decline in soil quality.

Annual per capita consumption of refined sugar has increased (British data) from 6 to 8 pounds in the 1750's to about 120 pounds in the 1950's. This quantity of sugar represents nearly 20 percent of the total caloric intake of the average individual. Since refined sugar is virtually devoid of vitamins and minerals, the increase in sucrose consumption has led to a 20 percent reduction, across the board, in micronutrient intake. Another important change that has come with industrialization is the refining of flour. White flour and white rice, which have been stripped of nutrient-rich germ and bran, constitute an additional 30 percent of calories in the typical Western diet. Large losses of nutrients occur in the refining of grains. For example, when whole wheat is refined to white flour the following losses result: thiamine (77%), niacin (80%), pyridoxine (72%), pantothenic acid (50%), folic acid (67%), vitamin E (86%), choline (30%), calcium (60%), magnesium (85%), potassium (77%), chromium (40%), copper (68%), zinc (78%), selenium (16 to

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80%). When the nutrient losses from refining sugar and grains are combined with those that result from canning, freezing, and vigorously processing foods, the overall reduction in micronutrient levels in the diet is substantial.

As noted elsewhere, even whole grain wheat is poor nutrition for human beings because of its increased ration of ω6 to ω3 fatty acids and its lack of most antioxidants. It should not constitute the mainstay of the diet which it has become for many people today who consume wheat-based breakfast cereals every morning, eat bread, cookies, cakes and crackers to the virtual exclusion of fresh fruits and vegetables. This is a dietary plan for disaster.

While modern food may not be so severely depleted as to cause classic nutritional deficiency diseases, chronic marginal intake of one or more nutrients could conceivably accelerate the development of degenerative cardiovascular disease. Evidence relating specific nutritional deficiencies today is that the chief risk factors are antioxidant deficiency and elevated Homocysteine and oxidative (free radical) damage.

Many physicians concerned about cholesterol levels in their patients and not aware of the recent research about inflammation, homocysteine, bacterial and viral infections and declining immune function with ageing have been prescribing drugs which are designed to lower cholesterol levels by blocking and inhibiting the normal metabolism of cholesterol. These drugs are dangerous to the patients as they interfere with many normal processes in the body and, as noted above, have been found in tests to produce more deaths from ischemic heart disease than are experienced by untreated patients.

The following drugs routinely used in an attempt to lower lipid levels, Atorvastatin,

Cerivastatin, Fluvastatin, Lovastatin, Pravastatin, and Simvastatin, all act by inhibiting HMG-CoA-Reductase which is a step in cholesterol biosynthesis. They have several properties. They block the dietary absorption of vitamins A, E, and K, which are all nutrients essential for proper mitochondrial function, energy production and antioxidant scavenging. They all produce ultra-structural damage to the DNA of mitochondria, which severely interferes with mitochondrial function and produce myopathies. They are administered on a long-term chronic basis and their chronic administration for months is destructive of energy production and free radical scavenging in numerous cells in the body including cardiac myosites. Upon chronic administration, these drugs may well produce myocardial infarction by inhibiting energy production - in other words, they can and do produce the very condition they are administered to prevent. By ill-advised attempts to block normal physiological function, they severely interfere with normal metabolism in the body which ultimately leads to cell, tissue and organ failure.

Attempts to treat the infections which are now believed to be involved in arteriosclerosis via the oral administration of antibiotics will have a similar effect which is even more devastating than the HMG-CoA inhibiting drugs listed above.

Mitochondria, no matter how long they have been endosymbionts in eucharytic cells are still bacteria and can be and are damaged in their structure and function by antibiotics, just like other bacteria are damaged by antibiotics. As a consequence, prolonged administration, particularly by the oral route, of such antibiotics will surely lead to cell, tissue and organ failure to an extent considerably greater than that failure produced by HMG-CoA Reductase Inhibitors. When the two are administered together, they rapidly produce severe myopathies. Therefore, if the millions of

patients suffering from ultra-chronic bacterial and viral infections of their circulatory system should be treated for their chronic infection. It is imperative that such treatment be by means other than antibiotics and statin drugs.

Several such treatments exist and have been used quite successfully for the last 30 years. The rationale for the use of many of them was speculative when their use began but they have produced regression of atherosclerotic plaque and reversal of symptoms without damage to the mitochondria or the cells of the heart, brain or circulatory system. It is now possible to understand, in light of the newly emerging data concerning the nutritional and microbial cause of atherosclerosis to finally completely understand the mode of action of these treatments.

Chelation with magnesium, calcium, ethylenediamine tetraacetic acid and intravenous nutrients has been used to treat and reverse arteriosclerotic plaques for the past 50 years with excellent results. This treatment has been used millions of times with an extremely low adverse incident rate. It has been used to treat and prevent coronary, carotid and femoral arteriosclerosis.

EDTA Chelation has been reported to stabilize mitochondrial function and increase ATP production. It is bacteriostatic and has the property of enhancing the effect of bacteriocidal drugs. It is a powerful viricidal drug itself. Plaque regression, as measured by angiogram and doppler ultrasound, has been regularly observed following repeated administrations of magnesium, calcium, EDTA Chelation. Physicians who are interested in administering this may undergo training and receive certification in this procedure from the American College for the Advancement of Medicine and the American and International Boards of Chelation Therapy. Protocols for Chelation therapy are available through the American College for the Advancement of Medicine. Physicians who

have not been trained and certified in Chelation therapy, should not administer this treatment before receiving this training. The entire process in its scientific basis is explained in Halstead and Rozema, *"The Scientific Basis of EDTA Chelation Therapy"*, 2nd Edition, Landrum, S.C., TRC Publishing, (1997). This book is published in cooperation with the American College for Advancement in Medicine, the Great Lakes College for Advancement in Medicine, the American Board of Chelation Therapy and the International Board of Chelation Therapy. This is also well covered in Chapter 175, Rubin, M., Magnesium EDTA Chelation, *Cardiovascular Drug Therapies* 1996 2nd Edition, Messerli, MD, Franz H., (Ed.) WB Saunders Company, Philadelphia, PA.

Patients at risk for developing arteriosclerosis should be placed on a diet which avoids empty calories such as refined wheat flour and sugar and includes meats, high protein, vegetables, fresh fruits and vegetables rich in antioxidants, vitamins, bioflavonoids and carotenoids which are only available in fresh fruits and vegetables. An excellent outline for such diet is found in Robert C. Atkins, M.D., *"Dr. Atkins Age Defying Diet"* Revolution, New York, St. Martin's Press, (2000).

There are effective homeopathic nosodes which are available and very effective for the treatment of chronic bacterial and viral infections, without the side effects and the mitochondrial damage incurred by the use of antibiotics.

In addition, many herbal preparations have been found to have powerful immune modulatory functions which are beneficial in preventing, treating and reversing arteriosclerosis. These will be discussed in detail in the Chapter on herbs.

In reviewing theories of the patho-etiology of arteriosclerosis we have come full circle. The original and now abandoned cholesterol hypothesis was nutritional; after a half century of intensive

research, we are back again to nutrition, not of fats and lipids but of antioxidants, bioflavonoids, carotenoids and other phytonutrients. There is little or no place for pharmaceuticals in the prevention, treatment or reversal of arteriosclerosis with the notable exception of EDTA chelation. Most of the pharmaceuticals available have adverse effects on mitochondrial structure, function and energy production and should be avoided. There are many nutritional, herbal, and bioenergetic resources for these purposes.

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BASIC SCIENCE ASPECTS OF THE MITOCHONDRIA
SECTION X

CANCER

Cancer is the second largest cause of death and disability in the United States. The trend has been a geometric progression in the incidence of cancer for the past half Century, which parallels several factors: the increase in environmental pollution, the decrease in the nutritional content of processed foods, the replacement of natural foods by such processed foods in the American diet, and the increase in the use of pharmaceuticals which block and inhibit natural bodily processes.

Mitochondria are pivotal in the causation and cure of cancer. The transformation of a normal cell to a malignant cell, the reversal of that process, and the destruction of transformed malignant cells are mitochondrial events, all mediated by changes in the form and function of mitochondria.

Cancer cannot be understood without some understanding of the mitochondria, their role in the life and death of the cell, the various functions they perform and how the breakdown of these functions can lead to malignant transformation of the cell.

Cancer can be and, for a long time, has been successfully treated and cured by people who have never heard of mitochondria, by using empirical treatments which affect mitochondria.

Life and health are dependent on a successful symbiotic relationship between our nucleated cells and the mitochondria which carry out a host of functions necessary for the processes of life.

HISTORY LESSONS

Otto Warburg first described the cancer cell as an essentially anaerobic cell, living by

glycolysis. Albert Szent-Gyorgi gave us a very lucid explanation of what happens to a cell when it divides and cannot find its way back to the oxidative state.

From these two insights, it is now possible to understand the mechanism by which a normal cell becomes malignant, through the loss of its symbiotic relationship with its mitochondria and reverts to its pre-symbiotic state of primordial function.

Dr. Warburg states it thusly:

> " There are prime and secondary causes of diseases. For example, the prime cause of the plaque is the plaque bacillus, but secondary causes of the plaque are filth, rats, and the fleas that transfer the plaque bacillus from rats to man. By a prime cause of a disease I mean one that is found in every case of the disease.
>
> Cancer, above all other diseases, has countless secondary causes. But, even for cancer, there is only one prime cause. Summarized in a few words, the prime cause of cancer is the replacement of the respiration of oxygen in normal body cells by a fermentation of sugar. All normal body cells meet their energy needs by respiration of oxygen, whereas cancer cells meet their energy needs in great part by fermentation. All normal body cells are thus obligate aerobes, whereas all cancer cells are partial anaerobes. From the standpoint of the physics and chemistry of life this difference between normal and cancer cells is so great that one can scarcely picture a greater difference. Oxygen gas, the donor of energy in plants and animals is dethroned in the cancer cells and replaced by an energy yielding reaction of the lowest living forms, namely, a fermentation of glucose. - - -
>
> If a lowered oxygen pressure during cell growth may cause cancer, or, more generally, if any inhibition of respiration during growth may cause cancer, then a next problem is to show why reduced respiration induces cancer. Since we already know that with a lowering of respiration fermentation results, we can re-express our question: Why does cancer result if oxygen-respiration is replaced by fermentation?
>
> The reverse process, the dedifferentiation of life, takes place today in greatest amount before our eyes in cancer development, which is another expression for dedifferentiation. To be sure, cancer development takes place even in the presence of free oxygen gas in the atmosphere, but this oxygen may not penetrate in sufficient quantity into the growing body cells, or the respiratory apo-enzymes of the growing body cells may not be saturated with the active groups. In any case, during the cancer development the oxygen-respiration always falls, fermentation appears, and the highly differentiated cells are transformed to fermenting anaerobes, which have lost all their body functions and retain only the now useless property of growth. Thus, when respiration disappears, life does not disappear, but the meaning of life disappears, and what remains are growing machines that destroy the body in which

they grow. - - -

Since the Lindau lecture of June 1966 many physicians have examined - not unsuccessfully - the practical consequences of the anaerobiosis of cancer cells. The more who participate in these examinations, the sooner will we know what can be achieved. It is a unique aspect of these examinations that they can be carried out on human patients, on the largest scale, without risk, whereas experiments on animals have been misleading many times. The cure of human cancer will be the resultant of biochemistry of cancer and of biochemistry of man. A list of selected active groups of respiratory enzymes will soon be published, to which we recently added cytohemin and d-amino-Levulinic acid, the precursor of oxygen-transferring hemins. In the meantime commercial vitamin preparations may be used that contain, besides other substances, many active groups of the respiratory enzymes. Most of these may be added to the food. Cytohemin and vitamin B 12 may be given subcutaneously. (A synonym of "active group" is prosthetic" group of an enzyme.)

There exists no alternative today to the prevention of cancer as proposed at Lindau. It is the way that attacks the prime cause of cancer most directly and that is experimentally most developed. Indeed millions of experiments in man, through the effectiveness of some vitamins, have shown, that cell respiration is impaired if the active groups of the respiratory enzymes are removed from the food; and that cell respiration is repaired at once, if these groups are added again to the food. No way can be imagined that is scientifically better founded to prevent and cure a disease, the prime cause of which is an impaired respiration. Neither genetic codes of anaerobiosis nor cancer viruses are alternatives today, *because no such codes and no such viruses in man have been discovered so far*[1]; but anaerobiosis has been discovered.)

What can be achieved by the active groups, when tumors have already developed? The answer is doubtful, because tumors live in the body almost anaerobically, that is under conditions that the active groups cannot act. On the other hand, because young metastases live in the body almost aerobically, inhibition by the active groups should be possible. Therefore we propose first to remove all compact tumors, which are the anaerobic foci of the metastasis. Then the active group should be added to the food, in the greatest possible amount, for many years, even forever. This is a promising task. If it succeeds, then cancer will be a harmless disease.

Moreover, we discovered recently a) in experiments with growing cancer cells in vitro that very low concentrations of some selected active groups inhibit fermentation and the growth of cancer cells completely, in the course of a few days. From these experiments it may be concluded that de-differentiated cells die if one tries to normalize their metabolism. It is a result that is unexpected and that encourages the task of inhibiting the growth of metastases with active enzyme

[1] These have now been discovered and are discussed below.

groups.

a) In press in Hoppe-Seylers Zeitschrift für Physiologische Chemie 1967. 10 g riboflavin per ccm or 10 g d-Aminolevulinic acid inhibit in vitro growth and fermentation completely but inhibit respiration less. As expected, ascites cancer in vivo is not cured.

As emphasized, it is the first precondition of the proposed treatment that all growing body cells be saturated with oxygen. It is a second precondition that exogenous carcinogens be kept away, at least during the treatment. All carcinogens impair respiration directly or indirectly by deranging capillary circulation, a statement that is proved by the fact that no cancer cell exists, the respiration of which is not impaired. Of course, respiration cannot be repaired if it is impaired at the same time by carcinogens.

A few years later, another Nobel Laureate, Albert Szent Gyorgi, in his treatise *Electronic Biology and Cancer* restated the problem thusly:

ORIGIN OF LIFE[2]

When life originated some three billion years ago, our globe must have been a very unpleasant place, hot and pitch dark, being surrounded by a heavy layer of water vapor. There was no light and no oxygen. We can only philosophize that under those conditions life could have built only the simplest forms, which, to make life continuous, had to proliferate as fast as conditions permitted. The protein molecules formed must have been rather stable, with no loose ends or unbalanced forces. They had to be "closed-shell molecules" with their electrons arranged in pairs. There must have been a strongly reducing atmosphere containing chiefly electron donors, but no electron acceptors. Among the donating groups the strongly reducing SH must have played an important role, involved in the process of proliferation. We can expect that under those conditions the electronic energy bands were saturated, nonconductant, and the protein dielectric.

As our globe cooled and the surrounding water vapor condensed, eventually, red light of long wavelength could reach the surface of the earth, whereupon life developed a green dyestuff which could capture the red photons, and still makes our meadows green. With the energy of the captured photons the living systems separated the elements of water, fixing the H to carbon, creating foodstuffs, while releasing the oxygen as O_2 into the atmosphere. Oxygen is an oxidizing agent, an electron acceptor, which could induce profound changes in the nature of the protein by separating its electron pairs, making highly reactive free radicals out of its inert

[2]Szent-Gyorgyi, Albert, Electronic Biology and Cancer, Marcel Dekker, NY (1976)

closed-shell molecules. It could desaturate the energy bands, thus making semiconductors out of dielectrics, creating unbalanced forces which could link protein molecules together to increasing complex structures which performed increasingly complex and subtle reactions, leading to differentiation and to a new state of the living systems which I called the "β state" to distinguish it from the α state which preceded the appearance of oxygen.

The unbridled proliferation of the α period was incompatible with the development of complex structures. To maintain the harmony of the whole it had to be arrested and regulated. But even without regulation the semisolid structures must have interfered with cell division, which involves a complete rearrangement of the cellular interior, and demands a more liquid state. To be able to divide, the cell has to dismount its structures to a great extent, dismounting first the most bulky structure, the nucleus, the membrane of which is dissolved and the chromatin condensed into a small number of mobile chromosomes. Also the oxidative mitochondria have to be disassembled, making the cell more dependent on fermentation for energy. All this means that the dividing cell has to dedifferentiate and return, to an extent, to the α state. After completed division the cell has to find its way back to the oxidative-resting β state, building up again its structures and electron transport chains. Should the cell find its road of return to the β state deranged, or should the β state be made unstable by some extraneous factor, then the cell would have to persist in the proliferative α state and tumor would result.

When nature creates new mechanisms she does not throw the old ones away but builds the new ones on top. So the building of solid structures in the β period did not mean that the system of soluble molecules was eliminated. It became the basis of metabolism and served as a matrix into which the new insoluble structures were embedded, and continued to perform its simple "vegetative function," fermentation, catering for the embedded structures which released the total energy of food, opening the way to the differentiation, the end product of which is us.

It should be remembered that at the time these writings were published, endosymbiont nature and origin of mitochondria had not yet been established and most biologists believed that mitochondria were synthesized from other membrane systems of the cell. In Albert Lehninger's classic treatise *The Mitochondrion, molecular basis of Structure and Function*, New York, W.A. Benjamin, 1965, the biogenesis of mitochondrion from other membrane systems is set forth completely and in passing, there is a suggestion that some scientists are beginning to contend that mitochondria may be of bacterial origin.

This latter concept was not fully accepted until 1981, after the demonstration of a mitochondrial genome and the publication by Margulis of *Symbiosis and Cell Evolution*, San Francisco, W. H. Freeman, 1981. Dr. Warburg and Szent-Gyorgi had arrived at their conclusions based entirely on chemical analysis without the benefit of electron microscopy and other techniques developed after their theories were published.

Actually, Dr. Warburg had made some of the pivotal discoveries about mitochondria much earlier. In 1913, he had found cellular respiration to be associated with granules, in soluble elements of cell structure, a finding he reported in Warburg, O., Arch Ges Physiol 154:599 (1913) and had made important contributions, the understanding of cytochromes in his book Warburg, O., *Schwermetalle als Wirkungsgruppen von Fermentation*, Verlag W. Saenger, Berlin, 1948, pp. 212 ff. and Szent-Gyorgi, S., *Discovery of a catalytic effect of the four-carbon dicarboxylic acids* had been the information that permitted Dr. Hans Krebs to elucidate the citric acid or Krebs cycle.

According to Lehninger's book:

> "These were the major events just preceding the confluence of the biochemical and cytological research on mitochondria. Actually, very few biochemists concerned themselves with the possible importance of the fact that respiratory enzymes were found to be associated with particulate matter of cells and tissues. It was a part of the biochemical *Zeitgeist* that particles were a nuisance and stood in the way of purification of the respiratory enzymes. Yet it almost seems paradoxical that it was two biochemists who had many years earlier made important discoveries on the occurrence of biological oxidation-reduction mechanisms in granular elements of the cell"

Drs. Warburg and Szent-Gyorgi were biochemists, not cytologists, and left the details of mitochondrial structure to others. Nevertheless, in principle, they were quite correct and this has been borne out by a large amount of research since that time.

