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PREFACE

This volume is a testament to the vitality and diversity of clinical chemistry as a scientific discipline. With an international editorial board and a distinguished complement of contributors, this series continues its efforts to serve the scientists and practitioners of this field. This is the first volume of the new millennium, and in this and subsequent ones, we will attempt to continue this series' tradition of publishing quality and relevant scientific subjects.

In order to reinforce the global nature of science in general and clinical chemistry in particular, two prominent Associate Regional Editors have accepted our invitation to join the Board. Their talents and perspectives will provide critical expertise in our efforts to provide the highest quality of publication possible.

There are six chapters in this volume, which cover the biochemistry of aging, kidney function, receptors in prostate cancer, oxidative changes in protein structures, markers of bone biochemistry, and markers of ovarian function. *Advances in Clinical Chemistry* will continue to incorporate the growing body of knowledge resulting from the ever-increasing identification of the molecular basis of human disease. The uniqueness of this field is the depth and diversity required for its practice. With the help and input of our readership, the editors will strive to address their expanding interests and the explosively growing science that is clinical chemistry.

Once again, the Board of Editors would like to acknowledge the talents and cooperation of the publisher, Academic Press. It is a privilege to work with such a professional and proficient group of people. My personal appreciation is expressed to my wife, Joanne, who is my unflagging cheerleader and unflinching supporter.

Finally, I would like to dedicate this volume to the hard work and intellectual curiosity of clinical laboratory scientists. Their dedication and discovery makes *Advances in Clinical Chemistry* possible.

HERBERT E. SPIEGEL

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THE BIOCHEMISTRY OF AGING

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1. Introduction

Aging is a fascinating topic that has interested philosophers and scientists for centuries. Indeed, interest in the aging process has markedly accelerated, particularly over the past two decades, primarily due to the realization that not only do the

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elderly form an ever-increasing percentage of the population, but they also utilize a disproportionate percent of health care costs.

Although the term "aging" is generally understood in broad terms, the aging process is extremely complex and multifaceted. As such, no single definition is universally accepted. Over 100 years ago (1895) Bichat was quoted as defining life as "the sum of the influences that resist death." (E1) More recently, Harman (H5) defined aging as "the progressive accumulation of changes with time associated with or responsible for the ever increasing susceptibility to disease and death which accompanies age." Thus, although aging and disease are different processes, aging predisposes one to various diseases. Strehler and North (S32) emphasized that regardless of how aging is defined, it is (a) deleterious, (b) progressive, (c) intrinsic, and (d) universal. As such, it is widely recognized that the effects of aging include all of the following: (a) a progressive decrease in vigor and efficiency of most, if not all, physiologic functions; (b) atrophy of most organs; (c) increased vulnerability to infections, trauma, and various immune abnormalities (autoimmune disorders, lymphoproliferative disorders, amyloidosis, etc.); (d) increased susceptibility to malignancy; and (e) a decreased capacity to extract oxygen from air and transmit it to the circulation (V_2O_{max}).

2. Life Expectancy versus Maximum Life Span

Do humans die of "old age"? Certainly, the bioscientific medical model assumes that death always results from a single disease/event (e.g., acute myocardial infarction) or a combination of diseases (e.g., stroke followed by pneumonia). Although this model is generally believed to be true, on occasion it is difficult to defend since several diseases may be identified at autopsy but none appear severe enough to cause death, at least in a younger person, either singly or in combination. Perhaps aging predisposes one to the extent that a combination of mild diseases can result in death.

Fries (F15) proposed that after traumatic deaths are excluded, the elimination of coronary heart disease, stroke, cancer, diabetes, and various other chronic diseases would result in a maximum average life expectancy of 85 years. As these diseases become uncommon, he proposed that (a) the number of very old would not increase; (b) the average period of decreased physical activity and vigor would decrease; (c) chronic diseases would involve a smaller percentage of life span; and (d) medical care needs in later life would decrease. Others, however, have argued against these predictions (S10). Nevertheless, from 1900 to 1990 there has been a definite rectangularization of the mortality curve (F15) in the United States, with a resultant compression of mortality (N6, N7). Thus, a person would ideally be healthy and both physically and mentally active until a very short time before a final illness strikes and death rapidly follows (Fig. 1).

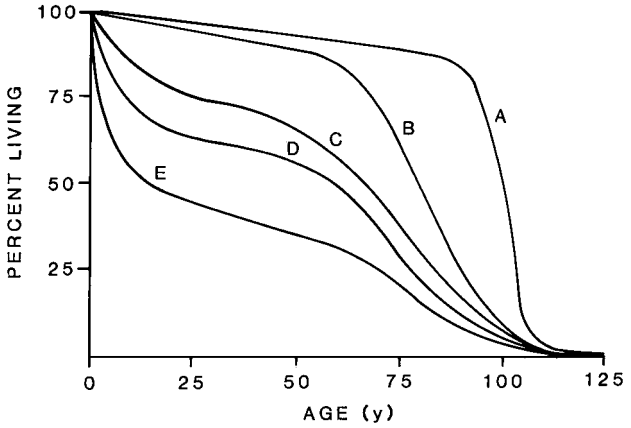

FIG. 1. Comparison of the percentage of people living at various ages and periods of time with the "ideal" maximum life span: A, "ideal"; B, 1980; C, 1920; D, 1900; E, 2000 BC (After F15.).

It is estimated that about 2000 years ago, the average life expectancy (birth to death) of a Roman citizen was 22 years (W6). From then to 1900 it increased to 47 years in the United States and over the subsequent nine decades (1992) increased to 75.8 years (G16) (Fig. 1). This remarkable increase in life expectancy since 1900 is due primarily to the prominent decline in neonatal, infant, and maternal mortality rates, along with the control of various infectious diseases. More recently, there has been a significant, albeit much less, reduction in early deaths due to coronary heart disease and stroke (i.e., due to atherosclerosis), as well as to improved management and treatment of diabetes mellitus, cancer, and various other chronic disorders. Nevertheless, the maximum theoretical life span has possibly increased slightly over the past many centuries. The oldest-ever documented person in the world, Jeanne Calment of France, died on August 4, 1997, at the age of 122 years, 5 months, and 14 days (W10). It has recently been suggested that the maximum life span could be extended to 130 years or more (M6).

3. Theories of Aging

Although the specific biologic basis of aging remains obscure, there is general agreement that its elucidation will be at the molecular level. Furthermore, it should be consistent, not only with the life span differences between species, but also with the fact that noncycling cells (e.g., neurons and myocytes) undergo a relatively uniform functional decline with age.

TABLE 1
THEORIES OF AGING

A. Stochastic (random event; "wear and tear")
1. Somatic mutation
2. Error catastrophe
3. Protein glycosylation
B. Developmental
1. Immune
2. Neuroendocrine
C. Genome-based
1. Intrinsic mutagenesis
2. Programmed
D. Free radical

There have been numerous theories to explain the aging process. However, current thought generally proposes that senescence results from various extrinsic events that lead progressively to cell damage and death and/or characteristric intrinsic events related to the genome-based theory. These general theories have been presented in a variety of ways. Table 1 outlines the classification scheme referred to in the current discussion. It should be emphasized, however, that no single theory is entirely satisfactory. Rather, it is suggested here that aging is due to a combination of several theories, the programmed and free radical presumably being the most important, while others may play a definite, albeit less critical, role. Moreover, some theories are probably based on phenomena that are of secondary importance.

3.1. STOCHASTIC THEORIES

3.1.1. *Somatic Mutation*

The somatic mutation theory is based, in part, on the idea that background radiation and/or various endogenous mutagens produce random chromosome damage in all cells. Over time, the genetic loci become sufficiently altered such that various critical functions fail and the cell dies. The fact that irradiation of laboratory animals results in accelerated aging and premature death lends some support to this hypothesis. However, since irradiation produces free radicals, it could be considered part of that theory.

Deoxyribonucleic acid (DNA) has limited chemical stability. As a result, oxidative damage, hydrolysis, and nonenzymatic DNA methylation occur *in vivo* at significant rates (L6). Thus, another aspect of this theory is that of intrinsic somatic mutations and the ability of cells to repair the damage to both mitochondrial and nuclear DNA. Indeed, mammalian cells have an elaborate system of DNA repair enzymes which become less efficient with time. Thus, failure to repair damaged DNA or to "misrepair" it could lead to gene inactivation or possible excision of

specific genes. Since there is some correlation between the efficiency of DNA repair and life span (H7), failure to completely repair the damaged DNA is of considerable importance for certain types of damage that could further accelerate the aging process (S31).

3.1.2. *Error Catastrophe*

The error catastrophe hypothesis suggests that through random errors in translation or transcription, erroneous copies of proteins associated with chromosomes lead to genetic abnormalities (O2). This, in turn, may result in persistently abnormal protein synthesis, and an eventual "error catastrophe" destroys the cell. As a result, the ability of a cell to produce its normal complement of functional proteins depends not only on the correct genetic specification of the various amino acid sequences, but also on the competence and fidelity of the protein-synthesizing apparatus (i.e., the information must be translated correctly).

Although it is true that abnormal proteins increase with age, most of them are a result of posttranslational changes. An example is the various isoforms of creatine kinase (CK). Here, the major isoenzyme, CK-MM (isoform CK-3_3), is normally synthesized in the heart and skeletal muscle. However, after its release into the circulation, carboxypeptidase hydrolyzes the terminal lysine from one of the M-peptides to form CK-3_2. Subsequent hydrolysis of the terminal lysine from the second M-peptide produces the third isoform, CK-3_1 (W8). Numerous similar posttranslational proteins are produced. Hence, the presence of "abnormal" proteins per se does not support this aging theory.

3.1.3. *Protein Glycosylation*

The protein glycosylation theory, although perhaps related to the error catastrophe theory, is somehat unique and therefore considered separately. It is based on the fact that glucose reacts nonenzymatically with numerous proteins including hemoglobin, enzymes, collagen, elastin, and many others to form advanced glycosylated end products. Glucose reacts similarly with nucleic acids. Although the exact chemical structures of the most advanced glycosylation end products are not known, the cross-link shown in Fig. 2 has been identified as 2-furanyl-4-(5)-(2)-furanyl)-1*H*-imidazole (C4). These authors proposed that this nonenzymatic reaction triggers a series of irreversible chemical reactions that result in the formation and accumulation of various adjacent cross-linked proteins.

These abnormal proteins are seen to increase progressively and thereby contribute to a wide variety of problems including stiffening of organs and tissues and the loss of elasticity which is so characteristic of aging. It has been further hypothesized that glycosylated proteins may decrease the elasticity and permeability of the extracellular compartment and thereby impair the passage of nutrients and waste products in and out of cells. In addition, both DNA and ribonucleic acid (RNA) glycosylation might result in serious cell impairment. It is further suggested that, in

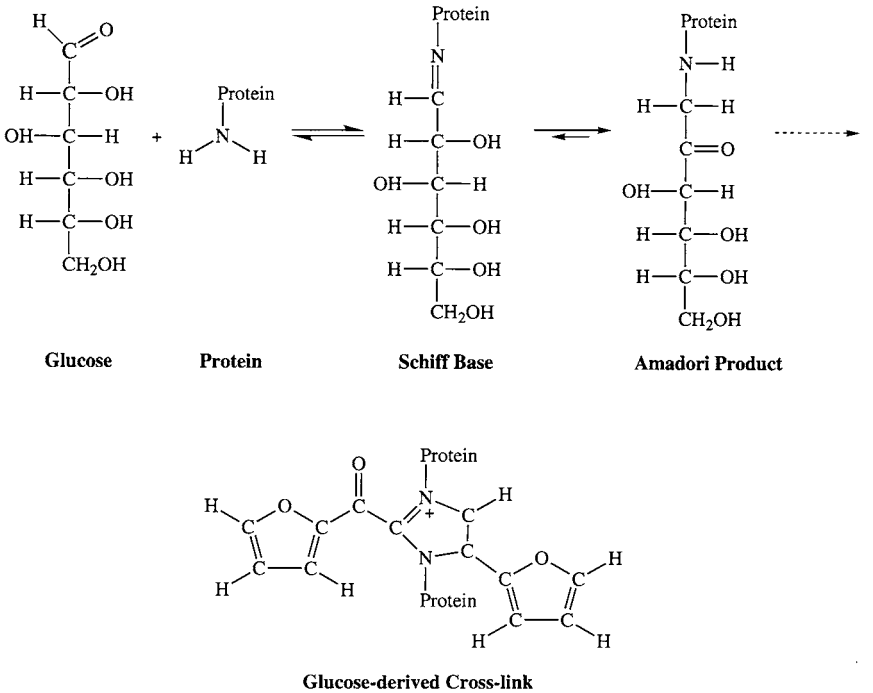

FIG. 2. The reaction of glucose with a protein amino group to form a glycoslylated protein (Amadori product) and subsequent glucose-derived cross-link (2-furanyl-4[5]-[2-furanyl]-1H-imidazole).

addition to glucose, other intracellular substances, including aldehydes, free radicals, quinones, and polybasic acids, could participate in protein cross-linkages.

Although there is little question that glycosylated proteins increase with age, there is no direct evidence that they affect the life span. Nevertheless, this important area needs further study in order to more fully understand its effects on aging and disease.

3.2. DEVELOPMENTAL THEORIES

3.2.1. *Immunity and Aging*

A decline in immunologic function with increasing age has long been recognized. Thus, older individuals are more prone to various infectious diseases, autoimmune phenomena, amyloidosis, myelomatosis, chronic lymphoproliferative disorders, and various forms of cancer. This decreased responsiveness of the immune system is primarily related to thymus-derived (T) lymphocytes (B17). However, B-lymphocytes are also affected since there is frequently a decreased

capacity to produce antibodies with advancing age (W1, W2). More specifically, the altered responsiveness of the immune system with age includes the following:

(a) Decreased interleukin-2 (IL-2) production
(b) Decreased T-cell response to IL-2
(c) Increased IL-6 production
(d) Decreased response to mitogen stimulation
(e) Increased frequency of autoantibody production
(f) Decreased antibody response to antigenic stimulation

Moreover, since B-cell function is dependent on T-cells, it is uncertain whether these are primary or secondary effects, or both. Thus, supplementation with various vitamin and mineral antioxidants have shown the following (C6, D13, E3, M12, P12):

(a) Increased absolute number of T-cells
(b) Increased various T-cell subsets
(c) Increased T4/T8 cell ratio (helper cell/cytotoxic cell)
(d) Increased number and activity of natural killer cells
(d) Increased mitogen response to phytohemagglutinins and concanavalin A
(e) Improved delayed-type hypersensitivity skin test response
(f) Increased IL-2 levels
(g) Decreased prostaglandin (PGE2) production
(h) Increased antibody response
(i) Decreased lipid peroxidation

It is also of importance to note that the major histocompatability complex is genetically linked with the antioxidant enzyme, superoxide dismutase (W9). Thus, it appears that there exists a relationship between the free radical and immune aging theories. As noted below, immunity is also influenced by the endocrine system.

The concentration of various interleukins (cytokines), as noted above, changes with age. These small protein messengers represent mediators and regulators of the innate immune system and play a role in lymphocyte growth and differentiation. In this regard, they may act as both pro-inflammatory and anti-inflammatory agents. Interleukin-6 (IL-6), a 25-kDa protein produced by a wide variety of cells, is a multifactorial inflammatory cytokine. Along with IL-1, it is an important mediator of acute-phase inflammatory responses, including B-cell proliferation and maturation, inflammatory cell activation, and the hepatic production of acute-phase proteins (E4). In the inflammatory response, as occurs with a wound or infection, leukocytes and their mediators concentrate at the involved site. Here, IL-6 and other cytokines (tumor necrosis factor-α, etc.) function as cell attractants; they bind to leukocytes and stimulate the expression of adhesion molecules. These latter agents allow leukocytes to bind to the endothelial cells and begin the healing process.

Serum IL-6 levels are usually very low or not measurable prior to middle age. However, subsequent IL-6 dysregulation results in increased production such that it is readily measurable in older persons even in the absence of inflammation. It has been suggested that dysregulation of IL-6 gene expression may be related to increased autoantibody production and the presence of benign paraproteinemia, both of which are commonly present in the elderly (R1). Moreover, increased IL-6 levels may be responsible for the age-associated development of malignant B-cell tumors. In addition, Ershler (E4) noted that increased IL-6 has been associated with alteration of amyloid precursor protein, as is present in Alzheimer's disease, and stimulation of postmenopausal bone resorption. IL-6 has also been implicated in multiple myeloma, lymphoma, rheumatoid arthritis, Castleman's disease, and cardiac myxoma (E4).

More recent studies also suggest that increased IL-6 production is associated with age-related depression (D6) and disability due to muscle atrophy and/or various specific diseases (F8). Moreover, Straub *et al.* (S30) suggest that increased IL-6 levels may be secondary to diminished levels of the steroid hormone, dehydroepiandrosterone (see below under neuroendocrine aging theory). The pathophysiologic role of IL-6 in human disease has been recently reviewed (P7).

3.2.2. *Neuroendocrine Theory*

The neuroendocrine theory, first proposed in 1928 (S31), emphasizes a role for the hypothalamic-pituitary system and the endocrine target organs in the aging process. Although age-related neuronal loss occurs in various areas of the brain (i.e., locus ceruleus, caudate nucleus, putamen, substantia nigra, hippocampus, and cerebral cortex), no similar loss of cells has been reported in the hypothalamus or pituitary. Moreover, a wide variety of neuroendocrine disorders (i.e., diabetes mellitus, hypo- and hyperthyroidism, muscle and organ atrophy due to decreased growth hormone production, gonadal dysfunction and hypertension) increase with advancing age. However, several of these are most likely due to secondary factors.

Interestingly, the endocrine system has been linked to aging through dehydroepiandrosterone (DHEA), an adrenal steroid hormone which has been referred to as a "fountain of youth" (S14). Although none of the described benefits has as yet been demonstrated in a large randomized placebo-controlled clinical trial, supplemented DHEA sulfate to mice reportedly decreases the incidence of cancer and atherosclerosis and increases longevity. It also improves the immune system. Thus, using a mouse model, Daynes and Araneo (D2) demonstrated that aging mice remain immunologically normal if they are regularly supplemented with DHEA-sulfate. Moreover, others (D5) reported that a one-time supplemental dose of DHEA-sulfate with influenza vaccination enhanced the immune response of aging humans.

Decreased DHEA production has also been linked to increased IL-6 levels in mice. Here, the administration of DHEA sulfate to old mice resulted in decreased

IL-6 levels (D3). In addition, Straub *et al.* (S30) reported that increased IL-6 levels were inversely related to serum DHEA levels in elderly humans. They also showed that DHEA and androstenedione inhibit IL-6 production by peripheral blood mononuclear cells in both males and females.

An alternative neuroendocrine postulate suggests that aging is a pathologic disorder due to decreased age-associated pineal gland synthesis of melatonin (*N*-acetyl-5-methoxytryptamine) from serotonin, leading to a syndrome manifested by the "diseases of the aged." That is, "aging is a syndrome due to relative melatonin deficiency resulting from the gradual failure of the pineal gland" (R8a). In this regard, melatonin levels are normally up to 10 times higher at night than during the day in young adults. Just as light is an exogenous timing signal, melatonin is an endogenous one. As such, light and melatonin are complementary, with melatonin providing a nighttime signal to the body's "master clock" while light is the daytime signal. Interestingly, Reiter (R3) reported that melatonin is a potent antioxidant, being particularly effective in neutralizing the hydroxyl radical (HO•), the most toxic and damaging naturally occurring free radical. Hence, if melatonin is of primary importance in the aging process, some of its positive effects may be due to its antioxidant actions.

Various other changes occur in the neuroendocrine system as one ages. Whether these alterations are primarily related to the aging process per se, or whether they are secondary to other events, is largely unknown and must await future studies.

3.3. Programmed Theory (Genome-Based)

There is a significant body of evidence suggesting that the aging process is, to a significant extent, under genetic control. That is, although everyone is programmed individually, an internal "clock" starts at conception and runs a specific period of time. Accordingly, genes carry specific instructions that control not only growth and maturation, but also decline and death. This theory has considerable support, including that obtained from both observational and laboratory studies.

3.3.1. *Observational Studies*

It is widely believed that there is a significant familial correlation with life expectancy. That is, we inherit many characteristics, including longevity, from our parents and grandparents. Indeed, several genealogical studies have shown some relationship here, but perhaps less than many have believed. For example, Abbott *et al.* (1974) published data from 9000 offspring of an original group who lived into their 90s. Here, the children of parents who lived to 80 years lived only 6 years longer than those whose parents both died before reaching 60 years. In addition, Kallman (K4) reported on 1700 pairs of twins, of which about one-third were identical. Regardless of whether they lived in different or similar environments, on average there was a difference of 3 years between the time of death of the identical

twin pairs. However, the same-sex fraternal twins died within 6.2 years of each other; the different-sex fraternal twins died within 8.8 years of each other.

3.3.2. *Accelerated Aging Syndromes*

A graphic overview of aging is seen in both early-onset (Hutchinson-Guillford syndrome) and adult-onset (Werner's syndrome) progeria, as well as in Down's syndrome. Other, less common forms of progeria have also been described (Cocayne syndrome, Wiedemann-Rautenstrauch syndrome, etc.). These disorders are all characterized by accelerated aging and a significantly reduced life span.

3.3.2.1. *Early-Onset Progeria.* Early-onset progeria (Hutchinson-Guillford syndrome) is a very rare disorder of unknown pathogenesis. The birth incidence is about 1 in 8 million; familial cases have not been reported. Males and females are equally affected, and there are no known racial differences. The lives of these individuals are quite characteristic and uniform in that they appear normal at birth but within a couple of years somatic growth slows markedly and clinically apparent aging features appear: baldness, loss of eyebrows, skin atrophy, and wrinkling. Before 10 years of age they usually begin to look old and frail. Although the average life expectancy is only about 13 years, they can live until 20 years or more. Death is most commonly secondary to atherosclerosis (i.e., heart attacks, congestive heart failure); diabetes, cancer, senility, and arthritis are all relatively uncommon. Most studies have indicated that the endocrine system remains functionally normal. More recently, however, Abdenur *et al.* (A3) presented data from five progeria patients suggesting that increased growth hormone levels are characteristic of this disorder. Moreover, they had an elevated basal metabolic rate, which was suggested as a cause for their failure to thrive.

There is some evidence that early-onset progeria is due to a dominant mutant gene. However, Oshima *et al.* (O4) reported an absence of mutations within the coding region of the Werner's syndrome gene. In addition, Wang *et al.* (W3) demonstrated, in the first case of progeria reported in China, ultraviolet light (UV)-induced unscheduled DNA synthesis in four progeria cell strains was only 33–50% of the normal level. In addition, the fraction of progeria cells surviving after UV irradiation was significantly lower than in normal cells. They suggested that the presence of a "built-in" defect in progeria cells is responsible for the reduced DNA repair capacity. Others (C14) studied two strains of fibroblasts derived from skin biopsies. The two cell populations initially multiplied normally but soon rapidly senesced and stopped proliferating after 14–15 population doubling levels (normal cells divide, on average, 50 times).

After reviewing the literature regarding a possible role for oxidative stress in early-onset progeria, Poot (P16) concluded the following: (a) a minor depletion in cellular reduced glutathione by exposure to a model lipophilic peroxide results in a significant decrease in DNA and protein synthesis, suggesting that the gluathione redox cycle is intrinsically fallible in protecting the DNA replication system;

(b) aging in culture cells results in partial uncoupling of the NADPH-producing and NADPH-consuming systems; and (c) the addition of antioxidants to culture media does not significantly extend the lifespan of cultured diploid somatic cells. Thus, the level of antioxidants appears to be a modulator rather than a primary determinant of cellular aging in culture. Although several lines of evidence suggest that DNA damages accumulate during aging, as yet no oxidant-related DNA damage has been specifically identified in progeria cultured cells.

3.3.2.2. *Werner's Syndrome.* Werner's syndrome (WS; late-onset progeria) is a rare autosomal recessive premature aging disorder which is usually apparent before 20 years. Goto and associates (G12) identified the gene linkage in this syndrome to five markers on chromosome 8. The incidence of WS is between 1 and 10 in a million. WS is clinically diagnosed in those individuals with at least three of the following criteria: (a) characteristic habitus and stature; (b) premature senescence; (c) scleroderma-like skin manifestations; and (d) endocrine abnormalities, especially diabetes mellitus. WS is also characterized by early graying and loss of hair, skin atrophy, and an "aged appearance." These individuals also prematurely develop the major age-associated diseases (i.e., atherosclerosis, malignancies, diabetes mellitus, osteoporosis, and cataracts). However, as with Hutchinson-Gilford syndrome, there is a striking tendency for premature atherosclerosis and acute myocardial infarction. Death occurs at a median age of about 47 years.

