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Review Article

Free radicals and low-level photon emission in human pathogenesis: State of the art

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Convincing evidence supports a role for oxidative stress in the pathogenesis of many chronic diseases. The model includes the formation of radical oxygen species (ROS) and the misassembly and aggregation of proteins when three tiers of cellular defence are insufficient: (a) direct antioxidative systems, (b) molecular damage repairing systems, and (c) compensatory chaperone synthesis. The aim of the present overview is to introduce (a) the basics of free radical and antioxidant metabolism, (b) the role of the protein quality control system in protecting cells from free radical damage and its relation to chronic diseases, (c) the basics of the ultraweak luminescence as marker of the oxidant status of biological systems, and (d) the research in human photon emission as a non-invasive marker of oxidant status in relation to chronic diseases. In considering the role of free radicals in disease, both their generation and their control by the antioxidant system are part of the story. Excessive free radical production leads to the production of heat shock proteins and chaperone proteins as a second line of protection against damage. Chaperones at the molecular level facilitate stress regulation vis-à-vis protein quality control mechanisms. The manifestation of misfolded proteins and aggregates is a hallmark of a range of neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, amylotrophic lateral sclerosis, polyglutamine (polyQ) diseases, diabetes and many others. Each of these disorders exhibits aging-dependent onset and a progressive, usually fatal clinical course.

The second part reviews the current status of human photon emission techniques and protocols for recording the human oxidative status. Sensitive photomultiplier tubes may provide a tool for non-invasive and continuous monitoring of oxidative metabolism. In that respect, recording ultraweak luminescence has been favored compared to other indirect assays. Several biological models have been used to illustrate the technique in cell cultures and organs *in vivo*. This initiated practical applications addressing specific human pathological issues. Systematic studies on human emission have presented information on: (a) procedures for reliable measurements, and spectral analysis, (b) anatomic intensity of emission and left-right symmetries, (c) biological rhythms in emission, (d) physical and psychological influences on emission, (e) novel physical characteristics of emission, and (f) the identification of ultraweak photon emission with the staging of ROS-related damage and disease.

It is concluded that both patterns and physical properties of ultraweak photon emission hold considerable promise as measure for the oxidative status.

Keywords: Antioxidants, Chaperones, Chronic disease, Free radicals, Heat shock proteins, Photon count distribution, Ultraweak photon emission

Introduction

In 1954 Gerschman and Gilbert proposed that most of the damaging effects of elevated oxygen concentrations in living organisms might be attributed to the formation of free radicals[1]. In 1956, Harman proposed the "free radical theory of aging" which suggested that free radical damage on cellular macromolecules is responsible for the aging process. However, this idea did not capture the interest of many biologists and clinicians until the discovery in 1969 of the enzyme, superoxide dismutase (SOD) with the function of catalytically removing a specific free radical[2,3]. During the 70's and 80's, many scientists, unfamiliar with free radicals, regarded the field as highly specialized or irrelevant to mainstream biology, biochemistry and medicine. In fact, however, it is just the opposite.

Much experimental data emphasizes that aerobic life is connected with the continuous production of free radicals, particularly reactive oxygen species (ROS) that may be dangerous for the living organism[4-12]. The reactive species attack biomolecules producing alterations in DNA, proteins and lipids, and were implicated in the pathogenesis of age-related disease[13].

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In recent years a wealth of experimental data was collected to clarify mechanisms that are critically involved in free radical damage resulting in pathologies. The data emphasize the role of heat stress proteins (HSP's) in protection against damage by free radicals. The HSP's are also named as to their function, such as "chaperone proteins", since they form complexes with proteinaceous structures in order to prevent deleterious interactions between proteins. Understanding the molecular mechanisms of cellular protection and recovery from damage in injured cells had steadily increased. In particular, how chaperones at the molecular level facilitate stress regulation vis-à-vis protein quality control mechanisms, and have become critical in development of a range of chronic diseases.

To record ROS, many techniques have been made available. Most of these techniques are invasive; they require the destruction of living cellular or tissue structures to estimate either specific ROS species or products derived from reactions between ROS and macromolecules, mostly lipids. Although these techniques are available to measure the progress of oxidation, none is applicable to all circumstances. In the present study, attention is drawn to the method of low-level chemiluminescence to detect electronically-excited states in biological systems. Low-level chemiluminescence was related to the direct utilization of molecular oxygen[14,15] and the production of electronically-excited states in biological systems[16]; in particular, the oxygen dependent chain reactions involving biological lipids[17-19]. This earlier research on low-level chemiluminescence was largely unnoticed in America and Europe, notwithstanding the reports by Stauff and Ostrowski on the chemiluminescence of mitochondria[20] as well as Howes and Steele on the chemiluminescence of microsomes[21,22], both from rat liver. This hesitation probably evolved because of earlier reports of the so-called "mutagenic radiation"[23,24] from living tissue which could not be observed with the then contemporary photon counting equipment[25].

In the meantime, data have demonstrated that spontaneous (natural) ultraweak photon emission originating from living organisms may be considered to reflect the state of oxidative stress *in vivo*. The aim of the present overview is to introduce (a) the basics of free radical and antioxidant metabolism, (b) the role of the protein quality control system in protecting cells from free radical damage and its relation to chronic diseases, (c) the basics of the ultraweak luminescence as marker of the oxidant status of biological systems, and (d) the research in human photon emission as a non-invasive marker of oxidant status in relation to chronic diseases. Perspectives in future research is presented that allow the evaluation of ultraweak luminescence as a method for recording *in vivo* and noninvasively the state of oxidative stress in human subjects vis-à-vis the development of chronic disease.

Free radicals and antioxidants

A "free radical" is defined as any atom, group of atoms or molecules containing one unpaired electron within an outer orbit. Molecular oxygen (O_2) is a triplet in its ground state because it contains two unpaired electrons within its outer orbits having parallel or unpaired spins. Singlet oxygen, by definition, is not a free radical; both electrons occupy the same orbit and the electron spins are paired. In O_2, parallel electron spin prevents the direct addition of electron pairs (this would include electron spins in both parallel and anti-parallel directions) unless an electron spin inversion occurs. A number of enzymatic systems have evolved that are capable of circumventing electron spin restriction by a one-electron reduction of O_2. This intermediate univalent pathway is an essential biological process that provides the pairing electron. The cytochrome oxidase complex localized at the inner mitochondrial membrane tetravalently reduces the majority of O_2 used by aerobic cells. It appears to be a major intercellular source of both O_2^- and H_2O_2.

Apart from the mitochondrial respiratory chain, all the monooxygenases, several dehydrogenases, cyto-chrome-P450, prostaglandin synthetase, leucotri-ene synthetase, vitamin K-dependent enzymes and many other enzymes normally generate radicals. The body not only produces radicals during normal metabolism but it also purposefully produces radicals, designed to be toxic, during immune and inflammatory responses. These radicals are deliberately generated during the respiratory burst of a macrophage in order to kill invading organisms.

In considering the role of free radicals in disease, their generation is only part of the story; the other part is their control, containment and safe disposal. Because radicals and their products are continuously generated and are so reactive chemically, they must physiologically be closely controlled and they must be released in an orderly fashion to avoid damage of vital

components. To maintain cell and tissue integrity, the "antioxidant system" maintains a "check and balance" with the production of reactive free radicals regarding their use in essential pathways and their effective clearance.

In their definition of antioxidant, Halliwell and Gutteridge[26] state, "any substance that, when present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate". The "antioxidant system" includes a number of enzymes and low molecular weight compounds, many dependent on essential nutrients including vitamin E (tocopherol), vitamin C (ascorbic acid), beta-carotene, zinc (Zn), copper (Cu), manganese (Mn), iron (Fe), and selenium (Se). The vitamins are not dependent on other factors that allow them to participate in free radical defence. However, the metals exert their action as antioxidants primarily via incorporation into specific enzymes. Most significant biologically reactive oxygen intermediates are superoxide radical, hydroxyl radical, lipid hydroperoxides and hydrogen peroxide. These oxygen intermediates are regularly discussed in the following paragraphs and, therefore, will be shortly introduced.

Superoxide radicals can be generated as part of many biological redox reactions. Approximately 1-4% of the total oxygen utilized by mitochondria is converted to superoxide and released from the mitochondria[27]. Thus, tissues such as muscle which increase their oxygen uptake during exercise generate larger amounts of superoxide[28]. The superoxide anion is also produced by several cellular redox systems including xanthine oxidase and membrane-associated NADPH oxidase. Phagocytic cells in particular demonstrate increased oxygen uptake and utilize NADPH oxidase to release large amounts of the superoxide anion into extracellular fluid[29]. Superoxide also appears to be produced during ischemia and reperfusion in tissues containing xanthine oxidase[30].

The accumulation of the superoxide anion is prevented by enzymes called superoxide dismutases which contain manganese or copper-zinc at their active site[31]. The superoxide radical is not very reactive. It is capable of slowly inactivating a number of essential macromolecules (including catalase and glutathione peroxidase). Since hydroxyl radical scavengers are capable of protecting damage induced by superoxide generation systems, hydroxyl rather than superoxide radicals are responsible for the damaging effect. Transformation of superoxide radical into a hydroxyl is possible because the superoxide radical is capable of diffusing throughout relatively large distances in the cell and undergoes, in the presence of iron or copper, a metal-catalysed Haber-Weiss reaction with the actual formation of the highly reactive hydroxyl radical (OH')[31,32].

The hydroxyl radical is very reactive[33]. It is the key radical species damaging tissue[31]. It readily reacts with almost every type of molecule (e.g., sugar, amino acid, phospholipids, nucleotides, and organic acids). On the other hand, hydroxyl radicals may be too reactive (see half life below) to survive collisions with compounds adjacent to the site of formation.

Lipid hydroperoxides are associated with the process of lipid peroxidation. In the presence of some transition metals, lipid hydroperoxides may also be cleaved homolytically to form more free radicals and thus accelerate peroxidation of membrane lipids. A variety of hydrophobic scavengers such as tocopherols, intercollated into cellular membranes, may prevent chain-propagating reactions[33]. Lipid hydroperoxides are injurious to cells; they may be detoxified and/or metabolized by glutathione peroxidase systems.

Hydrogen peroxide can be produced by (a) the enzymatic dismutating action of superoxide dismutase and (b) many other biological reactions involving molecular oxygen, including the divalent reduction of O_2 by enzymes such as urate oxidase, D-amino acid oxidase and xanthine oxidase. The majority of the divalent enzymes that result in H_2O_2 generation are localized in specialized organelles called peroxisomes[34]. Mitochondria are major intracellular sources of H_2O_2 generation although any intracellular source of O_2^- can result in H_2O_2 production. Hydrogen peroxide is decomposed to H_2O by catalase and a variety of peroxidases. Glutatione peroxidase (GSH-Px) has been the most intensely studied enzyme of this group[35]. Hydrogen peroxide is a weak oxidizing agent. However, it can inactivate sulfhydryl enzymes. Whereas the peroxide is not very reactive, it can cross biological membranes. Because of the possible involvement of hydrogen peroxide in the generation of hydroxyl radicals, this property places hydrogen peroxide in a more prominent role to initiate cytotoxicity than its chemical reactivity indicates.

The half life times of the major reactive oxygen species are vastly different. The highest rate constant for the reaction with target molecules is correlated with the hydroxyl radical; its reactions are diffusion

limited; i.e., they take place practically at the site of generation[36]. In contrast, other radicals are relatively stable with enzyme dependent half lives in the range of seconds. Such molecules may diffuse away from their site of generation and transport the radical or oxidant function to other target sites[37].

The repertoire of antioxidant protection constitutes antioxidants, protective enzymes, coenzymes and regenerating pathways. There are many essential nutrients involved. Table 1 overviews some of the antioxidants of biological interest[38].

Properties of an ideal free radical scavenger can be easily summarized as:

(a) it must be present in adequate amounts in the body;
(b) it must accumulate within compartments where a need for protection exists;
(c) it must be versatile in order to combine with a wide variety of free radicals. For example, a limitation of SOD in eliminating free radicals is its lack of versatility; it can interact with only one substrate;
(d) if some organisms are devoid of synthetic capability (such as ascorbic acid in primates), the compound must be eaten; therefore, it must exist in plant products and be stable for periods of days or weeks after harvest; and
(e) it might be suitable for regeneration. That is, the process of neutralizing a free radical results in the scavenger becoming oxidized. Thus, a scavenger would be particularly useful if it actually can be recycled. It must have a biologically convenient reducing mechanism, either a specific enzyme or a direct chemical reaction (Table 1).