Bradford and Allen in their *Primordial Thesis of Cancer* Med Hypoth 1992; 37:20-3, brought these concepts into harmony and later developments. So that while the details continue to be worked out, the principle that malignant transformation in cells results from a break down in mitochondrial structure and function remains not only viable but also essential to an understanding of the process.

REJECTION OF WARBURG HYPOTHESIS BY ESTABLISHMENT

Dr. Robert A.Weinberg is a founding member of the Whitehead Institute of Biomedical Research and Professor of Biology at the Massachusetts Institute of Technology, where he is head of the Oncology Lab and, as such, is a respected and preeminent establishment cancer researcher. In his book, *Racing to the Beginning of the Road: The Search for the Origin of Cancer,* NY Harmony Books 1996, he explains why Dr. Warburg's cancer thesis was ignored by the establishment thusly:

> " - - - So the problem of cancer, we agreed, needed to be understood in terms of the cells that invent their own, self-directed manifesto of growth and destruction. In fact, the problem could be reduced even further. The ultimate answer to the cancer problem would come from looking at the single ancestral cell that founds the colony of cancer cells by transforming itself from a normal, well- behaved member of a community into a renegade.
>
> How did the single ancestral cell, the renegade, make all this happen? What caused it to decide to strike out on its own—or, conversely, how did a normal cell know when to grow and when to hold back and remain quiet?
>
> The first big answer came long before my friends and I began our work. One of the most brilliant scientific minds of twentieth century science had a clear vision of the solution to the cancer problem, crafted with precision and powerful logic. He knew how the cancer problem would be solved. And he knew it with great—even total—certainty. He started this all. - - -
>
> 'Rubbish!' With a single word, the old man swept away a whole field of competitors. 'Forget everything they say. All you need to remember is what I have just told you.' He and he alone had uncovered the origin of cancer. The engine that drove normal cells to divide uncontrollably had now been revealed. Other ideas had been lying around on the workbench of science. But those ideas were, without

exception, inspired by ignorance and cobbled together by second-class minds.

'Rubbish' was the kindest word he could find. The verdict was clear-cut. So, too, was his own success. Already one of the greatest biochemists of the twentieth century—maybe the greatest—Otto Warburg had moved on from his early successes to conquer yet another important field of scientific research.

Many of those in his audience already knew Warburg. As the acknowledged leader of German biochemistry, he had come to visit them here in Stockholm a quarter of a century earlier. In December of 1931, he had come at the invitation of the Nobel Committee to collect the Prize. The award recognized his research on energy metabolism.

Warburg had figured out how cells harvest energy by burning sugars. His work was, by any standards, an extraordinary piece of research. Having conquered one major problem, he had gone on in the 1930s to attack two more: cancer and photosynthesis. Both had been waiting for unambiguous resolution. And both had yielded to the powerful tools that he had developed earlier to solve the problem of energy metabolism. It was 1955, and Warburg was seventy-two and back in town, still active, lecturing the cream of the Swedish scientific community on his advances, which had uncovered the deepest roots of cancer. The evening visit proved to be memorable. A full forty years later my Swedish friends would still be talking about it.

The researchers and physicians who came to hear Warburg that evening knew full well that the mystery of cancer would not be solved by studying tumors with the naked eye. Even the analysis of individual cancer cells under the microscope promised few satisfying explanations. The real answers seemed to lie far deeper, in the submicroscopic world of the molecules inside cancer cells. Few were as well equipped as Warburg to study the molecules of life that held the key to the cancer puzzle.

The German researchers who had preceded Warburg—his teachers and his teachers' teachers—had worked with the total certainty that their microscopes would lead them straight to the root cause of cancer. When their work began, in the first third of the nineteenth century, many of them believed that tumors arose from mucus and plasma that somehow aggregated into large masses. Only later did their microscopes show that tumors, like normal tissues, were assembled from the individual building blocks they now were calling cells. By mid-century their mindset was further shaped by Rudolf Virchow, one of the leaders of nineteenth-century German medical research. His dictum, omnis cellula e cellule—all cells arise from yet other cells—was applied at first to understand how a complex embryo arises from a fertilized egg. Subsequently they applied Virchow's law to the cells in a tumor, which seemed also to descend from cell-like ancestors.

Later they moved on to solve the question of where cancer cells originate. By about 1850 they had reached a definitive conclusion: cancer cells arose directly from the normal cells of the organ in which they were first discovered. Normal liver cells spawned hepatomas, stomach cells produced gastric carcinomas, brain cells

engendered glioblastomas. Some of the cells present in a normal tissue apparently decided to grow abnormally. Their descendants then formed huge cell populations, resulting in tumors large enough to be visible to the naked eye. *Omnis cellula e cellule* seemed to explain everything.

Then they hit a stone wall. Having reduced cancer to a disease of misbehaving cells, they could move no farther. No one knew what made cells grow normally or abnormally. No one knew how cells decided their own fates. Half a century passed.

In the 1920s, Warburg and a small group of other organic chemists appeared on the scene. They had an entirely new way of posing the cancer question. The real solution, they argued, lay in the chemistry of the cell, beyond the world visible through the microscope. The answers would come from analyzing the complex chemical machinery operating within the living cell. They meant the machinery of cellular metabolism: the hundreds—likely thousands—of chemical reactions that allowed cells to synthesize chemical building blocks and generate energy. Beyond or behind these biochemical reactions lay no further subtlety, no more hidden forces.

If the metabolism of a normal cell provided a complete explanation of its normal behavior, it followed that the life of the cancer cell could be explained by some type of abnormal metabolism. In effect, the cancer problem could really be reduced to something very simple: a key biochemical reaction governing cell proliferation. When this reaction fired properly, the cell around it would grow normally; when it misfired, runaway cell growth would ensue, and with that would come cancer—a straightforward story of cause and effect.

By 1955, the year of Warburg's Stockholm lecture, hundreds of biochemists had begun comparing the metabolism of cancer cells with that of normal cells, looking for the elusive malfunctioning reaction, the Holy Grail of cancer research. But Warburg had already found it, as he made abundantly clear during his memorable talk.

"The single ultimate root cause, from which all of cancer's aberrations can be traced, is anaerobiosis—life without oxygen. All normal cells have an absolute requirement for oxygen, but cancer cells can live without oxygen—a rule without any exceptions. "Cancer was ultimately a problem of how cells used or misused oxygen to burn sugars.

Warburg had succeeded in reducing the cancer problem to its primal cause. Those who proposed alternative explanations of cancer's origins were, as he said on frequent occasion, either incompetent or—worse—outright frauds. Their theories would fall by the wayside, as had a thousand other ideas that pretended to explain the origins of malignancy.

Warburg's explanation was based on a simple yet compelling proof: Cancer cells, unlike normal cells, could grow and divide without oxygen. More to the point, when he took normal cells from an embryo and forced them to grow in a Petri dish in the absence of oxygen, those oxygen-starved cells took on the traits of cancer cells. In itself, the observation of this transformation represented a milestone in

cancer research, since usually such conversions took place deep within the recesses of living tissues.

If a normal cell could be converted into a cancer cell at will, as he had now succeeded in doing, all the answers would fall quickly in place. The rest of cancer research that followed would, at best, be only a minor commentary on what he already accomplished.

Warburg's insight into the cause of cancer also led him to propose a powerful preventive treatment: By exposing animals or humans to the same biochemical compounds that cells normally use to catalyze oxygen-driven combustion, any tendency for cancerous outgrowths to appear could be blocked.

His own preliminary experiments gave indications that this trick would work. "How long cancer prevention will be avoided depends on how long the prophets of agnosticism will succeed in inhibiting the application of scientific knowledge in the cancer field. In the meantime, millions of men must die of cancer unnecessarily." Though the syntax was awkward, the message was, as always, crystal clear: Provide cells with oxygen and they will grow normally; deprive them and cancer will ensue. Rigorous science had finally broken open the age-old problem.

Warburg's Stockholm appearance was in all respects an extraordinary performance—a lecture given in a style that brooked no opposition. The answers to the major questions posed by him were arrayed on large charts that he had mounted in the front of the lecture hall. Warburg's faithful assistant and manservant of thirty years paced back and forth in front of the charts with a long wooden staff, pointing out key pieces of data and important conclusions as the lecture progressed.

When it was over, polite questions were ventured by several of the older Swedish professors. After all, a lecture by the most prominent biochemist of the century demanded some perfunctory follow-up. None of the questions were particularly probing. Warburg knew more about the subject than did anyone in his audience. Also, those who had come to hear him risked ridicule by provoking him with even mildly critical questions. No one had the stomach to take on the attack dog who happened to be their honored guest.

Rarely had any researcher working on the origins of cancer spoken with such certainty. And yet, in spite of the passion, the conviction, the voice of absolute authority, many leaving the lecture hall that evening did not want to believe Warburg. The skepticism of some had little to do with the details of Warburg's science; their motives centered on Warburg himself. They very much wanted him to be wrong, for whatever reason they or anyone else could find.

At the time of his Stockholm lecture, Otto Warburg had been a practicing biochemist for more than half a century. During his career, he had attracted a long list of enemies. Most anyone he encountered in the world of science had either failed to measure up to his own standards of scientific quality or had been unable to appreciate his ideas. His martinet style was learned in no small part in the Prussian military, where, as he said, he had learned "how to command and how to obey."

Warburg was a half-Jew who continued to work in the Third Reich while many of his relatives and colleagues fled for their lives or were shipped off in boxcars to the camps. He had only one item on his personal agenda: his own career.

His formula for survival was shrewd: he lived off Hitler's morbid fear of cancer. More than any man alive, he provided the Fuehrer with the hope of prevention and cure, so Hitler reclassified him as a quarter-Jew. That slight adjustment of his pedigree allowed him to pass under the wire and continue his research while the war raged around him. The Nazis even set him up with his own research institute.

Warburg's detractors knew all this history. Beyond that, they detested his style, his authoritarian voice, his imperial German certainty. Long before the 1955 performance, his style had become passé. Science was more democratic now, and for the first time in almost a century, there were many centers of power and influence outside the prestigious German research institutes. After two world wars, German science was a shell of its former self. The long rows of German scientists queuing up for their Nobel Prizes were no more than faint memories. That gave many in his Swedish audience great satisfaction. Hence, many who walked out of his 1955 lecture had come to view him as a relic, a detested one at that.

But there were also some who questioned the substance of his science. Was cancer actually triggered by the absence of oxygen in cells? His overall strategy of trying to understand cancer by puzzling out the biochemistry of the cancer cell seemed to represent the correct tack. The issue was whether the particular reaction that Warburg had identified lay at the heart of the cancer puzzle or represented a distracting side issue.

A few of his listeners were also troubled by the unusual coincidence that tied the different phases of his career together. First came his Nobel Prize work on oxygen and sugar combustion. Now, exactly the same thinking and techniques had been transferred directly to another, ostensibly unrelated problem, that of cancer. It seemed an unusual stroke of good luck that his monumental work on energy metabolism early in life would lead so directly and effortlessly to the solution of a second, equally monumental problem later on.

Maybe Warburg, by picking his favorite biochemical reaction, had bet on the wrong horse. Maybe, in his drive to find the root cause of cancer, he had chosen the wrong molecules among the thousands inside the cell. Maybe his credentials as the world's best biochemist did not guarantee him a sure ticket to solve the cancer problem.

There was yet another unspoken factor that influenced the skeptics in Warburg's audience: their increasing distaste for cancer research. Though greatly respected by the general public, this kind of science appeared to be attracting researchers whose credentials and credibility were less than impressive. Perhaps Warburg had been sucked into the swamp of cancer research together with a host of scientific mediocrities. Maybe he had even sunk in over his head.

Many scientists working on other medical problems had come to see the

cancer research field as a large garage filled with dozens of highly specialized mechanics. Each was expert in one or another automotive system, and had his own strongly held point of view on how to solve any problem brought before him. When a poorly running car was brought into this garage, the carburetor specialist would look it over and insist on a fault in its carburetor; the machinist knew that the cylinder bores needed to be remachined; the exhaust-system man would demand that a new muffler be installed. Each would assert that the large problem they all confronted was, by a stroke of good fortune, solvable through the particular expertise that he happened to possess.

Warburg, some feared, had become the mechanic specializing in energy metabolism, seeing all the problems of the biological world through the eyes of an energy specialist. They knew of another biochemist, an expert in RNA molecules, who insisted on defects in his favorite molecule as the explanation of cancer. A third, who studied damage to DNA molecules, was persuaded that this process provided the answer. A fourth, who looked at chromosomes, saw abnormal numbers of chromosomes in cancer cells. A fifth, who studied the breakdown of proteins in cancer cells, was convinced that this process provided a clear and unassailable explanation of runaway growth. There were as many explanations for cancer as there were subspecialties in the field of biochemistry.

Warburg remained aloof from the noisy crowd of cancer researchers. Why argue with them, when the answer was perfectly clear? Anyone having only a bit of insight into science could understand the essential difference between a cancer cell and a normal cell, "without knowing what life really is." "Imagine," he wrote, "two engines, the one being driven by complete, the other by incomplete, combustion of coal. A man who knows nothing at all about engines, their structure, and their purpose, may discover the difference. He may, for example, smell it." The smell of the engine inside the cancer cell seemed to suffice to explain all of its bizarre properties.

Still, Warburg lived with another problem that somehow, though quietly ignored, would not go away. There was no obvious reason why the abnormal combustion he described should lead directly to runaway cell growth. The connection seemed arbitrary. It made no more sense than a defective windshield wiper providing the underlying explanation of why an engine stalls or a brake fails. So Warburg's theory eventually fell short. The others fared no better. Their proponents had spent time looking around familiar lampposts for the answer. None of them could come up with a convincing reason why the patch around his particular lamppost held the key. They were all looking where they knew to look.

Their forays into cancer research, in which they tried to fit the disease into familiar molds, were not working. Cancer needed to be studied on its own terms. That made them very uncomfortable. In the early 1950s, some began to look at cancer in a totally different way. The newcomers turned their backs on sophisticated Nobel Prize research. Indeed, their approach was rather simpleminded: They looked at how often cancer struck different sub- populations of humanity. They soon found

> that common tumors appeared in different groups at dramatically different rates. That clue was far removed from the inner workings of cells, but unlike Warburg's smoking engines, it represented a solid start.
>
> Their finding that cancer struck in predictable ways was the springboard for all that followed, the foundation that ultimately allowed my friends and me to move the problem forward." - - -

The next forty-five years of rejection of these observations in favor of spending Billions of Dollars in diverse research instead of following these indisputable leads has produced the fiasco of the last "War on Cancer" by the Allopathic research establishment. During this time the mortality rate from cancer has risen steadily, despite 70 Billion Dollars spent on this research.

It is not likely that either Warburg or Szent-Gyorgi were aware of the pivotal role of free radical damage in mitochondria.

The free radical theory of aging was first described by Dr. Denham Harman, in 1954. He stated that a "single common process, modifiable by genetic and environmental factors, was responsible for aging and death of all living things", and identified this process saying, "Aging is caused by free radical reactions, which may be caused by the environment, from disease and intrinsic reactions within the aging process." Dr. Harman's conclusion, written more than forty years ago, sums up much of what is finally agreed upon today by scientists:

> The free radical theory of aging is supported by studies on the effect of ionized radiation on living things, the dietary manipulations of endogenous free radicals, the reasonable explanation that the free radical theory provides for aging, and finally the increasing number of studies which show that free radical reactions are involved in the pathogenesis of specific diseases.

Dr. Harman's theory was largely ignored and rejected; as late as 1977, authorities in the chemical field were still not convinced that superoxide could "Act as a deleterious or cytotoxic species in living cells". The idea that dangerous free radicals were present in the human biological

system was considered untenable by most biologists until 1969. They were convinced that disease must come from outside of man, not as a by-product of normal biological functions. At that time, a copper-containing protein had been isolated from red blood cells that had no known function. It was then discovered that this copper protein also contained zinc. It was an enzyme uniting two superoxide molecules to form one molecule of hydrogen peroxide and one molecule of oxygen. The protein was renamed superoxide dismutase (SOD) because of its ability to combine two molecules of superoxide.

Since the subunit for SOD was superoxide, a free radical, it became apparent that at least one free radical is normally found in biological systems. With this realization, research in this area of biology began opening a new avenue of research in which other free radicals were subsequently discovered. Their scavengers were discovered shortly thereafter.

It was recognized that superoxide and the hydroxyl radical were instrumental as causative factors not only in many degenerative diseases but in the aging process as well. In general, knowledge of the free radicals wasn't widespread in the scientific community until the 1980's and research in that era didn't really get stated until the late 1980's.

Dr. Harman anticipated the present interest in antioxidant prevention and treatment of cancer by 30 years with the publication of his paper *"Prolongation of the Normal Life Span and Inhibition of Spontaneous Cancer by Antioxidants"*, in the Journal of Gerontology 16:274 in 1961.

Warburg suggested feeding patients the chemicals which support the Krebs Cycle as a means of treating cancer, feeling that a deficiency of these might be the cause of the failure of oxidative phosphorylation in cancer cells. This helps where there are deficiencies, but at that time, detailed information about mitochondrial function was lacking.

If Warburg's and Harman's insights had been followed up on by research of the intensity and liberal funding done by the people described in Dr. Weinberg's book, by now the cancer plague would probably have been a thing of the past.

It may also be noted that Warburg's work all took place before the explosive growth of industrial and chemical production of the 1960's to 1990's, and that the world he lived in was not particularly polluted. The medical therapeutics of his day were, by and large, the synthetic petrochemicals which were developed after the end of World War II and were, to a large extent, still fairly natural. The antibiotic age has just dawned.

A current medical text of that time recommended the herb Condurango as a cancer treatment.[3] Antimitotic chemotherapy was still off in the future at that time and the incidence of cancer was still fairly low. The Materia Medica listed in that treatise contains over 50% herbal remedies.

Food was still generally locally produced without chemicals or artificial fertilizer, pesticides or herbicides, many of which were first produced during and immediately after World War II. While motor vehicles had been around for half a century, there were only about 25% as many as are operated today and rail transportation was very much in use for passengers as well as freight. There was far less particulate air pollution and smog. Many city water supplies were still potable and relatively unpolluted, not filled with toxic chemicals and the other toxins we add to mask them. Not surprisingly, there was far less cancer and far less of the factors known to produce oxidative damage than there is today.

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[3]Mullen, Edward, Handbook of Medical Treatment, Philadelphia, F. A Davis Company (1942)

For most of the 20th Century, at least until well into the 1970's, there was a social stigma attached to cancer and it was not openly discussed. Its increasing incidence in the population over the past twenty-five years seems to have overcome the stigma and open discussion brought the incidence to public attention.