Cultured fibroblasts from WS patients demonstrate a severely limited capacity to divide *in vitro* (i.e., <50). This limited *in vitro* division potential is similar to, albeit more extreme than, that displayed by fibroblasts from normal elderly people. Fukuchi *et al.* (F16) reported two lines of evidence suggesting that somatic mutations may be important in the pathogenesis of WS and of associated age-associated diseases: (a) somatic cells from WS patients have a propensity to develop chromosome translocations, inversions, and deletions; and (b) simian virus 40-transformed fibroblasts from unrelated WS patients display an elevated spontaneous mutation rate at the X chromosome-linked hypoxanthine phosphoribosyltransferase (HPRT) locus. They also reported the biochemical and molecular characterization of spontaneous mutations at the X chromosome-linked HPRT locus in 6-thioguanine-resistant WS and control cells. Analysis of 89 independent spontaneous HPRT mutations in WS and control mutants lacking HPRT activity revealed a high proportion of HPRT deletions in WS as compared with control cells (75% versus 39%, respectively). The authors suggested that the high somatic mutation rate, primarily deletions, may be pathogenetically important in WS and in several associated age-dependent human diseases.

Increased oxidative stress is apparently not a factor in WS since cultured cells from WS patients revealed normal levels of the antioxidative enzymes Cu-Zn and Mn superoxide dismutases, catalase, and glutathione peroxidase (M7). In addition, WS patients had normal lipid peroxidation levels.

3.3.2.3. *Down's syndrome.* Down's syndrome (DS), compared with the other accelerated aging syndromes, is relatively common; it occurs approximately once in every 700 live births; however, if the mother's age is 40 years or more, it occurs in about 1 in 25 live births. The syndrome is usually characterized by various dermatoglyphic features (epicanthal folds, broad flat nose, fissured tongue, palmer "simian crease," etc.), a distinctive sloping face with slanting eyes, short stature, short thick hands and feet, some degree of mental retardation (most DS subjects have an IQ less than 50), and a distinctly shortened life span (average about 48 years). In contrast to those with Werner's syndrome and early-onset progeria, these individuals often undergo marked mental deterioration with aging; postmortem studies reveal brain changes similar to those commonly found in the elderly, including those with Alzheimer's disease (i.e., increased neurofibrillary tangles, senile plaques, and vascular amyloid deposition). Individuals with DS also have an increased incidence of cancer, including leukemia, atherosclerosis, cataracts, autoimmunity, and age pigmentation.

Down's syndrome is the first clinical condition shown to be associated with a chromosomal abnormality; about 85% of these individuals have 47 chromosomes (three chromosomes 21) while in the remaining 15% the extra chromosome is borne by another chromosome (translocation), usally group D. As with the other accelerated aging syndromes, the factors responsible for accelerated aging in DS are not well understood. However, of probable importance here is that the gene encoding cytoplasmic superoxide dismutase (Cu/Zn-SOD; SOD-1) is on chromosome 21, resulting in three copies of this gene. Moreover, a literature review indicated an approximate 50% increase in SOD-1 activity in erythrocytes, blood platelets, leukocytes, and fibroblasts in accordance with a simple gene–dosage effect (K9). However, SOD-2 (mitochondrial SOD; Mn-SOD) activity was reportedly decreased by about one third in DS patients. The majority of studies indicate an increase in glutathione peroxidase (GPx) activity, while catalase activity is invariably normal. Thus, the major intracellular antioxidant enzymes are increased or normal in DS patients.

In spite of the increased activities of SOD-1 and GPx and normal catalase activity, increased lipid peroxides in the blood plasma of DS patients have been reported (K10), as has as an increased accumulation rate of "age pigments" (i.e., lipofuscin and ceroid, known products of lipid peroxidation) (K9). In addition, an early study showed increased lipid peroxides in the cerebral cortex of DS fetal brains (B15). More recently, cortical neurons from fetal DS and age-matched normal brains were shown to differentiate normally early in cell cultures. However, DS neurons subsequently degenerated and underwent apoptosis, whereas the normal cells remained viable (B18). In addition, the DS neurons exhibited a three- to fourfold increase in reactive oxygen species and increased lipid peroxidation that preceded cell death. Importantly, DS neuron degeneration could be prevented by treatment with the free radical spin trap *N*-*tert*-butyl-2-sulphophenylnitrone, the

free radical scavengers vitamin E, butylated hydroxyanisole and *N*-propyl gallate, and the glutathione precursor/radical scavenger *N*-acetyl-L-cysteine. In addition, catalase inhibited the degeneration of DS neurons, whereas SOD had no significant effect.

Odetti and others (O1) noted that early brain cortical dysgenesis and late neuronal degeneration are probably caused by amyloid beta-peptide overproduction followed by increased cellular oxidation. These workers studied DS fetal brain cortex and evaluated the presence and quantity of lipid and protein oxidation products (thiobarbituric acid reactive substances and 4-hydroxynonenal; protein carbonyl-2,4-dinitrophenylhydrazine reactive products, respectively). They also quantified two forms of glycation end products (pyrroline and pentosidine) known to be involved in cellular oxidation. These authors found that all of these parameters were significantly increased in DS fetal brains compared to controls.

Direct evidence for increased oxidative stress in DS was recently reported by Jovanovic *et al.* (J6). These workers analyzed urine samples from 166 individuals; samples from those with DS were matched with their siblings. Here, the levels of 8-hydroxy-2′-deoxyguanosine, an established biomarker of hydroxy radical reaction with DNA, and malondialdehyde, a lipid peroxidation product, were both significantly elevated compared with the controls. In this regard, recent evidence indicates that mitochondrial DNA (mtDNA) is about 10 times more susceptable to mutation than nuclear DNA. Druzhyna *et al.* (D12) hypothesized that defective repair of oxidative mtDNA damage would lead to defective electron transport and concomitant enhanced production of reactive oxygen species. To demonstrate this, these researchers treated fibroblasts from several DS patients with menadione, a reactive oxygen generator. Oxidative damage was then evaluated at 0, 2, and 6 h after exposure using a Southern blot technique and a specific mtDNA probe. Their results showed that DS cells are indeed impaired in their ability to repair mtDNA oxidative damage compared with age-matched control cells.

Although these findings are somewhat confusing in that the increased antioxidant enzyme levels might be expected to be protective, various theories have been proposed to explain the increased oxidative stress in DS. As suggested by Jovanovic *et al.* (J6), increased oxidative stress resulting from excess SOD-1 activity is due to the increased SOD-1 to catalase ratio plus GPx alteration such that SOD-1 generates more H_2O_2 than catalase and GPx can inactivate. This then results in oxidative imbalance. On the other hand, although they noted that increased H_2O_2 levels were reported by others, Teixeira *et al.* (T4) reported decreased H_2O_2 steady-state levels in transfected V79 Chinese hamster cells in which SOD-1 activities were 2.2- to 3.5-fold higher than in the parental cells. Thus, their data provided *in vivo* support for the hypothesis that superoxide dismutation prevents the formation of increased H_2O_2 levels by other reactions. Thus, it is apparent that considerably more work is needed to more clearly explain the phenomena of increased antioxidant enzyme activity and increased oxidative stress in DS.

In addition to the various complexities discussed above, DS is characterized by several other abnormalities which also frequently accomany normal aging. For example, DS is characterized by the following immune system deficiencies (N2):

(a) Decreased total number of T-lymphocytes
(b) Decreased in $CD4^+$ cells ("helper" cells)
(c) Increased $CD8^+$ cells in adult DS with resultant decreased $CD4^+/CD8^+$ ratio
(d) Accelerated increase in $CD57^+$ cells
(e) Decreased natural killer cell activity
(f) Altered immunoglobulin subclasses in older DS patients (increased levels of lgG1 and lgG3; decreased levels of lgG2 and lgG4)

Whether these changes are primary or secondary to other factors such as oxidative stress, poor nutrition (i.e., protein and/or micronutrient malnutrition) is currently unknown. Importantly, similar deficiencies have been significantly improved by antioxidant supplementation in the elderly (K13). In this regard, zinc deficiency is also common in the elderly and has been reported in DS patients. This topic is more fully discussed later.

3.3.3. *Finite Doubling Potential*

Thirty five years ago, Hayflick (H7a) demonstrated that serially cultured human diploid cells have a finite *in vitro* life span. That is, human cells dividing *in vitro* have a limited number of potential cell doublings (50 ± 10), presumably because of a built-in genetic senescence program. A rough correlation between the number of fibroblast doublings and life span has been noted in several animal species. Moreover, cells from individuals with progeria and Werner's syndrome have fewer doublings than cells from unaffected people. On the other hand, there is a relatively poor correlation between donor age and doubling potential (M9).

Following exposure to certain viruses and chemical agents, transformed cells become malignant and continue to replicate; they become "immortal" with respect to their replicative potential (L1). In this regard, the enzyme telomerase is expressed in some malignant cells but not in normal cells (C18). Each time a cell divides, the telomere (chromeric tip of the chromosome arm) chain shortens. Once critically shortened telomere length is reached, the cell stops dividing and presumably senesces. In some cancer cells, telomerase stimulates continued cell replication by maintaining the telomere length.

As recently reviewed by Fossel (F10), cell senescence can be reversed by transfection with a gene for the catalytic component of telomerase. He suggested that "telomeres are the clock of replicative aging." They not only shorten with cell aging, but relengthening the telomere appears to reset gene expression, cell morphology, and the replicative life span. Nevertheless, the author noted that there is, as yet, no evidence "that telomerase expression per se is resposible for malignant transformation."

3.4. Free Radical Theory

3.4.1. *Free Radicals*

According to the free radical theory, the production of highly reactive oxygen free radicals cause progressive, random damage to DNA, RNA, enzymes, and other proteins, as well as unsaturated fatty acids and phospholipids, which eventually leads to cell death (H4).

A free radical is an atom, molecule, or compound that contains one or more unpaired electrons. Common radicals include the hydrogen atom and most transition metal ions, as well as oxygen, which is a biradical since its outer two electrons have parallel spins and, therefore, are unpaired. The first reported free radical reaction was presumably by Fenton (F7), although free radicals were not known to exist at the time. The classic mechanism (Fenton reaction) with ferrous iron (as well as various other transition metal ions) predicts that hydrogen peroxide is reduced at the iron center with generation of the hydroxyl free radical (HO•).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + OH\bullet$$

Free radicals may also be formed by (a) homolysis of covalent bonds, (b) addition of an electron to a neutral atom, or (c) loss of a single electron from a neutral atom. These radicals, especially if they are of low molecular weight, are usually extremely reactive; hence, they are short-lived. Since they have an unpaired electron, they are highly electrophilic (i.e., "electron loving") and attack sites of increased electron density, as in compounds with nitrogen atoms (e.g., proteins, amino acids, DNA, RNA) and carbon–carbon double bonds (i.e., polyuunsaturated fatty acids and phospholipids which make up bilipid cell membranes).

Upon exposure to air, animal and vegetable fats and oils become rancid (i.e., develop color changes and a musty, rank taste and odor). Here, the hydrogen atoms of the —CH_2—groups located between alternating double bonds (i.e., —CH═CH—**CH_2**—CH═CH—) of a polyunsaturated phospholipid or fatty acid (LH) are very susceptible to abstraction by free radicals. This process can then lead to a general reaction known as autoxidation, which results in the formation of a lipid hydroperoxide (LOOH) and the generation of a new free radical; hence, an autocatalytic reaction results (lipid peroxidation).

$$LH + R\bullet \rightarrow RH + L\bullet$$
$$L\bullet + O_2 \rightarrow LOO\bullet$$
$$LOO\bullet + LH \rightarrow LOOH + L\bullet$$

Importantly, lipoperoxides can also result in the formation of various aldehydes (malondialdehyde, 4-hydroxynonenal, etc.), which are very reactive and can result in further damage by causing cross-links with proteins and lipids. A single free radical may result in the formation of hundreds of lipoperoxides before it is neutralized.

3.4.2. *Oxygen Toxicity*

Oxygen has often been referred to as "a double-edged sword." Although it is critical for life, many essential intracellular reactions in which it is required result in the formation of free radicals. Soon after oxygen was independently discovered by Priestley and Scheele in 1774, Lavosier reported that oxygen inhalation had poisonous effects. The "classic" studies of Bert in 1878 later documented that oxygen at high tension "is a powerful poison," leading to convulsions and death in sparrows, various laboratory animals, insects, and earthworms (B8). Lorrain Smith, a Belfast, Ireland, physician, carried out a series of experiments demonstrating that increased oxygen tension results in severe pulmonary congestion with pneumonia-like changes in mice, rats, and guinea pigs (S15). Nevertheless, this information was completely ignored by physicians for several decades, in spite of numerous similar studies, including that of Comroe and associates (C16), who reported on the toxic effects of oxygen inhalation at high concentrations in normal men at sea level and at the simulated altitude of 18,000 ft elevation.

Failure to recognize the potential toxic effects of increased oxygen tension led to an estimated 10,000 worldwide cases of blindness (retrolental fibroplasia) in newborns in the late 1940s and early 1950s (S19). Nevertheless, wide acceptance that increased oxygen tension was potentially toxic to humans did not occur until Nash *et al.* (N3) correlated the concentration and duration of inspired oxygen before death with the pathologic lung findings at autopsy; 1 year later, the formation of pulmonary hyaline membranes in adults was attributed to oxygen toxicity (S17).

4. Support for Free Radical Theory

4.1. Natural Production of Free Radicals

Because of their extreme reactivity, the existence of free radicals in biological systems was generally not considered possible until Gerschman and associates (G3) hypothesized that oxygen poisoning and X-irradiation have a common basis of action. Here, they reported the homolytic dissociation of water by ionizing radiation.

$$H_2O \xrightarrow{\text{ionizing radiation}} H^{\bullet} + HO^{\bullet}$$

It was subsequently learned that oxygen-derived radicals are normally produced in living organisms by a wide variety of reactions, especially in mitochondria and the electron transport chains in the endoplasmic reticulum, where the addition of an electron to molecular water produces superoxide ($O_2^{-\bullet}$).

$$O_2^{-\bullet} + e^- \rightarrow O_2^{-\bullet}$$

Superoxide is also a product of various enzyme reactions catalyzed by the flavin oxidases (e.g., xanthine oxidase and monoamine oxidase). In addition, $O_2^{-\bullet}$ is a product of the noncatalytic oxidation of oxyhemoglobin, of which about 3% is converted each day to methemoglobin. Moreover, $O_2^{-\bullet}$ is readily formed in phagocytic cells (i.e., neutrophils and monocytes) during the "respiratory burst." Furthermore, in addition to the Fenton reaction, the Haber-Weiss reaction results in the conversion of $O_2^{-\bullet}$ to the potent $HO^\bullet$ via the following reactions (H3):

$$Fe^{3+} + O_2^{-\bullet} \longrightarrow Fe^{2+} + O_2 \quad (1)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH\bullet + HO^- \quad (2)$$

$$\text{Sum:} \quad H_2O_2 + O_2^{-\bullet} \xrightarrow{\text{iron catalyst}} HO\bullet + OH^- + O_2$$

Various reduced transition metal ions (e.g., Cu^{1+}, Fe^{2+}) also react with O_2 to produce $O_2^{-\bullet}$.

Other important carbon-centered radicals also exist in biologic systems; these include peroxyl (ROO•), alkoxyl (RO•), and thiyl (RS•) radicals. Furthermore, the endothelium-derived relaxing factor [nitric oxide (NO•), a gaseous free radical] is the major signal for vascular smooth muscle relaxation (L10), NO• is produced by nitric oxide synthase (NOS), an enzyme that exists in three isoforms; one constitutive enzyme is restricted to endothelium, while the other one is found in the central and peripheral neurons, skeletal muscle, pancreatic beta-cells, and epithelial cells of broncheoli, uterus, and stomach (N5). The inducible isoform (iNOS) is calcium independent and expressed in many cell types after challenge with immunologic or inflammatory stimuli, including neutrophils, mast cells, endothelium, and vascular smooth muscle cells (A11). All isoforms catalyze the following reaction:

$$\text{L-Arginine} + O_2 + \text{NADPH} \rightarrow NO\bullet + \text{L-citrulline}$$

Although direct toxicity of NO• is very modest, it is significantly increased when NO• reacts with $O_2^{-\bullet}$ to form peroxynitrite ($ONOO^-$), a very strong oxidant.

$$NO\bullet + O_2^{-\bullet} \rightarrow ONOO^-$$

Importantly, the concentration of NO• under various inflammatory and immunologic conditions is enough to outcompete SOD for $O_2^{-\bullet}$ (B4).

Peroxynitrite is involved in various neurodegenerative disorders (K14) and several kidney diseases (K18), as well as in chronic inflammatory diseases such as rheumatoid arthritis (K8). Here, as well as in other conditions, peroxynitrite-mediated reactions with amino acid residues result in the formation of nitrotyrosine, which can lead to enzyme inactivation (V3).

4.2. Antioxidant Defenses

Free radicals are produced in abundance in all cells. In order to protect them against oxidative damage, a wide variety of natural mechanisms exist to either

TABLE 2
NATURAL ANTIOXIDANT DEFENSES

1. *Antioxidant enzymes*
   Catalase (Cat)
   Glutathione peroxidase (GPx)
   Glutathione reductase (GR)
   Glutathione S-transferase
   Superoxide dismutase (CuZn-SOD; MnSOD)
   Thioredoxin reductase (TR)
2. *Metal-binding proteins*
   Ceruloplasmin
   Ferritin
   Hemoglobin
   Lactoferrin
   Metallotheinein
   Myoglobin
   Transferrin
3. *Radical "scavengers"*
   Bilirubin
   Uric acid
   Thiols (R-SH)
   Vitamins A, C, E
   Carotenoids (beta-carotene, lycopene, etc.)
   Flavonoids (quercetin, rutin, catechin, etc.)
   Lipoic acid
   Melatonin
4. *Other antioxidants*
   Copper (as CuZn-SOD)
   Manganese (MnSOD)
   Reduced glutathione (GSH)
   Zinc (CuZn-SOD; other mechanisms)
   Selenium (GPx and TR)

prevent the formation of radicals or neutralize them after they have been produced (Table 2).

4.2.1. *Antioxidant Enzymes*

The general acceptance of free radicals in biological systems did not occur until the discovery (M11a) of superoxide dismutase (SOD), of which there are two enzymes, cytoplasmic CuZn-SOD and mitochondrial Mn-SOD. These enzymes catalyze the following reaction:

$$O_2^{-\bullet} + 2\,H^+ \rightarrow H_2O_2$$

Although it is not a free radical, H_2O_2 is rapidly converted to HO• in the presence of transition metal ions (Fenton reaction). However, both glutathione peroxidase

(GPx) and catalase (Cat) inactivate H_2O_2 as follows:

$$H_2O_2 + 2\,GSH \xrightarrow{GPx} GSSG + 2H_2O \quad (3)$$

$$2H_2O_2 + \xrightarrow{Cat} 2H_2O + O_2 \quad (4)$$

4.2.2. *Metal-Binding Proteins*

Although both GPx and Cat are very efficient in removing H_2O_2, HO• can still be formed in abundance (Fenton and Haber-Weiss chemistry). To partially offset the influence of transition metal ions on free radical production, there are numerous metal-binding proteins which prevent these reactions from taking place; these include, among others, ferritin, transferrin, ceruloplasmin, and metallotheinein (Table 2).

4.2.3. *Antioxidants (Radical "Scavengers")*

In spite of the protective effect of several antioxidant enzymes and metal-binding proteins, free radicals are still widely prevalent. Thus, Ames *et al.* (A10) estimated that in each rat cell there are 100,000 radical "hits" each day, while in every human cells there are 10,000/day. Importantly, there are numerous natural free radical scavengers/chain breakers, the most notable being vitamins C and E, various carotenoids (beta-carotene, lycopene, etc.), flavonoids (rutin, quercetin, catechin, etc.), uric acid, and bilirubin, among others (Table 2).

4.3. Free Radicals (Oxidative Stress) and Aging

The free radical theory of aging has gained significant credibility for the following reasons (Y8): (a) it satisfies most of the necessary critieria to define the aging process; (b) it provides an excellent model to investigate the best molecular insights and mechanisms; (c) it provides testable experimental approaches; and it (d) provides the best molecular explanations for the interaction between aging and various disease processes. However, these authors also suggested that recent studies have brought forth new roles for reactive oxygen species, including those for nitric oxide (S9), various enzyme reactions (Y1), and the HO• scavenging action of melatonin, a pineal gland hormone, in the defense systems (R4). As a result, they proposed that the free radical theory of aging be broadened to the "oxidative stress" theory of aging (Y8).

4.3.1. *General Observations*

It has been repeatedly demonstrated that caloric restriction in laboratory animals results in increased longevity. Thus, McCay and co-workers (M11) first demonstrated that by restricting food intake of young rats, their longevity could be significantly increased. Subsequent studies showed that food restriction in adult rats is also effective in increasing their life span (Y7). In fact, the life span of various

laboratory animals can be increased 30–50% by reducing their caloric intake to 60% of the amount normally consumed by those given food *ad libitum* (M10).

Based on data obtained from several recent studies, results strongly suggest that the mechanisms in the antiaging action of caloric restriction have a broad antioxidative effect. For example, long-term food restriction in mice selectively increases the activity of the antioxidative enzyme catalase, and a subsequent decrease in hepatic lipid peroxidation (K17). Dietary restriction also suppresses the production of reactive oxygen species in a variety of other tissues (L5, K12) including lymphocytes, liver, kidney, and heart; it also inhibits lipid peroxidation of brain synaptosomes (C9). Moreover, food restriction reportedly delays age-related neoplastic diseases in laboratory animals (M10). Furthermore, Chung and associates (C11) studied the effects of dietary restriction on DNA damage in both rat cell nuclei and mitochondria. Their work showed about 15 times greater damage to mitochondrial DNA than to nuclear DNA. Importantly, DNA damage in both nuclei and mitochondria was significantly reduced in diet-restricted rats compared to those who received food *ad libitum*.

Recent reports have also shown that caloric restriction lowers body temperature (L2), increases physical activity without exerting a negative influence on behavior (W5), and slows the postmaturational decline in serum dehydroepiandrosterone sulfate (DHEA) levels in rhesus monkeys, as was previously shown in rodents (L3).

Age pigments, primarily lipofuscin and ceroid lipopigments, have been recognized in various organs of elderly people for well over 100 years. Since the pigment accumulates principally in the lysosomes of nonreplicating cells, it is found primarily in cardiac, smooth, and skeletal muscle, brain, and liver. This yellow-brown, fluorescent pigment is formed as a by-product of lipid peroxidation. Although no definite functional derangements have been attributed to it, its presence is suggestive of continuing lipid peroxidation as a result of long-term inadequate defenses against the stresses of activated oxygen.

Several experimental studies adding further support for free radicals in aging were recently reviewed (R10). Here, in studies with laboratory-developed long-lived strains of the fruit fly (*Drosophila melanogaster*) and tiny soil-dwelling worm (*Caenorhabditis elegans*), the major demonstrated differences were the increased activities of some antioxidant enzymes. More specifically, the *age-1* mutation described in *C. elegans* increased the mean life span by 65% and the maximum life span by 110% (F14). Exactly how this single mutation results in the worm's highly increased life span is only partially understood. Nevertheless, the mutation does confer resistance to hydrogen peroxide and paraquat, both of which promote the generation of the potent hydroxyl radical. The mutant also accumulates fewer deletions of the mitochondrial genome, an age-related phenomenon believed to result from free radical damage. Importantly, this *C. elegans* strain has increased activities of the antioxidant enzymes, Cu/Zn superoxide dismutase and catalase. In

addition, when young worms were exposed to lethal heat shocks, the *age-1* mutants were 40% more resistant than are their wild-type counterparts; short, nonlethal heat shocks not only reduced thermotolerance, they extended the life span. More recently, transgenic flies with three copies of CuZn-SOD and catalase exhibited a one-third increase in life span, delayed loss of physical activity, and a lower amount of oxidative protein damage compared with the diploid controls (O3).

Although studies involving oxidative stress and aging in humans are few, Ceballos-Picot and others (C2) measured Cu-Zn superoxide dismutase (SOD-1), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-*S*-transferase (GST) activities in human erythrocytes from 167 healthy subjects (102 females, 65 males) ages 1 month to 63 years. They reported a significant inverse correlation between age and enzyme activities for SOD-1, GR, and GST; however, GPx correlated directly with increasing age. Upon dividing the participants into five groups (newborns to 1 year; >1–11 years; 12–25 years; 26–40 years, and 41–63 years), they found significant modifications in the 12- to 25-year-old group for SOD-1, GR, and GPx; modified GST activity occurred in the 1- to 11-year-old group. No sex-related differences for any of the enzymes were noted.