Free radicals and medical implications

The free radical "hype" often alluded to medical implications. Thus, based on research begun in the 80's, free radicals were implicated in ischemic-reperfusion damage and pathogenesis of cancer, atherosclerosis, and other chronic diseases. Some of the earlier experimental evidence will be shortly introduced.

Hypoxia, ischemia and reperfusion

Oxygen free radicals are important mediators of hypoxic or anoxic cell death in heart, lung, kidney, gastrointestinal tract and brain[9, 39-44]. Hypoxic injury can occur during respiratory failure, systemic hypotension and regional hypoperfusion of organs.

A simple model of hypoxia utilizes *in vitro* cell cultures wherein ATP depletion and the stress of hypoxia is mimicked by exposing cells to inhibitors of mitochondrial respiration and glycolysis, cyanide and iodoacetate, respectively[45-49]. Hepatocytes under the impact of such metabolic inhibition generate hydroperoxides and other reactive oxygen species both during hypoxia and before the onset of cell death. In this model, the loss of viability was delayed by antioxidants in an oxygen-dependent manner[50].

During severe hypoxia or ischemia, the reperfusion of the ischemic tissue can suffer additional injury. For instance, in the treatment of acute coronary thrombosis, reperfusion of ischemic myocardium tissue (a major therapeutic aim) can produce injury[51]. Such effects of temporary ischemia-reperfusion have also been documented during organ transplantation[52,53]. Direct and spin-trapping EPR (electron paramagnetic resonance) and other techniques including chemiluminescence[54-56] have demonstrated that there is a burst of oxygen free radical generation after post-ischemic reperfusion of the heart[57-67].

During severe hypoxia or ischemia, oxidation-reduction components that are normally oxidized in the aerobic state become reduced. When oxygen is

Table 1—Condensed list of antioxidant compounds and enzymes[38]

Non-enzymic	Enzymic (direct)
α-Tocopherol (Vitamin E) (radical chain-breaking)	Superoxide dismutase (CuZn enzyme, Mn enzyme)
β-Carotene (singlet oxygen quencher)	GSH peroxidases (GPx, PHGPx)
Lycopene (singlet oxygen quencher)	Catalase (heme protein, peroxisomes)
Ubiquinol-10 (radical scavenger)	Ancillary enzymes
Ascorbate (vitamin C) (diverse antioxidant function)	Conjugation enzymes (glutathione-S-transferases; UDP-glucuronosyl-transferases)
Glutathione (GSH) (diverse antioxidant function)	NADPH-quinone oxidoreductase (two-electron reduction)
Urate (radical scavenger)	GSSG reductase (maintaining GSH levels)
Bilirubin (plasma oxidant)	NADPH supply (NADPH for GSSG reductase)
Flavonoids (plant antioxidant e.g. rutin)	Transport systems (GSSG export; thioether (S-conjugate) export)
Plasma proteins (metal binding e.g. coeruloplasmin)	Repair systems (DNA repair systems; oxidized protein turnover; oxidized phospholipid turnover)
Chemical (food additives, drugs)	

restored, the components that are reduced may promote intracellular formation of ROS that can then attack lipids, thiols and other cellular components culminating in lethal cell injury[28,53]. Both oxygen-derived free radicals and radicals produced by xanthine oxidase (the other major source of such radicals) have been studied.

Many studies have focused on myocardium "reflow" injury producing cell death as well as mechanical dysfunction. Illustrative are *in vivo* studies of myocardium, either isolated or perfused. Reflow during reperfusion can cause either "stunning"[68] or arrhythmias[69].

The myocardium possesses a number of free radical scavenging systems (superoxide dismutase, catalase and glutathione peroxidase) that protect against injury under normal cellular conditions[70]. However, in presence of excessive radical formation, these systems become saturated and the cells become vulnerable to oxidative injury. Supplementing scavengers or other antioxidant agents, therefore, may enhance cellular protection against free radical injury. The role of oxygen-free radicals has been demonstrated with this indirect approach, utilizing xanthine oxidase inhibitor and radical scavengers such as SOD and catalase[68-70].

It is concluded from these earlier studies that: (a) hypoxia and ischemia followed by reperfusion results in free radical generation; (b) a variety of ROS sources exists, and (c) that the range of produced free radical species depends on the cellular or tissue complexity of the biological system.

Cancer and cancerogenesis

The metabolism of ROS in cancer cells is drastically altered. There is evidence favoring at least two mechanisms: (a) cancer cells produce larger amounts of ROS compared to non-neoplastic cells, and (b) suppression of the antioxidant system in cancer cells. Early evidence demonstrated diminished amounts of Mn superoxide dismutase of all tumors examined at that time[71]. Less Cu/Zn superoxide dismutase has also been documented in many, but not all tumors. Other studies have demonstrated that tumor cells frequently exhibit low catalase activity[72]. Therefore, the amount of superoxide or hydrogen peroxide (H_2O_2) contained in tumor cells should also be elevated. Indeed, most, if not all, hepatic tumours that were evaluated *in vivo* did exhibit more peroxidation then normal livers. In fact, in several human carcinoma cells including colon, pancreatic, breast and ovarian plus malignant melanoma and neuroblastoma demonstrated large amounts of hydrogen perioxide produced *in vitro* without exogenous stimulation[73].

However, the early studies with isolated cell fractions demonstrated that antioxidant systems are very complicated. Mitochondrial or microsomal suspensions prepared from cancer cells exhibited slow peroxidation[74-78] with some exceptions[79]. Data suggest that circuits might be differently regulated during tumor progression with a variety of patterns all characterized by persistent oxidative stress. The significance of such persistent oxidative stress in cancer has been debated. Perhaps, it may activate transcription factors[80] and induce expression of proto-oncogenes[81,82]. It may also induce damage such as modified base products and strand breaks that lead to further genomic instability[83].

Much research has been directed at clarifying the relationship between ROS and the development of neoplasias. If one considers the three-stage model of carcinogenesis (initiation, promotion, progression), the first phase is ROS mediated induction of several types of DNA damage including strand breakage, base modification and DNA-protein cross-linkage. Oxidative DNA damage can also be indirect; e.g., the action of peroxyl radicals freed by endogenous lipid peroxidation or derived from the metabolism of classical chemical carcinogens. Some chemicals are directly carcinogenic, but most require metabolic activation before they can react with genetic material. Free radicals are involved in these activation reactions. Metabolic activation of carcinogens in P450-mediated reactions is known to produce a variety of activated species. The formation of these free radicals is in the endoplasmic reticulum.

It is important to remember that highly reactive free radicals are essentially trapped in the immediate vicinity of their formation as a consequence of rapid interaction with neighbouring molecules. Therefore, their radius of diffusion is frequently small from cellular perspective. Reactive free radicals formed in the endoplasmic reticulum are unlikely to diffuse far enough to react with nuclear DNA. It has been postulated[84], therefore, that metabolically activated free radicals must involve an intermediate chemical reactivity to directly impact DNA with covalent adducts.

Therefore, the issue of location has led to the hypothesis that most cancer may originate in the mitochondrion rather than in the cell nucleus[85]. Mitochondria are self-regulating and contain their own DNA that directs the synthesis of some of the mitochondrial proteins. Mitochondrial DNA is a

single, circular molecule, much less protected than the coiled and chromatin-packaged nuclear DNA[86]. Mutagens bind to mitochondrial DNA up to 1,000 times more strongly than to nuclear DNA[87]. Also, DNA repair mechanisms are much less efficient in the mitochondrion[87,88]. Thus, both mitochondrial DNA and the organelle's inner and outer membranes, high in polyunsaturated fatty acids, are susceptible to attack by free radicals and electrophilic metabolites despite the impressive multilayer free radical defence system[87, 89,90]. It has been suggested that the damage to the mitochondrion by oxygen free radicals leaking from the electron transport chain may cause a baseline level of cancer ("natural" cancer), whereas damage resulting from mutagenic metabolites of chemicals may account for the remainder[91].

In the multi-step process of carcinogenesis, cell division is another critical factor[92-94]. When the cell divides, an unrepaired DNA lesion can give rise to a mutation. It is of interest that oxidants form one important class of agents that stimulate cell division[95-97]. This may be related to the stimulation of cell division that occurs during the inflammatory process, accompanying wound healing[92]. The idea of oxygen free radical involvement in tumor promotion is mostly supported by indirect evidence such as the ability of tumor promoters to induce the respiratory burst in phagocytic cells, the anti-promotor efficiency of antioxidants and free radical scavengers and the capacity of oxygen free radical generating compounds to promote tumors[98-106].

The relationship between chronic infection, inflammation and cancer is also of interest.

Leukocytes and other phagocytic cells combat bacteria, parasites and virus-infected cells by destroying them with a powerful oxidant mixture of NO, O_2, H_2O_2, and OCl^- [107,108]. These oxidants protect humans from immediate death vis-à-vis infection and simultaneously cause oxidative damage to DNA plus mutation[109,110] thereby contributing to the carcinogenic process. It is estimated that chronic infections contribute to about one-third of the world's cancer. Hepatitis B and C viri infect about 500 million people and are a major cause of hepatocellular carcinoma[111-113]. A chronic parasitic infection, schistosomiasis, may lead to cancer. It is prevalent in China and Egypt. It lays eggs in the colon producing inflammation that often leads to colon cancer[114]. It can also promote bladder cancer[115]. *Helicobacter pylori* bacteria infecting the stomachs of over one-third of the world population appear to be the major cause of gastritis, ulcers and stomach cancer[116-121]. Chronic inflammation resulting from non-infectious sources also contributes to various pathological conditions ultimately leading to cancer. For example, asbestos exposure causing chronic inflammation may be a significant risk factor in the development of lung cancer[122,123].

Atherosclerosis

The predominant role of atherosclerosis in causing human disease and death justifies a short discussion of the possible role played by ROS in such pathogenesis (for review see refs. 124, 125). The view that peroxidative processes are involved in the etiology of cardio-vascular diseases, particularly atherosclerosis was suggested by early experimental and clinical data[39,126-132]. Epidemiological studies have demonstrated an association with low plasma concentrations of ascorbate, tocopherol, and B-carotene[133-142]. Within this context, the pathogenetic role of lipid peroxidation in myocardial infarction and stroke was repeatedly discussed. However, evidence also was considered at that time as circumstantial[8,143-145]. The strongest evidence in favour of this assumption was the protective effect of radical scavengers, particularly enzymes or drugs.

Different mechanisms have been postulated wherein lipid peroxidation is involved in the development of artherosclerotic plaques causing thrombotic events including stroke or myocardial ischemia[146]. Lipid peroxidation especially that achieved via the production of ROS by activated monocytes/macrophages adhering to the arterial endothelium[147] could make an early and significant contribution to the development of atherosclerotic plaques[148].

It has been demonstrated that one of the earliest events, which occurs in atheroma formation is the accumulation of cholesterol-laden foam cells in the subendothelial space. Most of the cholesterol deposited in the cells is derived from low-density lipoproteins (LDL). Human LDL is not only rich in cholesterol but also in polyunsaturated fatty acids (PUFA) which are susceptible to lipid peroxidation

Free radical oxidation of LDL, is one of the biological modifications occurring *in vivo* that increases the rate at which LDLs are scavengered by macrophages; nonoxidized LDL is not scavengered at an increased rate[149-157]. Macrophages, the main precursors of the foam cells, do not take up low-

density lipoproteins at a rate rapid enough to cause lipid loading[149,158,159]. However, presence of Fe^{2+} in plaques following entry of blood through plaque fissures and subsequent local hemolysis enhances the oxidation of LDL and thus promotes the accumulation of foam cells. In addition, toxic products of lipid peroxidation favour local necrosis, which may, in concert with other factors, initiate an inflammatory process. Furthermore, oxidative modifications of LDL can, in conjunction with cytokines promote the attachment of even more monocytes to the endothelium. In line with this thinking, SOD has been found to inhibit the oxidation of LDL suggesting that the superoxide radical is responsible for the process. However, metal ion chelators and other general free radical scavenger can also prevent this oxidation[160-162].