The public attention resulted in the government's ill-fated "War on Cancer", which was waged in its entirety by the Allopathic cancer research establishment and ended in a humiliating defeat after years of astronomical expenditure of research funds.

Fortunately, not all scientists chose to join the cancer establishment and quite a few did follow up on Warburg and Szent-Gyorgi's leads. Most of these were not cancer researchers; they were biologists, biochemists, and other basic science researchers who soon discovered the missing link, which has been so studiously ignored by the cancer research establishment- mitochondria. They also discovered the genetic codes of anerobesis and the cancer viruses, which Dr. Warburg noted had not been discovered at the time he announced his hypothesis.

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"Studies described as early as 1930 by Warburg and most recently, by Kroemer's group, suggest mitochondrial involvement in cancer, perhaps through its central role in energy production and programmed cell death (apoptosis). Several other lines of evidence suggest a role of mitochondria in carcinogenesis. These include presence of mtDNA fragments into nuclear genomes, transmission of onco genic viral DNA, mitochondrial activation of chemical carcinogens and altered affects of mitochondrial Ca2+ homeostasis. In addition, mitochondria, being the primary site of ROS, contribute to spontaneous mutagenesis which may lead to neoplastic transformation and human cancer. A role for mitochondria in cancer is further supported by the presence of a tumor suppressor protein in mitochondria. The exact biochemical function of this tumor suppressor protein is not clear. However, it is believed to be involved in breast cancer and aging.

A mitochondrial role in carcinogenesis may also involve the Bc1-2 protein. Anti-apoptotic Bcl-2 protein is localized to the mitochondrial membrane, and it possesses anti-oxidative activity. Two mitochondrial proteins involved in the induction of apoptosis have been identified with the use of cell-free systems, proving a critical role for mitochondria in apoptosis. Cytochrome c and apoptosis-inducing factor (AIF) are located in the intermembranous space and are released upon initiation of apoptosis, coinciding with the permeability transition of mitochondria. Bcl-2 protein inhibits the release of the factors as well as transition in membrane permeability. Dysfunctional mitochondria alter the sensitivity of cells to apoptotic stimuli. A block in apoptosis is thought to be a major determinant of cellular transformation, thus aberrant mitochondria may contribute to carcinogenesis. The role of Bc1-2 in apoptosis is also evident in neurodegenerative diseases. - - - "

In this regard, see:

1. *Prevention of Apoptosis by Bcl-2: Release of Cytochrome c from Mitochondria Blocked"*, Yang, et al, Science, (1997)Vol 275 (5303):1129-1132.

2. *The Bcl-2 Protein Family: Arbiters of Cell Survival*, Adams and Cory, Science, (1998) 281(5381):1322-1326

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9. *Progress on the mitochondrial permeability transition pore: regulation by complex I and ubiquinone analogs.* Fontaine E, Bernardi P, J. Bioenerg Biomembr 1999;31(4):335-45

10. *Mitochondrial oxygen radical generation and leak: sites of production in states 4 and 3, organ specificity, and relation to aging and longevity.* Barja G., J. Bioenerg Biomembr 1999;31(4):347-66

11. *Mitochondrial genome mutation in cell death and aging.* Ozawa T., J. Bioenerg Biomembr 1999; 31(4):377-90

12. *The Release of Cytochrome c from Mitochondria: A Primary Site for Bcl-2 Regulation of Apoptosis.* Kluck, RM, et al., Science, 1997, 275(5303):1132-1136

13. *Mutations in SDHD, a Mitochondrial Complex II Gene, in Hereditary Paraganglioma*, Baysal, BE, et al., Science 2000, 287(5454):848-851

14. *Mitochondrial respiratory chain disorders I: mitochondrial DNA defects,* Leonard JV, Schapira AHV, Lancet 2000, 355:299-304

The Development of the Concept:

A healthy cell is a cell which possesses sufficient energy to carry out its functions and perform its work. Sickness is simply a lack of sufficient energy to carry out the functions of life. While many different kinds of insults and injuries can destroy cells. They do so by depleting its energy or by depleting its ability to utilize energy resources. A cell becomes sick when it does not

have enough energy to carry out its normal functions or is unable to meet the increased energy requirements influenced by various stresses; such a cell can be rejuvenated if its energy is somehow restored, regardless of the initial causes of its problems - cells die simply because they have no energy or life.

The main source of energy in cells comes from a molecule called ATP or Adenosine Triphosphate. Cells convert nutrients and products of metabolism to energy by manufacturing ATP, whose energy resides in high-energy phosphate bonds. As each phosphate is broken off, the bond connecting it to the rest of the molecule provides energy the cell can use for growth, reproduction, muscle contraction or anything it needs to do.

The process of making ATP is carried out in small organisms contained in each cell which are called mitochondria. They are found in every cell in the human body with the notable exception of red blood cells. The more work a cell has to perform, the more mitochondria it has. Mitochondria are generally felt by biologists to be symbionts since they do not share the DNA of the cells they inhabit but have their own DNA.

These endosymbionts, possessing their own DNA, reproduce independently of the cells reproduction and do not participate in the genetic inheritance which characterizes the cells; mitochondrial inheritance is solely by maternal inheritance, being passed to the offspring as a part of the ovum, as the sperm contain no mitochondria.

All aerobic life forms, plant, animal and fungal, contain mitochondria which are very similar.

The mitochondrial DNA of human mitochondria is said to have around 8 distinctive haplotypes which are believed to correspond to 8 human metabolic types.

Recently, Majid Ali shed light thusly:

Oxidative Injury to Mitochondrial Ecology[4]

"Mitochondria play a major role in human energy dynamics (ATP synthesis and related reactions), and a functional deficit may be expected to result in diminished supply of high energy phosphate bonds. In mitochondria, electron transfer is *coupled* to oxidative phosphorylation via a proton gradient, and the energy *released* from oxidation is transferred to the ATP synthase *trapping* system. A class of compounds called *uncouplers* can block electron-transfer-linked phosphorylation at any of the three stages (energy production, transfer, or trapping). Redox reactions may release free energy (delta G negative). Such energy may be dissipated as heat, as indicated earlier, or be trapped as a proton gradient. Whether energy is dissipated or trapped depends on the characteristics of biologic membranes rather than on the reaction itself. Any substance that allows protons to leak across membranes will play an uncoupling role, since it will block the transfer of energy between electron flow and ATP synthesis.

More than 90% of the oxygen used in the human body is utilized by mitochondrial cytochrome oxidase, which transfers four electrons into an oxygen molecule to produce two molecules of water. Under ordinary circumstances, reduction of oxygen by cytochrome oxidases in the above reaction does not release active oxygen radicals. This is assured by transitional metal ions such as iron, copper, vanadium, and titanium, which are carried in the active sites of cytochrome oxidases. Such metal ions occur in variable states of oxidation, and changes in such states facilitate transfer of single electrons in an orderly fashion in which various partially reduced forms of oxygen are held bound to the metal ions. These ions also play essential roles in spontaneous oxidation (autoxidation) of several nonradical compounds including ascorbic acid; thiols such as cysteine, homocysteine, and reduced glutathione; catecholamines such as epinephrine and norepinephrine; and a host of amines such as 3,4-dihydroxyphenylalanine (DOPA) and 6-hydroxydopamine.

Deficits in mitochondrial function arise by two mechanisms: (1) a block in electron flow or ATP synthesis which results in lactic acidosis; and (2) uncoupling electron flow from ATP synthesis (Luft's syndrome) in which oxidation occurs at a rapid rate but without a concomitant increase in ATP synthesis (energy released is dissipated as heat). In both conditions, mitochondria increase in numbers and are often deformed, and the muscle cells show ragged red fibers. Surprisingly, ATP measured in muscle tissue in such cases is often near normal; however, there is a marked reduction in creatine phosphate P_i ratio--from a normal value of 9:1 to 1:1 in some affected individuals.

Human mitochondrial DNA (mtDNA) is about 16,000 bases long. Of the 13 polypeptides that it encodes, six are for NADH dehydrogenase, three for cytochrome oxidase, two for ATP

[4]Ali, Majid, Oxidative Regression To Primordial Cellular Ecology, J. Integrative Medicine, 1998, 2:4-55

synthase, and one for cytochrome bc_1 complex. Point mutations in mtDNA of various degrees occur with predictably variable results, so that some cells may be produced with normal and others with defective mitochondria. For example, in one such mutation a defect in NADH dehydrogenase results in a block in electron flow from flavin to quinone and leads to fatal infantile myopathy. Other variants of mitochondrial malformations and malfunctions lead to certain types of cardiomyopathy, ophthalmoplegia, and encephalopathy. In Kearns-Sayre syndrome and related disorders, many body organs are clinically affected; however, in some cases, clinical symptomatology is limited to the skeletal muscle (fatal infantile myopathy) and brain (Leigh syndrome).

Oxidative mitochondrial injury leads to disturbance of cellular energy metabolism. Persistently elevated lactic acidosis levels indicate accelerated mitochondrial injury. Direct and indirect evidence that such injury is oxidative in nature may be drawn from the following: Reduction of mtDNA content, increased lactic acid production, and loss of mitochondrial cristae (indicating oxidatively-induced mitochondrial dysfunction) occurs when mitochondria are exposed to oxidizing influence of a variety of drugs. Bryostatin, a novel antineoplastin agent and protein kinase activator, causes myalgia by two mechanisms: 1) oxidative impairment of energy metabolism and 2) delayed proton washout from muscle indicating vasoconstrictive action. Norepinephrine and ADP stimulate Mg2+ efflux from intact cardiac myocytes and mitochondrial respectively. It has been proposed that direct hormonal regulation of myocardial Mg2+ plays a regulatory role in homeostasis. However, evidence for that remains inconclusive.

In McArdle's disease (myophosphorylase deficiency), impairment of glycogenolysis leads to exercise intolerance and exercise-induced myalgia--exercise related changes that are similar to CFS and fibromyalgia. The half-time for intracellular ADP recovery, an indication of maximal mitochondrial oxidative phosphorylation, is abnormally low in McArdle's disease and, predictably, will be shown to be low in CFS and fibromyalgia. Activities of respiratory-chain enzymes containing mitochondrial DNA (mtDNA)-encoded subunits are impaired in a patient with progressive weakness of extremities. In another 16-year-old with cytochrome c oxidase deficiency, high lactic acid levels indicated mitochondrial enzyme dysfunction.

In the past, the histologic and metabolic studies of mitochondrial function in CFS have been deemed inconclusive. However, recent studies show clear evidence of structural and functional derangements of mitochondria in the ORPEC state. Recently, Eisenger and colleagues reported glycolysis abnormalities in fibromyalgia. Plioplys and Plioplys described electron microscopic observations of muscle mitochondria in CFS patients. Veccheit et al reported reduction of some mitochondrial enzyme activities, inversion of cytochrome oxidase/succinate dehydrogenase ration, increments of common deletion of 4977bp, mitochondrial pleio/polymorphism, and "monstrosity" of mitochondria in CFS. Other lines of evidence for mitochondrial injury include damage observed with laser scanning confocal microscopy. It seems highly likely that further investigations into this matter will disclose additional evidence of mitochondrial dysfunction in clinical syndromes associated with the ORPEC state."

In 1997, Douglas C. Wallace of Emory University noted:[5]

"Increasingly, mitochondrial diseases are recognized as a relatively common cause of degenerative diseases in both children and adults. While the role of mitochondria as the power plants of the cell has been understood at the biochemical level for decades, the role of mitochondrial defects in human disease has only recently been recognized. The delay in recognizing the importance of mitochondrial disease is the result of their variable and complex signs and symptoms as well as the novel genetics and atypical inheritance of mitochondrial defects.

The mitochondria generate energy by breaking down carbohydrates and fats through a chain of chemical reactions. Since each organ in the body relies on mitochondrial energy to a different extent, the nature and severity of symptoms vary widely in patients, depending on the specific mutations in their nuclear and mitochondrial genes. Indeed, since the percentage of mutant mitochondrial DNAs …can differ among individuals in the same family and even among tissues of the same individual, the same mitochondrial DNA mutation can cause different symptoms even in members of the same family.

Though all tissues make and need mitochondrial energy, the areas of the body that are most reliant on mitochondrial energy are the central nervous system (brain and spinal cord), heart, skeletal muscle, endocrine systems (glands like the thyroid and pancreas) and kidneys. By their effects on these areas, mitochondrial diseases can cause certain forms of blindness, deafness, dementia, movement disorders, epilepsies, seizures, heart disease, muscle disease, diabetes and kidney problems.

Moreover, depending on the gene involved and the severity of the mutation, mitochondrial diseases can affect people of all ages, from newborns through adulthood. Well-known mitochondrial diseases of children are Leigh's syndrome, cardiomyopathy, and medium-chain acyl CoA dehydrogenase (MCAD) deficiency. Typical mitochondrial diseases of young adults include Leber's hereditary optic neuropathy and Kearns-Sayre syndrome. Mitochondrial defects have also been implicated in more common diseases such as diabetes, dystonia, and Alzheimer's disease …In fact, it has been suggested that mitochondrial DNA mutations accumulate with age in the different tissues of our bodies, progressively eroding energy production, and playing an important role in the progression of degenerative diseases and in aging.

Mitochondrial diseases, while only lately identified, are not rare. They may be an important component of some of the most common degenerative problems which plague our society. It is vital that this important new mechanism for disease be actively investigated and understood so that mitochondrial disorders can be routinely considered when diagnosing acute diseases of the newborn as well as the progressive disorders of aging."

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[5] Wallace, Douglas C. of Emory University, Exceptional Parent, Vol. 27, No. 6, June 1997:

Dr. Wallace recently expanded these remarks, as follows:[6]

Somatic mtDNA Mutations in Aging and Cancer

"The delayed onset and progressive course of mitochondrial diseases suggests that mitochondria function may decline with age. This hypothesis is supported by multiple reports of age-related declines in primate mitochondrial oxidative phosphorylation enzyme activities in skeletal muscle, liver and brain, and the associated accumulation of somatic mtDNA rearrangements in these same postmitotic tissues. For example, polymerase chain reaction (PCR) experiments have shown that skeletal muscle from human subjects under the age of 40 contains primarily intact mtDNAs, whereas skeletal muscle from subjects over the age of 50 shows an accumulation of a wide array of mtDNA rearrangements. In addition, the skeletal muscle of elderly subjects has been found to have RRFs, with each COX^- and SHD^+ fiber containing a different mtDNA mutation. This confirms that each of the mutations arose de novo and was selectively amplified within the cell to create the regional respiratory defects.

Somatic mtDNA mutations also occur in the brain. Quantitation of the common 5-kb mtDNA deletion has shown that mtDNA deletions accumulate markedly in the basal ganglia and various cortical regions in humans after age 75. An analogous age-related accumulation of somatic mtDNA rearrangements also occurs in mouse tissues, the extent of which is proportional to life-span rather than absolute time.

The cause of the somatic mtDNA mutations is likely to be oxidative damage, which increases with age in the mtDNA of both man and mouse. Patients with chronic ischemic heart disease, which is associated with cyclic bursts of mitochondrial ROS during ischemia and reperfusion, have been found to harbor 8 to 2000 times more mtDNA deletions in the heart than age-matched controls. Similarly, cortical mtDNA deletion levels are elevated in patients with Alzheimer's and Huntington's disease, and mtDNA from the former group shows increased oxidative damage.

These observations have led to the hypothesis that somatic mtDNA mutations accumulate in postmitotic tissues with age as a result of mitochondrial ROS damage. The resulting age-related decline in oxidative phosphorylation would ultimately degrade the tissue's bioenergetic capacity until it falls below a certain threshold, resulting in symptoms and senescence. This same age-related decline in oxidative phosphorylation could interact with inherited mitochondrial defects, which would account for the delayed onset and progression of mitochondrial diseases.

Somatic mtDNA mutations have also been identified in various tumors and tumor cell lines. These mutations include intragenic deletions, missense and chain-termination point mutations, and

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[6]Wallace, Douglas C., Mitochondrial Disease in Man and Mouse, Science, Vol. 283, pp 1482-1488, 5 March, 1999

alterations of homopolymeric sequences that result in frameshift mutations. In principle, these mutations could contribute to neoplastic transformation by changing cellular energy capacities, increasing mitochondrial oxidative stress, and/or modulating apoptosis.[7]

As can be seen from a review of the rapidly growing literature on Mitochondrial Diseases, quite a few diseases which are due to mtDNA deletions are manifest in infancy and early childhood, as syndromes, some of which are rapidly fatal, some of which can be treated. These distinct syndromes can be and should be treated in centers where this is diagnosed and treated. On the other hand, there is a growing awareness of much later onset of mitochondrial diseases which are due to decline in mitochondrial function with age due to mutations or to toxic influences with mitochondrial processes, or deficiencies in oxygenation due to repeated episodes of ischemia and reperfusion which lead to generation of Reactive Oxygen Species and other free radicals which can lead to mitochondrial damage and decline in mitochondrial function.

To distinguish these from the infant and early childhood diseases, we call these acquired mitochondrial diseases and disorders.

These acquired mitochondrial dysfunctions which lead to degeneration of tissues and organs can be treated, reversed and prevented by regenerative therapies on an outpatient basis.

The relatively slow onset of some of these disorders, due to a gradual diminution of tissue oxygenation rather than the abrupt onset of ischemic disorders, leads to damaging of tissues rather than the apoptosis seen after ischemia and reflow. The affected cells become dormant; their metabolic fires banked and slowly smoldering rather than amply oxygenated. Such dormant cells

[7]This is also addressed in another paper by Dr. Wallace and others in 1995 - Wallace, D.C., Shoffner, J.M., Trouce, I. et al 1995. Mitochondrial DNA mutations in human degenerative diseases and aging. *Biochem. Biophys. Acta* 1271:141-151.

can be brought back to normal or near normal function by appropriate measures.

Examples of such dormant cells are found in macular degeneration which can be reversed by electro-magnetic therapies; the penumbra surrounding infarcts in strokes which can be and are restored to function by hyperbaric oxygen therapy which avoids the excitotoxin induced apoptosis of reflow by supplying oxygen to the tissues, and the reversal of cardiac myopathy by pulsed electromagnetic therapy.

Antibiotics which attack bacteria and arrest their growth can have a profound effect on the structure and function of some of the mitochondria of the cells. Oral antibiotics affect not only mitochondria but also the gut flora adversely. This causes a dysfunctional gut ecology which is its turn allows toxins to reach the cells of various systems and interfere with mitochondria function.

Mitochondrial DNA encodes for the production of a number of proteins which are essential for carrying out oxidative phosphorylation. The production of these proteins is being studied by genetic biochemists who routinely utilize common antibiotics to block such protein processing. Some of the antibiotics used are chloramphenicol, tetracycline, and erythromycin. Other chemicals are capable of acting as uncouplers of phosphorylation, i.e., sodium fluoride, which in many places is routinely added to the drinking water.