Paolisso *et al.* (P6) recently measured plasma levels of malondialdehyde (a by-product of lipid peroxidation), lipid hydroperoxides, vitamins C and E, and reduced/oxidized gutathione ratios and compared the results in different age groups: adults (<50 years), aged subjects (70–90 years), and centenarians ($\geq$ 100 years). Their findings showed that the degree of oxidative stress was lower in healthy centenarians than in the aged subjects, but higher than in the adult group. The authors suggested that a greater daily intake of vegetables by the centenarians, along with various metabolic parameters, might be important differences, but they recognized that genetic factors may also play an important role (possible increased antioxidant enzyme activity in the centenarians?).

In a related study, Knight *et al.* (K16) measured malondialdehyde (MDA) levels in healthy adult blood donors aged 18–65 years and noted a significant increase in plasma MDA levels in a group of males aged 46–63 years compared with those aged less than 46 years. Moreover, increased levels were present in women over age 25 years compared with those under 25 years of age. Additionally, males had significantly higher plasma MDA levels than females.

5. Magnesium, Zinc, and Selenium

5.1. Magnesium (Mg)

After sodium, potassium, and calcium, Mg is the most abundant body cation and the second most prevalent intracellular cation after potassium. Only about 3% of total body Mg is present in the extracellular fluids; approximately 57% is in bone

and 40% is in soft tissues. Magnesium is a co-factor for more than 325 enzymes involving energy metabolism and nucelic acid and protein synthesis. Examples of important intracellular functions include a role in oxidative phosphorylation and potassium–hydrogen ion exchange. Extracellularly, Mg plays a significant role in the control of arterial vascular tone and modulation of the sodium–calcium pump; it also stimulates the vasodilator, prostaglandin (PGI_2). In addition, Mg is important in both DNA synthesis and degradation, as well as in the lymphokine-mediated effects of macrophage activation and migration inhibition.

Although serum Mg levels are probably not age-related per se, Touitou *et al.* (T8) reported that hypomagnesemia is common in the elderly. They measured both serum and erythrocyte Mg levels in 381 unselected healthy elderly men and women, most of whom were older than 75 years. They found that 10% of these individuals had decreased serum levels; however, 20% had low red cell levels. This latter finding is particularly important, since it is a more accurate measure of total body Mg. In this regard, the current minimum recommended daily Mg intake is 350–400 mg. It is estimated that prior to the Industrial Revolution, the average intake was 450–500 mg/day. Currently, however, the average adult intake in the United States is only 175–225 mg (A6).

Numerous studies have shown that hypo- and hypermagnesemia are common and of considerable importance in various cardiac dysrhythmias, myocardial infarction, increased overall mortality, diabetes mellitus, among others (K13).

5.2. Zinc (Zn)

Zinc is an essential element, being important in a wide variety of critical biochemical processes. Thus, zinc (a) is a component of about 200 enzymes including Cu-Zn superoxide dismutase (SOD-1), DNA and RNA polymerases, and reverse transcriptase; (b) competes directly with Cu^+ and Fe^{2+} to reduce hydroxyl radical formation by Fenton chemistry; (c) has a role in thyroid homeostasis; and (d) is important for various immune system functions. More direct evidence demonstrating the importance of Zn in reducing oxidative stress was reported recently by Tate *et al.* (T3). Here, cultured human retinal pigment epithelial (RPE) cells were maintained for 7 days in culture media containing either 14 or 0.55 μM total Zn. Conjugated dienes and thiobarbituric acid reactive substances (TBARS), measurements of lipid peroxidation, were measured in RPE cells treated with 0.5 mM H_2O_2, 10 μM $FeSO_4$ + 0.5 mM H_2O_2, or 10 μM $FeSO_4$ + xanthine/xanthine oxidase for 24 h or paraquat for 7 days. Oxidized proteins were determined by the production of carbonyl residues. The antioxidants metallothionein, catalase, superoxide dismutase, and glutathione peroxidase were also measured. Their results showed that Zn was protective of RPE cells from the oxidative toxicity of H_2O_2 and paraquat. RPE cells in 0.55 μM Zn medium contained higher levels of TBARS, conjugated dienes, and protein carbonyls compared to cells in 14 μM Zn. Catalase and metallothionein content were both reduced in cells exposed to 0.55 μM Zn.

TABLE 3
SIGNS/SYMPTOMS: ZINC DEFICIENCY VERSUS AGING

Signs/symptoms	Aging	Zinc deficiency
Anorexia	Yes	Yes
Poor wound healing	Yes	Yes
Taste/smell defects	Yes	Yes
T-cell deficiencies	Yes	Yes
Impotence	Yes	Yes

Plasma zinc levels are commonly decreased in the elderly (G11). These authors reported mean (±SD) plasma levels in normal young adults at 12.7 (±1.4) mmol/liter versus 10.5 (±4.7) mmol/liter in the elderly; intracellular levels (neutrophils) were 1.26 (±0.28) and 0.95 (±0.26) nmol/mg protein, respectively. This deficiency is most often due to lack of dietary lean meat, poultry, and fish. As a result, zinc intake is frequently less than the minimum recommended intake of 15 mg/day. Moreover, Zn deficiency is particularly common in individuals with diabetes mellitus, liver and renal diseases, malabsorption, alcohol abuse, and those taking diuretic medications (K13). Interestingly, many of the signs and symptoms of Zn deficiency are the same as those often attributed to the aging process (Table 3).

As discussed previously, the immune theory of aging is based, to a considerable degree, on the fact that the immune system becomes less efficient with aging. Importantly, Zn also plays a role in the immune system. For example, Zn supplementation in the elderly (a) increases the number of circulating T-lymphocytes; (b) improves delayed cutaneous hypersensitivity to various antigens; and (c) increases the immunoglobulin G antibody response to tetanus vaccine (D13). This early study is supported by additional recent reports which also demonstrate that Zn supplementation improves the immune system in the elderly (F9, R6).

Zinc deficiency may also be of importance in Down's syndrome (F11). Thus, oral supplementation of DS patients increased their serum thymic factor (TF) levels, decreased the levels of inactive zinc-free TF, and significantly increased the number of circulating T-lymphocytes. In addition, these individuals had fewer infections.

5.3. SELENIUM (SE)

Selenium is an essential trace element, being important in at least two critical enzymes, the antioxidant glutathione peroxidase (GPx), and type 1 iodothyronine deiodinase. GPx converts hydrogen peroxide to water, in the presence of reduced glutathione, while iodothyronine deiodinase catalyzes the conversion of thyroxine to triiodothyronine, the physiologically active hormone species.

Decreased plasma Se levels are commonly present in elderly people (B7). Moreover, low Se levels are associated with decreased cellular GPx activity. Although reports differ slightly, plasma reference values for "healthy" adults were reported as 1.38–153 μmol/liter, while "house-bound" elderly individuals had levels of 1.08–1.35 μmol/liter (K13).

In addition to Keshan disease, a dilative cardiomyopathy first described in the Keshan Province of China, Se deficiency has been associated with an increase in various malignant diseases. Thus, well over 50 studies have demonstrated significant reductions in experimental cancer in laboratory animals supplemented with Se. Moreover, reports from Finland and the United States support the hypothesis that Se deficiency results in decreased cellular GPx activity, increases oxidative stress, and increases the incidence of cancer, particularly carcinoma of the breast, prostate, and colon (K13).

6. Heat Shock Proteins

The heat shock protein (HSP) system is a ubiquitous cell defense mechanism involved in the response to acutely induced cell damage by numerous processes (i.e., heat, oxidative stress, hypoxia, fever, inflammation, ethanol, heavy metal ions, and metabolic deprivation). These stress proteins represent a large multigene family conserved from prokaryotes to eukaryotes; they are classified into several subgroups according to molecular weight. Although HSPs are rapidly induced in stressed cells, some are also constitutively expressed and are important in the maintenance of cellular integrity during basal conditions. Moreover, HSP may also slow the aging process (L7).

The most abundant of the HSPs is the 70-KDa subfamily (HSP70), represented in mammalian cells by several distinct proteins that exhibit both constitutive and inducible forms. Acting as chaperonin in protein folding, unfolding, and translocation across membranes, HSP70 also recognizes and interacts with nascent protein chains and forms complexes with malfolded proteins and plays a role in both the anabolic and catabolic phases of their metabolism. The major stimulation of HSP production is the intracellular accumulation of incomplete, damaged, or modified proteins. Thus, age-related alteration in the ability of cells to express HSP70 in response to stress could severely compromise the ability of a senescent organism to respond to changes in its environment.

During the period of aging, posttranslational modified proteins and advanced glycosylation end products, which accumulate in tissues, might chronically stimulate HSP production, and affect the basal synthesis of this cell defense mechanism. In this regard, acutely induced HSP synthesis does decrease with aging. However, since the effect of age on the basal expression of HSP70 had not previously been addressed, Maiello and associates (M3) studied the age-dependent basal

HSP70 mRNA in rat kidney from young (2–3 months), adult (6–11 months), and old (22–27 months) male rats by measuring steady-state levels of HSP70, gamma-actin mRNA, rRNA, and pentosidines, a measure of posttranslational modified proteins. The basal unstimulated results showed HSP70 mRNA was significantly increased in both young and old rats compared with adults rats and pentosidine levels increased progressively with age. The authors suggested that (a) different mechanisms are responsible for increased HSP70 basal synthesis in both young and old rats; (b) pentosidine accumulation chronically enhances HSP70 synthesis in aged animals; and (c) decreased synthesis of other proteins accompanying HSP-selective production might contribute to the impairment of specific cell functions in aging cells.

Gutsmann-Conrad *et al.* (G15) recently studied the effects that *in vitro* cellular senescence and cells cultured *in vitro* from young and old human donors have on the ability of cells to regulate the expression of HSP70. Here, the ability of early and late passage lung and skin fibroblasts and epidermal melanocytes obtained from young and old human donors to express HSP 70 was determined after a brief heat shock. Their results showed that the levels of HSP 70 protein and mRNA were lower in late passage cells and cells from old donors compared with early passage cells and cells from young donors. In addition, the binding activity of the heat shock transcription factor (HSF1) was significantly higher in early passage cells and cells from young donors compared to late passage cells and cells from old donors. Moreover, HSF1 was decreased in late passage cells and cells from old donors.

As is well established, the gaseous free radical nitric oxide (NO•; endothelium relaxing factor) is a double-edged sword that can stimulate smooth muscle cell (SMC) relaxation, resulting in vasodilation. It can also be cytotoxic, especially by reacting with superoxide anion ($O_2^{-\bullet}$) to form the potent oxidant, peroxynitrite ($ONOO^-$). Xu *et al.* (X1), noting that HSP70 is augmented in arterial SMCs during acute hypertension and atherosclerosis, demonstrated that NO• generated from several sources [i.e., sodium nitroprusside (SNP), *S*-nitroso-*N*-acetylpenicillamine, and spermine/NO• complex] led to HSP70 induction in cultured SMCs. Since induction of HSP70 mRNA was associated with activation of HSF1, this suggests that the response was regulated at the transcriptional level. Moreover, HSF1 activation was inhibited by hemoglobin, dithiothreitol, and cycloheximide, suggesting that NO•-induced protein damage and nascent polypeptide formation may initiate the activation. Importantly, SMCs pretreated with heat shock (42°C for 30 min) were protected from death induced by NO•.

As previously noted, caloric restriction (CR) is the only known effective experimental manipulation shown to retard the aging process. CR has also been shown to alter various age-related processes. Moore and co-workers (M14) studied the effects of aging and CR on the ability of alveolar macrophages to produce HSP70. Here, they isolated alveolar macrophages from young (4–6 months) and old

TABLE 4
MAJOR AGE-ASSOCIATED DISEASES/DISORDERS

Atherosclerosis
Cancer
Cataracts and macular degeneration
Diabetes mellitus
Immune system disorders
Neurodegenerative disorders
Neuroendocrine disorders
Nutritional deficiencies

(24–27 months) rats fed either *ad libitum* or a restricted diet (60% of *ad libitum* diet). Although there was no age-related change in the number of cells recovered from young and old rats on the *ad libitum* diet, the total number of macrophages recovered from the CR animals was reduced. HSP70 was measured under two conditions: in suspension and following cellular adherence to plastic culture plates. The macrophages incubated at 37°C in suspension showed no detectable HSP70 expression; however, HSP70 expression was induced at 37°C when the macrophages adhered to the plastic dishes. HSP70 levels were rapidly induced by heat shock (43°C for 1 h) in cells cultured both in suspension and on plastic. HSP70 expression did not change significantly with either age or CR in cells cultured in suspension. On the other hand, HSP70 levels in cells adherent to plastic decreased about 70% with age, and HSP70 induction was greater in macrophages isolated from CR rats. HSP70 induction by heat shock also decreased with age in the adherent cells. CR increased HSP70 expression three- to fourfold in adherent cells from both young and old rats.

7. Age-Associated Diseases/Disorders

Although aging and disease are distinctly separate processes, aging predisposes one to numerous diseases/disorders (Table 4). Several of the major age-related diseases will be briefly discussed in the following sections.

7.1. ATHEROSCLEROSIS

7.1.1. *Etiology/Pathogenesis*

Atherosclerosis ("hardening of the arteries") is clearly the most common disease process leading to disability and death in the industrialized countries. That is, coronary artery disease (CAD) was the number 1 cause of death in the United States, while cerebrovascular disease (stroke and ischemic dementia) was the third major

cause of death in 1995 (G16). In addition, common abnormalities in renal function tests, often inferred to be a result of "aging," are also due to atherosclerosis (arterionephrosclerosis).

The commonly recognized risk factors for atherosclerosis include increasing age, sex (males > females until menopause, after which the incidence is similar), serum lipid levels (increased total cholesterol and low-density lipoprotein cholesterol, decreased high-density lipoprotein cholesterol, etc.), diabetes mellitus, hypertension, and obesity. Other less well recognized but very important risk factors include increased plasma homocysteine, fibrinogen, and coagulation factor VII; increased blood hematocrit, leukocyte count (increased neutrophils), and C-reactive protein; and clinical depression.

Oxidative stress is now widely believed to be the major mechanism of atherogenesis. Interestingly, it was demonstrated 47 years ago that atheromatous plaques contain abundant lipoperoxides and other lipid peroxidation products (G9). More recently, our understanding of this process was advanced when evidence was provided for significant free radical activity and the lipid oxidative modification hypothesis was presented (P10). A subsequent study provided further evidence that oxidatively modified low-density lipoproteins (LDL) play a major role in the formation of the fatty streak, the earliest visible atherosclerotic lesion, and the subsequent production of the atheroscelrotic plaque (S27). The proposed sequence, which involves arterial endothelial and smooth muscle cells, as well as monocytes/macrophages, is as follows (Q1, S25).

(a) Circulating monocytes are recruited by a chemotactic factor produced by injured endothelial cells due to locally produced toxic oxidized LDL (ox-LDL).
(b) The recruited monocytes migrate through the endothelium and engulf the ox-LDL. Although monocytes/macrophages have LDL receptors, they take up very little normal LDL, but ox-LDL is taken up rapidly via acetyl-LDL receptors (scavenger receptors), leading to the formation of foam cells (as early as 3–4 years to age).
(c) Ox-LDL is also toxic to the monocytes/macrophages; they lose their motility and are unable to reenter the circulation.
(d) As ox-LDL increases, the arterial smooth muscle cells become activated, enter the submucosa, and ingest ox-LDL and become foam cells. In time, the foam cells progress to the fatty streak. The process continues with the subsequent formation of fibrous/calcified plaques.
(e) Endothelial integrity is eventually lost due to cytotoxic ox-LDL. P-selectin and von Willebrand factor may become exposed and trigger platelet activation, adhesion, and possible thrombosis.

Numerous subsequent reports further support this theory of atherosclerosis. Thus, it has been demonstrated in both laboratory animals and humans that *in vivo*

oxidation results in the modification of LDL (S26). Moreover, it was confirmed that iron and copper can catalyze the oxidation of LDL in smooth muscle cells (H9). It was also independently shown that LDL oxidation *in vitro* can be totally inhibited by antioxidants and that LDL oxidation is dependent on the presence of catalytic levels of iron or copper (M15, S28). Therefore, the oxidative process can be significantly inhibited by metal chelation. Likewise, it was demonstrated *in vitro* that oxidized LDL is cytotoxic to both endothelial and smooth muscle cells and that the cytoxicity depends on LDL oxidation during cellular incubation (M15). Further evidence was provided by several reports that showed an association between plasma autoantibodies against oxidatively modified LDL and the progression of CAD (P4, S2, M2). In addition, epitopes of the oxidatively modified LDL have been detected in human arterial fatty streaks (Y3, Y4).

The oxidants responsible for initiating LDL oxidation have been under intense investigation, and several possible mechanisms have been suggested. For example, $O_2^{-\bullet}$ has been implicated as a major contributor to LDL oxidation mediated by macrophages and smooth muscle cells (H8). Here, $O_2^{-\bullet}$ is converted to H_2O_2 by SOD, which in turn is acted upon by a transition metal ion with the formation of HO•. Another possible role for $O_2^{-\bullet}$ is its reaction with NO• to form $ONOO^-$, which is capable of oxidizing lipids and sulfhydryl groups, even in the presence of plasma antioxidants (V1). Moreover, *in vitro* studies have shown that $ONOO^-$ can induce the formation of F_2-isoprostanes, nonenzymatic products of the free radical-catalyzed oxidation of arachidonic acid (M13).

Numerous experimental studies have provided strong evidence that various antioxidants are antiatherogenic (P11, F13, J4, W15, V2). Representative of these studies is a recent report (P18) in which the antiatherogenic effects of supplemental vitamin E in rabbits were assessed in the presence of a high-cholesterol diet. Here, levels of total cholesterol, LDL-cholesterol, HDL-cholesterol, VLDL-cholesterol, triglycerides, and malondialdehyde (MDA), a product of lipid peroxidation, were measured. In addition, each rabbit aorta was evaluated by gross and microscopic examination, and MDA was measured in the aortic tissue. These parameters were compared with nonsupplemented control animals. The study showed that the vitamin E-supplemented rabbits had significantly lower blood and tissue MDA levels as well as significantly decreased gross and microscopic aortic atheromatous changes compared with the controls.

Several extensive human epidemiologic studies have also been published. For example, two U.S. studies, one involving 87,245 female nurses (S23) and the other 38,910 male physicians (R5), both concluded that vitamin E supplementation was directly associated with reduced risk for ischemic heart disease. In addition, Gey and associates (G6) reported on a large cross-cultural European population which differed sixfold in age-specific mortality from CAD. The data supported their conclusions that this highly significant difference in CAD was primarily due to increased plasma vitamin E levels in those with a relatively low incidence of

CAD. Furthermore, atherosclerosis has long been associated with increasing age. It is of importance to note here that Khalil *et al.* (K11) reported increased LDL susceptibility to oxidation by gamma-radiolysis with increasing age. Their results indicated that the greater LDL oxidation susceptibility in old subjects compared with a young group could be attributed to a fourfold lower LDL concentration of vitamin E in the elderly.

There is an accumulating amount of information that some infections may be linked to atherosclerotic disease. Infections may both augment atherogenesis and contribute to later manifestations of overt clinical disease by facilitating plaque rupture and thrombosis (D1). These workers presented data supporting the hypothesis that *Chlamydia pneumoniae,* a gram-negative bacterium, may be a causative agent for arterial disease. More recently, high titers of antibodies to *C. pneumoniae* have been associated with a risk for future coronary and cerebrovascular diseases (F2). This subject has been recently reviewed (M18). It should be noted that this theory, if true, does not change the basic ox-LDL theory, since infectious agents stimulate the inflammatory process which leads to increased numbers of neutrophils and macrophages. The "respiratory burst" follows phagocytosis, resulting in the formation of reactive oxygen species.

As a final note, an elevated plasma total homocysteine (tHcy) level is now recognized as a major independent risk factor for coronary, cerebrovascular, and peripheral vascular diseases. Although the mechanisms whereby tHcy induces atherosclerosis are only partially understood, oxidative stress and endothelial injury have been suggested. In this regard, F_2-isoprostanes, a recently described class of prostaglandin-like compounds, are produced by lipid peroxidation of arachidonic acid independent of cyclooxygenase (M17). Several additional studies have added evidence that the measurement of plasma F_2-isoprostanes is a reliable indicator of lipid peroxidation in patients with atherosclerosis. More recently, Voutilainen *et al.* (V5) tested the hypothesis that an elevated plasma tHcy concentration is associated with enhanced lipid peroxidation *in vivo* as measured by plasma F_2-isoprostane concentrations. In short, their data clearly suggested that increased fasting plasma tHcy levels is associated with enhanced *in vivo* lipid peroxidation.

7.2. Neoplasia

7.2.1. *General Considerations/Etiology*

Neoplasia is generally believed to result from one or more permanent cell changes. Although a single mutational event may lead to malignant transformation, in most cases the process is a complex multifactorial process that can broadly be categorized into five etiologic events: genetic, viral (e.g., oncogenes), chemical (e.g., xenobiotics), physical (e.g., irradiation), and inflammatory (chronic inflammation).

The vast majority of malignant tumors arise during mid–late adulthood from a complex interplay between genetic and environmental factors including life style, nutrition, microorganisms, radiation, metals, etc. In broad terms, cancer development involves several steps: initiation, promotion, and progression. Initiation is an irreversible specific alteration in the DNA of a target cell. Promotion involves the reversible stimulation of the expansion of the initiated cell or the reversible alteration of gene expression in a cell or its progeny. Progression is characterized by the development of aneuploidy and clonal variation in the tumor. In turn, these result in tumor invasion and metastasis.

Cancer is a genetic disease that arises from an accumulation of mutations that promote clonal cell selection with increasing aggressive behavior. The vast majority of cancer mutations are somatic and found only in an individual's cancer cells; only about 1% of all cancers are due to an unmistakable cancer syndrome (F4). However, mutations in several key genes can lead to neoplasia (V4). For example, mutation in the tumor-suppressor gene p53 is present in about 50% of human tumors. The p53 protein guards the check point of the cell cycle; its inactivation results in uncontrolled cell proliferation. Other critical factors in tumorigenesis include (a) the rate of excision by DNA repair enzymes; (b) the rate of cell division; and (c) defense systems (e.g., glutathione transferases protect DNA against mutagens; antioxidants prevent the formation of and/or neutralize free radicals) (A8).

7.2.2. *Oxyradicals and Cancer*

There is now abundant compelling evidence that free radicals and other reactive oxygen species (ROS) (e.g., singlet oxygen) are highly important factors in carcinogenesis. As noted earlier under "Antioxidants (Radical 'Scavengers')" Ames and associates (A10) estimated that in every rat cell there are about 100,000 free radical "hits" each day; in every human cell there are about 10,000/day. They noted further that although DNA enzymes can repair most of the oxyradical-produced lesions, there are still an estimated 2 million DNA lesions/cell in old rats (2 years) and about 1 million lesions/cell in young adult rats. Moreover, ROS result in the production of over 30 different DNA adducts, excluding protein and lipid addition products, as well as inter- and intrastrand cross-links (F5). DNA damage from free radicals and lipid peroxidation has been attributed to the following: (a) the action of free radicals produced from the decomposition of peroxidized lipids; (b) lipid hydroperoxides; (c) carbonyl derivatives; and (d) through a second messenger resulting in clastogenic activity.

Floyd *et al.* (F8a) were the first to present *in vitro* evidence for DNA alterations by a free radical-generating tumor promotor when they showed that 8-hydroxyguanine was a product of oxidatively damaged DNA.

$$\text{Guanine} + \text{HO}\bullet \rightarrow \text{8-hydroxyguanine}$$

This finding has now been repeatedly confirmed, and numerous subsequent studies

have added additional support for the carcinogenic effects of ROS (K5, K6, C5). Normally, guanine pairs with cytosine during DNA replication. However, 8-hydroxyguanine may pair instead with adenine, which results in a G→T transversion. Furthermore, misincorporation of 8-hydroxyguanine as substrate causes A→C substitution (C8). This reaction is believed to be mutagenic and may be responsible for tumorigenesis induced by DNA-damaging ROS (C8, W16). Additional recent reviews of oxidative stress and carcinogenesis have been published (A9, C7, G17, B3, K15).

7.2.3. *Diet*

Doll and Peto (D10) attributed about 35% of all cancer deaths in the United States to diet, both undernutriton (vitamin and mineral deficiencies) and overnutrition (obesity). More recently, Willett (W11) estimated this figure to be someplace between 20% and 40%. Overnutrition has repeatedly been shown to be a major risk factor for malignancy (P8). More specifically, one of many examples was that of Huang and associates (H15), who reported that women with a body mass index $\geq$31 kg/m^2 and/or recent weight gain were at significant risk for breast cancer. Tang *et al.* (T2) reported that the incidence of colon and mammary cancers increased rapidly when the dietary fat level increased from 15% to 30% of total calories. Similarly, an increased cancer risk has been reported in meat eaters (increased fat content) compared with vegetarians (T6). Others showed a direct correlation between fat intake and the development of prostatic carcinoma (G1). More specifically, this study found that those with increased plasma levels of alpha-linolenic acid (from animal fat) had a two- to threefold increase in risk for prostatic carcinoma compared with those whose levels were in the lowest quartile.