Brain pathologies

A third field of early interest came from biochemical studies suggesting that ROS is important in a number of brain pathologies[163-169]. The brain consumes a disproportionate amount of the body's O_2. It derives its energy, almost exclusively from the oxidative metabolism of the mitochondrial respiratory chain. Mitochondria are found in neuronal cell bodies but are also distributed throughout the neuritic structures.

Apart from high oxygen consumption, the brain is rich in oxidizable substrates, mainly unsaturated lipids and catecholamines. This initiated early interest regarding "oxygen radicals" as mediators of the action of certain neurotoxins, in the role of vitamin E in the nervous system and in the possible use of anti-oxidants in treating degenerative diseases of the nervous system as well as the consequences of ischemia.

The discovery of enzymes that specifically scavenge superoxide in aerobic cells (superoxide dismutases) led to the proposal that O_2 is a major agent of O_2 toxicity. This superoxide theory of O_2 toxicity[170-173] is based upon a mass of evidence demonstrating that superoxide dismutases are important for survival in the presence of O_2. SOD enzymes co-operate with other enzymes such as catalase and glutathione peroxidase that destroy H_2O_2 [173]. Catalase decomposes H_2O_2 directly. Very little catalase is present in brain as compared with liver, kidney and erythrocytes. Catalase in tissues is located in small subcellular particles known as "peroxisomes". The peroxisomes found in brain are very small as compared with liver peroxisomes and are often called "microperoxisomes"[174]. Most H_2O_2 generated in brain *in vivo* is probably disposed of by glutathione peroxidase[175]. This enzyme removes H_2O_2 by using it to oxidize glutathione (GSH). Glutathione peroxidase requires selenium for its action. Oxidized GSH (GSSG) is reconverted to GSH by a gluthathione reductase enzyme. Both glutathione peroxidase and reductase are present in all parts of the brain and nervous system. A role of GSH in neurodenegeneration is suggested by the observation that inborn defects in the ability to synthesize GSH produce severe mental and motor retardation and seizures[176]. It was also suggested that GSH depletion is involved in the Parkinson's disease-like syndrome induced by the meperidine analogue, MPTP[177].

Particular attention has been focused on a role of oxygen radicals in Alzheimer's disease. Alzheimer's disease is a progressive neurodegenerative disorder affecting >5% of the population over the age of 65. It is characterized pathologically by cortical atrophy, neuronal loss, glial proliferation, excessive neurofibrilary tangles, and deposition of B-amyloid in neuritic plages[178-181]. One hypothesis is that cellular events involving oxidative stress may lead to neurodegeneration[182-189]. Indeed, ROS may be involved in the production, aggregation and toxicity of B-amyloid[190] which is thought to contribute to neuronal damage in Alzheimer's disease[191].

Recently, attention has been focused on proteins exposed to reducing sugars. These proteins undergo nonenzymatic glycation and oxidation, which ultimately form irreversible advanced glycation end products (AGEs). AGEs-modified proteins form cross-links which result in aggregation and insolubility; they are also a continuing source of potentially damaging reactive oxygen species. The longstanding protein aggregates in Alzheimer's disease such as paired helical filament (PHF) tau and amyloid B-protein[192-194], could form AGEs and contribute to the development of neuronal dysfunction. It has been demonstrated that PHF tau contains AGEs. Other evidence emanates from a study comparing the levels of oxidative damage to proteins, lipids and DNA bases from seven different brain areas of Alzheimer's disease along with matched control tissues. No differences in levels of lipid peroxidation were found in any of the brain regions by using two different assay systems. However, both protein carbonyl levels and oxidized DNA bases were increased in Alzheimer's in several

areas. The documentation of increased damage to protein and DNA strengthens the possibility that oxidative damage may play a role in the pathogenesis of Alzheimer's disease.

A few epidemiological studies are consistent regarding a protective effect by fruits, vegetables or antioxidants[195-197] in a number of neurological pathologies including cerebral ischemia, Parkinson disease, familial amylotrophic lateral sclerosis (a chronic motor neuron degenerative disorder)[198,199].

The previous diseases are examples; data have demonstrated that many other diseases and clinical disturbances involve ROS reactions in mammalian systems. A list of such diseases is presented in Table 2[200].

Protein control quality and chronic disease development: Free radical damage and heat shock c.q. chaperone proteins

In this section a survey is presented regarding the mechanisms underlying the defence reactions following increased oxygen radical production. Stress conditions, including excessive free radical production, lead to the production of heat shock proteins (HSPs), able to protect against damage. The HSP or stress proteins are also named as to their function, such as "chaperone proteins", since they form complexes with proteinaceous and other cellular structures in order to prevent deleterious interactions between proteins. Understanding the molecular mechanisms of cellular protection and recovery from damage in injured cells had increased greatly in recent years. In particular, how chaperones at the molecular level facilitate stress regulation vis-à-vis protein quality control mechanisms, and have become hallmarks of a range of chronic diseases including neurodegenerative disorders, diabetes, atherosclerosis and many others.

ROS damage protected by heat stress

The suggestion that heat stress provides myocardial protection against ischemic-reperfusion injury has been extensively studied vis-à-vis cell cultures. When cells are exposed to a few degrees above their normal growth temperatures, inhibition of protein synthesis and cell death can occur[201]. However, when the treatment is sub-lethal, the cells exhibit a heat shock response[202]. The dramatic feature of this response is the massive and selective increase in synthesis of a small number of heat shock proteins[203,204].

Lee *et al*.[205] observed that heat shock and oxidative stress share a common effect on cells. Heat shock can increase levels of lipid peroxidation as determined by the formation of TBA-products. The supporting evidence was obtained from studies on the induction of heat shock proteins and increased antioxidant enzyme activity by heat shock and oxidant stress[206-208]. Furthermore, it was observed that (a) inhibition of antioxidant defences induce the production of heat shock proteins and increase lethal susceptibility to heat shock[209,210]; and (b) augmenting antioxidant defences decrease tissue damage that occurs during reoxygenation following a period of hypoxia[211].

Similar conclusions were derived from studies with lung slices exposed to oxidant and hyperthermic stresses. Heat and oxidants as well as reoxygenation following hypoxia at normal temperatures induced heat shock proteins. Heat shock protein synthesis was also induced in lung slices exposed to the Cu chelator diethyldithiocarbamate which decreases the activity of Cu/Zn superoxide dismutase[212].

In isolated rat[213] and rabbit[214] hearts, heat stress can provide myocardial protection against ischemic-reperfusion injury, reducing infarct size. In addition, heat stress can lead to an increase in cardiac catalase activity in the rat[213] providing an important pathway for hydrogen peroxide detoxification[160]. Inhibition of catalase abolishes the protection against post-ischemic dysfunction afforded by prior heat stress[215]. It has,

Table 2—List of diseases and clinical disturbances that involve ROS reactions in mammalian systems[200]

Adult respiratory distress syndrome	Contact dermatitis	Myasthenia gravis
Aging	Dermatomyositis	Pancreatitis
Alcoholism	Emphysema	Parkinson disease
Allergic encephalomyelitis	Favism	Psoriasis
Alzheimer disease	Glomerulonephritis	Retrolental fibroplasias
Arteriosclerosis	Gout	Rheumatoid arthritis
Autoimmune vasculitis	Haemachromatosis	Senile dementia
Bronchopulmonary dysplsia	Ischemia-reperfusion injury	Sickle cell anemia
Cancer	Lypofuscinosis	Stroke
Cataract	Malaria	Systemic lupus erythematosis
Chronic autoimmune gastritis	Multiple sclerosis	Thalassemia
Cirrhosis	Muscular dystrophy	Ulcerative colitis

therefore, been proposed that the benefit afforded by heat stress is due to an enhancement of cardiac anti-oxidant status[215] and HSP in facilitating cellular repair[216]. It can be concluded that both the induction of the anti-oxidant enzymes and the induction of HSP's may be considered as part of the second tier of defense that takes place at the level of gene expression. Its significance has become very clear nowadays.

Earlier work was perplexing in the way that many different agents were able to lead to the so-called 'stress response' which started as a molecular curiosity in fruit flies in the early sixties[217]. Following the nomenclature first used for fruit flies, various heat shock proteins in animal cells are referred to on the basis of their mode of induction and apparent molecular mass in kDa. Hence their designation as HSP70 or grp78 for example refers to heat shock proteins of 70kDa and glucose regulated proteins of 78kDa, respectively. Over the last 25 years, a number of observations provided support for the so-called abnormal protein or proteotoxicity hypothesis put forward to explain the induction of the heat shock response by a large variety of stress conditions[218,219]. When cells have been exposed to heat shock or to toxic substances such as ethanol, cadmium, arsenite or oxidative stress, the structure of many proteins is damaged. These abnormally shaped proteins become functionally inactive. Moreover, there is also a high risk that these abnormal protein molecules aggregate not only with other damaged proteins but also with still functional proteinaceous cellular structures.

Proteins, with their structural and functional complexity are fragile macromolecules. Already during their growth, when polypeptides mature stage by stage, the chains cannot fold correctly until a complete folding domain has been created raising the possibility that incomplete domains may misfold. These developments take place within highly crowded compartments. Such conditions compete with normal folding and may cause the phenomenon of misassembly. Misassembly is defined as the misguided association of two or more polypeptide chains to form nonfunctional structures[220]. These structures may be as small as dimers or large enough to be insoluble. The emphasis on function serves to distinguish misassembly from the formation of functional oligomers termed oligomerization. Misassembly should be distinguished from misfolding which is defined as the formation of a conformation which cannot proceed to a functional level within a biologically relevant time scale. Misassemblies are by definition misfolded.

Each protein in the cell has its own intrinsic propensity to unfold and misfold spontaneously, a tendency which increases with variations of environmental conditions. Thus, a continuous flux of toxic, misfolded proteins is spontaneously formed during the lifetime of a cell. Depending on their cellular concentration, misfolded species tend to assemble into stable protein aggregates in the cytoplasm which is also extremely crowded and viscous. The term 'crowded' is preferred to 'concentrated' because, generally no single, macromolecular species occurs at a high concentration. However, taken together, macromolecules occupy approximately 8-40% of the total volume[221]. The cytoplasm is a space, in which densely crowded proteins, each with a different complementary function, must be able to move randomly to meet and timely interact with rare specific partners. Most proteins native to a living system contain repulsing, negative charges on their surfaces and thus refrain from exposing hydrophobic segments; these proteins can optimally maneuver and avoid each other in the highly promiscuous environment of the cytoplasm. In this context, the spontaneous conversion of a functional native protein into a misfolded one, exposing positive charges and new hydrophobic surfaces, will greatly increase both the friction between the macromolecules and the viscosity of the cytoplasm. Increased cytoplasmic viscosity reduces freedom of movement and consequently impairs the function of many cytoplasmic proteins in addition to the above-mentioned cytotoxic effects of aggregates[222-224].

Bacteria and eukaryotes have developed defence mechanisms against "toxic" protein aggregation, utilizing two protein types: the molecular chaperones (typically HSP90, HSP70, HSP60, HSP27) and the ATP-dependent proteases (typically Lon, ClpC/X/P, FtsH, KslU/V, and the 26S proteasome)[225]. Laskey first proposed the term "molecular chaperone" for nucleoplasmin[226]. Ellis expanded the definition of molecular chaperone: a fully developed (stable) protein that escorts still developing proteins to prevent improper associations[227]. Presently, this definition of a protein with a simple escorting role is still applicable regarding some simple, binding chaperones such as the small HSP's. However, it has since been

demonstrated that chaperones[228] possess many active functions[229]: they convert part of the energy of ATP hydrolysis to repair structural damages in stable, misfolded, dysfunctional proteins. These chaperones forcefully disentangle stable dysfunctional aggregated proteins, unfold, refold and re-stabilize them into "re-educated and born again" native, functional proteins in the cell.