Synthetic compounds such as drugs, herbicides, pesticides, fertilizers or industrial pollutants are capable of interfering with or interrupting the life processes of mitochondria. These are routinely dumped into the aquifer from which they find their way into the human digestive system and eventually reach the mitochondria to disrupt mitochondrial function.

Antibiotics are singled out here along with fluoride as examples but there are thousands of synthetic compounds used therapeutically and industrially which are capable of affecting the

structure and function of mitochondria by interfering with the production of energy (ATP) as well as causing the generation of Reactive Oxygen Species and other free radicals.

Many food additives used to extend the shelf life of processed foods may be capable of producing such damage to the mitochondria as well.

Meats frequently contain the residues of antibiotics which are included in the feed. Some of these antibiotics' residues remain in the meat along with hormones used for promoting growth. When these reach the human digestive system, where bowel dysbiosis exists, they may easily reach the blood and then are carried to organs to the mitochondria of the cells.

The healthy cell is in what Szent-Gyorgi termed the β or oxidative resting state, producing abundant energy for its work through oxidative phosphorylation, and the electron transport chain of the mitochondria; when something interferes with this oxidative function to decrease energy production significantly, the cell begins to revert towards the α state, an anaerobic state. When it does so, it loses its functions and is barely able to maintain its structure. It may revert completely to the primordial state and resume the incessant mitosis characteristic of malignancy, or it may not reach that state and simply become dormant.

There is always the possibility that it can be returned to the healthy oxidative state and sometimes this occurs spontaneously.

THE NATURE OF CANCER AS A MITOCHONDRIAL DISEASE

Mitochondria have a large number of complex functions in the body, far beyond their role of producing energy. We also know that these endosymbionts possess their own genome, which is far less protected than is the genome contained in the nucleus and far more vulnerable to environmental toxins and pollutants than is the nuclear genome. We now know that when the

mitochondria become unable to adequately perform their functions, the cells will either die, become dormant, or undergo malignant transformation. The possibility exists to cause such cells to revert to normal aerobic cells, capable of normal function. This can be accomplished in a number of ways.

The allopathic approach has been to kill such cells rather than attempt to reconvert them to normal function. This has been consistently unsuccessful and, quite often, kills the patient rather than the tumor.

Anything which inhibits the production of antioxidants will eventually cause cancer or some other degenerative disease. Anything which promotes the production of antioxidants will help to prevent cancer and other degenerative diseases. Because of this realization, the use of antioxidants is becoming the medicine of the 21st Century.

Many of the traditional non-allopathic cancer therapies can now be understood in light of their activity as antioxidants and ability to promote antioxidant activity. The anticancer effects of a large number of herbs can be understood because of their activity as antioxidants.

The key to interruption of the function of mitochondria is through free radical damage to the mtDNA or other structures in the mitochondria. The proper antioxidants or combination of antioxidants and methylation enhancing factors would logically be therapeutic.

Most of the potent antioxidants are plant derived, and these make up an important part of the total antioxidant defense system. The antioxidant properties may be from vitamins, minerals, or compounds which possess the free radical scavenging property or promote the metabolic pathways of compounds which do have antioxidant properties.

For a supply of such antioxidants and the constituents which make them up, humans are

dependent on the foods they consume, which increasingly do not contain all the nutrients they should. Foods are processed by adding chemicals which have very deleterious effects on the body. It is generally necessary for all Americans to supplement their food intake with the vitamins, minerals and other non-nutritive components of foods in order to ensure that they have an adequate supply of antioxidants and the components which make up antioxidants. People should avoid some modern processed foods which contain deleterious substances.

Mitochondria age due to pollution-caused deletions and other mutations to their DNA or to the Nuclear DNA which encodes mitochondrial proteins. These mutations are due to free radicals produced in the mitochondria as a result of interference with the function of the complexes which transport electrons to produce ATP and, ultimately, combine with oxygen to produce water.

The high rate of cancer and coronary heart disease in the United States and other wealthy nations is largely due to industrial pollution and extremely high intake of pharmaceuticals, both prescribed and over-the-counter. All of these elements have the property of blocking or inhibiting some natural biochemical process in the body. In poorer nations with much less access to such drugs, the cancer rate is much lower.

The extremely high death rate from cancer is due to the toxic and ineffectual use of chemotherapy agents to aggressively treat cancers.

Cancer is one disease which manifests itself in different organs and tissues. It invariably results because of a breakdown of antioxidant defenses and free radical damage to the mitochondria of the cells making up the affected organ or system. Clumps of malignant cells may develop daily but are reversed by the body's immune system. Such small foci of malignancy may be routinely detected in CSCT scanning, and their spontaneous appearance and disappearance seems to be a

normal part of the body's functions. Those which occur and are not reversed become clinical cancers.

Since the appearance and spontaneous disappearance of small clumps of malignant cells produces no symptoms, and is generally unnoticed unless the individual undergoes some sort of electromagnetic scan, the precise incidence and rate of this is not known. There are thousands of recorded and reliably reported instances of the spontaneous remission of larger tumors which regressed without treatment. Many malignancies for years have been detected first at autopsies of individuals whose deaths were from unrelated causes.

Hundreds of thousands of individuals have been successfully cured of cancer at unorthodox cancer clinics, both in the United States and offshore. These cancers were reversed and their reversal resulted in return of the affected tissues to normal function.

Unorthodox cancer clinics rarely publish statistics concerning the cure rate. When they do, these are not published in the peer reviewed periodic literature. The only statistics available are those of clinics utilizing orthodox cancer therapies and these increasingly are dismal for most cancers.

The single most productive result of cancer research was the publication of the National Research Council's report on *Diet, Nutrition and Cancer*. This shed some light on the problem, and led to a considerable line of research into the role of nutrition and nutrients in both the causes and means of curing cancer.

The information that many foods, i.e., the cruciferous vegetables such as cabbage, broccoli, Brussels sprouts, all of which contain substances known as Beta Isothiocyanates, have a profound preventive effect on the development of cancer in those who regularly consume them. These

compounds are also highly effective antioxidants.

Ongoing research has discovered many other foods and food components which also have considerable antioxidant properties, such as Alpha Lipoic Acid, Cysteine, Glutamine, Methionine, N-Acetylcysteine, Pectin, Quercitin, Hesperidin, Diosmin, Resveratrol, Elegiac acid, Curcumin, Cinnamic acid, Lutein and Zeaxanthin, Lycopene, Coenzyme Q-10 and DHEA.

There are different species of free radicals, and each of these requires different types of antioxidants to scavage and control them. The body has an integrated system of antioxidant defenses, to suppress the various free radicals and this system is dependent on a variety of antioxidants and the nutritional precursors to antioxidants. This system can generally function adequately, but its operation can be interfered with by the buildup of toxins from environmental pollutants, food additives and therapy with pharmaceuticals. All of these may have an inhibitory and blocking effect on the processes responsible for mounting the antioxidant defense.

Prevention of cancer depends on two elements: the intake of the proper nutrients to act as a build-up of antioxidant defenses and the avoidance of chemicals and substances which can interfere with the antioxidant defense and lead to the excessive generation of free radicals capable of damaging and interfering with energy production in the mitochondria.

There is a form of liver cancer which at first seems to be connected to the virus which causes Hepatitis C. This disease produces cirrhosis in 50% of its sufferers and 10% of the sufferers go on to develop liver cancer. It is apparent that the cancer is not the direct result of the virus but of the disturbed liver metabolism resulting from the viral infection.

The immune system is believed by the Allopathic research establishment to consist of cells which destroy malignant cells by ingesting them. These cells contain a large amount of NADH

oxidase, an enzyme which promotes the antioxidant system. It may be that these cells, rather than destroying cancer cells, may be energizing them with their antioxidants, to help them return to normal function. Worn out cells are, phagocytized in the body by macrophages.

In 1997, Drs. L.C. Clark, O.F. Combs, Jr., B.W. Turnbull and others for the Nutritional Prevention of Cancer Group, published a Study in the Journal of the American Medical Association (227:1957-1963) reporting the results of a study of over a thousand people who consumed 200mcg Selenium in a high selenium yeast tablet daily for ten years. This was a controlled study, in which a comparable group of people who did not consume the selenium supplement was studied as well.

In the group taking the daily selenium supplement, there was a 37% drop in all cancers, and a 50% drop in cancer deaths, 46% fewer cases of lung cancer, 67% fewer cases of esophageal cancer, 62% fewer colon cancers and a 72% reduction in prostate cancer, as compared to the control group during that 10 years.

The primary antioxidant effect of Selenium is that it is a precursor to Glutathione Peroxidase. Selenium supplementation produces a 33% increase in glutathione activity. Whether or not this property was responsible for the reduction in cancer incidence observed in these studies, it certainly played a significant role in it.

This takes us back to the original Warburg Hypothesis of the 1950's in which Dr. Warburg and his co-workers first elucidated the cancer cell as an anaerobic throwback and to Albert Szent-Gyorgi's elucidation of malignancy as a reversion to the primordial state (Alpha State) from the oxidative or Beta State of normal function and validates these concepts by providing the information about the nature, etiology and function of mitochondria.

Antioxidant therapies will not, of course, cure every cancer patient; there are other factors in

the causation of cancer which cannot be addressed at the cellular level.

Most people with cancer, who genuinely want to be cured of the disease, can be cured with antioxidant therapy and the avoidance of the exposure to chemicals which interfere with the antioxidant defense system if the disease has not progressed to the point of incurability.

Patients who have undergone unsuccessful allopathic radiation and chemotherapy will be more difficult to treat and cure than those who have not undergone such assaults. Many of these people can be cured in unorthodox cancer clinics which employ one species or another of detox and antioxidant treatments.

There is absolutely no validity to the allopathic approach of using toxins to cure what is a toxic disease. Two toxins generate toxicity in a geometric progression. The only way to cure cancer is to rid the body of its toxic load and this simply cannot be accomplished by adding more toxins to the mix.

The human body has a very effective set of defenses against toxins. For millennia, it was fairly effective in a natural world where the toxins it encountered were themselves natural. The human body was not designed to deal with a world as loaded with artificial and synthetic toxins as the world has become over the past fifty years.

Dr. Allen J. Leibermann of Charleston S.C., in his Foreward to Krohn, et als "The Whole Way to Natural Detoxification", Pt. Roberts Washington, Hentley & Marks (1966) remarks:

> "Everyone realizes that our planet is increasingly polluted, but few recognize that humans are the final resting place for many of the toxic substances and materials to which we are exposed. Even fewer know what can be done to reduce the body's burden of toxins or exenobiotics, through a process known as detoxification. The population of the earth is faced with an increasing risk from toxic chemicals and physical forces, and now more than ever, humans may be facing a race between knowledge and extinction".

Sci-mito -8

The rise in the incidence of cancer and deaths from cancer not only parallels the rise in the development and use of toxic chemicals and materials in the environment, but also toxins in our food and water supplies and pharmaceuticals ingested by the ton. The rise in cancer incidence and deaths is directly caused by such toxic ingestion and the bodys' increasing inability to cope with this toxic overload of xenobiotics. Not only has allopathic medicine failed to realize this, but its very therapeutic system is the source of the ingestion of a large part of this toxic load. Therefore, allopathic medicine is not only unable to cure cancer, but responsible for the cause of much cancer.

The comments of Dr. James F. Balch in his recent treatise "The Super Antioxidants", New York, M.Evans & Co. (1998), state the problems succinctly:

> "Normally, enzyme free radical-scavenging activity occurs in every cell, but there are specific factors that may enhance or subdue this activity. The first of these factors to consider is the *total burden* of free radicals the body confronts. General Custer's troops lost the battle at Little Big Horn for a simple reason: too many Indians. In the same way, the limited number of antioxidants in your body can be overrun by too many oxidants. Free radicals are generated continuously, so it is essential to provide the cells with the best possible conditions for dealing with them. For each cellular component there is a specific concentration that must be reached before cellular damage occurs. Given the right circumstances, the activity of free radical-scavenging enzymes maintain free radical concentrations below this minimum toxic concentration (MTC). The safety margin between free radical concentration and the MTC is wide, but that safety margin decreases as the total free radical burden increases. The more oxidation occurring in a cell, the less efficiently the body is able to control it.
>
> Genetic regulation is another factor that controls both the absolute quantity and the efficiency of each free radical-scavenging enzyme. Each individual has a different capacity and rate of scavenging free radicals. Patients with genetically low absolute concentrations of free radical-scavenging enzymes are more susceptible to free radical damage at the cellular level. Consequently, they are more susceptible to free radical induced or mediated disease. This is one reason why longevity, certain diseases, and a specific rate of aging can be seen in families. Often it is equally evident that one family member ages more rapidly or has more disease and breaks the pattern because of tobacco and/or alcohol use. In such a case, that individual's

ability to deal with oxidation was the same as the other family members, but his or her intake of oxidants and antioxidant inhibitors was greater.

Nutritional status can decrease free radical-enzyme scavenging activity. Your body needs what it needs. You can't expect to be deficient in some vitamin or mineral and not be affected by it. Marginal vitamin deficiencies, particularly of vitamins C and E, can decrease the cell's ability to scavenge free radicals. Even cellular depletion of certain trace elements leads to decreased free radical-enzyme scavenging activity. Conversely, adequate amounts of these elements lead to efficient antioxidant functioning. These trace elements are essential cofactors for the synthesis and proper functioning of the free radical-scavenging enzymes. We have already mentioned selenium as a cofactor necessary for the production of glutathione peroxidase. Organic iron is needed for catalase to do its job. Inappropriate nutrition can lead to marginal deficiencies in some of the cofactors necessary for maximum free radical-scavenging activity. But even marginal deficiencies can lead to devastating effects if oxidation is not controlled.

Drug therapy affects enzymatic antioxidant systems in two ways. First, drugs, prescription or otherwise, can produce marginal deficiencies of trace elements by depleting cellular trace element concentrations. Also, drug metabolism can increase the total burden of free radicals. Increases in the total burden of free radicals often occur during drug administration. Most drugs are intended to shock the system into fighting certain symptoms, but this shock tends to pull trace elements away from their normal function and increase oxidation. Free radicals then are produced by almost all drugs as they are metabolized into less toxic compounds. For example, Adriamycin is an anticancer drug that is highly toxic to the heart because of the oxidative stress it creates. But Dr. Horie has found that the antioxidant properties of aged garlic extract nullifies its threat to the heart without interfering with its intended cancer-fighting properties. Therefore, drug metabolism places an excessive free radical load on the capacity of an individual's system of free radical-scavenging enzymes and depletes its ability to deal with that load.

Environmental factors can also increase the total burden of free radicals. Pollution, secondhand smoke, emotional stress, and many other factors may increase cellular oxidation. Any sudden excessive increase in the total burden of free radicals that is secondary to therapeutic, dietary, or environmental factors can lead to free radical cell membrane damage. Radiation, in the form of electromagnetic fields, is unavoidable in our culture ruled by computers, microwaves, and high-tension power lines. Even the radiation from sunlight increases oxidation. Pesticides and other contaminants in our food sources and water significantly affect the diseases prevalent here as compared to countries with simpler farming methods. Many of these environmental factors can be controlled if we are aware and take appropriate measures, like drinking purified water. Other factors cannot be

controlled, and the oxidation they cause must be accounted for in our supplementation.

All of the factors listed above can alter the efficiency of the free radical-scavenging systems. Regardless of the underlying cause, when the minimum toxic concentration (MTC) is exceeded, cellular damage occurs and the patient will ultimately exhibit clinical symptoms of the disease process. Which disease shows up will be determined by which enzyme system within which organs have failed and where the toxic concentration is. This means it is absolutely necessary to see to it that the system has everything it needs to function properly.

These enzyme-scavenging systems are designed as a fail-safe system to prevent the formation of hydroxyl radicals. Since there is no enzyme to scavenge the hydroxyl radical, failure of the free radical-scavenging system places more of a burden on other systems to rid the body of this toxin. The success of the enzyme system is dependent on a number of factors, including the presence of adequate amounts of selenium, glutathione, and glutathione reductase. When these factors are absent in a given patient, the probability that the patient will develop free radical-mediated disease increases significantly."

The average American is always gulping pills which control his or her muscle aches and pains, mask fatigue and depression. The average American has been brainwashed into believing that not only are pharmaceuticals generally harmless, but they are good for what ails you. A person cannot watch two hours of network TV without exposure to a half-dozen pharmaceutical ads. No one wants to believe that the family doctor is misguidedly prescribing poisons which can cause and contribute to the development of degenerative diseases.

There is the thought that surely the FDA wouldn't approve these drugs if they can cause or contribute to cancer - after all we have the DeLaney laws about carcinogenesis.

No one at the FDA has ever given the slightest thought to what effect the pharmaceuticals they regulate have on free radical production or whether or not they promote or inhibit antioxidant activity. Their purpose is the regulation of allopathic drugs. This is the same agency which routinely approves the addition of toxins to the food supply as preservatives.

Nevertheless, the person with cancer who will not stop ingesting synthetic pharmaceuticals will probably not be successfully cured with antioxidant therapy. There are natural alternatives for all pharmaceuticals.

People with cancer who feel they must consume alcoholic beverages should switch to moderate intake of red wines which have potent antioxidant properties.

Most foods containing antioxidants can be eaten raw. There are a few exceptions such as cooked tomatoes which are a rich source of Lycopenes, but raw tomatoes yield only a small fraction of the Lycopenes; Brussels sprouts have very decided antioxidant contents but this is obtainable only from cooked Brussels sprouts and not from those which are raw.

While fresh fruits and vegetables are important and have formed the basis for several successful cancer treatment diets, some considerations should also be given to the important nutritional and antioxidant aspects of soy and whey protein concentrates in anti-cancer diets. Soy products contain the isoflavonoids genisten and diadzen which have been shown to prevent prostate cancer, whey proteins are rich in glutathione and other amino acids which promote antioxidant metabolism.

The treatment program of Dr. Donald J. Kelley and later of Dr. Nick Gonzales for the treatment of pancreatic cancer was based upon the prescription of diets tailored to the patient's metabolic type. 100% of the patients who followed this program were cured of pancreatic cancer which is usually considered a fatal and untreatable condition.

There are reports from anthropologists who use mitochondrial DNA typology to trace the development and migration of humans throughout the world. There are 6 to 8 different DNA haplotypes in mitochondrial DNA, and these may have a lot to do with the individual's metabolic

type.

Diet, for people with cancer, should be prescribed by the treating physician based upon the individual's metabolic type as determined by trace mineral hair analysis.

The body's natural means of detoxification are through the liver, the bowels, the kidneys, the skin, and the respiratory system.

One of these which does not appear to have received much attention in cancer therapy is the kidney - recent reports from Nephrologists and Urologists indicate that one of the consequences of end-stage renal failure is the development of widely disseminated cancers in such patients, which may be caused by the accumulation of toxins ordinarily removed from the body by the kidneys.