Conversely, caloric restriction has been shown to retard the onset of several age-related malignancies, including leukemia in rodents (M1). In addition, Chung *et al.* (C11) demonstrated that dietary restriction significantly reduces both mitochondrial and nuclear DNA damage, as shown by a reduction in the production of 8-hydroxydeoxyguanosine in caloric-restricted rats. Although protein restriction has not been well studied, it apears to have the same effects (H14). In addition, decreased mitotic activity was noted in various tissues in dietary-restricted rodents compared to those fed *ad libitum* (L9, H11), a finding that presumably contributes to the decreased cancer incidence by preferentially enhancing apoptosis in preneoplastic cells (G14).

Numerous studies have demonstrated an inverse correlation between cancer rates and dietary intake of various antioxidants (foods rich in vitamins C and E, beta-carotene, lycopene, etc.) (C13, G5, E3, P2, H10, Y5). For example, a recent review (G8) showed a consistently lower risk of developing a variety of cancers in those with a higher consumption of tomatoes and tomato-based products (rich in lycopene, a potent carotenoid antioxidant), adding further support for the current recommendations to increase the consumption of fruits and vegetables. In addition

to these studies, numerous others have shown a protective effect of antioxidants against cancer. Thus, Block (B10) reported that of 46 studies, 33 showed a significant protective effect of antioxidants against various cancers. In addition, Blot *et al.* (B11), in a study involving 30,000 Chinese, noted a significant decrease in esophageal and gastric cancers in participants supplemented with vitamin E, beta-carotene, and selenium compared with the nonsupplemented control group. Moreover, over 55 published reports demonstrated a reduced incidence of experimental cancer in laboratory animals supplemented with selenium (a component of glutathione peroxidase). Furthermore, reports from both Finland (S1) and the United States (W12) showed that selenium was important in reducing human mammary, prostate, and colon cancers.

7.2.4. *Smoking*

The abuse of tobacco is the single most important risk factor for cancer and several other diseases. It contributes to about one-third of cancer cases, one-fourth of coronary artery disease, and an estimated 400,000 premature deaths each year in the United States (D10, P14). Smoking is a major risk factor not only for lung cancer, but for mouth, larynx, kidney, urinary bladder, esophagus, stomach, and pancreas cancers (A8).

Although the carcinogenic mechanism(s) of smoking is not fully understood, there is abundant evidence that free radicals are very important, along with other mutagens and rodent carcinogens. Cigarette smoke reportedly results in the formation of "more than a billion oxyradicals in each puff" (B12). As a result, it is not surprising that to achieve normal ascorbic acid blood levels, a smoker must ingest two to three times the normal intake (S7).

7.2.5. *Hormones*

Hormones are reportedly associated with a group of malignant tumors that comprised almost one-third of new cancer cases in 1990 (H13). The major tumors were endometrial, breast, prostate, and ovarian.

Endometrial cells divide in response to estrogen. Thus, it is not surprising that increased cancer risks of 10- to 20-fold have been reported following long-term exogeneous estrogen use (J5). Epithelial breast cells also proliferate in response to estrogens; the simultaneous presence of progesterone further increases the rate of cell division, thereby further increasing the rate of mammary cancer (H13). Conversely, exercise may decrease the incidence of breast cancer, presumably by its negative effect on hormone levels (B6).

Ovarian cancer is believed to develop primarily from the surface epithelial cells, which proliferate to cover the exposed surface following ovulation. As a result, factors which prevent ovulation (i.e., increasing numbers of live births, incomplete pregnancies, use of oral contraceptives) are protective against ovarian carcinoma.

Although prostatic cancer is widely regarded as being related to androgens, few studies have been reported. To complicate the matter, no definitive reproducible hormone markers are present in males. Nevertheless, testosterone administration has been reported to produce prostatic cancer in rats (N10). More recently, serum testosterone and estrogen levels in black males have been shown to be significantly higher than in white males, a finding that is presumed to account for the approximate twofold higher incidence of protaste cancer in black men compared with white men (R7).

7.2.6. *Inflammation*

The association of chronic inflammation with malignant diseases has been known for over a century (T9). Inflammatory cells produce large quantities of reactive oxygen species (ROS) during the respiratory burst; these ROS are generally considered to be carcinogenic (see later discussion). In their review of this topic, Weitzman and Gordon (W7) emphasized the following significant points: (a) DNA strand breaks rapidly develop in target cells exposed to either activated neutrophils or H_2O_2; (b) neutrophils and macrophages readily produce mutations in both bacteria and mammalian cells; (c) nontransformed C3H mouse fibroblast cell line, when exposed to neutropils, H_2O_2, or a cell-free enzymatic oxyradical-generating system (e.g., xanthine oxidase plus hypoxanthine), undergoes malignant transformation in tissue culture; (d) sister chromatid exchanges in Chinese hamster ovary cells were produced when exposed to activated phagocytes from normal donors but not in those exposed to phagocytes from patients with chronic granulomatous disease (their phagocytes are unable to undergo the respiratory burst and produce ROS); and (e) phagocytic activation can replace the mixed-function oxidase system (cytochrome P450 oxidases) to convert polycyclic aromatic hydrocarbons into potent carcinogens. Of further interest here is the recent report that nitric oxide ($NO^{\bullet}$), produced by macrophages, can stimulate mutations in the X-linked *hprt* gene (Z2).

7.2.7. *Heredity*

Heredity is primarily important in tumors that affect children and young adults. Although heredity presumably affects one's susceptibility to some cancers, its full extent is not known. Rather, current knowledge indicates that noninherited factors are of primary importance in most malignancies. For example, hereditary factors are important in only about 10% of breast cancers (W4). Similarly, more than 90% of colorectal carcinoma cases are considered to arise sporadically, with no identifiable genetic link (B14). Moreover, Fredrikson *et al.* (F12) investigated p53 gene mutations and colorectal cancer; there was no familial association. Others also reported that heredity plays a minor role in renal cell (S8) and lung (B13) cancers.

7.2.8. *Miscellaneous Factors*

In addition to the major etiologic causes of cancer described above, up to 20% of malignancies are caused by various infectious agents (viruses, bacteria, parasites), drugs, ultraviolet light, X-irradiation, and various environmental agents (ozone, radon gas, asbestos, and transition metals such as nickel and cadmium, among others). In addition, polluted air contains nitrogen oxide and ozone, which bring about the production of a wide variety of ROS.

7.3. Cataracts and Macular Degeneration

Ultraviolet light and lonizing radiation homolytically split water to form the hydroxyl radical and hydrogen.

$$H_2O \xrightarrow{\text{radiation/UV}} HO\bullet + H\bullet$$

As a result, the lens and macula are prone to damage, especially without adequate eye protection. In addition to appropriate external protection (e.g., sunglasses), the eye requires an efficient internal antioxidant system. In this regard, Galileo apparently became blind after repeatedly observing the sun. Centuries later, in 1912, an estimated 3500 Germans suffered visual impairment after watching a solar eclipse.

The retinal lesions caused by direct visual sun exposure are similar to those caused by less direct ultraviolet light, as well as by mild but repeated solar interactions over many years, which occur in many of the elderly. In addition, the recent diminishing level of atmospheric ozone may add to these problems, especially in the future if its concentration continues to diminish. Moreover, numerous drugs, including phenothiazines, tetracycline, and allopurinol, are photosensitizers, which may further add to the problem in some individuals.

The macula is particularly prone to direct light exposure in comparison to the more peripheral eye regions. Here, the metabolically active photoreceptor cells mediate light transduction into neuronal impulses. In addition, their cell membranes have the highest polyunsaturated fatty acid concentration of any known tissue (G4). Furthermore, retinal oxygen turnover is very high and its cellular mitochondria are abundant. Hence, the combination of light radiation, high mitochondrial concentration, and abundant polyunsaturated fatty acids makes the macula highly susceptible to free radical damage, especially via lipid peroxidation (G4). Further indirect evidence supporting a role for oxyradicals is that the concentration of lipofuscin ("age pigment"), a product of lipid peroxidation, is very high in age-related macular degeneration (K7, Y6). In addition, zinc, an important antioxidant (see below under immunity), has recently been shown to protect cultured human retinal pigment epithelial cells against oxidative damage (T3).

Cataract is the world's leading cause of visual impairment. As such, it is not surprising that cataract surgery is the most common surgical procedure performed in the United States. There is considerable evidence supporting a primary role for free radicals in the oxidation of lens proteins. Thus, deficiencies of vitamins C and E and the carotenoids have been linked to an increased incidence of cataracts. For example, Seddon and associates (S11) studied 22,071 adult men and reported that those who regularly took multivitamin supplements had a significantly decreased risk for cataract formation. Similarly, Jaques and Chylack (J2) found that deficiencies of both vitamin C and the carotenoids, and possibly vitamin E, are risk factors for senile cataract. Garland (G2) emphasized that vitamin C is protective against oxidative lens damage, "particularly photo-induced damage." Moreover, quercetin, an efficient flavonoid antioxidant, was recently shown to inhibit H_2O_2-induced oxidation of rat lens (S3). Several epidemiologic reviews showing a strong inverse relationship between the dietary intake of various antioxidants and cataract formation have been reported (G4, S11, J2, G2).

7.4. Immunity, Aging, and Nutrition

As noted earlier in this chapter, a decline in immune function has long been recognized with increasing age. Thus, older individuals are more susceptible to a variety of infectious disorders as well as various diseases, including amyloidosis, chronic lymphocytic leukemia, lymphoma, various forms of epithelial cancer, and autoimmune phenomena. More specifically, aging is associated with a decrease in the total number of lymphocytes and T-cell subsets (e.g., $CD3^+$, $CD4^+$, $CD8^+$) and a decreased mitogen response to concanavalin A and phytohemagglutinin (W10). In addition, there is a decrease in the number and percentage of suppressor/cytotoxic cells (N1). Studies in mice suggest that the common underlying element here is impaired production of interleukin-2 (IL-2) (T5), an essential growth factor for maintaining T-cell proliferation. Decreased IL-2 has also been reported in aged humans (G7). There is also considerable clinical evidence that both T- and B-cell activities are significantly related to the effects of oxidative stress. Several examples will be given.

As noted previously, Zn has an important role in numerous critical biochemical processes, including immunity. In addition to the positive effects of Zn supplementation, various antioxidant vitamins have been shown to stimulate the immune system. Thus, supplementation of elderly individuals with a combination of vitamins A, C, and E showed significant improvement in the following cell-mediated responses (P12): (a) increased total number of circulating T-cells; (b) increased the number of helper ($CD4^+$) T-cells; (c) increased helper-to-cytotoxic cell ratio ($CD4^+$ to $CD8^+$); and (d) increased total number of lymphocytes in response to phytohemagglutinin. Others (M12), in a double-blind, placebo-controlled study of an elderly group supplemented with vitamin E, demonstrated: (a) increased

positive antigen response to delayed-type hypersensitivity skin test; (b) increased IL-2 levels; (c) increased mutagenic response to concanavalin A; (d) decreased synthesis of prostaglandin E_2; and (e) decreased plasma lipoperoxide levels.

In addition to these and numerous other studies involving vitamins C and E, and the carotenoids, Chandra (C6) studied a large group of independently living, healthy older people who were randomly assigned to receive placebo or a multivitamin–mineral supplement for 12 months. Compared with the placebo group, the supplemented individuals demonstrated: (a) increased T-cell subsets; (b) increased number of natural killer cells; (c) increased killer cell activity; (d) increased IL-2 levels; (e) improved antibody response to an antigenic stumulus; and (f) decreased number of infectious sick days during the year.

Immunity is also influenced to some extent by the endocrine system. Thus, as noted previously, mice remain immunologically normal during aging if they are supplemented with dehydroepiandrosterone sulfate (D2).

7.5. Neurodegenerative Disorders

Our understanding of the basic causal factors of the age-related cognitive and motor function impairments has been relatively rudimentary. However, numerous recent studies suggest that our understanding of these disorders is rapidly accelerating. In this regard, numerous recent reports suggest that the oxidative effects of free radicals may be important in several of these diseases. As noted in a recent review (K14), the central nervous system (CNS) is vulnerable to oxidative stress for the following reasons: (a) relative to its size, the metabolic rate is high in comparison to other tissues; (b) the levels of natural critical antioxidants (e.g., GSH) and protective enzymes (Cat, GPx, SOD) are relatively low; (c) the endogenous generation of ROS via several specific chemical reactions is possible; (d) levels of polyunsaturated fatty acids (C22:6; C:20:4) are relatively high; (e) CNS neuronal cells are nonreplicating, and once damaged, they may remain dysfunctional; and (f) the CNS neural network is readily disrupted.

Although there are several biochemical mechanisms whereby various reactive oxygen species (ROS) can be generated, evidence for their role in CNS disorders is generally indirect, as briefly outlined here.

1. Increased lipid peroxidation (LP), as determined by the measurement of thiobarbituric acid reactive substances (TBARS), is significantly elevated in various CNS regions of aging rats compared with young rats (R2).
2. The brain has only moderate activity levels of SOD and GPx and very low Cat activity (C12). Furthermore, the lower cerebral cortex, striatum, thalamus, hippocampus, and cerebellum have low GSH levels (R2).
3. Brain homogenates rapidly undergo peroxidation. Using ox-brain, LP can be inhibited by several iron-chelating agents (S29). In addition, vitamin E prevents oxidative damage to lymphocyte and brain band 3 proteins during aging (P17).

TABLE 5
NEURODEGENERATIVE DISORDERS AND OXIDATIVE STRESS

Amyotrophic lateral sclerosis (Lou Gehrig's disease)
Alzheimer's disease
Down's syndrome
Ischemia/reperfusion injury
Mitochondrial DNA disorders
Multiple sclerosis
Parkinson's disease

4. The substantia nigra and globus pallidus are rich in iron; yet cerebrospinal fluid has very little iron-binding capacity (H2). Moreover, any injury to the CNS may result in the release of iron, which by Fenton/Haber-Weiss chemistry, catalyzes free radical production. Iron has also been shown to accelerate the production of CNS lipofuscin (increased in Alzheimer's disease), while various antioxidants (vitamin E, GSH, selenium) reduce its concentration (E5).

5. Phagocytes, when activated, greatly increase their oxygen uptake with the production of $O_2^{-\bullet}$ and subsequent production of H_2O_2 and hypochlorous acid. In addition, glial cells generate NO•, which can react with $O_2^{-\bullet}$ to produce $ONOO^-$, a potent oxidant. NO• also reacts with proteins to produce nitrotyrosines (J3).

A brief discussion of Parkinson's disease will serve as an example for the role of ROS in various other neurodegenerative diseases (Table 5).

7.5.1. *Parkinson's Disease*

Parkinson's disease (PD), also known as paralysis agitans, is a progressive neurodegenerative disorder which increases in frequency after the age of 50 years. PD is characterized by a stooped posture, slowness of voluntary movement, progressively shortened but accelerated gait, rigidity, an expressionless facies, and occasionally a "pill-rolling" tremor. Pathologically, the only gross finding is pallor of the substantia nigra and locus ceruleus. Microscopically, these areas show a loss of pigmented neurons; Lewy bodies (eosinophilic intracytoplasmic inclusions) may be present in some of the remaining neurons.

The major clinical disturbances in PD are a result of dopamine depletion in the corpus striatum, resulting from neuronal loss in the substantia nigra. This decrease in dopamine production is the result of severe degeneration of the dopaminergic nigro-striatal pathway. Although the neurochemical and physiologic effects of decreased dopamine levels are well known, little information regarding the underlying cause of cell death or the mechanism by which these cells degenerate is known. Several theories (P15) have been proposed, including the following in which PD is (a) the result of a random process; (b) secondary to defective DNA repair mechanisms; (c) due to a specific genetic defect; (d) secondary to a viral

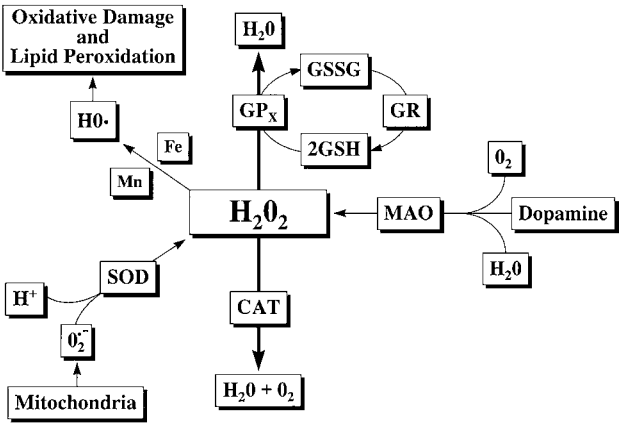

FIG. 3. Suggested schematic pathways involved in dopaminergic neurons CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; MAO, monoamine oxidase; SOD, superoxide dismutase; GSH and GSSG, reduced and oxidized glutathione, respectively; $O_2^{\bullet -}$, superoxide; HO•, hydroxyl radical; H_2O_2, hydrogen peroxide. (After P15.)

disorder; (e) due to a lack of a neurotrophic hormone; and (f) triggered by toxic compounds present in the environment. Although each of these hypotheses acounts for some of the abnormalities present in PD, none completely explains the cascade of events responsible for the initiation of PD, nor do any identify its cause. Rather, the pathogenesis of cell damage is very complex and involves several steps, each of which contributes to eventual cell death and decreased dopamine production (B1).

A wide variety of studies support a role for free radicals in PD. Thus, polyunsaturated fatty acid levels are reduced in the substantia nigra of patients dying of PD compared to other brain regions and to control tissue (Dexter *et al.,* 1989). Furthermore, basal malondialdehyde, a product of LP, is increased in parkinsonian nigra compared with other parkinsonian brain regions and corresponding control tissue. This study suggests that increased LP continues in the substantia nigra up to the time of death in those with PD owing to increased oxidative stress. Hence, the capacity to prevent the formation of or to neutralize free radicals after they are formed appears to be decreased in PD patients.

Figure 3 is a schematic representation of the major antioxidant systems in dopaminergic neurons.

7.5.1.1. *Antioxidant Enzymes.* Reports indicate that CNS catalase activity is very poor, and only moderate amounts of GPx and SOD are present (C12, H2). Since the copper–zinc SOD gene is preferentially expressed in the neuromelanin-containing neurons within the substantia nigra, these cells may be particularly vulnerable to oxidative stress and require a high SOD content to facilitate removal of superoxide radicals. Others have also reported that SOD and GPx levels are

further decreased in PD (A7, P13). Hence, elevated concentrations of hydrogen peroxide are presumably present in PD, thereby providing substrate for hydroxyl free radical formation via iron catalysis.

It is also of considerable interest that NADH-ubiquinone reductase (Complex 1) is reported to be significantly reduced in the substantia nigra of PD patients (S6). Importantly, this biochemical defect is the same as that produced in animal models by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (see below).

7.5.1.2. *Iron Storage and Iron-Binding Proteins.* Iron levels in the human brain are normally low at birth and increase rapidly over the next two or three decades, after which there is minimal further accumulation (H1). However, an increase of 31–35% in iron content in parkinsonian substantia nigra, compared to control tissue, has been reported (D8). This suggests that excessive free radicals may be produced in this brain region, thereby leading to the death of dopamine-producing cells. Furthermore, antioxidant enzymes and iron-binding proteins are normally very low in cerebrospinal fluid, further suggesting the possibility of increased metal-catalyzed hydroxyl radical production (C12).

7.5.1.3. *Glutathione (GSH) Tissue Levels.* Amino acid analysis of autopsied human brain showed that GSH levels are significantly lower in the substantia nigra than in other regions of the brain and is virtually absent in the substantia nigra of PD patients (P13). More recently, Sian and associates (S13) confirmed the decreased GSH levels of PD patients (40% less than control subjects). Conversely, oxidized glutathione (GSSG) was marginally increased. These latter authors concluded that the altered GSH/GSSG ratio in the nigra is consistent with the concept of significant oxidative damage in the pathogenesis of nigral neuronal death. Moreover, increased GSSG levels may be toxic (H2).

7.5.1.4. *Increased Dopamine Turnover.* Dopamine is normally stored in vesicles, where it is nonreactive. However, when released into the cytosol, it is metabolized in the mitochondria, primarily by type B monoamine oxidase (MAOb) to form H_2O_2.

$$\text{Dopamine} + O_2 + H_2O \xrightarrow{\text{MAO}} \text{3,4-dihydroxyphenylacetaldehyde} + H_2O_2 + NH_3$$

If dopamine turnover is increased, it may result in higher cytosolic levels and increased H_2O_2 concentrations. In this regard, the drug reserpine has been shown to increase dopamine release from the storage vesicles (S22). Furthermore, incubation of striatal synaptosomes with L-dopa, either with or without reserpine, resulted in increased levels of GSSG, suggesting increased H_2O_2 production and utilization of GSH in the presence of glutathione peroxidase (S21).

Dopaminergic neurons contain neuromelanin, a pigment composed of lipofuscin along with a complex mixture of polymers of the various catecholamines, metal ions, cysteine, and possibly other substances (A4). This waste pigment is presumed to be derived from the oxidation of dopamine and other catecholamines to produce quinones, semiquinones, and quinhydrones, some of which undergo

further oxidation to produce H_2O_2 (G13). These studies support the dopamine turnover theory, since the cells with the highest dopamine turnover have the highest cytosolic dopamaine levels and thereby produce the most neuromelanin.

7.5.1.5. *Environmental Toxins.* Several substances have been shown to produce a Parkinson's-like syndrome. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a by-product of various meperidine derivatives, has received considerable attention since the report by Langston *et al.* (L4). This neurotoxin has been studied in animal models and found to selectively destroy the dopaminergic substantia nigra. Further studies showed the MPTP was converted by MAOb to the toxic 1-methyl-4-phenylpyridinium cation (MPP^+). Following reduction by P450 reductase, the MPP free radical is formed, which reduces oxygen to $O_2^{-\bullet}$ (A4).

$$MPP^+ + e^- \rightarrow MPP\bullet + O_2 \rightarrow MPP^+ + O_2^{-\bullet}$$

MPTP toxicity has also been reported to be significantly increased by vitamin E deficiency (O1a). In addition, MPTP induces lipid peroxidation in the substantia nigra in vitamin E-deficient mice (A5); pretreatment with an MAO inhibitor prior to MPTP administration is protective from its parkinsonian-inducing effect (S16).

Oxidative stress has also been implicated in the potential neurotoxic effect of manganese (Mn). Thus, intoxication was first reported in miners involved in the extraction and processing of Mn (C19). Chronic Mn exposure was subsequently shown to produce a nonreversible PD-like syndrome (C17). Evidence suggests a free radical mechanism, since Mn has been shown to potentiate dopamine oxidation *in vitro* with the production of oxyradicals (D11). Furthermore, the accumulation of a dark pigment in the neurons of the caudate nucleus, which are normally melanin-free, is also suggestive of increased dopamine oxidation (C1). Mn also catalyzes the formation of $HO^\bullet$ from H_2O_2 produced during dopamine oxidation by MAO or Mn^{2+}.

7.5.1.6. *Therapeutic Supplementation.* If oxidative stress is involved in the pathogenesis of PD, then nonenzymatic CNS antioxidant defenses are of importance. Hence, therapeutic treatment with an oxidase inhibitor (e.g., deprenyl or selegiline; phenylisopropylmethylpropynylamine) and/or supplementation with vitamin E should slow disease progression. In this regard, uncontrolled studies suggest that these therapies may indeed be helpful in PD (F3, G10) and thereby extend the lives of these patients (B9). Indeed, a more recent multicenter study demonstrated the efficacy of deprenyl in reducing the rate of PD progression (P9).

7.6. Neuroendocrine Disorders

Since various neuroendocrine abnormalities increase with age, the neuroendocrine aging theory has received considerable attention. Nevertheless, some of these abnormalities are secondary phenomena, and others are due to disorders

which are readily controlled by hormone supplementation. As a result, it is often difficult to know which are age-dependent and which are due to secondary factors.

7.6.1. *Diabetes Mellitus*

Non-insulin-dependent diabetes mellitus (NIDDM), also known as "late-onset" or type II diabetes, affects over 12 million Americans, only about half of whom are aware of their disease (H6). Importantly, most diabetics have the disease for 4–7 years before it is diagnosed. About 50% of men and women aged 65–74 years demonstrate glucose intolerance (i.e., increased glucose levels but below that required for a definitive diagnosis); about 20% of these have NIDDM (C3, B16). NIDDM is a major cause of cardiovascular disease, stroke, renal failure, and blindness; it is also associated with accelerated aging.