When there is no appreciable stress, molecular chaperones and the proteases exist in cytoplasm at low concentrations. This is sufficient to perform physiologic housekeeping functions and to remove sporadically misfolded proteins. However, during extreme situations such as oxidative stress or heat shock, chaperone and protease systems become overloaded by toxic protein forms. Cells synthesize then massive amounts of molecular chaperones and proteases [230]. The stress-inducible nature of many molecular chaperones had led to early classification among the heat shock proteins (HSPs). They are categorized by molecular weight: HSP100, HSP90, HSP70 (HSP40, HSP20), HSP60 (HSP10) and HSP22/27 in eukaryotes (co-chaperones in brackets); and correspond to bacteria: ClpB, HtpG, DnaK (DnaJ, GrpE), GroEL (GroEs) and IbpA/B. Different chaperones display mutually non-exclusive properties. Some "binding" chaperones, e.g., HSP90, HSP70, HSP60, HSP40 and HSP22/27 can provide adhesive surfaces, which, upon interaction with partially denatured polypeptides or oligomerizing subunits, can passively reduce the extent of aggregation[231,232]. Unfolding chaperones, such as HSP100, HSP70 and HSP60 (possibly also HSP90) are involved in ATP-dependent unfolding (followed by the spontaneous native refolding) of denatured polypeptides[233, 234].

The literature regarding the roles of various chaperone types suggests two functionally different classes. Small chaperones (less than 20 kDa) bind transiently to short hydrophobic sequences on polypeptide chains and prevent them from both folding prematurely and misassembling by binding to these sequences for a period of time. Large chaperones, exemplified by GroEL, function basically by providing a molecular cage composed of one oligomer of GroEL capped by one oligomer of GroES [235]. Single, partly folded chains are encapsulated one at a time inside this cage. The enclosed chain continues to fold in the absence of other folded chains until the hydrophobic surfaces that cause misassembly are buried within the final folded structure. The time of folding inside this cage is set by the slow ATPase activity of the GroEL subunits and results ultimately in the release of the folded chain into the cytosol[220]. These data demonstrate that during one's lifetime, cells maintain a battery of defense that reduces the concentration of toxic, misfolded protein species, maintaining them below critical toxic concentrations.

As we age molecular chaperones and proteases are insufficiently produced. We may react poorly to environmental stress[236]. The levels of molecular chaperones and proteases are significantly decreased. Simultaneously, irreversibly damaged proteins accumulate[237, 238] due to decline in functional proteasomes and lysosomes. In addition to their general cytotoxic effect, irreversibly damaged proteins can inhibit the activity of the remaining minority of functional chaperones and proteases. At this stage, old cells often choose suicide, which may at times, be advantageous, for example with cancer cells. HSP70 has been shown to protect against cell death by directly interfering with the mitochondrial apoptosis pathway[239].

The occurrence of protein damage as the origin of cellular disorder is increasingly recognized as a main biomedical focus of interest since its occurrence not only has been observed as a result of physical and chemical stress but also upon exposure to pathogens as bacteria and viruses, during ischemia, inflammation, transplantation and upon neurodegenerative and other chronic diseases (see further in this chapter). The overproduction of molecular chaperones following treatments with various non steroidal anti-flammatory drugs (NSAIDs, e.g., sodium salicylate[230], ibuprofen[240]), and less classical HSP-inducers such as celastrol[241], resveratrol (french paradox)[242] and geranylgeranylacetone[243], may be responsible for the reduction of damages related to reactive oxygen and induced programmed cell death in various damaging contexts. Examples include ARDS[244], and post-ischemic reperfusion[245].

Regulation at gene expression level

A simple model for the regulation of availability of protector proteins in defense following damage is regulated at cell's DNA level. The quantity of free protector proteins available in the cell decreases under these adverse circumstances. As long as these essential proteins are available, damage is reduced to a minimum. However, when a shortage arises in the

case of an overload of damage, the originated abnormal protein molecules are capable of complexing with other cell structures. Cell damage can then only be avoided by production of new protector proteins. The replenishment of these protector proteins starts with activation of associated protector protein gene promoters on the cell's DNA. This highly specific event occurs by binding of specific DNA-binding factors, called heat shock transcription factors (HSF's) on specific (promoter) DNA-sites[246]. This binding constitutes the signal that triggers transfer of information from DNA into mRNA, leading eventually to synthesis of new protector proteins.

Whether or not these DNA-binding factors interact with the DNA depends on the existing quantity of protector proteins in the cell. The genome is only specifically activated to trigger this synthesis of additional protector proteins when their quantity falls below a certain threshold. Normally, at least one type of protective protein HSP70, forms a complex with HSF, which provides the basis for this regulation. If protector proteins are required to neutralize abnormal proteins, this complex dissociates, causing release of HSF which then binds to the promoters and induces mRNA production with the ensuing synthesis of new protector proteins. When sufficient new protector proteins have been produced, i.e. when their amount is raised above the threshold value, HSP70 will again form a complex with HSF molecules, uncoupling it from DNA, with a concomitant halt of mRNA production. This molecular reaction cycle can be indicated, in terms of systems theory, as the autoregulation loop which is the basis of damage-induced recovery processes.

However, cells do not use only one type of transcription factor (HSF) in response to stress conditions; they use multiple signalling pathways and transcription factors to fine-tune their response to specific circumstances. In addition to the heat shock factor, also nuclear factor-kB, nuclear factor erythroid-2 and activator protein-1 families have been recognized as important regulators of the cellular stress response. These different families of transcription factors are generally activated by different stress conditions. Although there is a functional overlap between these individual families and a given stimulus can activate members of more than one (and even all four) of these transcription factors, they broadly regulate different aspects of the cellular stress response by modulating specific target genes. As was described above, HSF is activated under stress conditions characterized by significant intracellular accumulation of non-native proteins and consequently activates genes whose products are capable of alleviating this condition and restoring the integrity of damaged proteins. NF-kB is an important regulator of cytokines and other mediators of the immune and inflammatory response that provides protection against bacterial and viral infections. Nrf2 is activated by various xenobiotics and oxidants and therefore regulates genes encoding proteins with xenobiotic detoxification and antioxidant activities. Finally, AP-1 factors control cellular fate by regulating production of proteins that mediate cell growth or cell death, the latter being the most drastic decision by a cell under extreme stress. Various stimuli may simultaneously cause multiple types of 'molecular' stress and therefore may activate two or more of the transcription factors, leading to a differential stimulus-specific gene expression. It has indeed been observed that a unique pattern of stress proteins is induced when cells are exposed to different stress conditions[247].

Heme oxygenase-1 (HO-1 or HSP32) appears to be the only protein which is induced by all four of the stress-responsive factors[248]. Upregulation of the HO-1 gene is associated with marked cytoprotection. Studies using HO-1 deficient cells and mice[249] have confirmed that the HO-1 system is indispensable to survival and, in particular, to protection from oxidant stress[250-252]. HO-1 is the rate-limiting enzyme in the breakdown of heme with bile pigments (biliverdin and bilirubin), iron and the gas CO as catalytic end products. Although initially viewed as obscure waste products with potential toxicological implications, they are currently seen as serving a critical physiological role in cytoprotection during cellular stress and organ pathology. Bilirubin is considered to be the most potent antioxidant molecule in serum[253]. CO also serves a clear physiological (hormetic) role in cellular defence ameliorating inflammatory and ischemic injuries[254], whereas iron stimulates the upregulation of the iron-binding ferritin protein which helps to prevent Fenton reactions leading to the highly damaging hydroxyl radical. A number of review papers have emphasized the importance as well as the clinical relevance of heme oxygenase since an

upregulation of HO-1 leads to an enhanced resistance against a broad range of (oxidative) stress conditions and alleviates a number of pathological conditions including cardiovascular disease, neurodegenerative diseases and inflamm-ation[251,252,254,255].

The unique pattern of stress proteins induced in cells that are exposed to different stress conditions has other highly interesting consequences. A brief and moderate heat shock to Reuber H35 hepatoma cells causes a rapid increase in the synthesis of heat shock proteins (HSP) and initiates the development of thermotolerance, which results in an increased ability to survive exposure to otherwise lethal temperatures. Low doses of various chemical stressors [arsenite, cadmium, mercury, lead, copper, menadione and diethyldithiocarbamate (ddtc)], at concentrations that do not exert any effect in control cultures, are able to enhance the synthesis of HSP's and to stimulate the development of thermotolerance when applied to cultures which were pretreated with a mild heat shock[256]. The degree of stimulation appears to be stressor-specific, which is not only observed in the ensuing development of thermotolerance but also in the enhancement of the heat shock-induced synthesis of stress proteins. The different HSP's that show an enhanced induction when heat shocked cultures are exposed to the various secondary applied low doses of chemical stressors, were found to resemble the HSP pattern that is characteristic for the secondary stressor and not for the initial heat shock. In other words, the nature of the post-treatment determines the observed pattern of enhanced synthesis of HSP's. In order to analyze the origin of the stimulation of survival capacity by low doses of the mentioned stressors, it was studied whether the degree of stimulation is determined by the degree of similarity between the overall stress response to heat shock and to the second stress condition when applied singly. The degree in which low doses of chemical stressors stimulate tolerance development and enhance the synthesis of HSP's in cells that were previously heat shocked, appears to be related to the degree of similarity in the HSP pattern induced by both stressors. The results support the notion that low doses of toxic compounds may, under certain conditions, have beneficial effects related to a stimulation of endogenous cytoprotective mechanisms.

Misfolded proteins and aggregates in disease

Misfolded proteins and aggregates are hallmarks of a range of neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD), amylotrophic lateral sclerosis (ALS), polyglutamine (polyQ) diseases that include Huntington's disease and related ataxias[257-259] as well as diabetes[260]. Each of these disorders exhibits aging-dependent onset and a progressive, usually fatal clinical course. Despite differences in the underlying genes and clinical presentation, similarities observed have led to the proposal that cellular protein quality control is the underlying common denominator of these diseases[261]. In this section this is first illustrated for a clinical situation, type 2 diabetes mellitus, and subsequently illustrated with basic research utilizing a model for polyQ pathogenesis.

Type 2 Diabetes mellitus (T2DM)

One of the most important cellular stressors in T2DM that contribute to protein misfolding and aggregation is redox stress. ROS may impact disulfide bond formation[262] and subsequently influence the development of Islet amyloid polypeptide (IAPP) misfolding. IAPP oligomers precede islet amyloid deposition. Disulfide bonds formed in newly synthesized proteins are important for proper protein folding, protein structure, biological activity, and stability of many secreted and membrane proteins[258,263,264]. Protein folding in eukaryotes takes place in the ER with assistance from many redox-sensitive chaperones and oxidoreductases (e.g., protein disulfide isomerase, Erp44, Erp57, Erp72, GRP58, HSP33)[264]. Growing evidence implicates both ROS and RNS (radical nitrogen species, such as the reaction of superoxide anion (O_2^-) with nitric oxide (NO) to form peroxynitrite and other RNS) could contribute to protein misfolding[265], and are important in the development of diabetes[266-270]. When the protein quality control system is overwhelmed and IAPP is not capable of being correctly refolded, this protein can become a soluble toxic monomer. Soluble IAPP oligomers have been shown to be cytotoxic and possibly responsible for beta cell apoptosis in T2DM[271-273]. Accumulation of mature islet amyloid is responsible for the space-occupying lesion with associated secretory and absorptive defects within the islet.

Thus, type 2 diabetes mellitus (T2DM) is an example of a conformational disease featuring a protein that aggregates in beta-pleated sheets that are linked by hydrogen bonding between their aligned pleated structures[260]. The contribution of islet amyloidosis to disease pathogenesis has been

vigorously debated [274]. IAPP oligomers that precede islet amyloid deposition are likely more toxic to beta cells than islet amyloid itself. The misfolded, soluble oligomeric proteins promote apoptosis[271,275]. Clinically, it is clear that aggregates of misfolded IAPP are a prominent pathological feature in the development of T2DM (reviewed by Hayden and Tyagi[276]). Islet amyloid is present at autopsy in as many as 96% of patients with T2DM[277]. In case of T2DM, amyloid fibrils are formed with subsequent stabilization by accessory molecules, such as serum amyloid P, perlecan, and apolipoprotein E [274].