Some herbs and hot water baths have the effect of promoting renal output. Since kidney cells are dependent on mitochondrial function, decreasing the total toxic load could conceivably influence renal function. Synthetic diuretics such as Lasix should be avoided but natural diuretics might well be helpful. Increasing water intake is helpful because many of the elderly are dehydrated. Non-steroidal Anti-Inflammatory Drugs, particularly Tylenol, should be strictly avoided by cancer patients because of its damaging effects on the kidney.

Cancer, as a disease, has a large spiritual and psychological component which is extremely difficult to address on an outpatient basis. Since socialization of cancer patients with each other has a profound effect on the outcome of treatment. This as well as the dietary component of cancer therapy are difficult to achieve on an outpatient basis for a large number of patients. Diet therapy should consist of more than handing a patient a list of foods they should or should not eat as a part of their treatment.

This problem is solved in a number of cancer clinics where the patients are fed three meals

per day at the clinic which insures that the patients get the proper diet as well as some occasions for socialization during meals.

These patients undergo an intensive course of treatments of 6 weeks to 3 months duration, during which they are treated on a daily basis, and spend 8 to 10 hours of the day at the clinic.

As in all mitochondrial diseases, the first step is detoxification. The second step is nutritional supplementation along with herbal and homeopathic support. Energy treatments, such as the Sodi Pallares protocol, as well as chiropractic, acupuncture and massage are all helpful. The next step is oxidative therapy with ozonized water baths.

In cancer treatment, metabolic typing and classification should be the beginning point for the initial workup and appraisal of every patient. The metabolic type can be determined accurately by hair analysis and such tests are routinely done during the first visit, along with a dietary history.

Mitochondrial DNA genotyping is a new technology which is proving to be important in understanding the individual's metabolic inheritance and capabilities, nutritional needs and is an important adjunct to hair analysis in detecting the diseases to which the individual may be prone to develop as well as preventive means to avoid these.

Energy medicine has been around for many decades. Oriental Medicine, Acupuncture, and Homeopathy are current forms of energy medicine.

Magnets have been used for centuries as treatments. Electricity was widely used from 1880 until about 1910, when "medical science" declared it to be quackery.

In the past two decades, Acupuncture and Homeopathy have been merged by the development of electroacupuncture, sometimes known as electrodermal testing, now commonly used by physicians, Chiropractors and Acupuncture practitioners.

Electromagnetic devices such as EAV are able to read signals from the acupuncture meridians. These signals originate from the ATP polarized cells of each organ and travel to the surface of the skin along well-defined meridians or pathways in the form of electrons. The strength of the signal can be determined by the operator who is able to test the response of the body to certain substances placed in the circuit of the device. In this way, problems in the body's energetic fields can be diagnosed and treated by Homeopathy, Herbs and Acupuncture techniques.

Very small electromagnetic vibrations can be very effective in treating problems which have a decreased energetic component. The art and science of such energy medicine is beyond the scope of understanding of "medical science" but is well understood by quantum physicists, acupuncturists and homeopaths.

Such energetic therapeutics have proven effective in restoring sight to sufferers from Macular Degeneration. This is a disorder for which "medical science" has no effective treatment, but which responds quite readily to energy treatments. Several other diseases which have no effective pharmacological treatment have been shown to respond well to the application of small electromagnetic vibrations applied to the body. The use of energy medicine shows great promise for the treatment of those chronic degenerative diseases which respond so poorly to pharmacological treatments.

Infectious Disease Resurgence

In America we once naively believed that "medical science" would wipe out all of the infectious diseases which had plagued the human race through the use of antibiotics. The antibiotics failed and antibiotic resistance has caused the re-emergence of new and more virulent infections which are difficult to treat in today's world. Several bacterial and viral infections are

prevalent in the American population today.

Hepatitis C Infection

It has been estimated that one in six Americans has an active viremia which will ultimately become manifested as Viral Hepatitis A, B, C, D, or G. The most pernicious is Hepatitis C, which has a relatively long period of asymptomatic development.

Patients with Hepatitis C are frequently found to have circulating autoantibodies and several immune mediated extra-hepatic manifestations of the virus have been reported including polyarteritis nodosa, thyroiditis, anemia, dermatitis, sicca syndrome, and non-Hodgkin's lymphoma. The extrahepatic manifestations that are most clearly associated with Hepatitis C infection are glomerulonephritis and mixed essential cryoglobulinemia.

Since there are now some newly developed effective treatments for viral hepatitis which may be able to eradicate the disease during the three decades long period between its contraction and the appearance of incurable damage, it would be advisable for testing for this disorder to become a routine part of comprehensive health assessment.

OVERVIEW OF CLINICAL MITOCHONDRIA DETOX, REOX AND EMT IN THE DIAGNOSIS AND TREATMENT OF CHRONIC DEGENERATIVE DISEASE

Most of the chronic degenerative diseases which are classified by the signs and symptoms they produce may become manifest at various times in the sufferer's life. These manifestations of a common disorder are created by impaired mitochondrial function and a progressive failure in the production of ATP. Because of the failure in the production of ATP in diverse tissues and organs, these tissues and organs began to regress back towards a primordial anaerobic metabolism in which

lactic acid builds up and the mitochondria become either dormant or the cell reverts to a malignant cell.

Some of these cells begin with genetically impaired function due to maternally inherited mtDNA; others acquire dysfunctions from new mutations in mtDNA as well as toxic interference with their function.

With the knowledge that a decrease in mitochondrial function leads to chronic diseases, then the therapeutic approaches should be aimed at restoring mitochondrial function, detoxification and regeneration of the tissues and organs.

Successful treatment of degenerative diseases consists of reversing the core process at the root of these disorders rather than attempting to suppress these diverse symptoms produced by the pathological process one by one as they appear.

It is estimated by the Mitochondrial Disease Foundation that one in every four thousand Americans born each year suffers from an identifiable mitochondrial DNA defect which is manifest at birth or in early childhood.

Well over 60% of Americans will, as they mature and age, acquire one or more mitochondrial dysfunctions through the impact of environmental factors. These accumulate and are eventually manifest by the diseases which are collectively called chronic degenerative diseases, ranging from arthritis, diabetes, cardiovascular disease, loss of vision and hearing, muscular diseases and cancer. These disorders are preventable and are reversible.

The only treatments which offer hope of return to normal function are those which remove the causes of the regression and restore the capacity to return to normal function. These appear to be:

(1) Detoxification

(2) Orthomolecular nutrition

(3) Oxidation

(4) Pulsed electromagnetic stimulation at an appropriate frequency

These measures have been highly successful in restoring healthy function by restoring the function of the mitochondria.

Detoxification is a multiphase process which involves nutrition. These have recently been described and in detail by Josephine Krohn, M.D. and her co-authors in their book "Natural Detoxification," Point Roberts, Washington, Hartley & Marks Publishers (1996) which constitutes a definitive manual of detoxification procedures.

This manual includes measures for the restoration of normal bowel ecology which is important since the bowel is the route for virtually all the toxins capable of interfering with mitochondrial function. Much nutritional therapy is involved in detoxing processes. Orthomolecular nutrition means a basic diet which is appropriate for the individual's metabolic type. The cancer therapy of William Donald Kelly, one of the most consistently successful alternative cancer therapies developed to date is based largely on this concept. There are certain nutrients which support oxidative phosphorylation and the known antioxidants should be supplied along with the basic diet appropriate to the individual's metabolic type. Appropriate nutrition means not only the intake of nutrients which are correct but also the avoidance of the substances which can and do adversely affect mitochondrial function. The diet, both food and drink, must not contain any of the mitochondrial toxins. The water must be free of any traces of fluoride as well as the hundreds of chemicals which are routinely found in certain water supplies, such as chlorine and

its derivatives. The foods must be free of herbicides, pesticides, inorganic fertilizers, food colors or additives, including those approved by the Department of Agriculture and the FDA for use as food additives. The tolerance for such substances in the treatment of mitochondrial dysfunction is zero. The foods and beverages must not contain aspartame or Nutrasweet - a product which is currently found in over 5,000 commercial foods and beverages. It is reported to cause Multiple Sclerosis and Systemic Lupus, both of which are mitochondrial disorders. When this product reaches a temperature exceeding 86 degrees F. it converts to Formaldehyde and Formic Acid and below that temperature, it metabolizes to methylalcohol. Some of the metabolic type diets include meats - these meats must be free of antibiotic residues; seafoods should be confined to those originating and living well away from coastal waters, particularly waters near the mouths of rivers and inland streams. Seafood consisting of Northern fish should be caught at least 50 miles off shore. Poultry should be of the free-range variety and not that raised in crowded cages and fed antibiotics and other chemicals. Fruits and vegetables should be thoroughly ozonated before consumption to eliminate all herbicide and pesticide residues, as well as to destroy pathogenic bacteria such as E Coli 0157-H7, Salmonella and similar organisms involved in Food-borne Diseases[8]. The toxins produced by such microorganisms can destroy mitochondrial function. All bathing water should be purified by ozonation and not chlorination.

Another highly successful alternative cancer treatment, Essiac Tea, is an herbal detoxification formula. The tea must be brewed from absolutely pure water. Green tea is also reported to be an extremely efficient preventive of cancer - it too must be brewed from absolutely

[8]See Fox, Nichols, SPOILED: The Dangerous Truth About Food Gone Haywire, New York, Basic Books (1997)

pure water.

Oxygenation: To the methods of oxygenation discussed in Krohn's et al's Natural Detoxification, the safest method of oxygen enhancement is by transdermal diffusion. The individual is immersed up to the neck in a tub of water through which Ozone is being bubbled. This leads to a rapid rise in tissue oxygenation which, reportedly, lasts longer than the increased tissue oxygenation achieved with hyperbaric oxygen and eliminates the drawbacks inherent in HBO. It can be used as often as necessary to maintain high levels of oxygen in the extracellular fluid where it is readily available to the cells, and it avoids problems such as oxy-hemoglobin disassociation shifts.

One pharmaceutical, Dichloroacetate, has been reported to be of any benefit in the treatment of mitochondrial diseases; all other pharmaceuticals should be suspected of being mitochondrial toxins. Some may not be, but until that is reliably established, they should empirically be handled as if they are. All synthetic compounds, those not occurring in nature, and synthesized from petroleum are capable of interfering with mitochondrial function.

Dental amalgam restorations are certainly capable of producing a mitochondrial toxin in the form of methylated mercury. Most other heavy metals should be avoided and where present in fat stores, should be removed by detoxification.

In the regenerative treatment of mitochondrial dysfunctions, if at all possible, pharmaceutical treatments should be replaced by herbal, nutritional, homeopathic or other natural therapies. During and after treatment, the principles of Natural Hygiene should be followed.

There is quite a lot that can be accomplished in this regard. Air and water can be filtered, oxygenated and sometimes electrostatically treated to remove pollutants.

Geopathic stress can be avoided. Bedding can be replaced by air mattresses, which should be placed on wooden platforms to avoid all coil springs and other metallic support which can generate or accumulate electromagnetic frequencies. Some household appliances which generated high gauss magnetic fields can be eliminated. Quartz watches should not be worn on the body. Areas of Geopathic stress should be avoided, particularly for work and sleeping areas.

Acupuncture by a skilled practitioner can support many systems and organs energetically and can be a valuable aid for maintaining good bowel function.

Spinal manipulation by a skilled Chiropractor or Osteopath can be crucial where the nerve supply to organs arises from the spinal nerve as well as the autonomic nervous system, the ganglia of which originate from the spinal nerves. Body work, Rolfing and massage therapies are also valuable.

Psychological and spiritual counselling are of inestimable importance in all treatment programs.

All psychopharmaceutical drugs should be completely avoided in persons being treated for mitochondrial dysfunction, particularly the Selective Serotonin Re-uptake Inhibitors and diazepam derivatives. These can be replaced by herbal remedies such as St. John's Wort. Drugs which alter brain chemistry almost certainly are mitochondrial toxins.

Blood pressure medications can and should be replaced by herbal and nutritional programs when possible.

Non-steroidal anti-inflammatory drugs are absolutely contraindicated in people suffering from mitochondrial dysfunctions.

Carbonated beverages should be avoided, especially those which are sweetened artificially

with Aspartame.

Alcohol should be avoided. If this is not done, the intake should be strictly limited to one ounce of alcohol daily, preferably red wine.

Electromagnetic Treatment: While there are a number of electromagnetic treatments and devices available, the one which appears to be most efficacious for the treatment of mitochondrial dysfunction is the treatment devised and successfully used by Dimetro Sodi Pallares, M.D. of Mexico City.

This treatment utilizes a pulsed electromagnetic pad which operates at 1 to 600 Gauss at 60 cycles. It is used in conjunction with a polarizing solution first developed several years ago by Dr. Sodi Pallares consisting of a high potassium, low sodium glucose solution with insulin.

Using the combination of the solution, pulsed electromagnetic therapy and a high potassium, low sodium diet, Dr. Sodi Pallares has successfully reversed cardiomyopathy, as well as reversed extensive metastatic cancer. Two fairly brief reports from the 4th International Symposium on Biologically Closed Electric Circuits serve to illustrate this:

> "This 32 year old female had a history of breast cancer treated by mastectomy. She subsequently developed metastasis to the right hip which failed to respond to radiation and chemotherapy. When first seen in February 1996, she complained of severe pain in several parts of her body, and X-rays confirmed spread of the cancer to the right femur, skull, and both hands and wrists.
>
> She was given polarizing solution twice weekly, pulsed magnetic field therapy for 4-5 hours daily, and started on a low sodium-high potassium diet. After ten days, her pain had significantly lessened, and her strength and energy were considerably improved. Four months later, she was completely free of all complaints, and, as noted below, her X-rays had essentially returned to normal. Unfortunately, this cannot be fully appreciated, since the size of these, as well as the other X-rays in this Newsletter are drastically reduced. This patient now leads a completely normal life, has no pain or any other symptoms and continues her daily

full time working activities as a biochemist.

> A 42-year old man had severe heart failure due to cardiomyopathy that was resistant to treatment. A heart muscle biopsy at a leading cardiovascular center confirmed myocardial necrosis, and he was told that unless he had a heart transplant, he would be dead in a few months. While waiting for a donor, he developed increasing shortness of breath, chest pain, and abdominal distention. When first seen, he was in severe heart failure, his heart was tremendously enlarged, and there were numerous abnormalities in his electrocardiogram. After only two weeks of treatment with polarizing solution, magnetotherapy, and diet, all of his symptoms had disappeared, and there was a remarkable reduction in heart size, as can be seen in the following X-rays. Almost four years later, this patient is in excellent health and lives a completely normal life. He continues to follow the diet, but requires no medication."

An additional example of the effects of electromagnetic treatment of degenerative diseases involving mitochondria is the reversal of Macular Degeneration by the use of Electromagnetic treatments using the Transcutaneous Electrical Nerve Stimulation (TENS) device.

Macular degeneration is a disorder of the Rods, Cones and Pigmented Epithelium of the retina in which these cells become dormant, leading to a loss of visual acuity and the accumulation of drusen over the macular area.

Electrical Nerve Stimulation of the macula as a treatment for macular degeneration thusly:

> "To create a closed circuit when stimulating the eyelids, a brass cylinder or rod is held in one hand of the patient, and a brass eyelid probe with a shielded handle is held in the other hand. The eye is treated by providing a micro-current to four points on each upper and lower eyelid. When stimulating the upper eyelid, the patient is asked to look downward with eyes closed. Four points are stimulated on the upper lid and lower lid using microamperage between 200 and 250 micro amps. The amperage is brought up until the patient sees light flashes and/or feels the tinge of electricity. It is then dropped until no detectable light or feeling is noted. This is the amperage then used. Each of the eight eyelid points are stimulated for 12 seconds using one of the three frequencies. Each of the eight eyelid points are stimulated for 12 seconds with frequency of 0.3 cycles/second, 9.1 cycles/second, and 30 cycles/second by a .5Hz biphasic pulse.
>
> A square waveform is used on the eye because square waveforms cause

better responses on neural tissue than sloped waveforms.

New patients are treated once a week for eight weeks and once a month thereafter. I feel more frequent long-term treatment would give us even better results.

The Michael-Allen study showed significant stability in the rate of visual acuity loss with the above regimen.

In my case studies, I have found significant visual acuity increases - sometimes 2-3 lines on the Snellen Chart - with patients who just begin to show a rapid drop in vision over 3 to 6 months. Every one of these patients showed an increase in visual acuity after one to two treatments. I will cover case studies later.

I feel that retinal function is lost for a time period before the macula cells are destroyed. *If we can intervene at the time of rapid acuity loss, we can recover significant retinal function.* If we wait too long before treatment, the macula cells are lost and cell function recovery will be minimal.

Normal retinal cell function is a photochemical reaction converting light energy to an electrical impulse which travels to the brain and vision occurs. Diseased, inflamed retinal cells eventually lose cell function, ATP levels drop, protein synthesis drops, the electric resistance goes up and cell electricity potential goes down. The cells seem to go dormant for a time before they die. *If we begin electric stimulation before the cells are lost, we re-establish a more normal cellular electrical potential, ATP levels increase, protein synthesis occurs and normal cell metabolism is restored.* With this, normal photochemical reactions again occur and visual acuity returns.

In 1982, Dr. Ngok Cheng studied the effect of microcurrent on Adenosine TriPhosphate (ATP) concentrations and protein synthesis in mammalian skin. ATP is the source of energy for normal cell activity. It is the carrier molecule for free energy derived from foodstuffs and sunlight. It is necessary for protein synthesis in a cell. ATP supplies the energy for the cellular sodium pump, which removes metabolic waste from a cell and transports metabolic substrates from the blood to the cell.

Dr. Cheng demonstrated the ATP concentration increased by as much as 300% to 400% in cells stimulated with microcurrents between 25 micro amps and 1,000 micro amps.

The electrical resistance of tissue with chronic pathology is higher than that of surrounding normal tissue. Regeneration is a series of endothermic,

electrochemical reactions. Electricity used in minuscule quantities is used by cells to regenerate.

I have found that to restore significant visual acuity, we have only a small window of time to begin treatment before the cells are permanently lost and good recovery of acuity is achievable. We must find and treat these patients at the proper time."

When the oxidative and energy producing functions of mitochondria suffer a loss of the effective function, they have a tendency to regress back toward the primordial state. This regression has been termed Oxidative Regression to Primordial Encoding - ORPEC.[9] The result of such regression is that the cells become dormant, decreasing energy production, becoming unable to function, until eventually, they either die or reach the ultimate primordial state, malignancy. Some cells can remain dormant for long periods of time - for years.

The regression to the primordial state can be reversed and normal function restored. The treatment is generally prolonged and return to normal or β state function can take quite a while to occur, and is being accomplished in progressive centers where Eclectic treatments are employed.

Since each sufferer is individual and possesses his or her own biochemical and electromagnetic individuality and the causes of the degeneration are highly individual, arising from personal lifestyles, experiences and living habits, the treatments must be individualized and treatment must be by a team or staff approach. Few practitioners of any discipline, medicine, chiropractic, Oriental medicine, Osteopathy possess all the skills needed to reverse the degeneration. The patient must be carefully examined, and an exhaustive toxic substance intake history must be taken. Laboratory analysis of blood, hair, urine, stool and other specimens must be

[9]Ali, Majid Op Cit Fn 4

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obtained. The patient's treatment plan must be coordinated by the staff of therapists who will participate, and monitoring of the patients response evaluated periodically.