Glucose tolerance is determined by the balance between insulin secretion and insulin action. The impairment of glucose tolerance in the elderly, known since the early 1920s (S20), generally begins in the third to fourth decade of life and continues throughout adulthood. Decreased glucose tolerance in response to a glucose challenge is characterized by a peak level of 10–14 mg/dl (0.6–0.8 mmol/l) per decade of life (J1). However, the fasting serum glucose is essentially unchanged with age, increasing no more than 1–2 mg/dl (0.06–0.11 mmol/l) per decade (Fig. 4). Figure 4 reveals several important points regarding glucose absorption and utilization with respect to age in normal people: (a) fasting serum glucose levels are minimally affected by age; (b) during the first 30 min the slope is similar for all ages, suggesting that absorption is unaffected by age; (c) glucose disposal (cell entry after about 75 min) is similar for all ages (lines are parallel); and

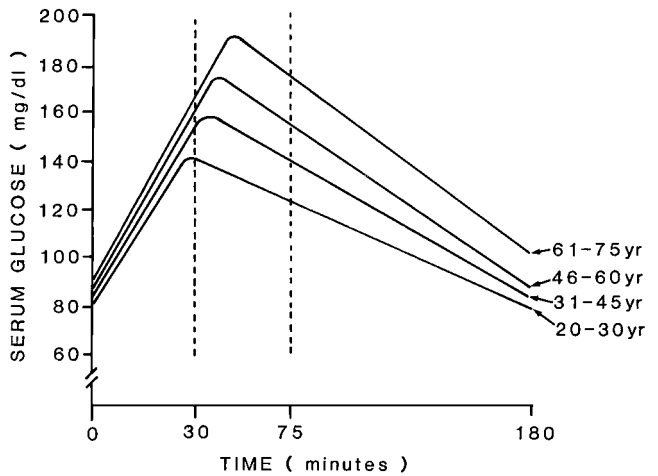

FIG. 4. Glucose tolerance response curves in normal people according to age. (After K13.)

(d) glucose levels peak at 50–75 min after oral glucose intake; serum levels increase 10–14 mg/dl per decade. Importantly, plasma insulin levels are also significantly higher than normal with increasing age; hence, the cells become insulin-resistant and glucose entry is slowed.

Is glucose intolerance secondary to aging, or to other factors such as heredity, obesity, inactivity, drug usage, concurrent disease, or a combination of these factors? Without question, family history and obesity have been repeatedly shown to be strong predisposing factors for NIDDM (Y2). In addition, increasing age and physical activity are risk factors for increased insulin resistance (B2), as is cigarette smoking (F1). A recent study examined familial and environmental variables associated with increased insulin levels in healthy premenopausal women (W14). Here, plasma insulin levels were related to weight, activity level, race, body fat distribution, and weight gain after age 20. They noted that increased plasma insulin levels were particularly related to weight gain. Numerous studies support the concept that insulin resistance precedes NIDDM. For example, Martin *et al.* (M8) studied 155 offspring of diabetic subjects for 6–25 years and found that those who eventually developed NIDDM were more insulin-resistant than those whose glucose levels remained normal. Moreover, in most cases insulin resistance began more than a decade before hyperglycemia developed.

The Baltimore Longitudinal Study on Aging (S12) studied 743 healthy men and women aged 17–92 years. They measured the body mass index, percentage body fat, physical activity, and calculated the maximum oxygen consumption. As indices of body fat distribution, the waist–hip ratio and subscapular triceps skinfold ratio were calculated. They concluded that (a) the differences between young (17–39 years) and middle-aged (40–59 years) groups were not significant, but the difference between these two groups and the elderly (60–92 years) were; and (b) fitness, fatness, and fat distribution accounted for the decline in glucose tolerance in the young and middle-aged groups; age remained a significant factor for further glucose intolerance in those 60 years and older. Others (P1) reported, however, no age-related changes in tissue insulin sensitivity or pancreatic beta-cell activity. These authors concluded that "age per se does not contribute to the deterioration of glucose tolerance when the insurgence of other age-related variables, eg, obesity, and physical activity, is precluded." Moreover, Helmrich and associates (H12) emphasized that increased physical activity had the greatest protective effect on those with the greatest risk, namely, the overweight and those with a family history of NIDDM. They also noted that physical activity (a) increases muscle mass; (b) retards fat accumulation; (c) lowers blood pressure; (d) opens blood vessels and speeds blood transit; (e) increases tissue insulin sensitivity; (f) lowers plasma insulin levels; and (g) lowers blood glucose levels. This and other reports (P3) emphasize that insulin resistance is related to decreased numbers of insulin cell receptors. Importantly, they noted that physical activity stimulates the production of insulin receptors. As a result, insulin sensitivity is increased and both plasma

insulin and glucose levels decrease. Two large epidemiologic studies also showed an inverse relationship between physical activity and NIDDM (M4, M5).

Although the basic pathogenesis of diabetes is not well understood, there is increasing evidence that free radicals are involved. For example, early studies showed that plasma lipid peroxide levels are consistently elevated in diabetic compared with nondiabetic (N9, S5) patients with NIDDM also have higher levels of plasma TBARS and conjugated dienes than normal (C15). In addition, NIDDM patients with retinopathy have increased plasma levels of TBARS (U1). Moreover, neutrophils from diabetics contain significantly higher levels of $O_2^{-\bullet}$ due to their decreased SOD activity (N4). Numerous other studies have been published on this topic, and it has been recently reviewed (K15).

7.6.2. *Pituitary Hormones*

Numerous studies have shown that pituitary function is negatively affected with aging. Although the causal relationship between aging and pituitary function is obscure, the production of various hormones, including growth hormone (GH), adrenocorticotropic hormone (ACTH), prolactin, and gonadotropins, decreases with advancing age.

The anterior pituitary releases GH in 6 to 8 pulsatile bursts over a 24 h period; the major portion is released just prior to deep sleep. Its secretion is controlled by hypothalamic peptides; GH-releasing hormone stimulates GH secretion, while somatostatin inhibits it. GH stimulates the synthesis of insulin-like growth factor-1 (IGF-1; somatomedin C) mainly, but not solely, in the liver. GH and IGF-1 receptors are widely scattered throughout the body, and both hormones exert important metabolic actions in various tissues, especially muscle and bone.

By 35–40 years of age, everyone begins to undergo progressive changes in body composition, including expansion of adipose tissue, decrease in lean muscle mass, and organ atrophy. The changes have been considered unavoidable and a natural part of aging. However, recent reports suggest that reduced availability of GH in late adulthood may contribute to these changes, since its concentration begins to decline in some as early as age 30 years and in most by age 50. Thus, about 70–80% of older people have reduced serum levels of IGF-1 (R8a) and essentially all elderly men and women have decreased GH levels compared with young adults (Z1). However, the current availability of recombinant GH may result in successful therapy in some individuals, especially the chronically malnourished and possibly those who are physically inactive.

Kaiser and associates (K3) reported that GH treatment in individuals with low GH blood levels improves caloric intake, increases muscle and bone mass and bone density, decreases fat tissue, increases the basal metabolic rate, thickens the skin, and improves the immune system. Rudman *et al.* (R8) studied the effects of supplemental GH on men 61–81 years of age with plasma IGF-1 levels less than 350 U/liter (reference values for young adult males are about 500–1500 U/liter).

The men were divided into two groups, one receiving 0.03 mg of GH/kg body weight three times a week for 6 months; the other group was not treated and served as controls. The group receiving GH increased their plasma IGF-1 levels to the young-adult range, increased the mean lean body mass by 8.89%, and decreased the adipose tissue mass by 7.1%; the control group remained at less than 350 U/liter, and there were no physical changes. Importantly, it has been emphasized that before widespread use of GH is accepted, the possibility of increased cancer rates, hypertension, arthralgia, acromegaly, or other disorders must be more fully investigated (E2). The relationship of GH to aging, nutrition, and its possible use in the frail elderly has been reviewed (K2).

7.6.3. *Gonadal Hormones*

7.6.3.1. *Androgens.* The androgens are a group of C-19 steroid hormones that exert a profound influence on the male genital tract. They are involved with the development and maintainance of secondary male characteristics—deepening of the voice at puberty, beard growth, sexual drive, body strength, and muscle and bone development. Although the adrenal glands secrete a small amount of testosterone, this hormone is primarily produced in the testes. Healthy males aged 20–30 years have plasma testosterone levels of 480–1270 ng/dl (A1). Using this reference interval, about 85% of "healthy" elderly men over 60 years are hypogonadal (R9).

Although estrogens are well known to be important in the pathogeneis of osteoporosis in women, the effect of testosterone on osteoporosis in men is less well studied. However, Stanley *et al.* (S24) reported a significant relationship between reduced plasma testosterone levels in elderly men and the occcurrence of minimal-trauma hip fractures. Others (M16) reported that elderly men supplemented with testosterone had increased right-hand muscle strength, osteocalcin levels, and hematocrit compared with the pretreatment data. Although these preliminary results suggest that testosterone replacement may have some positive effects, there are several possible negative effects: fluid retention, behavior disorders, prostatic hypertrophy, and cancer (R9). In addition, testosterone therapy may produce polycythemia, resulting in increased blood viscosity, stagnant blood flow, and vascular occlusion (K19).

7.6.3.2. *Estrogens.* The somatotropic axis in aging women declines in the same manner as in older men. Moreover, the favorable effects of GH on hyposomatotropic young adults with regard to muscle function, body composition, and quality of life are gender-neutral (R9). Although the major ovarian hormone is estradiol, estriol and 2-hydroxyestrone are also produced in significant amounts (over 30 estrogens have been identified). In addition, small quantities of estrogens are produced in the adrenals and testes.

The major clinical effects of estrogen deficiency are coronary heart disease and osteoporosis. Estrogen supplements in postmenopausal women have been directed mainly at slowing the process of osteoporosis. Nevertheless, atherosclerotic heart disease is the leading cause of death in women, accounting for more than 30%

of deaths. It is also important to note that atherosclerotic cerebrovascular disease is the third leading cause of death. In this regard, women supplemented with estrogens have half the risk of acute myocardial infarction as women not taking the hormone (S4). The hormonal treatment of postmenopausal women has been reviewed (B5).

7.6.4. *Adrenal Cortical Hormones*

ACTH, produced in the anterior pituitary, stumulates the synthesis of cortisol and various androgens by the adrenal cortex. ACTH release is, in turn, controlled by corticotropin-releasing factor (CRF), a peptide produced in the hypothalamus. Both CRF and A CTH are released in a diurinal pulsatile fashion which is maximum in the early morning hours (peaks at about 6–8 AM). Cortisol, the major adrenal glucocorticoid, stimulates the apetite and sense of well-being; it also helps maintain blood glucose levels. All disorders secondary to cortisol excess are classified under the general title of Cushing's syndrome.

The mineral corticoids influence renal sodium and potassium exchanges and play a major role in the regulation of extracellular volume. Aldosterone, the major steroid hormone in this group, is secreted primarily through the control of the renin-angiotensin system. Renin, a proteolytic enzyme produced in the juxtaglomerular renal cells, is released as a result of decreased kidney perfusion pressure and negative sodium balance. Upon release, it catalyses the conversion of angiotensinogen to angiotensin I, which is rapidly converted to angiotensin II by angiotensin-converting enzyme (particularly rich in the lungs). Angiotensin II stimulates the adrenal zona glomerulosa cells to produce aldosterone, which promotes sodium reabsorption and potassium excretion by the renal tubules.

The urinary excretion of aldosterone decreases with increasing age. If sodium intake is unrestricted, aldosterone is decreased by about 50%. In addition, although sodium restriction results in an increase in aldosterone secretion, those over 60 years of age show urinary increases that are still only 30–40% of that in young adults.

Overall, other adrenal androgens also show a progressive decrease in urinary excretion in both men and women. Thus, the mean 17-ketosteroid urine levels of elderly people are about 50% of those in young adults. This is primarily secondary to decreased dehydroepiandrosterone (DHEA) and androsterone production. In men, about one-third of the daily 17-ketosteroids are of testicular origen, the remainder being mainly from the adrenals. Androstenedione is a prehormone for testosterone.

The serum levels of both DHEA and androstendione decline markedly with age in both men and women (R9). DHEA and its sulfate (DHEAS), the major circulating form of DHEA, are considered to be hormone markers for aging, including cross-sectional and longitudinal linear decreases, as well as having significant stability of individual differences over time. In addition, the administration of DHEAS has been shown to enhance immune function in aging mice (D2, D4). In elderly women, serum DHEAS levels are directly correlated with bone density

(N11, T1). DHEAS also reportedly has an antiobesity effect (N8). More recently, Lane and associates (L3) reported that, as with humans, male and female rhesus monkeys exhibit a steady, age-related decrease in serum DHEAS concentration. They also noted that the proportional age-related loss of DHEAS in rhesus monkeys is more than twice the decline rate observed in humans. More importantly, they found that caloric restriction slows the postmaturational decline in serum DHEAS levels, thereby providing the first evidence that nutritional intervention has the potential to alter certain aspects of postmaturation aging in a long-lived species.

Other adrenal disorders are relatively uncommon in the elderly. For example, both ACTH and cortisol synthesis remain constant throughout life. Thus, in general, blood levels are not altered during aging (W13).

7.6.5. *Parathyroid Hormone*

The major function of the parathyroid hormone (PTH; parathormone) is to regulate the concentration of extracellular calcium. PTH is heterogeneous and circulates as an intact polypeptide and as fragments. The major circulating biologically active peptide is intact PTH, which is rapidly cleared from the circulation (half-life less than 10 min). The N-terminal portion is responsible for its biologic activity.

PTH secretion is regulated by the serum ionized calcium concentration via a negative feedback mechanism. PTH secreting benign or malignant parathyroid tumors result in primary hypercalcemia (primary hyperparathyroidism). In those with chronic renal disease, the parathyroid glands become hyperplastic, PTH synthesis is increased, and hypercalcemia follows (secondary hyperparathyroidism). Conversely, absent or nonfunctioning glands result in hypoparathyroidism, decreased PTH synthesis, and hypocalcemia.

The incidence of hypercalcemia and primary hyperparathyroidism increases with age. Interestingly, older patients with primary hyperparathyroidism often present to their physican with depression or acute organic psychosis. The major complaint, in up to 50% of cases, is a lack of initiative. Most patients are diagnosed with hyperparathyroidism following the discovery of hypercalcemia on a screening chemistry profile. Tibblin *et al.* (T7) reported that 1.5% of 1129 elderly patients were hypercalcemic secondary to a parathyroid tumor. The authors of an earlier report, after screening 15,903 subjects, concluded that "at least 3%" suffered from "asymptomatic hypercalcemia" (C10). Others (S18) concluded that hyperparathyroidism requiring clinical attention was about 3% for women but less than 1% in men. These and other studies indicate that regular screening of the elderly for hypercalcemia is important and will lead to the diagnosis of hyperparathyroidism in about 2–3% of elderly patients, most of whom will have a parathyroid tumor.

7.6.6. *Thyroid Hormones*

7.6.6.1. *Hypothyroidism.* Decreased thyroid function is significantly more common in older than younger people. Unfortunately, hypothyroidism is often not recognized in the elderly because the signs and symptoms may be atypical or

mild, thought to be merely signs of "old age," or interpreted as mild psychiatric features. Although early studies suggested that decreased thyroid activity was "natural" and secondary to "aging," more recent studies indicate that thyroid hormone alterations are secondary to disease rather than the aging process (K1, S33).

Livingston and co-workers (L8) studied a group a geriatric patients, mainly men, for thyroid disease by measuring their serum levels of thyroid-stimulating hormone (TSH), thyroxine (T_4), free T_4, triiodothyronine (T_3), and the T_4 and T_3 indexes and compared them with a similar group of ambulatory outpatients. Hypothyriodism was present in 7.8% of male inpatients compared with 0.7% of outpatient males. Although the number of women studied was small, hypothyroidism was present in 17% of the female inpatients but only 2.4% of the female outpatients. Overall, 9.4% of the geriatric inpatients were hypothyroid. If those with decreased thyroid reserve were included (subclinical hypothyroidism), then 14.1% were abnormal.

7.6.6.2. *Hyperthyroidism.* Although once considered very uncommon, hyperthyroidism in the elderly occurs considerably more often than previously thought. Moreover, age-based studies consistently indicate that of all patients with hyperthyroidism, 10–30% are elderly. An early study, which included all new admissions to a geriatric service, showed a prevalence of 3.6% (P5). Other survey studies reported an overall incidence that varied from 0.7% to 6% (F6). In elderly women, aged 70–79 years, hyperthyroidism occurred half as often as hypothyroidism (S33). Thus, both hypo- and hyperthyroidism are relatively common in the elderly. As a result, all middle-aged adults and older, especially women, should be regularly screened for these disorders.

8. Summary

Although philosophers and scientists have long been interested in the aging process, general interest in this fascinating and highly important topic was minimal before the 1960s. In recent decades, however, interest in aging has greatly accelerated, not only since the elderly form an ever-increasing percentage of the population, but because they utilize a significant proportion of the national expenditures. In addition, many people have come to the realization that one can now lead a very happy, active, and productive life well beyond the usual retirement age.

Scientifically, aging is an extremely complex, multifactorial process, and numerous aging theories have been proposed; the most important of these are probably the genomic and free radical theories. Although it is abundantly clear that our genes influence aging and longevity, exactly how this takes place on a chemical level is only partially understood. For example, what kinds of genes are these, and what proteins do they control? Certainly they include, among others, those that regulate the processes of somatic maintenance and repair, such as the stress–response systems.

The accelerated aging syndromes (i.e., Hutchinson-Gilford, Werner's, and Down's syndromes) are genetically controlled, and studies of them have decidedly

increased our understanding of aging. In addition, *C. elegans* and *D. melanogaster* are important systems for studying aging. This is especially true for the former, in which the *age-1* mutant has been shown to greatly increase the life span over the wild-type strain. This genetic mutation results in increased activities of the antioxidative enzymes, Cu-Zn superoxide dismutase and catalase. Thus, the genomic and free radical theories are closely linked. In addition, trisomy 21 (Down's syndrome) is characterized by a significantly shortened life span; it is also plagued by increased oxidative stress which results in various free radical-related disturbances. Exactly how this extra chromosome results in an increased production of reactive oxygen species is, however, only partially understood.

There is considerable additional indirect evidence supporting the free radical theory of aging. Not only are several major age-associated diseases clearly affected by increased oxidative stress (atherosclerosis, cancer, etc.), but the fact that there are numerous natural protective mechanisms to prevent oxyradical-induced cellular damage speaks loudly that this theory has a key role in aging [the presence of superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase, among others; various important intrinsic (uric acid, bilirubin, -SH proteins, glutathione, etc.) and extrinsic (vitamins C, E, carotenoids, flavonoids, etc.) antioxidants; and metal chelating proteins to prevent Fenton and Haber-Weiss chemistry]. In addition, a major part of the free radical theory involves the damaging role of reactive oxygen species and various toxins on mitochondria. These lead to numerous mitochondrial DNA mutations which result in a progressive reduction in energy output, significantly below that needed in body tissues. This can result in various signs of aging, such as loss of memory, hearing, vision, and stamina. Oxidative stress also inactivates critical enzymes and other proteins. In addition to these factors, caloric restriction is the only known method that increases the life span of rodents; studies currently underway suggest that this also applies to primates, and presumably to humans. Certainly, oxidative stress plays an important role here, although other, as yet unknown, factors are also presumably involved.

Exactly how the other major theories (i.e., immune, neuroendocrine, somatic mutation, error catastrophe) control aging is more difficult to define. The immune and neuroendocrine systems clearly deteriorate with age. However, are these changes primary causes of aging, or are they secondary to other, more basic processes? For example, many deficient immune functions can be reversed by improved diet (increased protein intake) and antioxidant supplementation (Zn, vitamins C, E, beta-carotene and other carotenoids, flavonoids, etc.). Moreover, certain neuroendocrine disorders, common in the elderly (hyper- and hypothyroidism, diabetes mellitus, etc.), are clearly not due to the aging process per se. On the other hand, growth hormone (GH) decreases with age. As such, there is a change in body composition as one ages (increased fat accumulation, decreased muscle mass, abnormal bone turnover, etc.); however, these can be significantly reversed with GH supplementation. The concentration of dehydroepiandrostenedione also decreases

with age; again, supplementation reverses some of the age-related disturbances, including immune system deficiencies.

There are numerous areas for future research to more clearly understand the basic factors involved in aging, as well to clarify the relationship between aging and the age-associated degenerative diseases, including the following, among others.

1. Both clinical and basic studies of oxidative stress are needed to further clarify their role in aging and age-related diseases. Here, it is important to more clearly define the dietary needs of various antioxidants to slow aging and lower disease incidence. Previous research has focused on slowing the rates of adverse reactions; future efforts should probably be directed toward decreasing free radical initiation rates.
2. Studies need to be designed to more clearly explain the specific biochemical processes whereby caloric restriction results in increased longevity.
3. In-depth studies of the accelerated aging syndromes, especially Down's syndrome, will further clarify the relationship between the genomic and free radical theories.
4. Studies are needed to understand the age-associated deficiencies in the immune and neuroendocrine systems. More information is sorely needed as to which of these, if any, are etiologically related to aging per se, and which are secondary phenomena.
5. Studies of cytokines, various kinases, etc., will clarify the complex area of cell signaling, which varies from cell adhesion to differentiation, and apoptosis. As progress continues, useful clinical methods will probably be developed whereby various age-related diseases can be controlled and/or prevented.
6. A more thorough understanding of the role of genetics, oxidative stress, diet, environmental toxins, and the endocrine system is clearly important to understand why most cancers occur after age 70 years.
7. Although glycosylated proteins increase significantly with age, are they primarily involved in aging and age-related diseases or are they merely biochemical markers of aging and have no primary role in the aging process? Future studies should resolve this important question.
8. There is a need to increase our knowledge with respect to the basic changes that occur as a result of exercise, and how it improves various biologic systems.

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CYSTATIN C—PROPERTIES AND USE AS DIAGNOSTIC MARKER

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1. Protease–Antiprotease Equilibria

The healthy human body might be described schematically as being composed of several dynamic equilibria. All diseases might be considered as disturbances in one or more of these dynamic equilibria. The balance between protein production and degradation is one of these equilibria, which are crucial to health, and several systems for control of both production and degradation are known. Degradation of

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proteins is brought about by proteolytic enzymes, proteases, which, based on their catalytic mechanisms, can be assorted to four major classes: the serine-, cysteine-, aspartic-, and metallo-proteases. The activities of human serine proteases of, *inter alia,* the coagulation, fibrinolytic, and complement systems have for several decades been known to be regulated by a large number of proteinaceous serine protease inhibitors of, for examples, the Kunitz, Kazal, and serpin types. In contrast, relatively few inhibitors of the other three major classes of proteases have been described. However, extensive research during the last 15 years has identified a group of human inhibitors for papain-like cysteine proteases comprising at least 11 different inhibitors. This group of inhibitors constitutes a new superfamily of human proteins, which is named the cystatin superfamily. The present discourse will focus on one of the most well characterized inhibitors, cystatin C, and provide some information on its biochemical properties, its role in normal and abnormal physiological processes, as well as on its use as a diagnostic marker.

2. Biological Roles of Papain-like Cysteine Proteases

A major part of the cysteine proteases are evolutionary related to the structurally well-defined cysteine protease papain and are therefore called papain-like cysteine proteases (B6, B7, R4). The human cysteine proteases of this family are mainly localized in lysosomes and play key roles in the intracellular degradation of proteins and peptides (cathepsins B, H, and L) (B4, G3). They also participate in the proteolytic processing of prohormones (D5) and proenzymes (T1) and seem to be involved in the penetration of normal tissues by macrophages (I1, R5) as well as by several types of malignant cells (C5, P8, S8, S9). Such proteases (e.g., cathepsin K) are also pivotal in the degradation and remodeling of bone (D2, D4, G1, L5) and may be instrumental in controlling the MHC class II trafficking in dendritic cells (cathepsin S) (P6).

Papain-like cysteine proteases are present not only in animals and plants but also in bacteria, fungi, and protozoa (R4), and the proteases of, for example, *Entamoeba histolytica, Trypanosoma congolese, Leischmania mexicana, Trichomonas vaginalis,* and *Plasmodium falciparum* seem to be involved in the replication, migration, and food digestion of these organisms (B24, C2, L17, N8, R4, R9, R10). Proteolytic processing of polyproteins by virally encoded proteases, *inter alia* cysteine proteases (R4) is required for replication of several viruses (K7, L4, O2).