An additional factor in disease development is that mitochondrial respiratory function has been demonstrated to decline in various human tissues during the aging process[278,279]. Mitochondria are the major intracellular source and primary target of ROS, which are generated under normal conditions as by-products of aerobic metabolism in animal and human cells. It has been established that defects in the respiratory chain lead to increased production of ROS and free radicals in mitochondria[280-282]. Mitochondrial biology is one of the fastest growing areas in molecular genetics and medicine. Mitochondrial diseases are very numerous and different. Apart from diseases definitely caused by abnormalities in mitochondrial DNA, many diseases are suspected to be caused in part by dysfunction of mitochondria, such as diabetes mellitus, forms of cancer and cardiovascular disease, lactic acidosis, specific forms of myopathy, osteoporosis, Alzheimer's disease, Parkinsons's disease, stroke, and many more. The decline in functioning is caused, at least partly, by oxidative damage and mutation of mitochondrial DNA (mtDNA) and lipid peroxidation in somatic tissues of aged individuals[279, 283-287]. Recently, it was found that mtDNA copy number is increased in the tissues of elderly human subjects[283]. Taken together, these findings suggest that the increase in mitochondrial mass and mtDNA content are the early molecular events of human cells in response to endogenous or exogenous oxidative stress through cell cycle arrest and it was thought to compensate for respiratory function decline during the aging process[288,289].

PolyQ disease: Caenorhabditis elegans in basic research

There is growing evidence for genes involved in protein folding and degradation that modulate onset, development and progression in models of multiple neurodegenrative disease[290-292]. Some of the disorders, including the polyQ diseases, exhibit familial inheritance that facilitates the identification of single gene alterations underlying the disorders[293-296]. Other diseases are sporadic and yet, they too have helped to identify candidate genes that could reveal insights into pathology. These include mutations of amyloid precursor protein in Alzheimer's disease, parkin and alpha-synuclein in Parkinson's disease and superoxide dismutase in amylotrophic lateral sclerosis[297-302]. Identification of these genes has led to the development of transgenic mouse, cell culture models as well as models using Drosophila and *C. elegans* to study neurodegenerative disease[303-307].

In a few animal models it can be demonstrated that aggregation is accompanied by cellular dysfunction and formation of polyQ aggregates visible by light microscopy[308,309]. An illustrative research line is the study of polyQ-length-dependent aggregation in neuronal dysfunction by Morimoto and colleagues utilizing *C. elegans*. Behavioral phenotypes of *C. elegans* were examined to test whether polyQ aggregation in neurons was accompanied by neurotoxicity. There was a polyQ length-dependent loss of coordinated movements leading to nearly complete paralysis. Animals with no visible polyQ aggregates (Q0 animals) demonstrated rapid movements similar to wild type animals. Animals with visible aggregates, Q67 and Q86, had limited capacity for coordinated movements. Animals with intermediate polyQ length, for instance Q19, showed an intermediate situation with slight decrease of movement. These data suggest that formation of visible polyQ aggregates correlates with neuronal dysfunction. Studies on the influences of aging regarding the threshold for polyQ aggregation and toxicity focused on the behavior of polyQ proteins[309]. Individual animals were examined daily for the appearance of protein aggregates and motility. Q40 and Q82 animals quickly accumulated aggregates of protein and exhibited a rapid decline in motility; Q33 and Q35 animals exhibited an initial lag prior to the gradual accumulation of aggregates demonstrating ultimately lower levels. This data reveal that the threshold for polyQ aggregation and toxicity is age-dependent[309]. The molecular link between these pathways is regulated, in part, by factors that detect and respond to misfolded proteins: namely, heat shock transcription factor (HSF) and molecular chaperones/heat shock proteins. For example, it has

been shown that inhibition of HSF-1 function leads to decreased lifespan and an accelerated aging phenotype in *C. elegans*[310-312]. Conversely, overexpression of HSF-1 in *C.elegans* extends lifespan[311,312].

In summary, there is convincing evidence to support a role for oxidative stress in the pathogenesis of many chronic diseases. The model includes misassembly and aggregation of proteins when three major tiers of defense are insufficient: (a) direct antioxidative systems, (b) molecular damage repair systems (like chaperones), and (c) capacity of the compensatory chaperone synthesis. Aggregates of amyloid proteins are commonly novel producers of ROS.

This has resulted in the hypothesis that during life time depending on both, predisposition and stressful conditions, the defence system can fail and an increase of ROS occurs in the early period of development of chronic diseases. The implications of this hypothesis for diagnostic purposes has raised interest in the use of noninvasive procedures to record human oxidative in relation to development of pathology of chronic diseases (such as Alzheimer's disease and diabetes) that are supposed to be linked to non-linear progression in ROS production. A number of patients with these diseases are also taking antioxidant therapy on the recommendation of their caregivers or physicians in the belief that such therapy may offer some protective benefit. Along this line, the development of a noninvasive tool for detection of human emission and its validation is crucial.

The next section reviews the current status and future issues of the human photon emission techniques and protocols for recording the human oxidative status, i.e., recent knowledge regarding uniformity and variation in anatomical pattern of photon emission, its dynamics in vis-à-vis internal physiology and psychophysiology, and its relation to health and disease.

Low level luminescence as marker of oxidant status of biological systems

Many techniques are available to measure the progress of oxidation, but none is applicable to all circumstances. A summary of techniques as biomarkers of oxidative stress in tissue damage, focusing predominantly on the measurement of biological lipid peroxidation has been reviewed by Gutteridge and Halliwell[313] and summarized in Table 3. The table contains the techniques to detect lipid peroxidation as evidence most frequently cited to support the involvement of free-radical reactions in toxicology and disease. However, no single method is adequate for all stages of lipid peroxidation in a biological system and few have the desired specificity. Although most techniques have focused on lipids, it is evident that proteins are also targets for oxidation in biological systems under oxidative stress. Both aspects of oxidative stress can be recorded with the chemiluminescence method. Both lipid[314] and protein oxidation[315] are accompanied by spontaneous light emission that may be easily detected with sensitive photomultipliers[316].

Luminescence of living organisms has fascinated scientists since antiquity[313,317,318]. Until 1961 it was thought to be restricted to organisms with "light organs" containing luciferin-luciferase systems. At that time, Tarusov *et al.*[17] used photon counting to identify a weak blue-green light emission from mouse liver *in situ*. This observation was later extended to brain, muscle, intestine, tissue homogenates and lipid extracts[17-19, 319-321]. The existence of such light emission was labeled "low-level" chemiluminescence or "dark" chemiluminescence to differentiate it from the more effective photoemission of the luciferin/ luciferase systems which is 10^3-10^6 times brighter[16,19]. In early studies, yeast cells[322, 323], phagocytosing leukocytes/ macrophages[324-326] and hepatocytes[327] exhibited "low level" luminescence[16]. This luminescence cannot be seen by the dark adapted human eye because retinal illumination of 3×10^3 photons sec^{-1} cm^{-2} is required to perceive a luminous signal[328].

Low level chemiluminescence in production of electronically-excited states

Low-level chemiluminescence was soon related to the direct utilization of molecular oxygen and the production of electronically-excited states in biological systems; in particular, the oxygen dependent chain reactions involving biological lipids[14,15,17-19,321]. This earlier research on low-level chemiluminescence was largely unnoticed in America and Europe, but reports by Nakano and colleagues on light emission during lipid peroxidation, both in isolated microsomes and during other oxidative reactions, revived the interest in chemiluminescence and suggested its use as a tool for the investigation of the radical reactions of lipid peroxidation under physiological conditions[329-331]. The simplified systems employed in these earlier studies demonstrated a remarkable rule: during the early stage of lipid

Table 3—Methods used to detect and measure biological lipid peroxidation.

What is measured	Method	Remarks
Loss of unsaturated fatty	Analysis of fatty acids by GC or HPLC	Very useful for assessing lipid peroxidation stimulated by different metal complexes that give different product distributions.
Uptake of oxygen by carbon-centered radicals	Oxygen electrode	Dissolved oxygen concentration is measured. Useful in vitro when spectrophometric interference occurs or when chemicals interfere. Not very sensitive.
Lipid peroxides	Iodine liberation	Lipid peroxides oxidize I^- to I_2 for titration with thiosulfate. Useful for bulk lipids, e.g., foodstuffs. H_2O_2 also oxidizes I^- to I_2. In the presence of excess iodine the triiodine anion (I_3^-) can be measured at 353 nm.
Lipid peroxides	Heme degradation of peroxides	Heme moiety of proteins can decompose lipid peroxides with formation of reactive intermediates. Radicals produced can be reacted with isoluminol to produce light.
Lipid peroxides	GSHPx	GSHPx reacts with H_20_2 and hydroperoxide, oxidizing GSH to GSSG. Addition of glutathione reductase and NADPH to reduce GSSG back to GSH results in consumption of NADPH, which can be related to peroxide content. Cannot measure peroxides within membranes; they must first be cleaved out by phospholipases.
Lipid peroxides	Cyclooxygenase	Stimulation of cyclooxygenase activity can be used to measure trace amounts of peroxide in biological fluids. This assay cannot be used to identify specific peroxides.
Lipid peroxides/ aldehydes	GC-MS	Extraction, reduction (e.g., by borohydride) to alcohols, separation by GC, identification by MS. Several variations of these methods exist.
Pentane and ethane	Hydrocarbon gases	GC measurement of gases formed during lipid peroxide decomposition. Only a minor reaction pathway but can be used as a noninvasive in vivo measure of peroxidation. Results in practice have been variable. Rigorous controls are required.
Excited carbonyls, singlet oxygen	Light emission	Reaction of peroxyl radicals can produce excited-state carbonyls and singlet 0_2. Both species emit light as they decay to the ground state. Measurement of low-level chemiluminescence is a method for measuring generation of reactive oxygen species in whole organs, but the light appears to arise from several sources.
Aldehydes	Fluorescence	Aldehydes such as malondialdehyde can react with amino groups to form Schiff bases (at acid pH only). At neutral pH, fluorescent dihydropyridines may be formed. Formation of fluorescent products is a minor reaction pathway and has very complex chemistry. It should never be assumed, without detailed characterization, that fluorescent products accumulating in vivo are end products of lipid peroxidation.
TBARS	TBA test	The test material is heated at low pH with TBA, and the resulting pink chromogen is measured by absorbance at ~532 nm or by fluorescence at 553 nm. The chromogen can be extracted into butan-1-ol. Most of the aldehydes that react with TBA are derived from peroxides and unsaturated fatty acids during the test procedure. Simple and nonspecific assay, rigorous controls required.
Aldehydes	Antibody techniques, GC-MS, HPLC	Hydroxyalkenals such as 4-hydroxynonenal are products of lipid peroxidation that are cytotoxic at nanomolar concentrations. They can be measured by HPLC or GC-MS. Several techniques have been developed that involve antibodies to detect proteins modified by lipid peroxidation products.
Diene conjugation	UV spectrophotometry	Oxidation of unsaturated fatty acids is accompanied by an increase in UV absorbance at 230-235 nm. Useful for bulk lipids. Requires extraction or separation techniques for biological use. Serious problems can arise when used on human body fluids.
Octadeca-9,11-dieonic acid	Linoleic acid isomer	This isomer accounts for most of the diene conjugation present in human plasma and tissues but has not been produced in oxidatively stressed animal and model lipid systems. A single isomer of one polyunsaturated fatty acid is more indicative of an enzymic reaction than random free radical attack.
Nitrone adducts of reactive short-lived free radicals	Spin trapping	Spin traps allow the formation of stable nitroxides, which can be examined by electron spin resonance. Spin traps can be used in animal experiments in vivo to detect carbon-centered radicals as well as alkoxyl and peroxyl radicals.
F_2-isoprostanes	GC-MS HPLC	Peroxidation of polyunsaturated fatty acids produces a complex mixture of non-specific rostaglandin isomers.

GC, gas chromatography; MS. mass spectrometry; GSH, glutathione; GSHPx, glutathione peroxidase; GSSG, oxidized glutathione; UV, ultraviolet; TBARS, thiobarbituric acid-reactive substance.

peroxidation, the intensity of chemiluminescence is proportional to the square of the concentration of lipid peroxide, suggesting that singlet oxygen and a compound in the triplet state (probably a carbonyl compound) are both generated by a self-reaction of lipid peroxy radicals[330].