The treatment's four general phases, must go on simultaneously. Dietary and life style must be changed and a new healthier lifestyle be adopted. If the stage of malignancy is in the later stages, the treatment must be intensive. If the malignancy is at the beginning stages, a less intensive program may be indicated.

The treatments outlined are generic, and the total extent the program is followed is a decision which must be made on a case-by-case basis. The ultimate decision in the end after advice and guidance will be made by the patient or patient's family.

The treatments outlined here are outpatient treatments for chronic degenerative diseases which are not immediately life threatening. The treatment of cancer, in advanced stages, should probably be undertaken at a residential facility.

Over the past seventy-five years, a large number of what are known as Alternative Cancer Therapies have been developed. Many of these have demonstrated to be very effective.

Since the Allopathically-oriented "War on Cancer" began in the 1970s has now been conceded to have been less than successful, a significant percentage of patients have sought and received such alternative therapies with a success rate not completely known, but generally conceded to be significantly higher than has been realized by the allopathic approach.

There are a number of books about alternative cancer therapies currently in press, for the purpose of this discussion, three of these which seem to use an even-handed and analytical approach have been reviewed; these are:

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1. Falcone, Ron, THE COMPLETE GUIDE TO ALTERNATIVE CANCER THERAPIES, New York, Citadel Press (1994)
2. Moss, Ralph, CANCER THERAPY, New York, Equinox Press, (1992)
3. Walters, Richard, OPTIONS, THE ALTERNATIVE CANCER THERAPY BOOK, New York, Avery Publishing Group (1993)

These three books cover the following therapies:

Burzynski's Anti-Neoplastin Therapy
Gaston Naessens 714X
Revicci Therapy
Burton's Immune Augmentative Therapy
Livingston Therapy
Issels Whole Body Therapy
Hoxsey Therapy
Essiac Tea
Wheatgress Therapy
Macrobiotic Therapy
Moermann's Anti-Cancer Diet
Gerson Therapy
Kelley Nutritional-Metabolic Therapy
Hans Nieper's Therapy
Oxygen Therapy
Hyperthermia
DMSO Therapy
Live Cell Therapy
Bioelectric Therapies
Ayurvedia
Chinese Medicine
Amygdalin
Arginine
BGC and Coley's Toxin
Gonzales Therapy

When analyzed, all of these therapies can be classified as falling into one or more of the four approaches to chronic disease treatment.

The recent emergence of Cell Specific Cancer Therapy on electromagnetic treatment and the electromagnetic therapies which are reported at the Fourth International Symposium on

Biologically Closed Electric Circuits, October 26-29, 1977, amplify the information on the electromagnetic approaches.

However, there remains a very important aspect of cancer therapy which is exhaustively analyzed in the PhD Dissertation of Alice Rose at Georgia State University in 1979. This is the psychological and spiritual aspects of cancer treatment. The main thrust is that in many cases, cancer is associated with an attitude of psychological or spiritual despair, which must be alleviated if any therapy is to be successful.

Experience in a large number of alternative cancer clinics both in the United States and off-shore has demonstrated the crucial importance of residential cancer patients living in and around the facility forming friendships and bonding during the treatment experience to provide a form of psychological and spiritual support not possible to obtain in other circumstances.

Its impact on the success or failure of the treatment programs cannot be overestimated in overcoming the psychological and spiritual despair which characterizes the disease.

With their interactions each with the other, these patients facing an identical problem literally seem to heal each other.

Therefore, a residential facility where such interaction can occur during a cancer treatment program may be essential to its success.

In the studies of David Spiegel, a psychiatrist at Stanford University,[10] Dr. Spiegel's treatment of terminally ill breast cancer patients included mutual support and discussion groups among such patients. Those who served as untreated controls received no such treatment. The

[10]Spiegel, D.A., A psychosocial Intervention and Survival Time of Patients with Metastatic Breast Cancer advances: The Journal of Mind-Body-Health, volume 7, pp. 10-19, 1991.

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results showed that the treatment group lived twice as long after the time they entered the study as the control group. Survival on average was 18.9 months for the controls and 36.6 months for those in the program. The time between the recurrence of illness and death was significantly prolonged in the treatment group and the more patients who participated in the group, the greater the effect.

An Example of the Analysis of One Treatment

Perhaps the most hotly debated and contested alternative cancer therapy to have emerged in the past half-Century is the use of Amygdalin or Laetrile, which continues to be a mainstay of therapy at most offshore cancer therapies.

The use of Laetrile was the subject of one of the most prolonged lawsuits in America, during which the FDA was enjoined by the Court from interfering with its use by cancer patients for over a decade. Some 26 states passed legislation legalizing the use of Laetrile within those states.

One dispassionate and thoughtfully analytical appraisal of the possible role of Laetrile in the treatment of cancer, contained in an Expert's Affidavit used in that case, which in applicable part reads:

"Perhaps the clearest evidence accumulated since 1977 of the effectiveness of Laetrile in the treatment of cancer is the report of the National Research Council, Committee on Diet, Nutrition and Cancer, "Diet, Nutrition and Cancer", Executive Summary, page 11 states:

> Foods and numerous …nonnutritive components of the diet have been examined for their potential to protect against carcinogenesis …In laboratory experiments, vitamins, trace elements, nonnutritive food additives, and other organic constituents of food, …indoles, phenols, flavones, and isothiocyanates have been tested for their ability to inhibit neoplasia.
>
> …A number of nonnutritive…compounds that are present in these vegetables also inhibit carcinogenesis in laboratory animals.

Section A The Relationship Between Nutrients and Cancer, page 51 states:

> Yet, as the data reviewed in Chapters 13 and 14 indicate, at least some of the compounds in food (e.g., flavones, isothiocyanates) that have been implicated in the causation or prevention of cancer are food constituents other than nutrients …

On page 52, it is stated:

> …For example, the constituents of cruciferae responsible for their apparent effect on the occurrence of cancer may be, as Chapter 15 suggests, indoles, isothiocyanates, or other nonnutritive substances demonstrated to affect carcinogenesis in the laboratory.

Page 358 states:

> In recent years, a number of …constituents of foods have been studied for their inhibitory effects on carcinogenesis.

Chapter 15, "Inhibitors of Carcinogenesis, pp. 362:

> Aromatic Isothiocyanates. Benzyl isothiocyanates and phenethyl isothiocyanate are also constituents of cruciferous plants. These aromatic isothiocyanates have been shown to inhibit neoplasia induced by polycyclic aromatic hydrocarbons (PAH's) when they were administered during the initiation phase under several different experimental conditions. These results were obtained when the aromatic isothiocyanate was fed both before and during administration of the PAH's (Wattenberg, 1977, 1979b).[11] Little is known about their mechanism of inhibition other than the fact that benzyl isothiocyanate is a potent inducer of glutathione X-transferase activity. In further studies, mammary tumor formation resulting from exposure to DMBA was inhibited by the administration of benzyl isothiocyanate subsequent to the carcinogen. It has also been demonstrated that this compound inhibited 1,2-dimethylhydrazine-induced neoplasia of the large intestine when the exposures were begun 1 week after administration of the carcinogen. (Wattenberg, 1981b)[12] The mechanism of these inhibitory effects is not known.

The report in Life Sciences Vol. 27, pp. 659 (1980) by Heikkila and Cabbatt, entitled: "The Prevention of Alloxan-induced Diabetes by Amygdalin" provides the clue to understanding the anticarcinogenic properties of Laetrile. That report states:

> "Firstly, amygdalin most likely was a good hydroxyl radical scavenger since amygdalin contains both a benzene ring and a sugar moiety and compounds with these groups have very high rate constants for reactivity with the hydroxyl radical.[13] Secondly, amygdalin like all of the other agents which we have found to be protective, can be tolerated by experimental animals at rather high doses - - -

[11]Wattenberg, L.W. (1977) Inhibition of carcinogenic effects of polycyclic hydrocarbons by benzyl isothiocyanates and related compounds. J. Natl. Cancer Inst. 58:395

[12]Wattenberg, L.W. Inhibition of carcinogen-induced neoplasia by sodium cyanate, tert-butyl isocyanate and benzyl isothiocyanate administered subsequent to carcinogen exposure. Cancer Res. 41:2991-2994.

[13]Dorfman, L.M. and Adams, G.E., National Standard Reference Data System, NBS, 4 1-59 (1973)

It has previously shown that high doses of several good hydroxyl radical scavengers…dimethylsulfoxide …given to mice at various times prior to alloxan, were able to protect against the diabetogenic action of alloxan …in a recent report it was shown that catalase, superoxide dismutase, and several potent hydroxyl radical scavengers could prevent some of the toxic actions of alloxan on isolated islet cells. - - - In the present study we report that amygdalin can protect against alloxan. Although, as mentioned above direct rate constants for amygdalin's reactivity with the hydroxyl radical, to our knowledge have not been published, one would expect that the rate constant for reactivity of amygdalin with the hydroxyl radical is very high. - - - The rate constant for amygdalin has been recently directly determined with a pulse radiolysis technique and found to be $4.1 \times 10^{9} M^{-1} S^{-1}$. Thus, one might reasonably add amygdalin to the list of hydroxyl radical scavengers able to protect against alloxan. It is interesting that the chemical structure of amygdalin is quite different from most of the other hydroxyl radical scavengers previously reported to protect against alloxan. - - -

It should be mentioned, however, that hydroxyl radical scavengers in general are known to be radioprotective due to their scavenging of the deleterious and reactive hydroxyl radical, which is thought to be the damaging species generated by ionizing radiation …"

Levine and Kidd[14] report:

- - - There is a great deal of evidence that nutrient-derived and synthetic antioxidant factors can successfully intervene to halt the progression of chemical carcinogenesis at any point. Various antioxidants protect against the actions of initiator carcinogens in the "model" skin tumor system (Slaga, 1984). Antioxidant compounds also can block the promotion process (Demopoulos, et al., 1980, Slaga, 1984). Progression past promotion to neoplasia can also be protective against antioxidants. Vitamin A and its related retinoid derivatives are widely recognized as tissue growth regulators and have also been shown to protect by antioxidant mechanisms.

Dietary antioxidant factors can be potent inhibitors of malignant progression. Exposure of all-trans-retinoic acid (a synthetic vitamin A analog) can effectively block further progression of preneoplastic skin growths, skin (basal cell) carcinoma, and bladder papillomas. Other retinoids caused the regression of chemically induced pulmonary adenomas. Glutathione, the tripeptide nucleosorbophylic antioxidants, may cause the regression of established malignant tumors. Rovi reported in Science that dietary supplementation with reduced glutathione (GSH) caused the regression of hepatocellular carcinomas induced in rodents by the highly potent carcinogen aflatoxin. Two subsequent studies failed to

[14]Levine, Stephen A., Ph.D. and Kidd, Parris M., Ph.D., Beyond Antioxidant Adaptation - A Free Radical-Hypoxia-Clonal Thesis of Cancer Causation, Journal of Orthomolecular Psychiatry, Vol. 14, No. 3 (1984)

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confirm this finding, Novi, et al (1982) cautioned that the route by which glutathione is administered is important; a second group has reported that reduced glutathione causes hepatocellular carcinomas to regress. - - -

In the cancer cell the major mode of ATP generation appears to be non-mitochondrial, i.e., glycolytic. Cancer cells respiring glycolytically may encounter a major limiting factor in their capacity to generate ATP by this means: a relative glut in the reduced electron carriers NADH and NADPH, and a relative deficiency in their corresponding oxidized forms NADP+ and NAD+. These occur as a result of the impaired cellular ability to utilize these electron carriers in electron transfer to the ultimate electron acceptor - oxygen. Hence an extra-reduced cellular state has developed, i.e., a state beyond antioxidant adaptation. The cell in this state has markedly lower reliance on oxygen for metabolism, and fewer oxygen radicals are available to present an oxidizing challenge to the cell. The drawback for the cancer cell is that it now lacks a full complement of antioxidant enzymes and the flexibility to mount an effective antioxidant response to oxidant attack.

While other hypotheses for the anticarcinogenic properties of Laetrile have been reported and there may, of course, be several modes of action, it can now be reasonably deduced from the available scientific literature that one of the chief effects of Laetrile and its metabolites responsible for its ability to shrink tumors is its effect as a free radical scavenger in cancer cells, which are known to be largely developed in anaerobic glycolysis and have severely restricted oxidative phosphorylation.

Frank after an elegant series of experiments demonstrated that the high rate of mitosis in cancer cells is due to the cytoplasmic accumulation of reduced pyridines, which become available for various synthetic processes of tumor growth and drive mitosis.[15]

Tumors having a relatively anaerobic metabolism, with a much higher rate of glycolysis than normal tissues, at least in part due to a relative inhibition of pyruvate dehydrogenase, pool Lactic acid which serves as a reservoir of hydrogen for cytoplasmic reduced pyridines, which do not efficiently cross the mitochondrial membrane by the glycero-phosphate and malate-aspartate shuttles. These non-mitochondrial reduced pyridines are the critical stimulus for the abnormal growth (mitosis) of cancer cells, particularly NADH and $NADPH_2$. Tumors in which this pool of extra mitochondrial pyridines is decreased shrink rapidly, probably by reduction of the pool of reduced pyridines as $NADPH_2$ and NADPH which then decrease mitosis in such cases.

It is likely that the interference with glycero-phosphate and malate aspartate

[15]Frank, Benjamin, Nucleic acid as antioxidant therapy of Aging and Degeneration. N4, Royal Health Books, Ltd., New York (1977)

shuttles necessary to transport the hydrogen ions across the mitochondrial membrane is due to free radicals in the cytoplasm and that Amygdalin which is a very potent free radical scavenger may assist in making pyruvate more accessible to the Krebs cycle by assisting in the oxidation of NADH to NAD and thus reduce the cytoplasmic pools of reduced pyridine.

If this is the case, then Laetrile can be considered a non-toxic antimitotic therapeutic which achieves its results not by damaging the apparatus of mitosis but by diverting to oxidative phosphorylation the excess pool of reduced pyridines which fuel excessive mitosis, and that this is achieved with no deleterious and indeed, perhaps a salubrious effect on non-malignant cells so that the toxicity and interruption of mitosis in normal cells prevalent in the use of toxic antimitotics does not require the interrupted administration necessary in toxic chemotherapeutic agents.

Therefore, Laetrile can exert its antimitotic and anticarcinogenic effects in a prolonged and continuous fashion and thereby bring about rapid tumor regression without adversely impacting the immunological status of the patient, indeed, perhaps improving that status.

It may be significant in this regard to note that leukocytes contain significant amounts of NADH oxidase which may account for some of the ability of leukocytes to produce tumor shrinkage through a similar mechanism as NADH oxidase could likewise shrink the pool of reduced pyridine available to drive abnormal mitosis.

A great deal of research during the past decade has been focused on the probability that cancer cells can be converted back to normal or near normal cells by agents which influence intracellular biochemistry and restore the cells to aerobic metabolism.

Booyens, et al[16] have demonstrated that human melanoma cells can be reversed by addition of gamma linolenic acid and have successfully treated a number of cases of malignant melanoma by the administration of gamma linolenic acid.

Indeed, the FDA has granted Phase I approval to E. I. Dupont, Co. for testing of n-methylformamide, a solvent similar to Dimethylsulfoxide, both of which were discovered by David Dexer and his colleagues at Brown University to have the property of transforming malignant cells into near normal cells.

In this regard Heikkila and Cabbert note that dimethylsulfoxide is a good hydroxyl radical scavenger while, like amygdalin, protects against the effects of alloxan when administered prior to alloxan.

Thus it may be reasonable to investigate whether or not Amygdalin and DMSO, as well as dimethylfourmamide achieve their effects by restoring normal or near normal oxidative phosphorylation and decreasing the malignant cells

[16]Booyens, et al, Second International Congress on Essential Fatty Acids, Prostaglandins and Leukotrienes (London) March 24-27, 1985 (Abst).

dependence on anaerobic glycolysis for energy production and thereby depleting the cytoplasmic pooling of reduced pyridines which appear to drive abnormal mitosis in malignant cells.

Certainly all of the hydroxyl-scavenging agents may also help normalize other enzyme systems, as does gammalinolenic acid and improve the metabolism of malignant cells in other fashions as well.

For instance, it is reasonable to assert that GLA achieves its effects by conversion to Prostaglandin E_1 which influences intracellular adenyl cyclase and cyclic AMP.

For this reason it would be well to test Amygdalin together with other nutrients which have been found clinically to produce tumor shrinkage as their action may be synergistic.

This synergism could be expected since both GLA and Amygdalin occur in nature predominately as components of seeds.

In "Nutritional Factors with the Potential to Inhibit Critical Pathways of Tumor Promotion[17] a Chapter in Modulation and Prevention of Cancer by Vitamins, Flavin and Kolbye of the Nutrition Education and Food and Drug Administration Bureau of Food, state:

"Nutritional factors potentially can inhibit critical phases of tumor promotion. The proper combination of these factors is more effective in this inhibition than isolated substances because of their ability to complement each other in their mechanisms of action".

The hypothesis advanced here also finds support in the report of Shearer in "Modulation and Mediation of Cancer by Vitamins"[18]

"Amygdalin use by humans with cancer indicates that the effect of the 'laetrile' regimen must be on controlling growth of the cancer cells rather than killing them.

Effect on Cancer Initiation. The ability of vitamin A or amygdalin to alter the rate of cancer initiation by 3'me DAB in rat liver was assayed by competitive hybridization of normal rat liver nuclear RNA to normal fetal rat DNA.

Effect on Cancer Growth. The ability of vitamin A or amygdalin to alter the rate of growth of hepatocellular carcinomas previously initiated and promoted by 3 months of feeding 0.06% 3'meDAB was assayed by lifetime feeding studies.

…Rats on the high vitamin A diet and amygdalin diet survived on the average somewhat longer than those on the control diet (93 days, 99 days, and 76

[17]Meyskens, F.L. and Prasad, K.N. (Eds), Modulation and Mediation of Cancer by Vitamins, (Karger, Basel 1983) Flavin, D.F., Kolbye, A.C., Jr., Nutritional Factors with the Potential to Inhibit Critical Pathways of Tumor Promotion, pp. 24-38.

[18]Modulation and Mediation of Cancer by Vitamins, Editors: Meyskens, Prasad (1983) Karger, Basel , Chemoprevention of Azo-Dye-Induced Liver Carcinogenesis in the Rat by a Natural Carotenoid, R. W. Shearer, pp. 89-94.

days, respectively, after stopping the carcinogen) and rats fed both high vitamin A and amygdalin survived significantly longer (128 days). 4 rats in these groups which died early were cannibalized, but all of the rest were either dead or moribund with huge liver tumors when the experiment was terminated 7 months after stopping the carcinogen treatment, with the exception of 1 rat on the amygdalin diet which had only small cancers and would have lived probably another month.