3. The Cystatin Superfamily of Inhibitors of Papain-like Cysteine Proteases

The occurrence of proteins which inhibit papain-like cysteine proteases has been known at least since 1946, when Grob demonstrated that blood serum (G7) and

isolated fractions thereof (G8) inhibit papain. In retrospect, it might be concluded that Grob detected the inhibitory capacities of the plasma proteins cystatin C, low- and high-molecular-weight kininogens, and α_2-macroglobulin. However, it was not until the 1980s and 1990s that the majority of the multitude of presently identified cysteine protease inhibitors were isolated and structurally and functionally characterized (A1, B1, B5, F2, F3, I2, N3, N4, R6, R7, T9). The most comprehensive class of cysteine protease inhibitors is the cystatin superfamily of inhibitors. The name "cystatin" was originally given to a low-molecular-weight inhibitor of papain isolated from chicken egg white (B5, B8), since this inhibitor was a *cyst*eine protease *in*hibitor. The middle section, *stat,* of cystatin might also be considered as alluding to the capacity of cystatins to arrest the activity of cysteine proteases, since *stat-* is the supine stem of the Latin verb *sisto,* meaning "to arrest." In 1986 it was agreed that "cystatins" should be used to denote the entire superfamily of cysteine protease inhibitors structurally and functionally related to chicken cystatin (B3).

Cystatins have been demonstrated not only in higher organisms but also in a large number of plants and lower organisms including rice seeds, *Drosophila,* and *Candida albicans* (D3, K6, T8).

4. The Human Cystatins

The amino acid sequence of human cystatin C (Fig. 1) was determined in 1981 (G11) and, since it did not display any significant homology with the sequences of any protein of the superfamilies known then, it was evident that it belonged to a new protein superfamily. Retrospectively, it can be seen that the amino acid sequence of cystatin C was the first sequence of a cystatin to be determined (B5). The function of cystatin C as an inhibitor of cysteine proteases was identified about 2 years later, when the sequence of chicken cystatin was determined, showing that the two proteins had a sequence identity of 44% (B2, B25, S2, T10). Studies during the two last decades have identified 10 further human cysteine protease inhibitors, which display strong sequence homologies to cystatin C and chicken cystatin and, consequently, belong to the human cystatin superfamily. The human cystatin family therefore presently comprises 11 identified proteins (Table 1). Two of these, cystatins A and B, form the family 1 cystatins and are mainly, or exclusively, intracellular proteins, while cystatins C, D, E, F, S, SA, and SN are mainly extracellular and/or transcellular proteins and constitute the family 2 cystatins. The family 3 cystatins, high- and low-molecular-weight kininogen, contain three cystatin domains each (S1) and are mainly intravascular proteins, which, in addition to being inhibitors of cysteine proteases, are precursor molecules for the production of the vasoactive kinins, bradykinin and kallidin (M9). High-molecular-weight kininogen also participates in the contact-phase activation of the endogenous blood coagulation cascade (M9). The three cystatin domains (called D1–D3)

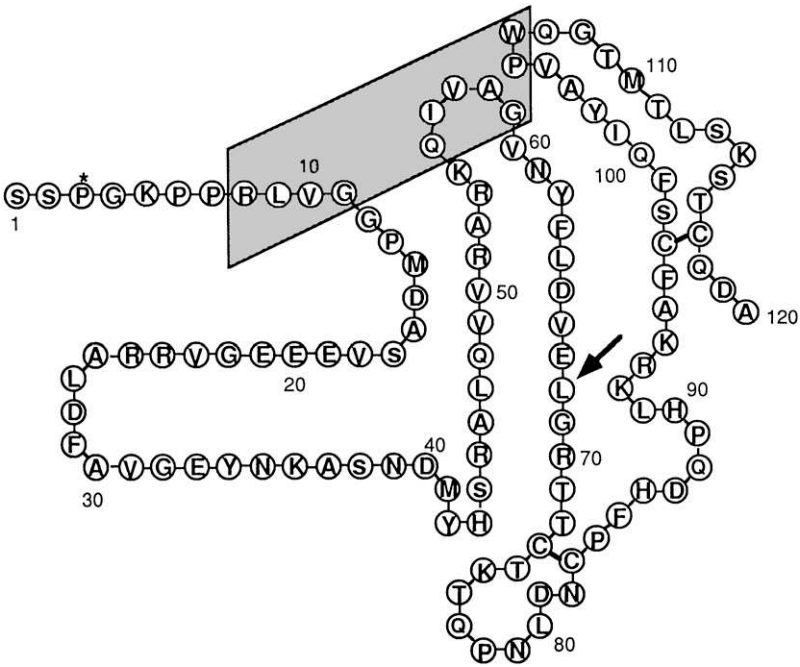

FIG. 1. Amino acid sequence and schematic structure of human cystatin C. The shaded area marks the inhibitory site for papain-like cysteine proteases, which does not overlap with the inhibitory site for mammalian legumains comprising, *inter alia,* the Asn^{39} residue. The arrow indicates the Leu^{68} residue, which is replaced with a Gln residue in the cerebral hemorrhage producing cystatin C variant. The asterisk marks the Pro^{3} residue, which is partly hydroxylated.

of the kininogens inhibit not only papain-like cysteine proteases (D2 and D3) but also calpains (D2). Figure 2 displays a schematic illustration of the evolutionary relationships among all known inhibitory active cystatins and kininogen cystatin domains.

5. Identification of Target Enzymes for Cystatins

All human cystatins are assumed to have major biological roles as inhibitors of one or more target proteases of human and/or nonhuman origin. Identification of target proteases of biomedical relevance is, however, difficult. Only few examples of clear-cut identifications of target proteases for inhibitors are known, and these identifications usually rely on experiments by Nature and not by Man. Two examples are the identifications of granulocyte elastase as a target enzyme

TABLE 1
THE HUMAN CYSTATIN SUPERFAMILY

Family 1 (intracellular cystatins)	Family 2 (extracellular and/or transcellular cystatins)	Family 3 (intravascular cystatins)
Cystatin A	Cystatin C	LMW-kininogen
Cystatin B	Cystatin D	HMW-kininogen
	Cystatin E	
	Cystatin F	
	Cystatin S	
	Cystatin SA	
	Cystatin SN	

Synonyms: cystatin A (R7): Acid cysteine proteinase inhibitor, epidermal SH-proteinase inhibitor, Stefin A; cystatin B (R6): neutral cysteine proteinase inhibitor, Stefin B; cystatin C: See Section 8 in main text; cystatin E: cystatin M (S10); cystatin F: leukocystatin (H2); cystatins S, SA, and SN (I2): salivary cysteine proteinase inhibitor; cystatin SN: cystatin SU (A1) (Grubb, A., unpublished results); LMW- and HMW-kininogens (M9): α-cysteine proteinase inhibitor, α-thiol proteinase inhibitor.

for α_1-antitrypsin revealed by the pathophysiology of α_1-antitrypsin deficiency (emphysema and liver cirrhosis) (O3) and of thrombin as a target enzyme for antithrombin revealed by the pathophysiology of antithrombin deficiency (thromboembolism) (C3). No serious disease states in which the major pathophysiological events can be ascribed to the lack of the specific inhibitory capacity of a cystatin have so far been described. Individuals with complete deficiencies of high- and low-molecular-weight kininogens have been described, but they do not seem to suffer from any serious pathophysiological abnormality (C7). Mutations in the genes for cystatins B and C produce progressive myoclonus epilepsy (B12, L1, L2, P2, P3) and hereditary cerebral hemorrhage (A6, G2, P1), respectively, but the present knowledge of the pathophysiology of these disorders does not allow the identification of target proteases for any of these cystatins. Although therefore no unequivocal identification of target proteases for human cystatins has been feasible yet, it is possible to select, or exclude, candidate target proteases for the different cystatins based upon theoretical considerations (B13) as well as upon comparisons with known examples of target proteases for, *inter alia,* those serine protease inhibitors mentioned above. One requirement that must be fulfilled by a cystatin to render it a biomedical significant inhibitor of a potential target enzyme is that the molar concentration of the cystatin must be higher than that of the active protease at the site in the body where the protease is released. A second requirement is that the equilibrium constant for dissociation of a protease–cystatin complex must be low to secure a negligible amount of free proteolytic activity at equilibrium. Finally, the association rate constant for the formation of the protease–cystatin complex

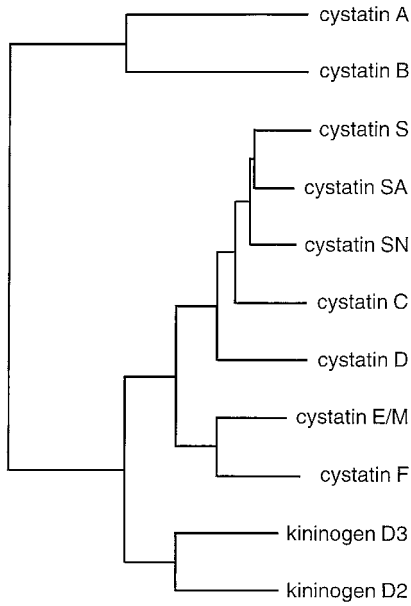

FIG. 2. Schematic diagram of the evolutionary relationships among all known inhibitory active human cystatins and kininogen cystatin domains. The phylogenetic tree was constructed using "Growtree," included in the GCG software package (version 8.1; Genetic Computer Group, Inc., Madison, WI). The phylogenic distances were obtained according to the method of Kimura (K4). The reconstruction of the tree was done by the unweighted-pair group method using arithmetic averages.

must be high so that any free protease activity is rapidly quenched. Since all human cystatins display a unique set of equilibrium and association rate constants when tested against a limited collection of cysteine proteases, each cystatin has a unique inhibitory spectrum (Table 2). However, these inhibitory spectra are usually overlapping, and it is quite probable that some cystatins might share target proteases.

6. Distribution of Cystatins in Body Fluids

The distribution in body fluids of the different cystatins is remarkably different (Fig. 3). For example, while cystatin C is present in appreciable amounts in all investigated body fluids, cystatins S, SN, and SA are virtually confined to saliva, tears, and seminal plasma (A1). Cystatin D is present only in saliva and tear fluid (A1, F3). In some body compartments, e.g., spinal fluid, cystatin C represents more than 90% of the total molar concentration of cysteine protease

TABLE 2
EQUILIBRIUM CONSTANTS FOR DISSOCIATION OF COMPLEXES BETWEEN HUMAN CYSTATINS AND CYSTEINE PROTEASES K_i (nM)

Cystatin	Cathepsin B	Cathepsin L	Cathepsin S	Cathepsin H	Papain
A	8.2	1.3	0.05	0.31	0.019
B	73	0.23	0.07	0.58	0.12
C	0.25	< 0.005	0.008	0.28	0.00001
D	>1000	25	0.24	0.28	1.2
E	32	—	—	—	0.39
F	>1000	0.31	—	—	1.1
S	—	—	—	—	108
SN	19	—	—	—	0.016
SA	—	—	—	—	0.32
L-kininogen	600	0.017	—	0.72	0.015

Data from (A9, L11, N3, N4, S1).

inhibitors, while in other compartments, e.g., blood plasma, it only represents a few percent of the total cysteine protease inhibitory capacity (A1). Moreover, the total cysteine protease-inhibiting capacity varies also considerably among different body compartments. For example, the total papain-inhibiting capacity of blood plasma is about 12 μmol/L, while that of cerebrospinal fluid is less than 1 μmol/L (A1). Since each body fluid displays a unique set of cystatins, it is also clear that the different body fluids display unique cysteine protease inhibitory spectra, although these partially overlap, like the inhibitory spectra of the individual cystatins. Table 3 shows that different cysteine proteases with different catalytic properties are controlled by separate cystatins in the various body fluids.

7. Additional Functions Attributed to Cystatin C

In addition to being an inhibitor of papain-like cysteine proteases, cystatin C has recently been shown be an efficient inhibitor of some of the cysteine proteases of another family of cysteine proteases, called the peptidase family C13, with human legumain as a typical enzyme (C6). Human legumain has, like cathepsin S, been proposed to be involved in the class II MHC presentation of antigens (M3). It has also been shown that the cystatin C inhibitory site for mammalian legumain does not overlap with the cystatin C inhibitory site for papain-like cysteine proteases (Fig. 1) and that the same cystatin C molecule therefore is able to simultaneously inhibit one cysteine protease of each type (A10).

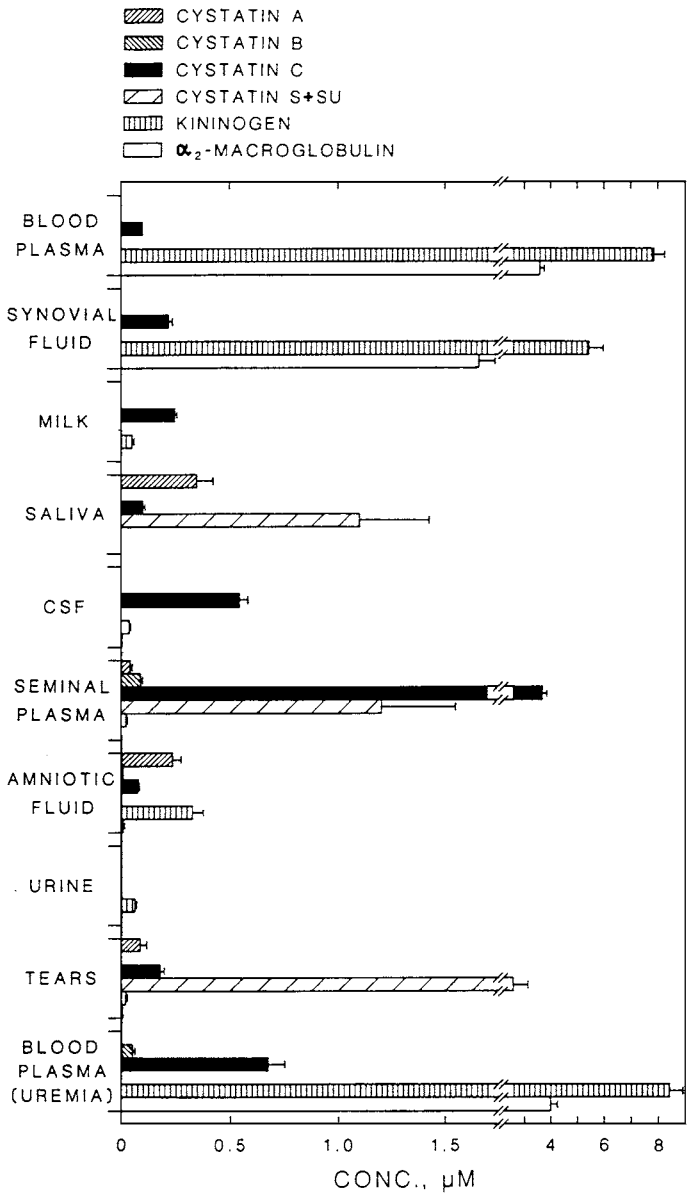

FIG. 3. Molar concentrations of cystatins and α_2-macroglobulin in 10 human body fluids.

TABLE 3
HALF-LIVES ($t_{1/2}$, S) OF FREE (A) HUMAN CATHEPSIN B AND (B) PAPAIN ON INTERACTIONS WITH CYSTATINS IN HUMAN BODY FLUIDS

	$t_{1/2}$ (s)				
	Cystatin A (12,000)	Cystatin B (12,000)	Cystatin C (15,000)	Cystatin S/SN (15,000)	Kininogen (60,000)
(a) Cathepsin B					
Blood plasma	> 740	> 1900	3.5	> 650	6.3
Synovial fluid	> 740	> 1900	1.7	> 650	9.1
Milk	> 740	> 1900	1.5	> 650	960
Saliva	39	> 1900	3.8	4.6	> 5900
Cerebrospinal fluid	> 740	> 1900	0.69	> 650	1200
Seminal plasma	320	190	0.10	4.3	1500
Amniotic fluid	59	2100	4.9	> 650	150
Urine	> 740	> 1900	> 9.9	> 650	760
Tears	150	3900	2.1	2.0	3000
Blood plasma (uremia)	> 740	330	0.56	> 650	5.9
(b) Papain					
Blood plasma	> 11	> 2.1	0.74	> 5.5	0.006
Synovial fluid	> 11	> 2.1	0.36	> 5.5	0.008
Milk	> 11	> 2.1	0.31	> 5.5	0.84
Saliva	0.6	> 2.1	0.80	0.039	> 5.2
Cerebrospinal fluid	> 11	> 2.1	0.14	> 5.5	1.1
Seminal plasma	4.9	0.21	0.021	0.036	1.3
Amniotic fluid	0.90	2.4	1.0	> 5.5	0.13
Urine	> 11	> 2.1	> 2.1	> 5.5	0.67
Tears	2.2	4.3	0.43	0.017	2.6
Blood plasma (uremia)	> 11	0.37	0.12	> 5.5	0.005

Calculations were made according to the equation $t_{1/2} = \ln 2/(k_{+1} \times [I])$, where k_{+1} denotes association rate constant and $[I]$ is inhibitor concentration. The molecular weights used in the calculations are given in parentheses. Cystatin S was assumed to have the same k_{+1} value as cystatin SN. A k_{+1} value of 1.0×10^7 M^{-1} s^{-1} for cystatin C was used for calculations of the $t_{1/2}$ for free papain at the cystatin C concentrations found in the fluids. Data from (A1).

Cystatin C has also been suggested to possess biological functions, presumably unrelated to its protease-inhibiting potential. For example, human cystatin C has been described to play a regulatory role in inflammatory processes, *inter alia,* by down-regulation of the phagocytosis-associated respiratory burst reaction displayed by polymorphonuclear neutrophils as well as by down-regulation of their chemotactic response (L6, L7). Chicken cystatin, and thus probably human cystatin C, has also been shown to up-regulate nitric oxide release from peritoneal macrophages (V1). However, these suggested additional functions of cystatin C remain to be confirmed.

8. Previous Designations for Cystatin C

The protein now generally designated with the functional name cystatin C was first discovered in 1961 (B26, C8, M1) and its function as a cysteine protease inhibitor was thus unidentified for more than 20 years. As a consequence, several trivial names were used for the same protein, and it is important to know these for complete retrieval of data on cystatin C by bibliographic studies. The following trivial names can be found in the literature: γ-trace, post-γ-globulin, gamma-CSF, post-gamma protein, γ_c-globulin, δ aT, and high alkaline fraction (HAF) (B26, C1, C8, H9, H10, K5, L3, M2, M4, S11).

9. Structure of Human Cystatin C and Its Concentration in Body Fluids

The complete amino acid sequence of the single polypeptide chain of human cystatin C was determined in 1981 (G11) and later corroborated by identification and sequencing of the corresponding cDNA (Fig. 4) (A3) and gene (A5, A7).

The three-dimensional structure of cystatin C is not yet determined, although some crystallographic data are available (K8), but it can be presumed that it is similar to that described for the homologous protein chicken cystatin (B19) and this has, at least partially, been confirmed by NMR studies (E1). A schematic structure for human cystatin C is given in Fig. 1.

Studies of truncated forms of cystatin C (A8) and of cystatin C variants produced by site-directed mutagenesis (B18, H4, H5, H6, L12, M7), as well as identification of sequence similarities between all cystatins, have indicated that the inhibitory center of cystatin C for papain-like cysteine proteases comprises three peptide segments, Arg^{8}-Leu^{9}-Val^{10}-Gly^{11}, Gln^{55}-Ile^{56}-Val^{57}-Ala^{58}-Gly^{59}, and Pro^{105}-Trp^{106}. Peptidyl derivatives, structurally based upon the aminoterminal segment of the inhibitory center, have been synthesized and shown to be efficient inhibitors of cysteine proteases (G9, H3). Some of these peptidyl derivatives have also displayed antibacterial and antiviral properties (B16, B17).

Table 4 displays some of the physicochemical properties of cystatin C as well as its normal concentration in body fluids.

10. Serum/Plasma Cystatin C as a Marker for Glomerular Filtration Rate (GFR)

No investigations have demonstrated that the diagnostic usefulness of the serum level of cystatin C is different from that of the plasma level of cystatin C. The term

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                                                                    22
                                                  GGGCGCAGCGGGTCCTCTCTAT

                                                                    95
CTAGCTCCAGCCTCTCGCCTGCGCCCCACTCCCCGCGTCCCGCTCCTAGCCGACC ATG GCC GGG CCC CTG CGC
                                                  Met Ala Gly Pro Leu Arg
                                                  -26                 -21

                                                                   155
GCC CCG CTG CTC CTG CTG GCC ATC CTG GCC GTG GCC CTG GCC GTG AGC CCC GCG GCC GGC
Ala Pro Leu Leu Leu Leu Ala Ile Leu Ala Val Ala Leu Ala Val Ser Pro Ala Ala Gly
                                                                     -1

                                                                   215
TCC AGT CCC GGC AAG CCG CCG CGC CTG GTG GGA GGC CCC ATG GAC GCC AGC GTG GAG GAG
Ser Ser Pro Gly Lys Pro Pro Arg Leu Val Gly Gly Pro Met Asp Ala Ser Val Glu Glu
1                                                                   20

                                                                   275
GAG GGT GTG CGG CGT GCA CTG GAC TTT GCC GTC GGC GAG TAC AAC AAA GCC AGC AAC GAC
Glu Gly Val Arg Arg Ala Leu Asp Phe Ala Val Gly Glu Tyr Asn Lys Ala Ser Asn Asp
                                                                    40

                                                                   335
ATG TAC CAC AGC CGC GCG CTG CAG GTG GTG CGC GCC CGC AAG CAG ATC GTA GCT GGG GTG
Met Tyr His Ser Arg Ala Leu Gln Val Val Arg Ala Arg Lys Gln Ile Val Ala Gly Val
                                                                    60

                                                                   395
AAC TAC TTC TTG GAC GTG GAG CTG GGC CGA ACC ACG TGT ACC AAG ACC CAG CCC AAC TTG
Asn Tyr Phe Leu Asp Val Glu Leu Gly Arg Thr Thr Cys Thr Lys Thr Gln Pro Asn Leu
                                                                    80

                                                                   455
GAC AAC TGC CCC TTC CAT GAC CAG CCA CAT CTG AAA AGG AAA GCA TTC TGC TCT TTC CAG
Asp Asn Cys Pro Phe His Asp Gln Pro His Leu Lys Arg Lys Ala Phe Cys Ser Phe Gln
                                                                   100

                                                                   515
ATC TAC GCT GTG CCT TGG CAG GGC ACA ATG ACC TTG TCG AAA TCC ACC TGT CAG GAC GCC
Ile Tyr Ala Val Pro Trp Gln Gly Thr Met Thr Leu Ser Lys Ser Thr Cys Gln Asp Ala
                                                                   120