Other reports on chemiluminescence directed attention to mitochondria as the second important contributor to cellular chemiluminescence. Data collected in experiments with perfused liver, isolated liver mitochondria and isolated submitochondrial particles suggested that (a) intensity of light emission by mitochondria was dependent on the metabolic state, (b) singlet molecular oxygen was mainly responsible, and (c) chemiluminescence integratively measures radical reactions involved in lipid peroxidation and related processes[332].

In this sense, mitochondrial and microsomal fractions[332, 333] behave similarly with respect to light-emission: in both cases, singlet molecular oxygen appears mainly responsible for the observed chemiluminescence. The experimental evidence for the generation of singlet oxygen was obtained mainly through the effect of specific quenchers[329,333,334] or spectral analysis[329]. In mitochondria, microsomes and submitochondrial particles, optimization of light emission requires (a) a membrane-bound electron transfer system, (b) added hydroperoxide and (c) the presence of O_2 [332,333]. Oxygen containing species (O_2^-, H_2O_2, HO^-, RO´, ROO´ and singlet oxygen) were generated either by an interaction of oxygen with the components of the respiratory chain or by the homolytic scission of hydroperoxides by hemeproteins [332,333,335-337]. These species were able to initiate free radical reactions, primarily through increased HO' and RO´ formation, ending in lipid peroxidation[28,338-341].

Despite many experiments demonstrating that spontaneous chemiluminescence increases when using extracellular stimuli such as hydroperoxides or increased oxygen tension[342], it took more time to understand how chemiluminescence is affected when changes in the intracellular steady-state concentration of hydroperoxides occur. However, this problem was eventually addressed with additional knowledge about inhibitors of enzymatic and nonenzymatic intracellular defences against partially reduced oxygen species that, as a consequence, could lead to an increased intracellular concentration of oxygen radicals. The intracellular steady-state concentrations of hydrogen peroxide or the superoxide anion were increased by inhibiting either catalase, glutathione peroxidase or superoxide dismutase activities. This information explained the increased spontaneous chemiluminescence after inhibition of any antioxidant enzyme[343].

The most important aspect of organ chemiluminescence is that it provides, on a non-invasive basis, a signal of oxidative metabolism and the (overall) free radical, steady-state concentration that is readily and continuously detectable. It is possible to continuously monitor the metabolism of organs *in vivo* with chemiluminescent techniques. In that respect, chemiluminescence has been favored compared to other indirect assays of lipid peroxidation such as glutathione release[28], evolution of hydrocarbons[344,345] or malondialdehyde accumulation[327,346].

Many comparative studies on different assays have been completed utilizing chemiluminescence compared to other assays regarding lipid peroxidation. One study focused on doxorubicin (DXR), a widely used antineoplastic agent that is known to induce cardiotoxicity. This toxicity is mediated by reactive free radicals produced by DXR. DXR undergoes NADH dehydogenase-catalyzed one-electron reduction to a semiquinone free radical in mitochondria[347,348]. Subsequently, these free radicals participate in DXR induced lipid peroxidation of mitochondrial membranes. DXR induced lipid peroxidation is generally evaluated by TBARS formation. However, fluorescent substances and high molecular weight protein aggregates, both non-specific indicators of lipid peroxidation, have also been employed[349]. ESR measurements have been used to specify the molecular nature of reactive oxygen species generated during DXR redox cycling[350,351]. An additional chemiluminescence study confirmed an increased free radical generation utilizing noninvasive, continuous monitoring of chemiluminescence produced during DXR redox cycling and its analysis with chemiluminescence spectroscopy.

Both chemiluminescence and one other type of assays for detecting free radical generation were utilized to study ischemia and reoxygenation (reperfusion) in heart and liver.

In the late 80's and early 90's, the rapidly growing interest in oxygen toxicity and free radical reactions in biology and medicine led to the hypothesis that reoxygenation damage may be produced by increased free radical generation[352-355]. The possibility that myocardial ischemia followed by an attempt to

therapeutically reoxygenate, generates free radicals was, in fact, supported by the direct Electron Spin Resonance (EPR) technique[59,356-358]. An additional chemiluminescence study confirmed an increased free radical generation rate during post ischemic reoxygenation of the heart utilizing non-invasive, continuous monitoring of ultra-weak chemiluminescence at the surface of the heart[359].

A similar involvement of oxygen radicals was proposed in the pathogenesis of hepatic ischemia-reperfusion injury[360]. One related potential practical clinical application is when a liver has been preserved for transplantation and in the process subjected to a prolonged period of anoxia[361]. Reperfusion (reoxygenation) is required for the graft to function; however, paradoxically, a sequence of events may occur during reimplantation that leads to increased injury (reperfusion injury). The major support for this theory was first based on experiments that demonstrated protective effects of superoxide dismutase, catalase or other oxygen radical scavengers[362,363]. However, a sensitive biochemical methodology to measure free radical formation in the liver is not easily available. The short half-lives and broad line widths of many of the oxygen radicals make direct measurement with ESR within physiologic conditions difficult, if not impossible. The use of spin traps such as 5,5-dimethyl-1-pyrroline N-oxide (DMPO) have been used in biological systems to study oxyradical formation, but they may alter the very processes that are being measured. Other commonly used parameters such as lipid peroxidation or efflux of oxidized glutathione in the bile[316] lack sensitivity; the latter situation is limited by a dearth of biliary effect during hypoxia / anoxia. Monitoring oxygen radicals in a continuous and non-invasive manner by the chemiluminescence technique in an isolated liver perfusion model has, in fact, provided the essential data[361,364]. It has clearly demonstrated superoxide radical formation during hepatic reperfusion.

Summarizing these developments, 4 points can be made:

(1) Substantial information has been gathered regarding what reactions lead to increased light emission. Several models have been used to characterize the nature of the emissive species in *in vitro* systems. *In vivo,* antioxidant enzymes are commonly utilized to manipulate spontaneous light emission.

(2) Spontaneous light emission is useful as a non-invasive technique to monitor changes in the steady-state oxidative conditions of intact cells and organs without adding reagents that could interfere with the process[343,365-368]. Chemiluminescence seems to be one of the earliest physiologic responses to oxidative stress[367]. It precedes other parameters of oxidative stress, other parameters being based on metabolic end products of oxidative reactions (i.e. carbonyls, lipoperoxide derivatives, pentane and ethane release, capillary permeability, etc).

(3) A few factors have limited the application of the technique. (a) The low intensity of emitted light limits the use of interference filters to better characterize emission wavelength and provide information on chemical nature of specific oxidized intermediates[369]; (b) Moreover, photo-multipliers are not equally sensitive to light emitted at different wavelengths. Most sensitive photomultipliers detect light between approximately 350 and 850 nm, with maximal quantum efficiency around 24% at 400–500 nm. However, knowledge about species specificity is more interesting for scientific purposes than for diagnostic purposes. It is also interesting to observe the increasing interest to develop these methods as part of biophotonics research methodology[370].

(4) Taking the technical limitations into account, spontaneous (natural) ultraweak photon emission originating from living organisms offers significant information on physiological and functional conditions of vital systems and may be considered to reflect the state of oxidative stress *in vivo*.

Technical developments in low-level luminescence recording

The spontaneous chemiluminescence technique offers additional perspectives since such a system can also be developed for highly sensitive imaging and spatiotemporal analysis. A two-dimensional photon counting imaging of a rat's brain was technically achieved in 1999[371]. The equipment used in this first experiment consisted of a two-dimensional photon-counting tube with a photocathode measuring 40 mm in diameter, a highly efficient lens system, and an electronic device to record time series of a photoelectron train with spatial information. Utilizing the imaging system, regional time courses of emission intensity have been demonstrated, indicating the potential usefulness of spatiotemporal characterization

regarding physiological information on oxidative stress. Spontaneous photon emission was further imaged from a rat's cortex *in vivo* during cardiac arrest. The intensity after cardiac arrest was depressed to approximately 60%. This technology constitutes a novel method with the potential to extract pathophysiological information from the central nervous system[372].

Another application has been in the field of transplanted tumors, both with photomultipliers and imaging equipment. Early interest focused on the liver of tumor-bearing animals. The liver of tumor-bearing animals is subjected, during the early phase after tumor implantation, to an increased oxidative stress. The increased steady-state levels of peroxyl radicals are essentially responsible for the increased photoemission observed *in vivo*. Utilizing integrative studies on tumor-bearing animals, the *in situ* liver chemiluminescence[316] was measured simultaneously along with the activity of antioxidant enzymes and the content of endogenous antioxidants. The increased *in situ* liver chemiluminescence in the early phase after tumor implantation in tumor-bearing mice is associated with (a) decreased activity of the protective antioxidant enzymes in the liver and with (b) increased hydroperoxide initiated chemiluminescence in the homogenates and mitochondria of the liver[373]. Other data demonstrate that spontaneous light emission was not only enhanced within *in situ* liver but also within *in situ* brain in tumor bearing animals[374], an observation that was reminiscent of the decrease in catalase activity found in most tumors[375].

In the first report of a two-dimensional imaging and photon counting of ultraweak light emission from transplanted cancer, attention was focused on bladder cancer transplanted into the feet of nude mice. The photon emission of the developing cancer was followed. During the early log phase of cancer cell growth, necrosis, hemorrhage, leukocyte infiltration or crusta formation are not observed[376]. Observations in that early period suggested that increased photon emission was soon observed in the implanted tumor region indicating development of the actively proliferating cancer. In other studies, ultraweak photon intensity from different transplanted malignant tumors was recorded[377-379].

Recently, ultraweak photon detection was reported utilizing a novel technique for cancer imaging[380], a highly sensitive and ultra-low-noise charge-coupled device (CCD) camera system that records two-dimensional biophoton images from tumors transplanted in mice. In addition, a procedure for whole body scanning of mice was developed utilizing a small, mobile and sensitive photomultiplier tube (PMT) operated at room temperature in a dark box. The investigations focused on scanning, ultraweak photon emission from mice that were transplanted with ovarian cancer cells. This scanning procedure is a potentially cost effective method for detecting tumors compared to the cooled CCD system. Data confirm the increased photon emission of tumors.

This, then, begins to initiate discussion regarding practical applications addressing specific pathological issues. Unsolved is the depth from which photons are able to penetrate tissue. In some papers it is suggested that a recorded value of 10 cps/cm^2 corresponds to approximately 20 cps/cm^3.sec if the light originates through a 5 mm thick tissue. However, studies on transparency of tissue are required. Photon emission seems to transfer through rather large tissue distances as documented by studies utilizing paraquat, tumor transplants, and the recording of emissions through the skull. Furthermore, two-dimensional data show more gradient-like pictures without sharp boundaries. Data suggest chemiluminescence spreads from areas of high emission. These data might help to explain (a) the observed patterns of oxidative activity and (b) the quantitative evaluation of oxygen radical activity.

Human photon emission

In recent years, there has been increasing interest in the use of human luminescence to record ROS in the development of pathology of chronic diseases (such as Alzheimer's disease and diabetes) that are supposed to be linked to non-linear progression in ROS production. The method is also interesting for recording the status of patients with these diseases that are taking antioxidant therapy. Along this line, in particular the development of a noninvasive tool for detection of human photon emission and its validation is crucial. This section reviews the current status of human photon emission recording techniques and protocols, the knowledge regarding both uniformity and variation in the anatomical pattern of photon emission, its dynamics in internal physiology and psychophysiology, and its relation to health and disease. Future perspectives deal with applications in physiologic and patho-physiologic conditions where the technique can be rapidly and easily implemented. Such conditions can be found in (a) extreme exercise (in sports physiology), (b) shift work that leads to

alteration of the circadian rhythm e.g., jet lag or alternating work schedules (in industrial/company medical office), and (c) during development of chronic diseases (in clinical practice).

Historical aspects

Research in human photon emission with single photon counting devices started about three decades ago[381,382], utilizing a setup consisting of a photon detection system mounted in a darkroom in which subjects placed different anatomical part under the photomultiplier opening. Shortly after these reports, the first publications appeared utilizing more advanced devices to deal with large anatomic surfaces. In the Inaba Biophoton Project (funded by Research corporation of Japan (presently, Japan Science and Technology Corporation) human photon emission was investigated with two-dimensional photomultipliers in order to record the two-dimensional pattern of ultra weak photon emission (UPE) from human body surfaces. In Germany, Popp started pioneering research in human biophoton emission in 1993 by building a darkroom for the installment of a detector head that by hanging on runners, could be moved over the whole body of a subject lying on a bed underneath.