The fetalism of malignant tumors must be explained by any potentially valid theory of the fundamental nature of the malignant process. The currently popular mutation theory is inadequate for this reason. These results suggest the usefulness of both vitamin A and amygdalin in modulating rat liver carcinogenesis in the study of tumor fetalism and other promotion-specific events". "

Since that time, Laetrile and other Betaisothiocyanates which are commonly found in cruciferous vegetables have been acknowledged to be of great value in the prevention of cancer by the National Research Council as well as the American Cancer Society.

As early as 40-50 years ago, scientists felt that measures which supported mitochondrial function, the production of ATP were crucial in the treatment of cancer. It appears that laetrile may do this by acting as a highly efficient antioxidant.

The cancer therapies listed in these three books must of course, undergo similar penetrating analysis before being incorporated into an integrated cancer treatment program designed to cure cancer.

The metabolic typing and dietary adjustments based on such typing are discussed in Moss's book in conjunction with Nicholas Gonzales' approach. This originated as an integral part of the highly successful cancer therapies of William Donald Kelley.

Nicholas J. Gonzales, M.D., became interested in Kelley's method while a student at Cornell University Medical College in the late 1970's. After graduating, Dr. Gonzales did post-graduate work with Robert A. Good, M.D., Ph.D., then president of Sloan-Kettering Institute, and thoroughly investigated Kelley's treatments. In a lengthy unpublished monograph, One Man Alone,

Dr. Gonzales reports that the Kelley method, when used properly, achieved a remarkably high success rate, even in such seemingly incurable forms of cancer as carcinoma of the pancreas and liver. Robert G. Houston, author of Repression and Reform, an Evaluation of Alternative Cancer Therapies, argues that this report constitutes proof of the effectiveness of Kelley's method.

"The Gonzales Monograph documents results dramatic enough to constitute formal proof even with a single arm study. The survival of 5 or more years for all 5 pancreatic cancer patients on the full program was significant statistically compared to the standard 5-year survival of 3%.

These results mandate that Kelly's method of metabolic typing and prescription of an appropriate diet for each metabolic type should be an integral part of any alternative cancer program which relies to any measure on nutritional therapies.

Such metabolic typing into the two main classifications and the four subclassifications of each can be determined by analysis of mineral patterns during mineral hair analysis. This type of metabolic analysis is performed at Trace Elements, Inc., 4501 Sunbelt Drive, Addison, Texas, 75001, Telephone: 1-800-824-2314.

Diet therapy for people with cancer is variable; diet should be determined by the individual's metabolic type. Research has developed the ability to recognize metabolic types through tissue mineral patterns of the hair. Dr. David L. Watts has found that certain mineral patterns reveal metabolic characteristics that correlate well with the descriptions of earlier investigators.

> *Metabolism is a term used to describe nutrient utilization or efficiency on a cellular level resulting in energy production and maintenance. Cellular metabolism is controlled by neurological and endocrine function, which will affect nutrient absorption, retention, and excretion.*

Clinical research, conducted by Dr. Watts correlated over 100,000 tissue mineral analyses

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with specific physical and biochemical characteristics. Through a properly obtained and assayed sample, eight distinct metabolic categories were identified. These include, the fast and slow metabolic types, each with their four sub-types.

The metabolic types with their sub-categories can generally be associated with the various stage of stress, whether acute or chronic in nature. Developed by Hans Selye, these stages are the alarm, resistance, recovery and/or exhaustion stage. Metabolic typing through TMA allows these stages of stress to be more easily determined, and therapy can then be made more specific by working with rather than against the body's normal responses to stress.

The following descriptions briefly define the characteristics of Fast and Slow Metabolism and the neuroendocrine combinations of the sub-categories.

FAST METABOLISM:

Fast Metabolism is synonymous with Sympathetic Dominance, Fast Oxidation, and Type A Personality. Excessive sympathetic nervous system activity increases the availability of glucose for rapid metabolism via epinephrine release from the adrenal medulla. The adrenal medulla stimulates other areas of the body that are not directly innervated by sympathetic nerve fibers and can increase the metabolic rate by as much as 100 percent.

The fast metabolizer's cellular oxidation is more than adequate in pyruvate and oxaloacetic acid production, but inadequate in the production of acetates. This results in incomplete energy production in the Krebs cycle. The fast metabolizer is in a state of rapid glycolysis, which accounts for the high metabolic rate. High HCL with tissue acidity and low pancreatic enzyme production are usually present also.

The fast metabolizer is usually experiencing a considerable amount of stress (physical,

emotional, or a combination of both). He or she often enjoys stressful situations and may even seek them. This type of person is usually late for appointments, somewhat agitated or hyperexcitable, and is often considered a workaholic. If the metabolism becomes too fast, he begins to experience more emotional stress, especially anxiety about the future. The blood pressure may become elevated, with accompanying dental problems and excessive perspiration. Frequently, a increased need to eat develops in order to maintain high energy levels. Weight gain will usually occur in the abdominal region.

FAST METABOLISM TYPE # 1: Classified as sympathetic dominant with increased adrenal activity and increased thyroid function. This synchronous neuro-endocrine combination will frequently result in increased energy levels. However, if an imbalance develops between the adrenal and thyroid glands, the ability to sustain energy levels may become diminished. The Fast Metabolizer Type # 1 can develop TMA patterns associated with alarm, resistance, or recovery stage of stress.

FAST METABOLISM TYPE # 2: Classified as sympathetic dominant with increased adrenal cortical activity and lowered thyroid function. This imbalanced neuro-endocrine combination reflects the alarm stage of stress. When the adrenal cortex becomes dominant over thyroid activity, energy fluctuation may become dramatic. Often the Type #2 individual will experience an increased then a decrease in energy levels, which can contribute to significant mood swings.

FAST METABOLISM TYPE # 3: Classified as sympathetic dominant with decreased adrenal cortical activity in conjunction with increased thyroid function. This imbalanced neuro-endocrine combination is indicative of the resistance or exhaustion stage of stress and is often

associated with depression and irritability if chronic.

FAST METABOLISM TYPE # 4: Classified as sympathetic neurological dominance with decreased activity and decreased thyroid glandular function. This neuro-endocrine combination is associated with the exhaustion stage of stress, often reflected in extreme fatigue, depression, and anxiety.

SLOW METABOLISM

Slow metabolism is synonymous with Para-sympathetic Dominance, Slow Oxidation, and Type B personality. Generally speaking, the slow metabolic types metabolize glucose at a reduced rate. If slow metabolism is severe, energy production and maintenance of normal energy levels will become inadequate. This is a result of the inability to split glucose molecules to form adequate amounts of pyruvates and oxaloacetic acid in the glycolysis cycle. This leads then to the inability to produce citric acid in the Krebs cycle. Low HCL and tissue alkalinity are also usually present.

Slow metabolizers are most often well organized and methodical. They tend to start projects and see them through to completion. Somewhat regarded as perfectionists, they perform best when not under stress. If the metabolic rate becomes excessively reduced, they become subject to fatigue, requiring extra amounts of rest. They eventually experience depression, often dwelling upon the past. Blood pressure may decrease below normal, along with the development of cold hands and feet. Weight gain will usually be noticed on the thighs and hips. If the metabolism continues to decrease, protein foods, especially meats will become poorly tolerated which may then increase their tendency toward vegetarianism.

SLOW METABOLISM TYPE # 1: Classified as para-sympathetic dominant with decreased adrenal medullary activity and decreased thyroid function. This synchronous neuro-

endocrine combination will result in sustained energy levels (endurance); however, the production of energy will be below optimum. The Slow Metabolizer Type # 1 can experience any one of the four stages of stress.

SLOW METABOLISM TYPE # 2: Classified as para-sympathetic dominant with increased adrenal cortical activity and decreased thyroid function. This imbalanced neuro-endocrine combination is indicative of the alarm stage of stress. When the adrenal cortex is dominant relative to the thyroid, energy fluctuations may become pronounced. The slow metabolizer Type # 2 will normally experience both elevated and depressed energy levels, which can contribute to significant mood swings.

SLOW METABOLISM TYPE # 3: Classified as parasympathetic dominant with decreased adrenal cortical activity and increased thyroid function. This imbalanced neuro-endocrine combination is indicative of the resistance or exhaustion stages of stress. When chronic, slow metabolism Type # 3 is often associated with depression and irritability.

SLOW METABOLISM TYPE # 4: Classified as para-sympathetic dominant with high adrenal activity in conjunction with elevated thyroid function. This imbalanced neuro-endocrine combination is usually a result of an acute alarm stage of stress that has progressed into the stage of resistance.

The alternative cancer therapies do not lend themselves well to double blind studies, since they are integrated therapies, not dependent on one substance or approach which can be isolated for such tests.

Many of the alternative modalities discussed in these three books as well as the electromagnetic therapies discussed above should be integrated into the program.

The results will be appraised by outcome rather than by process analysis and the only control will be the patient's progress as contrasted to outcomes from conventional treatments which are well documented and well known.

Since the aim of these therapies is the conversion of malignant cells back to normal function, outcome analysis is the only method of comparing the relative efficacy of such therapies.

Cancer has been cured in hundreds of thousands of patients, both in the United States and in offshore clinics, for the past 30 years. While formal statistics are not available, the reports of many patient-oriented support groups such as the Cancer Control Society, People Against Cancer, and Cancer Victors and Friends constitute an impressive selection of experience and data concerning such therapies which are significant statistically when compared with the known survival rates of conventional therapies. These therapies integrated into the newly emerging knowledge about electromagnetic treatments and the possibility of reversing degeneration due to mitochondrial dysfunction are sufficient to mandate widespread adoption of alternative therapies by anyone who prefers such treatments over the surgery, radiation and toxic chemotherapy which has consistently failed to produce significant cure rates.

Symptoms of Metabolic Disease

Seizures
Pre-existing developmental delay
Hypotonia or weakness, particularly if there is no evident cause
Combined central and peripheral cause for hypotonia or weakness
Increased anion gap and/or acidosis
Unexplained myopathy or cardiomyopathy
Retinopathy
Unusual MRI findings, particularly basal ganglia or unusual white matter abnormalities
Recurrent or cyclic episodes of vomiting and dehydration
Feeding difficulties, vomiting or reflux
Fasting intolerance

Failure to thrive despite adequate caloric intake
Unexplained hearing loss
Recurrent episodes with changes in mental status, particularly if associated with vomiting or ataxia
Liver or kidney disease
Peculiar body odor
Short stature when combined with any of the above signs and symptoms

Family history of:
Known mitochondrial or metabolic problems
Muscle or brain diseases
Unexplained hearing loss
Unusual heart diseases
Developmental delay or regression
Epilepsy
SIDS
Diabetes
Blindness or retinopathy
Parkinson disease
Alzheimer disease
Unexplained illness or death

THE ANTIOXIDANT SYMPHONY

There is an array of substances which have a biologically antioxidant and free radical scavenging property in the body and there is an array of free radicals produced during the process of electron transport and oxidative phosphorylation with the conversion of ADP to ATP in the mitochondria, in normal and under abnormal conditions.

There are several metabolic types and several haplotypes of mitochondrial DNA, as well as several point deletions and mutations in mitochondrial DNA which are passed along with mitochondria from mother to offspring. The interplay of these as well as some nuclear DNA mutations all have an influence on who and under what circumstances will develop arteriosclerosis and subsequent coronary heart disease or stroke.

Heart function is determined mitochondrially, and the health and function of the

myocardium is dependant on the state of function of its mitochondria.

The cardiovascular system does not exist in splendid isolation from the body; it is a vital and essential part of it and is subject to the same nutritional and bioenergetic influences which affect all the organs.

Nutrients are far from standardized in the American diet in raw fruits and vegetables grown in American soil. The only way of ensuring a constant and dependable level of nutrient intake is through supplementation.

Antioxidant and mitochondrial medicine will dominate the early years of the 21st century. Nutritional supplementation, herbs, energy medicine and detoxification will replace pharmacology in the prevention and treatment of the major killer diseases.

The medicine of the 21st century will be eclectic in its philosophy; health oriented, not disease oriented, and far more effective than anything developed and used in the 20th Century.

Clinical trials and meta-analysis of clinical trials, conducted according to standards set by regulatory agency requirements, are used extensively in allopathic medicine. They have little to do with the treatment or prevention of chronic degenerative diseases.

Such trials are sponsored and paid for by manufacturers of pharmaceuticals. Clinical trials are uniquely designed to determine whether or not a particular pharmaceutical is or is not effective in the treatment of a particular disease in relatively large population of people who have been diagnosed as suffering from a particular disorder. They are useful to determine which of two drugs may be the most efficacious for the pharmaceutical treatment of a particular disease.

For testing hypotheses about the long-term use of multiple nutrients on multifactorial diseases, such trials are inappropriate.

In treatment programs which do not include the use of pharmaceuticals, other approaches to approve the efficacy must be derived. Laboratory research together with epidemiological evidence and careful appraisal of treatment results, will be the most effective to determine outcome.

Chinese medicine represents the accumulation of thousands of years of experience in the treatment of human diseases. Because its methodologies are largely non-pharmaceutical and deal with modalities which are never considered in allopathic medicine, there has been a tendency to ignore its impressive results.

A Western tradition and accumulation of hundreds of years of empirical experience in the treatment of diseases, has likewise been ignored in the enthusiasm of 20th Century Allopathic teachers who felt that all the answers were to be found in synthetic chemical compounds which block one or more life processes in the body.

The accumulated results of 20th Century Allopathic medicine in the treatment and prevention of chronic degenerative diseases is far from impressive.

Chinese medicine on the other hand, has been working well for thousands of years and the same may be said of the Western empirical approaches.

The shift away from Allopathic towards more experienced based medical practice is underway throughout American medicine. This internal movement within the medical profession is characterized by the laying aside of Allopathic gold standards which simply do not work and an open willingness to learn about older methods which were cast aside in the mid-20th Century.

Traditional Chinese medicine is based upon herbology and energy, two subjects which were excluded from 20th Century Allopathic medicine in the United States. The rich traditions of Chinese Medicine continue to be practiced there, as it has for thousands of years. It has much to

offer in the management of chronic degenerative disease.

While Chinese Medicine has been practiced predominantly within Chinese populations for well over a Century and a half, it was little known or appreciated in this country until the late 1970's when an influential American journalist, James Reston, accompanying President Nixon on a State visit to the Chinese mainland. He developed appendicitis and was treated in a Chinese hospital where he underwent acupuncture treatment for post-operative pain. Mr. Reston was impressed and wrote glowing reports about acupuncture and Chinese medicine. This focused national attention on Chinese medicine and led in a few years to the licensing of Oriental medical practitioners or Acupuncturists in several states such as California, New York, Nevada and Arizona. Now acupuncture and Oriental medicine is a licensed profession in most states, and there are several colleges of Oriental Medicine in the United States.

CONCLUSION

Presently, there are a number of excellent books in print which describe the use of antioxidant and herbal remedies in the treatment of cancer by nationally known experts. These are listed below at the beginning of the bibliography and should be of great benefit in designing specific antioxidant and herbal treatments for individuals being treated for cancer.

What Drs. Warburg and Szent-Gyorgi told us around a half-century ago is that when the respiratory apparatus of the cell is compromised sufficiently, that cell will either die or become malignant. That if it does become malignant, it may eventually find its way back to normal function but it may not. If it becomes malignant it usually spreads until it kills the organism. They offered us clues to how to help these cells find their way back to normal function. If their ideas had been accepted, rather than rejected, by the cancer research establishment a half-century ago, the

cancer problem would probably have been solved by now. Since it was not accepted at that time, it will be necessary to go back to that turning point and armed with a half-century of research on mitochondria which has taken place since that time, carry their ideas out to fruition, if the problem is going to be solved.

BASIC SCIENCE ASPECTS OF THE MITOCHONDRIA
SECTION XI

CELLULAR AND MITOCHONDRIAL PERTURBED ENERGY METABOLISM AND NEURONAL DEGENERATION IN ALZHEIMER'S AND PARKINSON'S DISEASES

Synaptic degeneration and death of nerve cells are seen in both Alzheimer's disease and Parkinson's disease, the two most prevalent age-related neurodegenerative disorders. In Alzheimer's Disease, neurons in the hippocampus and basal forebrain which subserve learning and memory are invoked. In Parkinson's Disease, dopamine-producing neurons in the substantia nigra striatum which control body movements degenerate. Studies of postmortem brain tissue from both Alzheimer's and Parkinson's Disease patients show increased oxidative stress, mitochondrial dysfunction and impaired glucose uptake in involved neurons.

Recently, Mark A. Smith and co-workers at the Institute of Pathology in Case Western Reserve University in their review on Oxidative Stress and Alzheimer's disease (Biochemica et Biophysica Acta 1502 (2000) 139-144) noted:

> "For Alzheimer's disease (AD), the majority of research resources have been dedicated to studies on the pathogenesis of the intraneuronal filamentous inclusions, known as neurofibrillary tangles (NFT), and the extracellular senile plaques. This focus has often been detrimental to the advancement of other theories. Consequently, there is a large void in our understanding of the

> pathogenesis of AD, namely the underlying mechanism of the disease. Nonetheless, in recent years, research has clearly pointed to the importance of oxidative imbalance in AD."

Parkinson's Disease is better understood due to the availability of animal models and the ability to create a model of Parkinson's Disease with some toxins. This has recently been described in great detail by Sanberg, P, Nishino, HH, Borlongan CV (Eds), in "Mitochondrial Inhibitors and Neurodegenerative Disorders", Humana Press, Totowa, NJ (2000).

Recent data has shown that both diseases manifest profound alterations in energy metabolism, increased insulin resistance, and dysregulation of glucose metabolism. Current research shows that dietary restrictions can forestall the development of both diseases. This tends to show a metabolic component for these diseases, as well as offering the possibility of prevention for both of these diseases. Evidence consisting of brain imaging studies documents reduced glucose uptake in brain cells of living Alzheimer's sufferers. Cellular studies show reduced mitochondrial function in affected brain regions, as well as fibroblasts, in both Alzheimer's and Parkinson's Disease patients. Any consideration of the pathogenesis of Parkinson's and Alzheimer's Disease has to include the fact that, in both, increasing age is a major risk factor and increased

oxidative stress, mitochondrial dysfunction and metabolic abnormalities are prominent features of aging in all body systems, including the brain.

In Alzheimer's Disease, the hippocampal, entorhinal cortex, basal forebrain, and neocortical association areas degenerate whereas, in Parkinson's Disease, there is degeneration of dopaminergic neurons in the substantia nigra.

Currently, the fashion in research is an attempt to find genetic explanations for all diseases and genetic theories of the causation of Alzheimer's and Parkinson's Disease abounds in all the current literature. This is frequently to the exclusion of consideration of older, well-established data concerning both nutritional deficiencies and accumulated toxicities from both heavy metals and organophosphate pesticides.