                                                                   593
TAG GGGTCTGTACCGGGCTGGCCTGTGCCTATCACCTCTTATGCACACCTCCCACCCCCTGTATTCCCACCCCTGGAC

                                                                   672
TGGTGGCCCCTGCCTTGGGGAAGGTCTCCCCATGTGCCTGCACCAGGAGACAGACAGAGAAGGCAGCAGGCGGCCTTTG

                                                                   751
TTGCTCAGCAAGGGGCTCTGCCCTCCCTCCTTCCTTCTTGCTTCTCATAGCCCCGGTGTGCGGTGCATACACCCCCACC

                          777
TCCTGCAATAAAATAGTAGCATCCCC
```

FIG. 4. Nucleotide and deduced amino acid sequence of a cDNA clone encoding human precystatin C. Numbering of the nucleotide sequence starts at the first nucleotide and proceeds in the 5′ to 3′ direction. Amino acid numbering begins with residue 1 of the mature protein (G11) and the putative hydrophobic signal sequence thus comprises residues −26 to −1. The Kozak initiation consensus and the polyadenylation signal are underlined.

TABLE 4
PHYSICOCHEMICAL PROPERTIES OF HUMAN CYSTATIN C AND ITS CONCENTRATION IN BODY FLUIDS

Polypeptide chains: One, with 120 amino acid residues
Glycosylation: None
Molecular mass: 13,343 Da (nonhydroxylated); 13,359 Da (hydroxylated proline residue at position 3)
Isoelectric point: 9.3
Electrophoretic mobility: γ_3 (agarose gel electrophoresis at pH 8.6)
Extinction coefficient: 1.22×10^4 (mol^{-1} liter cm^{-1}) = 9.1 (280 nm, 1%, 1 cm)
Amino acid sequence: SSPGK PPRLV GGPMD ASVEE EGVRR ALDFA VGEYN KASND MYHSR ALQVV RARKQ IVAGV NYFLD VELGR TTCTK TQPNL DNCPF HDQPH LKRKA FCSFQ IYAVP WQGTM TLSKS TCQDA
Disulfide bonds: Between residues 73 and 83 and between residues 97 and 117
Gene location: Chromosome 20 at p.11.2
DNA sequence: The nucleotide sequence data are available from the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under accession number X52255
Half-life: About 20 min (experimentally determined for human cystatin C in rat plasma. The similarity in distribution volume and renal clearance between human cystatin C and acknowledged markers of human glomerular filtration, i.e., iohexol and ^{51}Cr-EDTA, suggests that the substances are eliminated at the same rate in humans with a half-life of approximately 2 h in individuals with normal renal function)
Concentrations in body fluids of healthy adults (mg/liter; mean and range):
  Blood plasma: 0.96; 0.57–1.79
  Cerebrospinal fluid: 5.8; 3.2–12.5
  Urine: 0.095; 0.033–0.29
  Saliva: 1.8; 0.36–4.8
  Seminal plasma: 51.0; 41.2–61.8
  Amniotic fluid: 1.0; 0.8–1.4
  Tears: 2.4; 1.3–7.4
  Milk: 3.4; 2.2–3.9

"serum cystatin C" will therefore in the following paragraphs, unless otherwise indicated, also refer to the plasma level of cystatin C.

10.1. PRODUCTION OF CYSTATIN C

Determination of the structure of the human cystatin C gene and its promoter has demonstrated that the gene is of the housekeeping type, which indicates a stable production rate of cystatin C by most nucleated cell types (A7). The presence of a hydrophobic leader sequence in precystatin C (Fig. 4) strongly indicates that the protein normally is secreted (A3, A7). Indeed, immunochemical and Northern blot studies of human tissues and cell lines have shown that cystatin C and/or its mRNA is present in virtually all investigated cell types (A7, J1, L9, L10, S7). Likewise, investigations of the production of cystatin C by human cell lines in culture have displayed that nearly all cell lines investigated secrete cystatin C

(B14, C4, N4) (Abrahamson, M., personal communication). Studies of the serum level of cystatin C in large patient cohorts have failed to correlate the serum level to any pathophysiological state besides those affecting the glomerular filtration rate, which also is compatible with a stable secretion of cystatin C from most human tissues (G12, K9, N2, S6). However, some reports have described that stimulation of macrophages *in vitro* down-regulates their secretion of cystatin C (C4, W1), but inflammatory conditions are not generally associated with decreased serum levels of cystatin C.

10.2. Catabolism of Cystatin C

Blood plasma proteins with molecular masses below 15–25 kDa are generally almost freely filtered through the normal glomerular membrane and then almost completely reabsorbed and degraded by the normal proximal tubular cells. This should consequently also be true for cystatin C with a molecular mass of 13 kDa and with a probable ellipsoid shape with axes of about 30 and 45 Å (B19). Indeed, studies of the handling of human cystatin C in the rat have shown that the plasma renal clearance of cystatin C is 94% of that of the generally used GFR-marker ^{51}Cr-EDTA and that cystatin C thus is practically freely filtered in the glomeruli (T4). At least 99% of the filtered cystatin C was found to be degraded in the tubular cells. Figure 5 shows the rat plasma concentration of intact human ^{125}I-cystatin C and ^{51}Cr-EDTA relative to the initial concentrations after intravenous injection. Figure 6 displays the plasma disappearance of cystatin C in normal and nephrectomized rats and indicates that the renal plasma clearance of cystatin C is about 85% of the total plasma clearance (renal + extrarenal). When the GFR of a set of rats was variably lowered by constricting their aortas above the renal arteries, the renal plasma clearance of cystatin C correlated strongly with that of ^{51}Cr-EDTA (Fig. 7), with a linear regression coefficient of 0.99 and with the y intercept not being statistically different from 0 (T4). This observation clearly implied an insignificant peritubular uptake of cystatin C. Immunohistochemical and Northern blot studies of human kidneys have also strongly indicated that human cystatin C normally is degraded by proximal tubular cells after its passage through the glomerular membrane (J1).

10.3. Clinical Use of Serum Cystatin C as a GFR Marker

The knowledge that most human tissues produce cystatin C and that it, being a low-molecular-mass protein, is removed from plasma by glomerular filtration, suggested that its plasma, or serum, level might be a potentially good marker for GFR. Early investigations demonstrated that serum cystatin C, indeed, was a marker for GFR, at least as good as serum creatinine in the populations investigated (G12, S6). These studies also showed that the serum cystatin C level was a

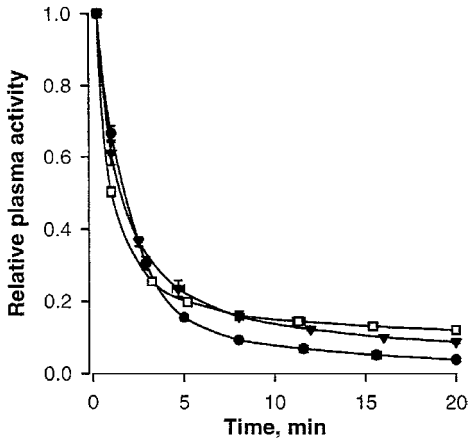

FIG. 5. Plasma concentration of intact ^{125}I-cystatin C(•), ^{51}Cr-EDTA (□) and ^{131}I-aprotinin(▼) relative to the initial plasma concentration after intravenous injection in 12 rats. Error bars show ±1 SEM, when larger than the symbols. Aprotinin is a 6.5-kDa microprotein with a pI of 10.5.

better GFR marker than the serum levels of the other low-molecular-mass proteins investigated, β_2-microglobulin, retinol-binding protein, and complement factor D (G12, S6). However, in these early studies the cystatin C concentration was determined by enzyme amplified single radial immunodiffusion (L15). This procedure is slow, requiring at least 10–20 h, and has a relatively high coefficient of variation (about 10%), which decrease the usefulness of the obtained serum cystatin C value

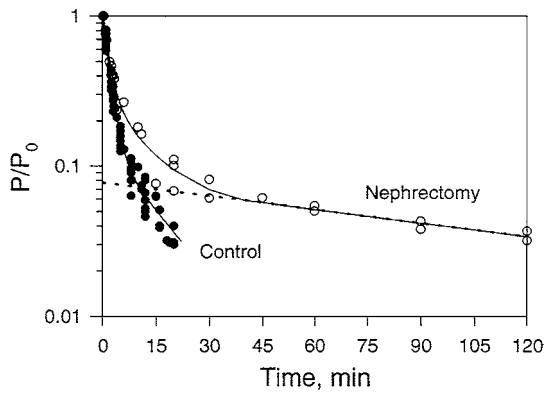

FIG. 6. Plasma disappearance of intact ^{125}I-cystatin C in nephrectomized (○) and control (•) rats. The monoexponential regression line of the plasma concentration, P/P_0, against the time, t, between 60 and 120 min in nephrectomized rats is indicated by a dotted line.

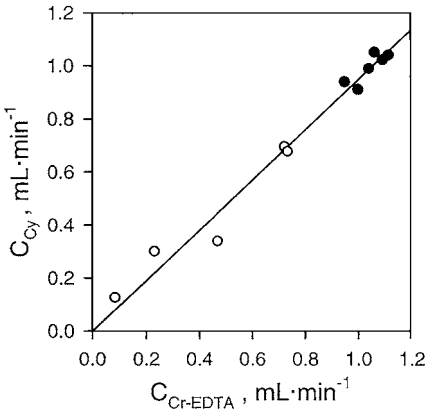

FIG. 7. Renal plasma clearance of ^{125}I-cystatin C (C_{cy}) compared to that of ^{51}Cr-EDTA ($C_{Cr-EDTA}$) in rats with normal glomerular filtration (•), and in rats with renal blood flow reduced to 25–50% of control by constricting the aorta above the renal arteries (○). The clearance measurements were completed 2.5–6.0 min after tracer injection. $C_{cy} = 0.944 \times C_{Cr\text{-}EDTA}$. $r = 0.989$.

as a GFR marker in the clinical routine. The development, about 10 years later, of automated particle-enhanced immunoturbidimetric methods, which were rapid as well as more precise, therefore significantly improved the possibility of using serum cystatin C as a GFR marker in clinical routine work (K9, N2). So did the introduction of a sandwich enzyme immunoassay for the determination of serum cystatin C (P4). Since the automated particle-enhanced immunoturbidimetric procedure for determination of serum cystatin C was introduced in 1994, the vast majority of all studies of the use of serum cystatin C as a GFR marker have relied upon the commercially available version of this procedure. A commercially available automated particle-enhanced immunonephelometric method has also recently been described (E2, F1, M10).

Serum creatinine is ubiquitously used as an indicator for GFR despite the knowledge that a substantial proportion of patients with reduced GFR display serum creatinine levels within the normal range and that even a 50% reduction of GFR not infrequently is associated with a normal concentration of serum creatinine (L8, P5, S3). The usefulness of serum creatinine as a marker for GFR is limited by, *inter alia,* the influence of an individual's muscle mass on the production rate of creatinine (H8, P5, S5), by the tubular secretion and reabsorption of creatinine, by the dietary intake of creatine and creatinine, and by analytical difficulties (P5). These significant limitations in the use of serum creatinine as an indicator for GFR has made it of interest to search for better indicators for GFR. Several recent studies have compared the use of serum cystatin C and creatinine as markers for GFR as determined by "golden standard" procedures based upon determinations of the

plasma clearance of injected low-molecular-mass substances such as Cr^{51}-EDTA, ^{99m}Tc-DTPA, and iohexol. These studies have indicated either that serum cystatin C is a better GFR marker than serum creatinine, particularly for individuals with small to moderate decreases in GFR, in the so-called creatinine-blind GFR range, or that the two parameters are of equal value as GFR indicators (B20, B22, H7, J4, K9, N2, N6, P4, P7, R2, R3, S12, S13, T6, V2). Figures 8 and 9 illustrate one

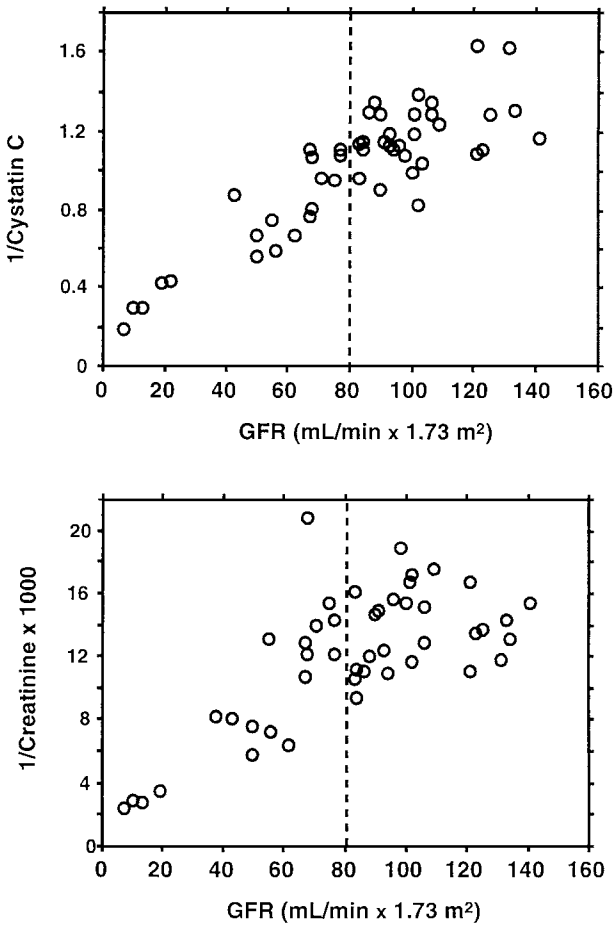

FIG. 8. Correlation between glomerular filtration rate and (top) reciprocal serum cystatin C (mg/liter) or (bottom) reciprocal serum creatinine (μmol/liter) in 27 male and 24 female patients;- - - - is the lower reference limit for glomerular filtration rate. The difference in the diagnostic capacity of serum cystatin C and serum creatinine to identify patients in the "creatinine-blind" area with a glomerular filtration rate of 60–80 ml/min $\times 1.73$ m^2 is obvious in this investigation of a population of patients with various renal conditions.

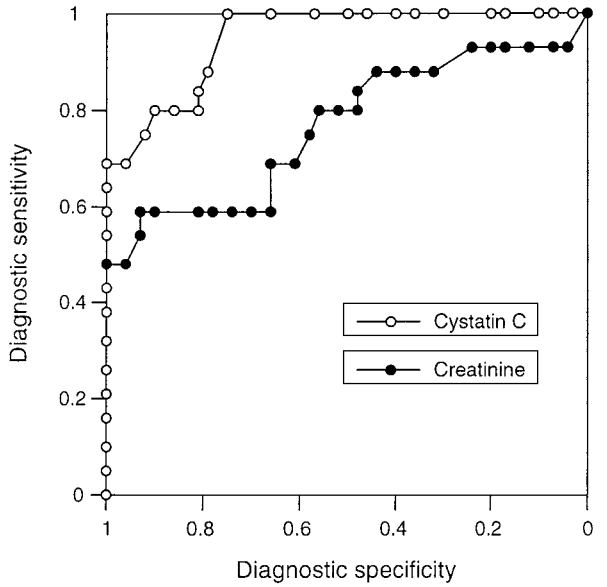

FIG. 9. Nonparametric ROC plots for the diagnostic accuracy of serum cystatin C and creatinine in distinguishing between normal and reduced glomerular filtration rate ($\geq$ and $<$ 80 ml/min$\times$1.73 m^2, respectively) in 51 patients with various renal conditions (the same population as the one displayed in Fig. 8).

study indicating the usefulness of serum cystatin C in the creatinine-blind GFR range. Nearly all investigations have emphasized that serum cystatin C, in contrast to serum creatinine, is uninfluenced by gender and muscle mass. Several studies indicate that virtually the same reference values might be used for serum cystatin C for males and females from 1 year of age, up to 50 years of age, when the age-related decline in GFR becomes significant (B20, B22, H7, L14, N6, R3).

Serum cystatin C has also been described to be a better predictor than serum creatinine of fasting total homocysteine serum levels, probably because of its closer correlation to GFR (B23, N7).

10.4. REFERENCE VALUES FOR SERUM CYSTATIN C

Establishment of reference values of general use requires general availability of a well-defined calibrator. The availability of such a calibrator also facilitates accreditation of procedures for quantitative determination of the corresponding analyte. Recombinant human cystatin C can easily be produced and isolated and used for establishing reliable calibrators (A2, D1). A first step toward an international calibrator for cystatin C has been taken by the production of a solution of recombinant human cystatin C of high purity, determining the concentration of this

TABLE 5
REFERENCE INTERVALS FOR SERUM CYSTATIN C

Adults (male + female; 20–50 years): 0.70–1.21 mg/liter[a]
Adults (male + female; above 50 years): 0.84–1.55 mg/liter[a]
Children (male + female; 1–18 years): 0.70–1.38 mg/liter[b]
Children (male + female; 1–16 years): 0.63–1.33 mg/liter[c]

[a]Reference (N6).
[b]Reference (B20).
[c]Reference (H7).

solution by quantitative amino acid analysis and spectrophotometric analysis and then diluting it with cystatin C-free human serum to physiological concentrations (K9). Based upon the use of such a cystatin C calibrator and a commercially available automated particle-enhanced immunoturbidimetric method, several studies of reference values for serum cystatin C, comprising populations of both adults and children have been published (B20, B22, H7, K9, L14, N6). The results for adults have generally shown that there is no sex differences for any age group and that the well-known decrease in GFR with age is mirrored by an increase in the cystatin C level with age (Fig. 10). However, the decrease of GFR with age is slow before 50 years of age, and it has been suggested (N6) that it is sufficient for most practical purposes to use separate reference values only for the age groups 20–50 and above 50 years of age (Table 5).

The results for children (Fig. 11) have demonstrated that the cystatin C level, in contrast to the creatinine level, was constant for children beyond the first year and with no difference between the sexes (B20, B22, H7, R3). The recommended reference values for children beyond 1 year of age were virtually identical to those

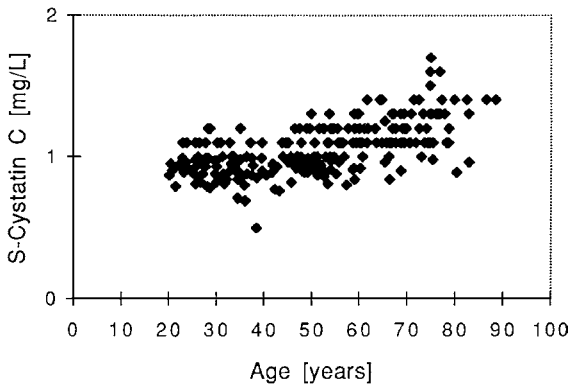

FIG. 10. Serum cystatin C in relation to age in a population of 121 healthy women and 121 healthy men, 20–89 years old. No sex difference was found.

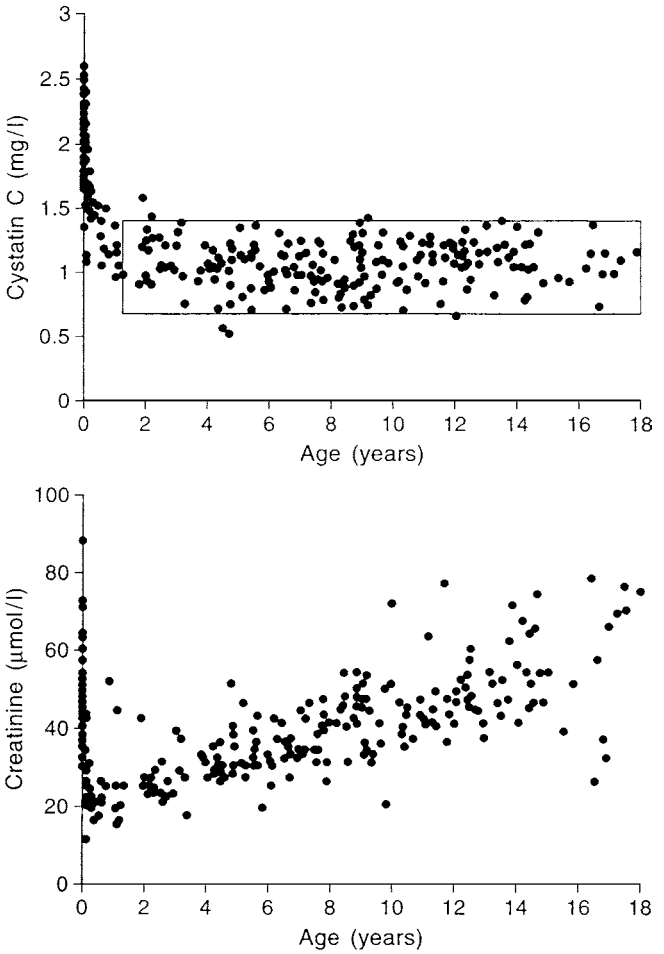

FIG. 11. Serum cystatin C (top) and creatinine (bottom) in relation to age in a population of 258 children, 1 day–18 years old, and without evidence of kidney disease. The boxed area represents the serum cystatin C reference interval for children over 1 year.

recommended for adults 20–50 years of age, so it might be justifiable to use the same values for both age groups (Table 5). It should be emphasized, however, that efficient international use of serum cystatin C as a GFR marker requires the establishment of a generally available international cystatin C calibrator. The relatively large variation in the reference values for serum cystatin C suggested so far (R1, R3) is most probably due to the use of different, often ill-described, cystatin C calibrators.

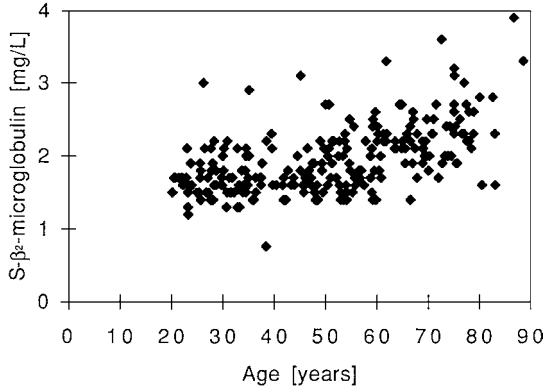

FIG. 12. Serum β_2-microglobulin in relation to age in a population of 121 healthy women and 121 healthy men, 20–89 years old. No sex difference was found, but the influence of age on glomerular filtration rate and thus on serum β_2-microglobulin is evident.

Since all plasma proteins with molecular masses below 15–25 kDa are almost freely filtered through the normal glomerular membrane, their serum concentrations in a person will be strongly influenced not only by their production rates, but also, and often to at least the same extent, by the person's GFR. Thus, when the production rates of such low-molecular-mass proteins are of interest for evaluation of biomedical processes, the diagnostic specificity of the ratios between the serum concentrations of such proteins and cystatin C can be expected to be higher than that of the serum concentration of each specific protein. For example, the influence of age on GFR and thus on the serum β_2-microglobulin level (Fig. 12) is not seen for the ratio between the serum levels of β_2-microglobulin and cystatin C (Fig. 13) (N6). This ratio might thus be a more specific marker for cell proliferation than the isolated serum β_2-microglobulin level.

10.5. LIMITATIONS IN THE USE OF SERUM CYSTATIN C AS A MARKER FOR GFR

When the first automated particle-enhanced immunoturbidimetric method was introduced, it was claimed to be undisturbed by hypertriglyceridemia (K9). However, widespread clinical use of it since then has demonstrated that the results are influenced by sample turbidity caused by, *inter alia,* chylomicronemia, which might produce both falsely low and high values for serum cystatin C (Nilsson-Ehle, P., submitted manuscript; Grubb, A., unpublished results). The influence of chylomicronemia on the analytical procedure might partly explain the relatively large biological variation reported for serum cystatin C (K3) as well as the outcome of some studies which have failed to show any advantage of serum cystatin C over serum creatinine as a GFR marker. Studies of the biological variation of serum

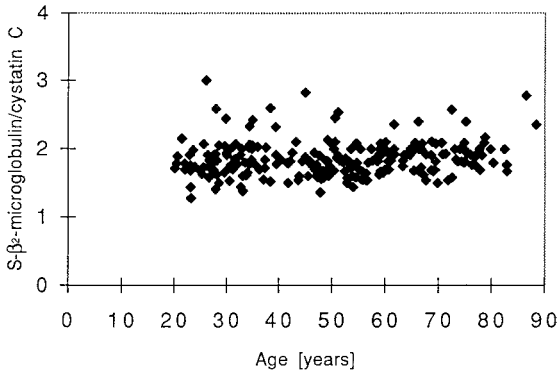

FIG. 13. Serum β_2-microglobulin/cystatin C ratio in relation to age in a population of 121 healthy women and 121 healthy men, 20–89 years old. The serum β_2-microglobulin/cystatin C ratio is, in contrast to the serum levels of each separate protein, not influenced by the age-dependent decrease in glomerular filtration rate.

cystatin C, using nonturbid samples, indicate that it is comparable to that of serum creatinine (N5) (Nilsson-Ehle, P., submitted manuscript).

It has also been observed that some, but not all, rheumatoid factors interfere in some of the presently used particle-enhanced immunoturbidimetric methods and produce erroneously high results (Grubb, A., unpublished results).

It should also be emphasized that although the precision of the automated particle-enhanced immunometric methods is higher than that of the enzyme-amplified single radial immunodiffusion first used to quantitate serum cystatin C, it is still lower than that for most methods for determination of serum creatinine. Moreover, the data for the intraindividual variation of serum cystatin C strongly indicate that a higher precision of the method would markedly improve the clinical usefulness of serum cystatin C determinations according to the criterion of Cotlove (N6).

10.6. RECOMMENDED USE OF SERUM CYSTATIN C AS A GFR MARKER

Available evidence indicates that serum cystatin C is a better marker for GFR than serum creatinine, particularly for the identification of an initial small decrease in GFR, i.e., in the so-called creatinine-blind GFR range. The most efficient use of this knowledge in clinical practice requires that quantitative methods of good precision, undisturbed by sample turbidity, are used. At least some of the presently available particle-enhanced immunometric methods seem to fulfil the first criterion of acceptable precision, but nonturbid fasting samples should preferably be used until new methods undisturbed by turbidity are developed.

Combined use of serum cystatin C and creatinine produces the best possible information on GFR in situations where more accurate, but invasive and more expensive, clearance determinations cannot be performed for biomedical or economic reasons. If both serum cystatin C and creatinine are within the relevant reference limits, the risk of missing a decrease in GFR will be minimal.

In situations when the GFR has been determined by accurate invasive clearance methods, either serum cystatin C or creatinine might be used to follow changes in GFR. However, as long as the precision of the methods for creatinine determination is higher than that of the methods for cystatin C determination, there are presently no valid reasons to use serum cystatin C for this purpose, particularly since creatinine determinations generally are cheaper than cystatin C determinations.

It should be observed that there is a clear possibility that some renal disease processes might differently affect the filtration of cystatin C, a positively charged 13,000-Da molecule, and the filtration of creatinine, an uncharged 113-Da molecule. Indeed, although serum cystatin C has been described to be a better GFR marker than serum creatinine for patients with renal transplants (R8), transplanted and untransplanted patients with the same reduction in inulin clearance have been reported to display different levels of serum cystatin C (B21). Future studies might therefore show that the use of several GFR markers, differing in physicochemical properties, might be optimal for the noninvasive monitoring of kidney function.

11. Urine Cystatin C as a Marker for Proximal Tubular Damage

Since cystatin C is a low-molecular-mass protein, it is almost freely filtered through the normal glomerular membrane and then nearly completely reabsorbed and degraded by the normal proximal tubular cells. The urine level of cystatin C is therefore low in healthy individuals. The mean urine cystatin C in an adult healthy population has been estimated to 0.095 mg/liter or 8.0 mg/mol creatinine with a range of 0.033–0.29 mg/liter or 5.2–13.3 mg/mol creatinine (L15). Proximal tubular dysfunction results in impaired reabsorption of low-molecular-mass proteins and increased urinary excretion of cystatin C can therefore be used as a sensitive marker for disease processes affecting proximal tubular cells (A1, C10, K1, K2, L15, L16, T5). However, the practical use of the urine level of cystatin C as a marker for tubular dysfunction is hampered by two facts. First, the upper reference limit for the urine concentration of cystatin C is so low (L15) that presently available rapid and cheap immunochemical methods cannot be used to demonstrate the small increases in the urinary levels of cystatin C which signal initial, and often reversible, stages of proximal tubular dysfunction. Second, cystatin C is proteolytically degraded in a significant proportion of native urine samples