In the next 10 years, only a few systematic studies were performed and published. Cohen and Popp[383] considered long-term periodicity in a systematic study on photon emission from hands and forehead using the moveable photon detector. The authors examined both the palms of the hands and the forehead of one subject, daily, over a period of 9 months. Recordings demonstrate a clear preference for left and right hand correlation. Long-term biological rhythms of spontaneous emission of that subject became evident with Fourier analysis. Influence of age on photon emission of hands was investigated[384]. Spontaneous photon emission was increased in elderly subjects. A few studies have focused on photon emission from the hands in relation to disease. A study with 7 hemiparesis patients demonstrated that left and right differences of photon emission rates from the palm and the dorsum of the hands were large for 4 out of those 7 patients, compared to 20 healthy subjects[385].

The limited number of studies did not allow hard conclusions about the implications and significance of biophotons in relation to health and disease or mental state. Still, the presented experimental data make clear that these aspects need attention and were the reason for a large systematic study that started in 2003 and was supported by Samueli Institute for Information Biology. At present, the studies contain information on: (a) procedures for reliable measurements, and spectral analysis, (b) anatomic intensity of emission and left-right symmetries, (c) biological rhythms in emission, (d) physical characteristics of emission, (e) physical and psychological influences on emission, and (f) emission in health and disease.

Reliable multi-anatomical site recording of UPE and spectral analysis

The goal of the initial studies was to describe a protocol for management of subjects that (a) avoids interference with light-induced (long-term) delayed luminescence, and (b) includes the time slots for recording photon emission. The protocol was utilized to discriminate photon emissions from anatomical locations within a subject and to complete spectral analysis of emission from different body locations. The accuracy was sufficient: photon counts ranged between 6 and 40 counts per second (cps) wherein a difference of 1.1 cps is significant. The thorax-abdomen region demonstrates the lowest emission. The upper extremities and head region emit the highest levels [386-389]. Spectral analysis in the 200-650 nm range was possible with cut-off filters and repetitive measurements. Analysis documents major spontaneous emission at 470–570 nm. This indicates specific electron-excited states[386].

In subsequent studies, a newly developed and highly sensitive charge-coupled device (CCD) imaging system was utilized in collaboration with Kobayashi and coworkers to fundamentally characterize spatial distribution of ultra-weak photon emission. The CCD images from the upper frontal torso, head and neck and upper extremities corresponded with the data of multi-site recordings utilizing the moveable photomultiplier system[390-392]. Systematic, multi-site recording with a group of 60 healthy males of ultra weak photon emission over high- and low anatomic emission locations presented evidence for a “common” human anatomic intensity emission pattern[391,392]. The common pattern opened a possibility to measure photon emission characteristics utilizing a few representative anatomic locations. Attention was focused on the hands. The hands emit high strength photon signals. CCD images of hand UPE were statistically analysed (manuscript in preparation). The study demonstrated that UPE was equally distributed over the palm and dorsum of the hand. To learn about the anatomic origin of UPE, the

patterns of UPE are currently compared with anatomic characteristics of the corresponding locations.

(Diurnal) fluctuations

The stability of UPE at a single anatomic location was studied. Recording of 29 selected, body sites demonstrated that emission over the body is systematically lower in the morning than in the evening at all locations[386-388]. Data registered during daytime hours demonstrated anatomic locations on the right side had higher photon strength than corresponding locations on the left. The right-left asymmetry was subsequently confirmed with a larger number of male (n=20) and female (n=20) subjects by recording over a smaller number of locations (manuscript in preparation).

In a subsequent study fluctuations in UPE were studied during 24 hr by recording ventral and dorsal sites of both right and left hands every 2 hr in 5 separate experiments. Data demonstrated that intensity as well as left-right symmetry varies diurnally. Emission intensity is low during the day, rises during the evening and is high at night. Time patterns for left and right hand are different. Although the fluctuations in UPE during the course of 24 hr were more over dorsal than ventral sites, all were highly significant. Correlations of fluctuations over ventral and dorsal sides are not apparent. During the 24 hr period, a change in left-right symmetry occurred at night. Photon emission over left locations was high at night, whereas the right sides emitted primarily during the day. Specific parameters have been developed to represent laterality[393-395].

Temperature and photon emission

Human photon emission is a product of biochemical processes. Therefore, environmental temperature is expected to influence photon emission when it originates from anatomic layers that are not kept at 37°C and instead depends on environmental temperatures. Indeed, as temperature of hand declines, the intensity of UPE decreased, whereas UPE increased at increasing temperature[396].

Another study led to a similar conclusion. In the study, subjects participated in cold exposure experiments after being dark-adapted. They lay in supine position to stabilize their body temperatures in a dark room at temperature of 17°–18°C for 15 min. Subsequently they disrobed for 30 min to only T-shirts, shorts and sport shoes. Both skin temperature and photon emission were recorded from several anatomic locations. Temperature decrease during cooling roughly correlated with the photon emission of a specific location[397].

Initially, the question was whether anatomic locations with different photon emission intensities reflected different skin temperature. Multi-site recordings of both photon emission intensities and skin temperature demonstrated that temperature at different anatomic locations ranged between 26° and 34°C. However, a relationship with photon emission was not observed[397]. In a second study, both UPE and temperature were recorded at the body location in different subjects in order to evaluate whether differences between subjects correlate with temperature. Between subjects, temperature of any of the recorded anatomic sites was not correlated with the UPE[397].

It was concluded that differences between both anatomic locations and subjects could not be explained by differences in temperatures. At a given location, however, photon emission intensity is temperature-sensitive, suggesting that temperature recording at anatomic locations concomitant with photon emission is an improvement in comparative studies between different subjects and within a subject.

Hypoxia, exercise and photon emission

In three types of experiments the effect of hypoxia on photon emission was investigated. In the first study a tourniquet was placed around the upper arm to depress the supply of oxygen and nutrients to the hand. Photon emission of the hand was recorded during periods of increasing degree of tourniquet tightness. Data demonstrate that photon emission progressively decreased during blood flow limitation. After removing the tourniquet, photon emission returned to the former level within minutes. Data confirm that photon emission is oxygen dependent[386,388,398]. Direct exposure of the hand also resulted in some decreased photon emission[396]. Data suggest that generation mechanisms of photons emitted from the hand are both from interior sources and from skin.

Daily tasks and photon emission

Subjects engaged in cognitive tasks, e.g., filling out forms and addressing mathematical challenges, were also recorded for ultra weak photon emission. These tasks did not influence photon emission of hands. The results led to the selection of a new, more difficult

challenge in a second study. The more sophisticated mathematical challenges were addressed in the dark. Data demonstrated that cortisol levels were higher after the completion of the challenges, and decreased after relaxation. However, statistical significant changes in ultra weak photon emission during the challenges were not observed. It was concluded that common daily tasks had no statistically significant effect on photon strength and related parameters.

Procedure for advanced photon count analysis of human ultra weak photon emission

Spontaneous photon signals demonstrated that variance was higher than the mean suggesting that photocount distributions were not normal. Skewness was not zero implying a skewed distribution. Kurtosis was non-zero and large, thus also ruling out normal distribution of photon counts. However, this was true for both photon signals and background. To find the appropriate correction for background signals, a novel method to physically characterize UPE was utilized. The procedure was based on quantum optics[399] and has been applied in studies on non-human living systems[400-402]. The procedure describes the fluctuations in the signals by assuming the signal in a quantum squeezed state of photons. This state is specified by four real parameters (magnitude of displacement, $|\alpha|$; magnitude of squeezing, r; and phase angles θ and Φ)[399]. Utilizing this approach, a procedure for correcting background noise was developed.

A novel method was utilized to characterize human photon signals of low, intermediate and high intensities. Fluctuations in these signals are measured utilizing probabilities of detecting different numbers of photons in a bin, and establishing the optimal bin size. These measurements suggested that this set of parameters is quite useful. The subsequent study utilizing the measurements parameter compared additional anatomic locations between subjects. Photon count distribution over 12 different locations in 20 male subjects was examined. The fluctuation of each signal was characterized by the parameters $|\alpha|$, r, θ and Φ. The possibility of systematic differences of squeezed state parameters between different locations was studied. There were differences between the 12 locations for $|\alpha|$, r and Φ, but not for θ. The question then arose for each parameter whether correlations existed between locations within subjects. Anatomical locations were grouped in three regions: (a) torso region, including abdomen right and left, stomach region and heart; (b) head region, including throat, cheeks right and left and forehead and (c) hand regions, including ventral right and left and dorsum right and left. Data demonstrated for the parameters $|\alpha|$ and r significant correlations (with a trend for Φ) between hand regions and the anatomic region of torso plus head [403]. It was concluded that the novel analysis was able to discover a squeezed state structure in the fluctuating photon emission; the squeezed state parameters can be expressed for each individual subject as mean values of measurements at all anatomic sites. Correlations in signals of different locations implied that squeezed states can differ between subjects. Differences between individuals in the fluctuations of the photon emission can thus be expressed by utilizing the combination of these parameters. The relationship between the structure in the fluctuating photon emission and the underlying elementary biochemical processes is part of the basic studies[403].

Application of photon emission in health research

There is mounting evidence indicating that reactive free radical species are involved in initiation and development of many different forms of human pathologies, including psychiatric disorders. The utilization of ultraweak photon detection to evaluate the oxygen radical activity has increased the interest in a study on human photon emission in health and disease. In this section, preliminary data of two types of studies are presented:

a. Comparative studies on subjects ranging from healthy to clinically diseased.
b. Influence of long-term meditation on intensity and pattern of ultra weak photon emission.

Ultraweak photon emission in disease

Several studies suggest that the intensity of photon emission changes in a state of disease. Japanese studies of the two-dimensional pattern from the index and middle finger indicated that intensities could be used to differentiate hypothyroidism, lower state of metabolic activity[404-406]. Ultraweak photon emission in patients with hyperthyroidism was less intense than normal. The lower emission was also found in patients whose thyroid glands had been removed. Another study reported of several multiple sclerosis patients who emitted more photons than ordinary healthy subjects[383,407,408]. In this study, the authors introduced a second parameter for disease, e.g.,

percentage of difference in emission between left and right hand. They suggested that in certain diseases left-right symmetry was broken. In another study, left-right symmetry of photon emission from the palm and the dorsum of the hands of hemiparesis patients was compared with similar data from the hands of 20 self-reportedly healthy subjects. The variation in left-right symmetry among healthy subjects was not large. In hemiparesis patients though, the left and right differences were reported very large in 4 out of 7 patients both for the palm and dorsum of the hand. In the 3 other patients the differences were within normal range[409].

Quantitative data on ultraweak photon emission of ROS-related diseased state require a study design with at least three defined stages from health to disease: (a) healthy state, (b) early state of disregulation in which an impaired cell function is detectable, and (c) overt diseased state. The putative model is that accumulation of aggregates increased in subsequent stages with corresponding oxygen radical activity. Well-defined markers for most chronic diseases are available only for the stage that the disease is overt and the subject has contacted the medical circuit for specific symptoms. Relative few markers are available for earlier stages. An extra difficulty is that the many ROS-related chronic diseases are final manifestations of early stages of a common disregulation (increased ROS activity).

As a first approach we have chosen for a descriptive, explorative study, in which no treatments are provided. The aim is to describe photon emission of 150 subjects divided into three groups. The two extreme groups are the (a) healthy young subject without severe disease history, and (b) chronic disease subject that ask for CAM after long-term disease history. The intermediate group are pre-diabetic subjects. Each group will include 50 subjects. The 50 healthy subjects participated in the study according to the inclusion criteria (a) healthy (assessed by questionnaires), (b) normal Dutch eating habits, (c) age > 20 and < 30 years.

The 50 chronic diseased subjects are included according to the criteria (a) chronically diseased with a history of medical events and having a general practitioner, (b) normal Dutch eating habits, (c) age > 30 and < 65 years. The inclusion criteria for the pre-diabetic group of 50 subjects are: (a) healthy (assessed by questionnaires and physical examination), (b) normal Dutch eating habits, (c) age > 30 and < 65 years, (d) body mass index >26 and <35 kg/m^2, and (e) pre-diabetic as established by fasting glucose blood values >6.0 to < 7.0.