To the extent that genetic factors may come into play in selective vulnerability brain areas as well as in populations, these genetic studies should, of course, be pursued, but not to the extent of loosing sight of nutritional and environmental factors, which have been shown for years to influence both of these diseases.

The first and most obvious nutritional deficiency involved in the pathogenesis of dementia is B vitamin deficiency in general, and thiamine deficiency in particular. Thiamine is a necessary co-enzyme for several steps for ATP production and mitochondria.

A decade ago, Butterworth and Besnard, University of Montreal reported that brains of Alzheimer's Disease patients showed significant decreases in pyruvate dehydrogenase, alphaketoglutarase, and transketerase. (*Metabolic Brain Diseases* (1990) 5(4):179-84, "Thiamine Dependent Enzyme Changes in the Temporal Cortex of Patients with Alzheimer's Disease")

It has been reported that Alzheimer's patients had significantly lowered thiamine levels. (Gold, et al, *Archives of Neurology,* (1995) "Plasma and Red Blood Cell Deficiency in patients with Dementia of the Alzheimer's Type") and Kish, *Annals of the NY Academy of Science*, (1997) 826:218-28, "Brain Energy Metabolising Enzymes in Alzheimer's Disease, Alpha ketoglutarate dehydrogenase complex and Cytochrome Oxidase")

In 1997, Calingasan and co-workers at Cornell Medical College" reported that Thiamine deficiency alters amyloid precursor protein metabolism and is involved in the pathogenesis of Alzheimer's Disease. (*Neuroreport* 8(11):2631-4, "Thiamine deficiency alters APP but not presenilin-1 immunoreactivity in vulnerable brain regions")

In 1988, Gibson and Co-workers, Department of Neurology, Cornell University Medical College, reported that in Thiamine deficient Alzheimer's patients, activities of the alpha-ketoglutarate dehydrogenase complex were reduced more than 75 % and transketolase was reduced more than 45%. They

concluded that activities of thiamine dependent enzymes in brains are involved in the pathogenesis of Alzheimer's disease. (*Archives of Neurology*, 45(8):836-40, "Reduced Activities of Thiamine-dependent enzymes in the brains and peripheral tissues of patients with Alzheimer's disease")

This sampling of literature should suffice to demonstrate the pivotal role of thiamine deficiency in the pathogenesis of brain neurodegenerative disorders.

What has not been appreciated is the number of factors which can produce relative thiamine deficiency, particularly in the elderly. Some of these are alcohol ingestion in which the alcohol dehydrogenase in the liver manditorily uses thiamine as a co-enzyme and will deplete thiamine from all tissues for this purpose. Another is the presence of thiaminase in several foods, most notably in raw fish. Up until 10 years ago, the presence of large amounts of heat-labile thiaminase in raw fish was a matter of importance, largely in animal feeding. Presently, however, there has been a large increase in the consumption of raw fish by human beings in the form of sushi. There are now sushi bars in most towns and cities and a large number of Americans consume sushi usually, together with alcoholic beverages, without any consideration of the fact that raw fish and alcohol consumption can almost completely deplete thiamine in human beings. High carbohydrate diets increase the need for thiamine. In the elderly, particularly those living alone, there is an increased reliance on tea and toast to the virtual

exclusion of other foods. Thiamine absorption is frequently impaired in the elderly due to intestinal problems and diarrhea. Alpha ketoglutarate dehydrogenase complex is an essential step in the Krebs Cycle and is solely dependent on thiamine as a co-enzyme. Thiamine deficiency produces a severe disruption in electron transport and ATP production and, eventually, leads to apoptosis of neurons which is a hallmark of both Alzheimer's Disease and Parkinson's Disease.

Alzheimer's disease is a nutritional deficiency disease. It is at the end of the spectrum of thiamine deficiency diseases, including Beriberi and Wernicke-Korsakoff Syndrome. It is the chronic, subclinical manifestation of protracted thiamine deficiency, superimposed on chronic aluminum and other metal toxicity, all of which, ultimately, combine to produce loss of mitochondrial function and cell death in cholinergic neurons.

Thiamine supplementation cannot restore neurons lost in this fashion but can restore adequate energy production in remaining brain cells and can do much to prevent these diseases. Thiamine deficiency is commonly seen in people who are also victims of environmental toxins, particularly aluminum toxicity, and particularly the toxicity of aluminum fluoride, which is commonly seen in municipal water supplies. These water supplies are treated with aluminum and fluoride, which combine to produce aluminum fluoride. People who consume

water from such municipal water supplies accumulate aluminum fluoride, which is poorly excreted. People who consume foods prepared in aluminum cookware and who consume aluminum containing antacids are also at high risk. Individuals who have both thiamine deficiency and high levels of aluminum fluoride are at high risk for development of neurodegenerative diseases. To this must be added the effects of pesticides, such as organophosphates which inhibit Complex I and IV of the electron transport chain. People with dental restorations containing mercury are also at high risk.

The first insult is aluminum toxicity which, frequently, begins in the first days of life because most infant formulas contain aluminum. (McGraw, M.D., Bishop, N.; and Jameson, R., et al. "Aluminum content of milk formulae and intravenous fluids used in infants." *Lancet* 1:157, 1986); (Bishop, N.; McGraw, M, and Ward N. "Aluminum in infant formulas." *Lancet*. March 4, 1989); (Freundlich, M.; Zillervelo, G.; Abitbol, C; Strauss, J.; Faugere, M.C.; and Malluche, H.H. "Infant formula as a cause of aluminum toxicity in neonatal uraemia." *Lancet* ii:527-5299, 1985); and (American Committee on Nutrition. "Aluminum toxicity in infants and children: *Pediatrics* 78:1150-1154, 1986).

From this beginning, exposure to aluminum ingestion is fairly consistent and unrelenting throughout life. A hypothetical individual born in 1940, after his or her initial exposure in infant formulas was exposed to aluminum in the drinking

water, oftimes in the form of aluminum fluoride complexes. His or her food was very likely to have been cooked in aluminum pots and pans. When this produced gastric upset, the relief was by consumption of antacid which contained as their main ingredient, aluminum hydroxide. The first visit to the dentist compounded the problem by the addition of mercury in the form of amalgam dental restorations, and lead was added from the daily water piped into the home through lead pipes. After 1960, the introduction of aluminum foil added to the contamination of most foodstuffs.

By 1970, when our hypothetical individual was 30 years of age, 18 million kilograms (39.6 million pounds) of sodium aluminum phosphate was used in the American food supply. That same year 3.6 million kilograms of sodium aluminum sulfate, 510,000 kilograms of aluminum sulfate, 230,000 kilograms of aluminum ammonium sulfate, and 3,800 kilograms of aluminum potassium sulfate were added to the American food supply as direct additives. (Weiner, M.A., "Reducing the Risk of Alzheimer's", New York, Stein and Day, 1987, pp. 63).

The manufacture of aluminum produces a toxic by-product, fluoride, which is sold to municipalities to be added to the municipal water supply, allegedly to prevent dental decay. As noted above, this is added to water supplies which have been processed with aluminum compounds. The result is an aluminum-fluoride compound, so that if our hypothetical individual only ate bread and drank water,

his or her exposure to aluminum ingestion was extremely high. (Shore D, Stuart M, Sprague G, Mayor GH, Moreno C, Apostoles PS and Wyatt RJ, Sept./Oct. 1985. "Aluminum-Floride Complexes: Preclinical Studies". *J. Envir., Path., Tox., & Onc*., 6:9), (Spencer H, Kramer L, Norris C, Osis D, and Wiatroski E., 1980a. "Effect of aluminum on fluoride and calcium metabolism in man". In: *Trace Substances in Environmental Health XIV;*.Hemphill DD (Ed.) Univ. of Missouri, Columbia), Spencer H, Kramer L., Norris C., and Wiatroski E. 1980b. "Effect of aluminum hydroxide on fluoride metabolism". *Clinical Pharmacology and Therapeutics*, 28(4):529-35)

Aluminum and all of its compounds are extremely neurotoxic. It is concentrated in neurons, where it interferes with the balanced production of proteins and enzymes in brain cells.

When B vitamin and, particularly, thiamine deficiency is added to this situation, metabolic disorder is set in motion in the form of progressive neurodegeneration, particularly of cholinergic neurons, those involved in Alzheimer's disease.

Depending on variables, our hypothetical individual born in 1940 and living in the 20th Century, would begin to exhibit the early signs of Alzheimer's Disease at somewhere between 55 and 65 years of age. Some of the variables concern whether or not the individual consumed significant amounts of antacid tablets or

liquids, whether or not the individual developed heart failure for which he or she was treated with diuretics which deplete thiamine; whether or not the individual takes Digoxin which further depletes thiamine; whether they drink alcoholic beverages, or eat raw fish, or other foods which contain thiaminase. In summary, development of Alzheimer's Disease depends on how much aluminum he or she ingested and how little or how much thiamine was available to serve as essential co-enzymes in the Krebs cycle.

An individual born in 1940, probably has had far less exposure than his or her children are now experiencing. In the future, it is to be expected that Alzheimer's Disease will make its appearance at an earlier age and more individuals will be affected than individuals born before 1960.

Currently, most research in the pathophysiology of Alzheimer's Disease is concentrated in genetics which, to date, has produced no useful information and is not likely to do so in the future, since Alzheimer's is simply not a genetic disease or disorder but simply a nutritional disorder compounded by aluminum and other metal toxicants.

The thiamine deficiency contribution to Alzheimer's etiology is well documented and it is highly likely that the other B vitamins contribute as well.

Metal toxicity includes many more metals than aluminum; mercury, lead, cadmium, zinc, copper and iron are also involved.

Genes are involved, since toxic metals, particularly aluminum, in neurons, concentrate in the nucleus where they interfere with the proper function of genes and the production of RNA.

It should be remembered that 80% of the mitochondrial respiratory function is controlled from the nucleus DNA where proteins and enzymes are encoded. Most of the proteins and enzymes used in mitochondrial function are produced in cytoplasmic ribosomes and imported into the mitochondria; these pre-proteins must be transported through the contact points of the mitochondria where chaperones aid in the unfolding and folding of these proteins. Amyloid deposits are essentially proteins misfolded and unable to be imported into the mitochondria. They remain outside the mitochondria to produce neurofibrillary tangles and amyloid bodies. They are a by-product of the effects of toxins on that portion of the nucleus which encodes proteins for the mitochondria. Their presence is indicative of the presence of Alzheimer's Disease. The amyloid bodies and tangles interfere with energy production through a deficiency of thiamine and other B complex vitamins which are essential co-enzymes in the Krebs cycle and electron transfer chain. (Serpell, LC; Sunde, M; Benson, MD; Tennet, GA, Pepys, MB, Fraser PE, "The Protofilament Substructure of Amyloid Fibrils", *J. Mol. Biol.* (2000) 300:1033-1039), (Parker, WD Jr., Haas R, Stumpf DA, Parks, J, Eguren LA and Jackson C., "Brain mitochondrial metabolism in experimental thiamine

deficiency", *Neurology* 34 (11): 1477-1481); (Parker WD Jr., Parks J, Filley CM, and Kleinschmidt-DeMasters BK, "Electron transport chain defects in Alzheimer's disease brain", *Neurology* 44(6):1090-1096); (Todd, KG and Butterworth RF, "Mechanisms of Selective Neuronal Cell Death due to Thiamine Deficiency" *in* "Oxidative/Energy Metabolism in Neurodegenerative Disorders", Bass JP, McDowell, FH (Eds), Annals of the New York Academy of Sciences, Vol 893 (1999)); (Mantyh PW, Ghilardi JR, Rogers S, DeMaster E, Allen CJ, Stimson ER, and Maggio JE, "Aluminum, iron, and zinc ions promote aggregation of physiological concentrations of beta-amyloid peptide", *J. Neurochemistry*, 61(3):1171-1174 (1993));

In the elderly, an additional risk factor is polypharmacy. Many pharmaceuticals directly affect thiamine. The administration of antibiotics, in particular, increases thiamine requirements. Since pharmaceuticals are seldom monitored for their effect on thiamine levels, the effect of many pharmaceuticals on this is completely unknown. Many Americans over 60 consume 12 to 15 pharmaceuticals daily. When this is added to inadequate thiamine intake, increased thiamine requirement and years of accumulation of neurotoxins, the question becomes not, *will* they suffer neurodegeneration but, *when they will* suffer neurodegeneration. The next question is whether their neurodegeneration

will first manifest itself as Alzheimer's Disease or Parkinson's Disease and, for many unfortunates, perhaps both.

Post-menopausal women with estrogen deficiency are particularly vulnerable to Alzheimer's Disease. It is well-established that high caffeine intake and nicotine intake bear an inverse relationship to the incidence of Parkinson's and Alzheimer's Disease. The incidence of neurodegeneration in people who drink a lot of coffee and smoke cigarettes is considerably smaller than those people who, for reasons of health concerns, avoid caffeine and nicotine, both of which are mitochondrial stimulants. Oddly, the administration of nicotine by skin patch is not nearly as effective as cigarette smoking in preventing neurodegenerative diseases.

It should be emphasized that the thiamine deficiency referred to here, is a relative thiamine deficiency, which is far less than that required to produced frank Beriberi or Wernicke-Korsakoff Syndrome. The effect of this is slow and cumulative, as are the effects of slowly accumulated neurotoxins.

It is highly likely that both Alzheimer's Disease and Parkinson's Disease occurred rarely before the 20th Century. Prior to the 20th Century, most people did not over-eat. Since caloric restriction prevents or long-delays the appearance of these neurodegenerative diseases, much of the blame for the appearance of these

diseases in the 20th Century may be the result of over-consumption under-nutrition, which was seldom seen prior to the 20th Century.

It is highly likely that the processes leading to the development of Parkinson's and Alzheimer's Disease are present and, to some degree, operative in all Americans and Central Europeans. For this reason, a concerted effort should be made to halt these processes before they result in frank dementia, movement disorders, or both. To this end, everyone should avoid the use of foods and drinks to which aluminum have been added. This includes baked goods which use aluminum-based rising agents; over-the-counter and prescription drugs containing aluminum; intravenous fluids containing aluminum and all liquids containing water from municipal water supplies. In addition, the ingestion of B vitamins, in much larger quantities than current recommended daily allowances should be regularly consumed. This entails the entire B vitamin complex. Ginkgo Bilobia extracts are also helpful. (Zhu L Wu J Liao H Gao J Zhao XN Zhang ZX "Antagonistic effects of extract from leaves of Ginkgo biloba on glutamate neurotoxicity". Acta Pharmacol Sin 1997 JUL;18(4):344) Klein J Chatterjee SS Loffelholz K, "Phospholipid breakdown and choline release under hypoxic conditions: Inhibition by bilobalide, a constituent of Ginkgo biloba",Brain Res 1997 MAY 2;755(2):347-350), (Kanowski S Herrmann WM Stephan K Wierich W Horr R, "Proof of efficacy of the Ginkgo biloba special extract EGb 761 in

outpatients suffering from mild to moderate primary degenerative dementia of the Alzheimer type or multi- infarct dementia (Reprinted f rom Pharmacopsychiat, vol 29, pg 47-56, 1996)." Phytomedicine 1997 MAR;4(1):3-13), (Nathan, P., "Can the cognitive enhancing effects of Ginkgo biloba be explained by its pharmacology", *Medical Hypotheses* (2000) Dec; 55(6):491-493)

Persons over 50 should seriously consider undergoing a course of EDTA Chelation and a serious, prolonged, detoxification process such as that entailed in the use of Badmaev 269 and Ecomer. They should regularly consume supplements containing N-acetyl cysteine, N-acetyl inositol, choline, and daily supplementation of all essential enzymes, including Pancreatin, Pepsin, Rutin, Bromelain, Trypsin, Papain, Soy Isoflavones, Chymotrypsin, Shitake Mushroom Powder, Dionaea Muscipula Extract (Venus Fly Trap).

Real cheese is, however, an excellent source of nutrition. However, processed cheese food contains large amounts of aluminum to enhance its melting point. Therefore, commercially prepared cheeseburgers must be strictly avoided. A diet high in fibers and relatively low in calories should be maintained. Persons should avoid excessive alcohol intake, and should practice eating foods rich in B vitamins while consuming alcohol. The intake of alcohol should be limited to 2 ounces per day, if alcohol is to be consumed at all. This regimen cannot restore nerve cells that have been destroyed, but brain cells can be and are replaced at a

slow rate by natural bodily processes. This can only occur if the remaining neurons are kept in a state of optimal mitochondrial function and energy production.

Excellent dietary advice with specimen menu's for avoidance of Alzheimer's and Parkinson's Disease are contained in Casdorph and Walker, "Toxic Metal Syndrome", Garden City Park, New York, Avery Publishing Company, (1995).

The pivotal role of mitochondrial function in the pathogenesis of neurodegeneration has only recently come to be recognized by researchers and is virtually never considered by clinicians.

The emphasis here on thiamine deficiency should not serve to diminish the importance of other B vitamins, deficiencies of which commonly occur together with thiamine deficiency. It is highly likely that all B complex vitamins play a pivotal role in maintaining adequate mitochondrial function. Deficiencies in niacin, vitamin B 12, are well known to produce dementia. An example is Pellagra, (B3 deficiency) which, in its classic form, is characterized by dementia and diarrhea, as well as skin diseases.

Attempts to prevent and reverse these dementia's likely should include adequate intake of all B vitamins, elimination of neurotoxic substances, the

elimination of accumulated stores of these toxic substances in the body, and the absolute avoidance of water from municipal water supplies.

A supplement protocol based on the following recommendations: 1) High Potency Multiple Vitamins containing the entire B vitamin complex in amounts greater than the RDA, 2) Vitamin C 500 – 1,000 mg three times daily, 3) Vitamin E 400-800 I.U. daily, 4) Fish Oil containing Omega 3 and Omega 6 Fatty Acids, 5) Thiamine 3-8 mg daily, 6) Phosphatidylserine 100 mg 3 times daily, 6) Phosphatidylcholine 100 mg 3 times daily, Phosphatidylinositol 100mg 3 times daily, 7) Ginkgo Biloba Extract (24% ginkgo flavoglycosides), 80 mg three times daily, 8) Badmaev, 1-2 tablets three times daily with meals, 9) Ecomer one to two capsules two to three times daily, 10) Coenzyme Q10 100-200 mg per day, 11) Regular hair mineral analysis to rule out aluminum or other heavy metals, 12) EDTA Chelation where indicated by hair mineral analysis, 13) A diet containing generous servings of deep sea fish from Northern waters, 14) Never, under any circumstances, consume water from municipal water supplies, including coffee, tea and other beverages prepared from such water supplies, 15) Methylcobalamin (active B12), 1,000 micrograms twice daily and, 16) antioxidants.

This basic regimen must, of course, be modified to suit the individual needs and conditions of the patient to be treated. It is a guideline, not an inflexible standard.

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