For this study a new mobile device was constructed, since the original scientific equipment for human research is too large and bound to a specific research sites. The new equipment is smaller and more appropriate for laboratories and clinical practices. It is built in such manner that both hands can be recorded simultaneously, if required. Data collection protocol allows the analysis for: (a) strength of photon signal, (b) squeezed state parameters, and (c) left right symmetry. The selection for hand recordings was based on data discussed above. Such data have demonstrated that ventral and dorsal surfaces commonly demonstrate high emissions compared to other anatomical locations. A higher signal results in more reliable estimations of squeezed state parameters, whereas mean values for the four locations can be considered as representative for the subject.

The preliminary data support the hypothesis that photon strength ($|\alpha|$) in chronic diseased subjects has increased compared to healthy subjects, whereas mean photon emission for the pre-disease group was intermediate. The squeezing parameter (r) is small in chronic diseased subjects compared to healthy subjects. It has an intermediate value for the pre-disease group (manuscript in preparation).

Long-term meditation and free radicals

It is generally accepted that meditation, if practiced for a long time, induces a greater state of self-awareness and inner calm in its practitioners. Techniques of meditation include attention to one's breath, repeating a mantra and detaching from various thought processes in order to focus one's attention. The resulting "inner calm" implies reduction of stress which may have prophylactic and therapeutic health benefits. The hypothesis suggesting a possible link between meditation and its therapeutic effect utilizes the information about the initiating role of free radical-mediated oxidations in disease and proposes that oxidized lipids may reflect free radical induced damage that may contribute to pathophysiology[410,411]. The hypothesis has stimulated considerable curiosity in the scientific community. The measurement of serum lipid peroxide fluctuations indicates that chronic psychosocial stress probably does increase oxidative stress[412,413]. In addition, findings suggest the presence of lower lipid peroxide levels in the plasma

of practitioners of transcendental meditation (TM)[414], Zen meditation[415] and yoga practitioners[416].

Recent studies have focused on ultraweak photon emission of long-term practitioners of meditation. The studies utilized the system that is capable of multi-site recordings[407] according to a defined protocol[386]. The comparison between 10 TM practitioners and 10 subjects without experience in meditation indicated an intensity discrimination of ultraweak photon emission in meditation practitioners compared to control subjects[417].

A follow up study examined the ultraweak photn emission from the hands of three groups of subjects: control group having no experience in meditation, TM group practicing Transcendental meditation, and a different group practicing a form of meditation other than TM (OTM). Each group consisted of 20 healthy, non-smoking subjects. Data demonstrated that the intensity of ultraweak photon emission by subjects of both meditation groups is lower by 15-33% for the TM group and 4-15% for the OTM group compared to the control group. All subjects demonstrated a high degree of symmetry[418]. Additionally, the photon signal was described according to a quantum optical approach utilizing the four parameters ($|\alpha|$, φ, r, θ) that determine the signal[419]. Both the squeezed state parameters and asymmetries suggest that the control group is different from both meditation groups. The difference between TM and control group is more than that between OTM and control group. The data support the conclusion that persistent meditation influence metabolic activities responsible for photon emission.

Conclusion and future perspectives

In the present review both the emergence and development of two separate lines of research and their convergence into one model have been discussed. The first line focused on biochemical hallmarks of ROS related chronic diseases: the biological defence tiers that, if overwhelmed, result in persistent conformational changes and the progression of ROS-related chronic diseases. The second line focused on ultraweak photon emission as an overall measure to monitor the oxidative status from enzyme level to man. The review illustrates the wealth of experimental data for both lines of research. The convergence of both lines has resulted in research on the application of ultraweak photon emission to monitor the oxidative status in human subjects both under physiologic and pathophysiologic conditions.

The wide array of recognized ROS related and conformational diseases have in common that they arise from secondary or tertiary structural changes within constituent proteins, with subsequent aggregation of those altered proteins. Examples are the systemic amyloidosis, neurodegenerative diseases and type 2 diabetes mellitus as discussed before. A human study focusing on the ultraweak photon emission in the development of these diseases can thus be performed with any of these diseases. Empirical foundations that make the application of ultraweak photon emission research in health and disease transitions possible have already been discussed and can be shortly summarized:

1. Components and origins of ultraweak photon emission. Variability in photon emission has been defined and parameters to describe the non-classical aspects of photon emission have been given. Technical requirements for recording have been established and both dynamic and steady state characteristics of the ultraweak photon emission have been studied. The significance of additional measures (e.g., temperature) has been discussed.
2. Guidelines, recommendations and caveats. Meaningful analysis of ultraweak photon emission is dependent on the integrity of the basic photon signal corrected for (technical) background. With modern methods the fluctuations in the signal is analyzed to identify the integrity of the signal, to identify abnormal peaks and for artefact editing. Artefacts from a variety of sources may contaminate the photon signal. Therefore, it is generally preferable to use a distribution-based artefact-detection algorithm. In practice, a combination of both automated and visual approaches is optimal. Detection and processing of abnormal gross fluctuations in the signal are more problematic in individuals. This happens not very often (less than 2% of the cases).
3. Deriving inferences from ultraweak photon emission. Current scientific interest in ultraweak photon emission emphasizes the potential relation of photon signal components to functional dimensions that presently cannot be measured directly in a non-invasive manner. Given this, it is essential to identify reference criteria against which these measures may be validated. The relevant functional dimension may very from discipline to discipline. In biochemistry, reference criteria have included molecular reactions involving ROS. In

medicine, reference criteria have included organ (e.g., cardiovascular) damage and risk stratification for disease. In human health and sports, ultraweak photon emission has been proposed as markers of physical and psychological stress and workload. Whatever the focus of the study is, however, putative autonomic mechanisms are generally invoked as mediators of this relationship. Consequently, false interferences about ROS mediation, based on inadequate evaluation of validity, can hamper a valuable line of research. In fact, the pattern of ROS control is often the primary interest in many biomedical studies of ultraweak photon emission. It is at this most fundamental level that ultraweak photon emission measures must be further validated.

Unfortunately, most chemical measures of ROS activity have limited applicability and are associated with methodological and interpretative problems of their own. The invasiveness of the procedures, indirectness of the reactive processes, and limited applicability to broader functional contexts (from blood to tissues) restrict the utility of these approaches. Despite the technical difficulties of the non-invasive luminescence procedure, confirmatory approaches are now available and applicable to humans. These approaches can utilize the adequate recording and data processing procedures, control or correction for temperature, and the appropriate selection of squeezed state parameters, that provide a selective index of ROS.

As is with most (psycho-) physiological measures ultraweak photon emission is a more accurate index of a change than in absolute level. Consequently, within-subject differences among experimental conditions are likely to be more accurate than of absolute level of ROS. Potential contributions of age, sex, stress, and diet parameters and other individual characteristics need to be considered carefully in interpreting between-subject differences in ultraweak photon emission. In such aggregate, the findings suggest caution in inferring absolute levels across individuals. The identification of ultraweak photon emission with the staging of ROS-related damage and. disease fosters the development of different experimental approaches. This convergence must offer perspectives for (a) basic research utilizing appropriate models that increase our understanding, and (b) applied research including possibilities and limitations of the application of human ultraweak photon emission as a tool. To illustrate this, several lines of research are proposed.

Basic research: *Caenorhabditis elegans*

The *C. elegans* model for studying loss of coordination in neurodegenrative diseases is a promising tool. Both coordination in movement and ultraweak photon emission can be monitored. In a previous paragraph the utilization of *C. elegans* in polyQ aggregation is discussed. PolyQ diversity and corresponding loss of coordination of movement (from normal movement to nearly complete paralysis) can be studied using a variety of strains. The model allows a parallel study in time (e.g., duration of lag period and rate of development) of loss of coordination and ultraweak photon emission. The resulting data allow the testing (or present crucial arguments) on non-linear development of ROS production. The model can be further used to study the influence of ROS scavenging system.

Biophoton emission in health and disease (Type 2 diabetes)

Diabetes mellitus is a complex syndrome of hyperglycaemia in association with metabolic and vascular abnormalities. Despite problems identifying the cause of these diseases, the concept that free radicals mediate pancreatic B-cell destruction and retinal vascular damage was already debated since the early 1980's. Several lines of evidence suggested that plama lipid peroxide levels are significantly higher in diabetic patients than in control subjects and that the levels in diabetic patients with vascular complications were markedly raised as compared to diabetics without angiopathy[420-423]. Interestingly, studies on ultraweak photon emission of plasma of Type 2 diabetes patients showed that emission increased with the duration of overt diabetes[424]. In the meantime hardly any additional data were collected utilizing ultraweak photon emission.

In diabetic patients, the observed risk for complications in the vascular system exceeds that expected from the classic risk factors, which are known also as metabolic syndrome Experimental and clinical findings have suggested that enhanced levels of free radicals found in Type 1 as well as in Type 2 diabetic patients could be the risk factor explaining the excess of mortality in these individuals. The disease can be considered as a good model to explore the role of oxidative stress in the development of late diabetic complications and the implications for therapy[425].

We propose a subject (patient)-oriented research in which not the fulfilments of inclusion criteria are central. All patients are considered to provide valuable extra information and patients enrol in the study by the basic criteria for diabetes 2. Patients are not too old or too young, too illiterate, or suffer from co morbidity or concurrent psychiatric disturbances. Normally, these subjects are excluded from the study if it is disease-oriented in random clinical trials (RCT). Without wanting to undermine the enormous relevance RTC has for scientific development, the major drawback is that it does not allow maximum insight in the ROS production under conditions that the disease pattern becomes more and more complicated.

The patient-centered approach means that health care providers are directed to the illness, rather than to the disease, and have to explore and value – predominantly by questionnaires – the patients' relevant history (age, duration of diabetes, development of complications), biopsychosocial context in which biological (exercise, food, etc), psychological (stress coping, etc.) and social elements as important as the strictly biomedical (blood glucose, fructosamine, HbA1c, lipid peroxide, etc) elements.

Exercise in sport and revalidation medicine

There are limited data in literature concerning oxidative stress in hypokinesia and hyperkinesia. Extreme hypokinesia occurs with spaceflight, chronic bed rest, and immobilization. Extreme hyperkinesia occurs with extreme, long-duration, exercises.

Non-damaging habitual exercise using resistive or endurance regimens provides some protection against age-related contractile function and risk of muscle injury [426-428].

A potential mechanism that would trigger increased protein degradation and atrophy in skeletal muscle is oxidative stress, where antioxidant proteins and scavenger protection are overwhelmed by oxidant production. The problem is particular interesting in relation with heart failure and corresponding anoxia.

Growing evidence indicates that impaired stress protein (e.g., antioxidant enzymes, heat shock proteins and other chaperones, IGF-1) may play a role in regulating muscle dysfunction that occurs in heart disease and chronic heart failure (CHF)[429-431]. Exercise training improves work capacity, tolerance to fatigue, reduces risk of myocardial infarction in cardiac patients, and reduces the risk of heart disease in healthy adults[432-434]. In contrast with healthy peers, heart disease patients respond to endurance exercise training with primarily peripheral adaptations, rather than changes in central (i.e., cardiac) function. In healthy adults, exercise training increases protective stress proteins in skeletal muscle including antioxidant enzymes and chaperones[435,436]. Other data indicate that exercise results in a partial reversal of the reduction in antioxidant activity in heart failure patients[430]. Therefore, an interesting focus of application of non-invasive ultraweak recording to test free radical status is research in hypo- and hyperkinesias in healthy and heart patients, in particular a study of the combined effects of coronary ischemia and subsequent exercise training on free radical levels. It is evident that such measurements will be conducted in combination with the common techniques (e.g., heart beat rate measurements, oxygen consumption, body temperature).

In summary, patterns of ultraweak photon emission hold considerable promise as measure for the oxidative status. Further developments in measurement and analysis and advances in concepts and metrics of ROS-related diseases would foster further biomedical applications of ultraweak photon emission. A multifactorial interdisciplinary approach, at both biomedical and psychosocial levels, would undoubtedly contribute to this development of the field.

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