(G13, T7). Although these two difficulties can be overcome by the use of sensitive, more sophisticated quantitative methods and the addition of preservatives to urine samples (A1, T2), respectively, this state of affairs argues against the practical use of urine cystatin C as a marker for proximal tubular dysfunction (G13). The urine level of free protein HC (*alias* α_1-microglobulin) seems to be a more practical marker for proximal tubular dysfunction as the upper reference limit is high (about 8 mg/liter or 700 mg/mol creatinine) and since protein HC is stable in most native urine samples (G13, T2, T3). But it cannot be excluded that future studies will display that the diagnostic potentials of urine cystatin C and urine protein HC might differ in some disease states, since cystatin C is positively charged at the pH range of urine, while protein HC is negatively charged.

12. Cystatin C and Cerebral Hemorrhage

Cystatin C amyloid deposits have been demonstrated to be associated with two types of brain hemorrhage. One type is the dominantly inherited form of cerebral hemorrhage, which is caused by a mutation in the cystatin C gene and displays amyloid deposition of the cystatin C variant but no co-deposition of amyloid β-protein. The other type comprises the cerebral hemorrhage conditions, which are connected with cerebral deposition of amyloid β-protein and display co-deposition of wildtype cystatin C in the amyloid.

12.1. Cystatin C and Hereditary Cystatin C Amyloid Angiopathy

The Icelandic physician Árni Árnason described in 1935 several families suffering from high incidences of cerebral hemorrhage affecting young adults and showed that the disease displayed autosomal dominant inheritance (Fig. 14) (A12). Afflicted individuals generally suffered from their first cerebral bleeding before 40 years of age and subsequently from recurrent multifocal cerebral hemorrhages. It was not possible to identify carriers of the trait in the affected families, but Gudmundsson *et al*. showed in 1972 that the disease was associated with amyloid deposits mainly located in the media of the walls of cerebral medium-sized arteries and suggested "hereditary cerebral hemorrhage with amyloidosis (HCHWA)" as a suitable name for the syndrome (G14). The N-terminal sequence of a main component of the amyloid fibril was determined in 1983 and found to be identical to the sequence of cystatin C starting at residue 11 (C9). Subsequent immunohistochemical studies corroborated that cystatin C was a major component of the amyloid deposits (L13) and showed that also vessels and other tissues outside the central nervous system contained cystatin C amyloid deposits (B9, L13). In 1984 immunochemical quantitation of the level of cystatin C in cerebrospinal fluid demonstrated that virtually all carriers of the trait for the disease, independent of whether they

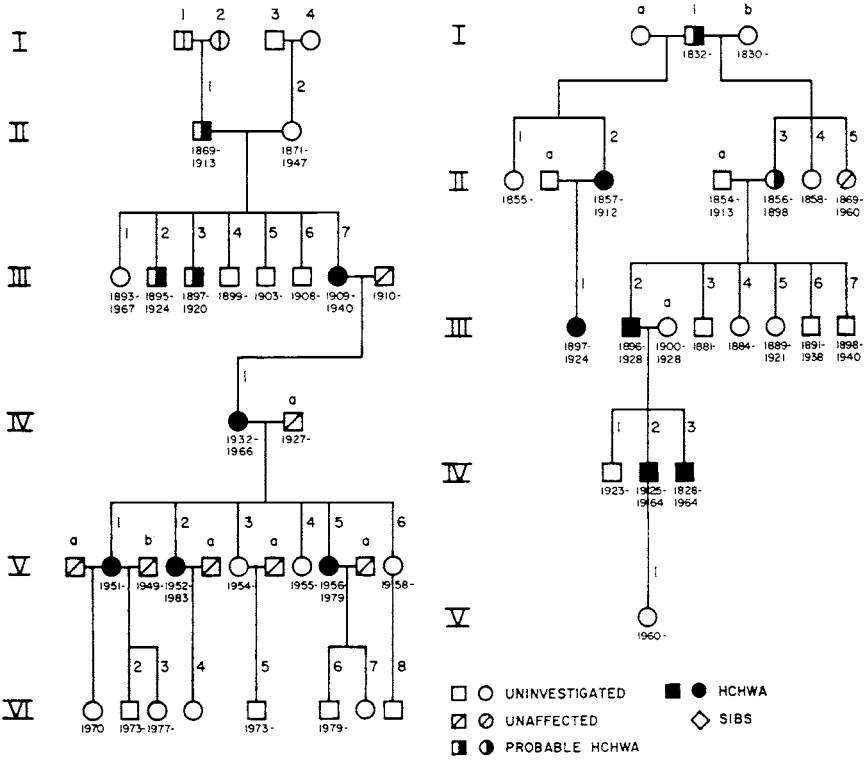

FIG. 14. Transmission in two families of the allele causing hereditary cystatin C amyloid angiopathy, HCCAA (also called hereditary cerebral hemorrhage with amyloidosis, HCHWA).

had suffered from their first cerebral hemorrhage or not, could be identified by their low cystatin C concentration (G10, J3). Not only did this observation allow the identification of healthy carriers of the disease trait, but also suggested that the pivotal pathophysiological process was an abnormal metabolism of cystatin C. Subsequent studies showed that the complete sequence of the cystatin C polypeptide chain deposited as amyloid in one individual with brain hemorrhage differed in one position from the sequence previously determined for cystatin C isolated from a Swedish patient without cerebral hemorrhage (G2). It was therefore suggested that a point mutation responsible for this amino acid substitution, a glutamine residue replacing a leucine residue at position 68 of the cystatin C polypeptide chain, could constitute the genetic background to the disease, although it also might represent a disease-unrelated polymorphism (G2). The potential disease-causing mutation would, according to the sequence later determined for a full-length cDNA encoding cystatin C, destroy an *Alu* I-cleavage site in the cystatin C gene (A3). This

observation allowed the identification of a restriction fragment length polymorphism permitting identification of carriers of the mutation (P1) as well as the construction of a simple and rapid polymerase chain reaction (PCR)-based procedure for the same purpose (A6). When individuals from seven afflicted families were studied, it was observed that all individuals suffering from early-onset cerebral hemorrhage carried one mutated allele, while most healthy relatives and all unrelated persons did not (A6). The results of these studies therefore permitted the conclusion that the investigated mutation causes the disease and that all patients are heterozygous (A6, P1).

12.1.1. *Nomenclature*

Two designations for the disease originally described by Árnason are presently used, *viz.,* hereditary cystatin C amyloid angiopathy (HCCAA) and "hereditary cerebral hemorrhage with amyloidosis, Icelandic" (HCHWA-I). The first mentioned might be the preferred one, since it indicates the amyloid-forming protein and agrees with the recent observations that the amyloid depositions are neither confined to the cerebral vasculture (B9, L13), nor to Icelandic patients (G5). It is also an appropriate designation for the condition before the first cerebral hemorrhage has occurred.

12.1.2. *Clinical Considerations*

The disease should be considered when a normotensive, previously healthy individual below 40 years of age is hit by a severe cerebral hemorrhage and particularly if young relatives previously have suffered from brain hemorrhage. Recurrent cerebral hemorrhages generally occur and result in increasing motor disability and gradual loss of mental functions, but in some individuals the disease does not progress for several years and a few carriers of the mutated allele attain a normal life span (J2, O1). In a few cases the presenting symptom may be dementia rather than brain hemorrhage (J3). The disease is not uncommon in the Icelandic population, with more than 150 cases described during the last 70 years, but it is rare (G5) or not described in other populations (G4, M8).

12.1.3. *Diagnosis*

When HCCAA is suspected because of clinical observations and/or family history, the diagnosis can easily be verified by demonstrating a low cerebrospinal fluid level of cystatin C (G10, J3) or the presence of the mutated cystatin C allele producing the Leu 68→Gln cystatin C variant (A6, P1). The first-mentioned procedure requires lumbar puncture and, to secure a stable level of cystatin C in the sample, the addition of a serine protease inhibitor, e.g., benzamidinium chloride, directly when the sample is drawn (Grubb, A., unpublished results). The PCR-based procedure is more robust, does not require lumbar puncture, and can be used for prenatal diagnosis and is therefore presently the preferable diagnostic method.

12.1.4. *Molecular Pathophysiology*

It has been possible to produce the disease-causing Leu68→Gln cystatin C variant in an *Escherichia coli* expression system (A4). Parallel studies of Leu68→Gln cystatin C and the wild-type protein have revealed that although both are efficient inhibitors of cysteine proteases, they differ considerably in their tendency to dimerize and form aggregates. While wild-type cystatin C is monomeric and functionally active even after prolonged storage at elevated temperatures, Leu68→Gln cystatin C starts to dimerize and lose inhibitory capacity immediately after its isolation. The dimerization of Leu68→Gln cystatin C is highly temperature-dependent, with a rise in incubation temperature from 37 to 40°C resulting in a 150% increase in dimerization rate and a considerable concomitant rise in the formation of larger aggregates (Fig. 15). These observations might suggest

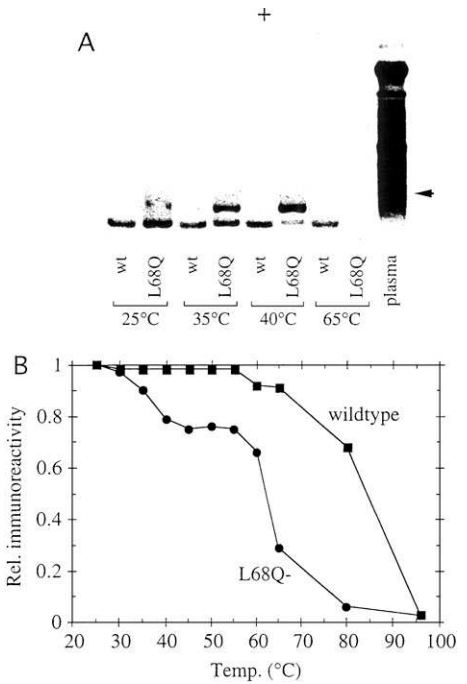

FIG. 15. Temperature stability of wild-type cystatin C and of L68Q-cystatin C, the cerebral hemorrhage-producing cystatin C variant. Samples of L68Q- and wild-type (wt) cystatin C were incubated for 30 min at various temperatures. (A) shows agarose gel electrophoresis at pH 8.6 of selected samples. The point of application and the anode are marked by an arrow and a plus sign, respectively. (B) shows the remaining cystatin C-immunoreactivity of sample supernatants after incubation and centrifugation as determined by single radial immunodiffusion.

a pathophysiological process in which Leu68→Gln cystatin C, due to its tendency to spontaneously aggregate, is partly trapped intracellularly and not secreted from the cell as efficiently as the wild-type protein. Continuous intracellular accumulation of Leu68→Gln cystatin C would, in combination with its specific physicochemical properties, lead to amyloid formation, cell damage, and death (A4). Recent *in vitro* studies have indeed shown that the intracellular processing of Leu68→Gln cystatin C differs from that of wild-type cystatin C (B15) and results in the formation of stable dimers that are partially retained in the endoplasmic reticulum (B11). It has also been asserted that no Leu68→Gln cystatin C is secreted from the cells of HCCAA patients (A13), but this has been questioned (B11). The temperature dependence of the aggregation of Leu68→Gln cystatin C might be of clinical relevance, since medical intervention to abort febrile episodes might reduce the *in vivo* formation of aggregates in carriers of the disease trait and thus possibly delay the point of time for their initial brain hemorrhage (A4).

12.2. Cystatin C and Cerebral Hemorrhage Conditions Connected with Deposition of Amyloid β-Protein

Cerebral amyloid angiopathy with wild-type, or a variant of, amyloid □-protein as the major amyloid constituent, is a condition with a high prevalence in the elderly and is also commonly found in patients with Alzhemier's disease or Down's syndrome (G6, H1, I3, M5, V3). The condition is associated with cerebral hemorrhage and may account for more than 10% of the brain hemorrhage cases in the elderly (I3). Immunohistochemical investigations of the amyloid deposits have demonstrated that all, or a considerable portion of them, display cystatin C immunoreactivity in addition to their amyloid β-protein immunoreactivity (B10, H1, I3, M5, M6, V3). Quantitative estimations have generally indicated that cystatin C is a minor constituent of the deposits, however (I3, M5, M6, V3). Efforts to demonstrate the presence of cystatin C variants, e.g., the one producing HCCAA, in the amyloid deposits have so far been unfruitful (A11, I4, M6, N1). It has been reported that the cerebrospinal fluid level of cystatin C is low in some of these conditions (S4), but if this observation can be used for diagnostic purposes is still uncertain. It is evident that the pathophysiological significance of the occurrence of cystatin C as a minor constituent in the amyloid deposits of these conditions remains to be determined.

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THE ROLE OF RECEPTORS IN PROSTATE CANCER

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1. Introduction

The living body is receptive to a wide variety of external disease-causing agents and also to the fallibility of its own composition, which increases with age. These factors are demonstrated in one of the most common diseases of modern society, cancer. Adding to the profound physical effects experienced by a patient are the intense psychological and emotional distress, which could in some ways be eased by the support of loved ones. Prostate cancer is the most commonly diagnosed male cancer and one of the leading causes of cancer-related deaths in men in the United States. In 1997 alone, more that 200,000 new cases of prostate cancer were diagnosed and more than 40,000 men died because of it (V10). What makes it even more scary is the inadequate knowledge of the development and progression of this disease and the fact that prostatic tumors grow slowly and silently, and in some cases, malignancy was detected only when metastasis had already occurred, thereby complicating treatment. Fortunately, during recent years there has been a stage shift in the diagnosis of prostate cancer, and many cases are detected as soon as stage B (confined) or early stage C (B12).

1.1. Receptor Role

Communication plays an essential role in normal daily living, not only in the modern external environment, but also in the human body itself, between different body parts and between neighboring cells. Ineffective or defective communication can create immense hazards in the outside modern world as well as inside the living body. Communication revolves around sending, receiving, and responding to different signals or messages. The ability of specific target cells to receive and respond to a specific signal is dependent on the binding of a ligand to a receptor found in the target cell. Ligands are usually secreted in the circulating blood or in the cellular environment, and can travel over varying distances to reach local or distant receptors (A2). On the other hand, receptors are found only in cells that respond to specific signals. When ligands travel from a different organ to reach their receptors, it is called endocrine control; when neighboring cells are responsible, it is called paracrine control; and when the same cell secrete and bind the ligand, it is called autocrine control. In the prostate, for example, circulating testosterone has an endocrine action. Various growth factors have a paracrine action with the receptors on the epithelial cells and the ligands secreted by the stromal cells. In malignant prostate cells, growth factors frequently show an autocrine action by secreting and binding the ligands (L14, S17).

The microenvironment of the prostate gland in which ligands act on their receptors consists of the epithelium and the stroma. The three major different types

of cells in the prostatic epithelium are the secretory epithelial cells, the basal/stem cells, and the neuroendocrine (NE) cells. The alveoli of the prostate are lined with tall, columnar secretory epithelial cells that secrete prostate specific antigen (PSA), prostate acid phosphatase (PAcP), other enzymes, and secretory granules. Though they are not essential for fertilization, prostate secretions provide part of the volume of the ejaculate and may play a role in providing optimal fertilization conditions by buffering the sperm, protecting it against microorganisms, providing energy for the sperm, increasing sperm motility, survival, and transport in male and female reproductive tracts. Secretory epithelial cells also have microvilli and are connected to each other via cell adhesion molecules (CAM), with their bases attached via integrin receptors to a basement membrane that separates it from the prostatic stroma. Basal/stem cells are small undifferentiated cells, without secretory function, and they are situated between the bases of adjacent tall columnar epithelial cells. They may act as stem cells for secretory epithelial cells and for both benign and malignant neoplasias. The NE cells are found in the epithelium of the accini and in ducts of the glands, as well as in the urothelium of the prostate urethral mucosa. Their function is probably to regulate prostatic secretory activity and cell growth via secretion of hormonal polypeptides such as serotonin. Receptors that play a role are α- and β-adrenergic receptors, muscarrinic cholenergic receptors, and acetylcholine esterase nerves. The prostatic stroma consists of an extracellular matrix, ground substance, and stromal cells. The latter includes smooth muscle cells around the acinar structures, fibroblasts, capillary and lymphatic endothelial cells, and NE cells (L16). The extracellular matrix is an extracellular scaffolding or residual skeleton that organizes and interacts with cells. Structurally it is linked to the cell nuclear matrix by the cytoskeleton or cytomatrix. Interactions between the extracellular matrix and the cell may play a major role in maintenance of cellular function by regulating cell shape, functional differentiation, gene expression, cell growth, and hormone responsiveness (G8).

Receptors can be found in the cell nucleus, the cytoplasm, or on the cell membrane. Hydrophobic ligands such as steroids and vitamins bind to receptors in the cytoplasm, whereafter this complex is translocated across the nuclear membrane to the nucleus. Cytoplasmic receptors may be artifacts of receptor assays, however, and if this is true, ligands bind directly to nuclear receptors. In the nucleus the receptor–ligand complex is phosphorylated and binds to specific sites on the DNA called hormone response elements (HREs). After binding with transcription factors, the specific gene are activated, mRNA are transcribed and eventually proteins, which activate the cell for a specific function, are expressed. Receptors of protein hormones and growth factors are usually present on the cell surface or plasma membrane and can trigger different types of cellular responses. Binding of the ligand to these receptors usually results in enzymatic release of second messengers which include cAMP, cGMP, 1,2-diacylglycerol, inositol 1,4,5-triphosphate,

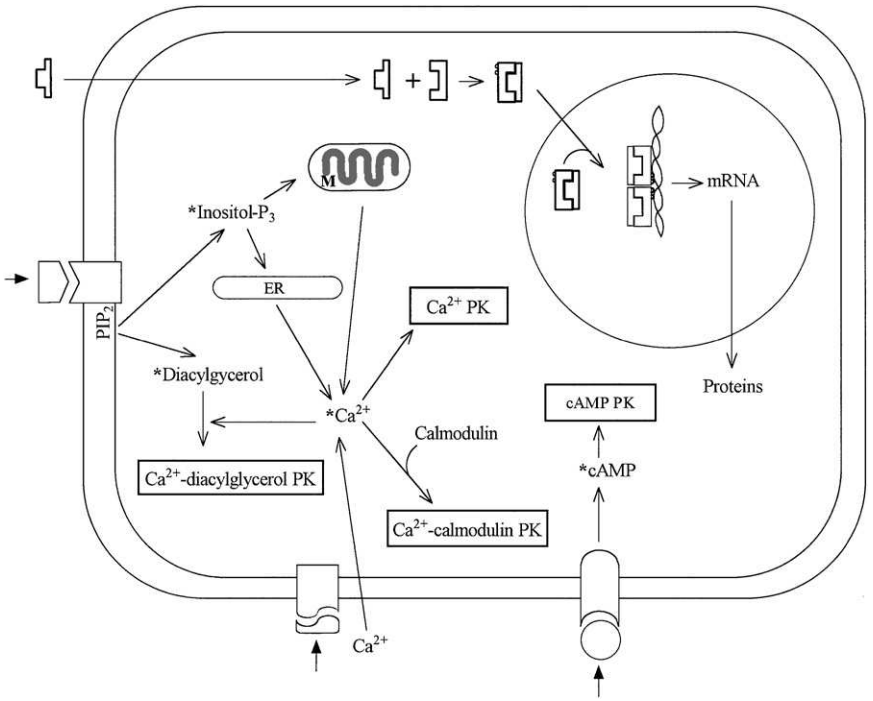

FIG. 1. A compact visual summary of the signaling of cytoplasmic/nuclear and cell surface receptors. ER, endoplasmic reticulum; M, mitochondrion; PIP_2, phosphatidylinositol-4,5-diphosphate; IP_3, inositoltriphosphate; PK, protein kinase*, second messenger.

and Ca^{2+}. These second messengers activate protein kinases (PK), which promote a cascade of cell signaling reactions (Fig. 1).

Binding of the same ligand to the same receptor in different cells mediates different responses due to different signaling mechanisms inside the cell. Androgens, for example, are involved in prostate specific antigen production in the epithelial cells of the prostate, while androgens are essential for sperm maturation associated with the Sertoli cells in the testes. Normal and tumor cells could therefore respond differently to the same ligand, causing uncontrollable cell proliferation in malignant cells, as in the effect of androgens on androgen-sensitive prostate cancer (H21). For the cell to respond differently to a steroid, the receptor–ligand complex must activate different genes. This is accomplished by binding to different hormone response elements (HREs) on the exposed DNA sites. The selection mechanism for different HREs is largely unknown, but it has been proposed that the tissue matrix plays a major role in this process, by organizing the cell for directed intracellular transport and spatial coupling of cell signaling systems (G8).

1.2. Receptors and Prostate Cancer Risk Factors

Aging is an important factor in the development of prostate cancer, since it causes various changes in the body composition and function, especially in reproduction, which is regulated by hormones and hormone receptors. The incidence of prostate cancer in men over the age of 50 increases rapidly. In men younger than 39 the probability of developing clinically significant prostate cancer is 1 in 100,000, in men aged 40–59 the probability is 1 in 78, and for men aged 60–79 the probability is as high as 1 in 6 (V10.) The reason for developing a clinically significant cancer with age is not clearly understood, but it could somehow be linked to the altering serum hormone and growth factor levels in the aging male. Although data are scarce, some hormone and growth factor levels decrease with age, while others increase or show no alteration (K16, M4, M26, T11). In the case of testosterone, it is generally accepted that it is not so much the total serum testosterone levels that decrease with age, but rather the serum testosterone not bound to the sex hormone-binding globulins, the bioavailable testosterone. In healthy men 55 and older, total serum testosterone may be lower in only 20% of men, while bioavailable testosterone may be decreased in as much as 50% of men (T3). This decrease in bioavailable testosterone has prompted the issue of androgen therapy in the elderly.

Advantages of androgen supplementation may include an increase in lean body mass, an increase in strength, and a decrease in fat mass. No evidence supports the benefits in sexual behavior, cognition, mood, and bone density (T3). At this stage the disadvantages of androgen therapy, such as potentiation of cardiovascular problems, BPH, and prostate cancer, surpass the advantages, and any man considering androgen therapy should carefully think it over. Bioavailable testosterone, dehydroepiandrosterone, dehydroepiandrosterone sulfate, prolactin, growth hormone, and insulin-like growth factor-I (IGF-I) levels decrease in the aging male, while levels of follicle stimulating hormone (FSH) and luteinizing hormone (LH) increase and dihydrotestosterone (DHT) and estrogen (E) levels stay the same (K16, M4, M26, T11). This causes changes in hormone and growth factor ratios—for example, the testoterone:estrogen (T:E) ratio decreases with age (S28). Hormone levels may not merely be increased or decreased, but irregular and asynchronous secretion of hormones such as LH and testosterone may play a major role (P14). Since serum hormone-binding proteins bind and transport hormones from the producing organs to the target tissue, an increase in the serum hormone-binding proteins reduces the bioavailability of androgens, estrogens, and IGF-I (B4). Receptor status can also be affected by age. An example of this is that, in the prostate, cytoplasmic AR increases with age while nuclear AR stays the same (S2).

Prostate cancer incidence and mortality vary in different countries and racial groups, and this implicates not only genetic or hereditary differences but also environmental and dietary factors. American Blacks have the highest incidence and mortality, followed by Whites and lastly Asians. Black and Asian Americans

also have a higher incidence of prostate cancer compared to the incidence in their countries of origin, due to altered lifestyles which can at least be associated with a higher intake of saturated fats. It has been suggested that prostate cancer is associated with lower production and intake of vitamin D, which could explain the tendency of increased risk in areas with a lower sunlight intensity or exposure (F3, H4). The geographic distribution of ultraviolet (UV) radiation in 3073 counties of the United States was examined and the amount of UV radiation correlated inversely ($p < 0.0001$) with prostate cancer mortality (H4). Other racial differences may be serum testosterone, being higher in Blacks than in Whites, 5α-reductase activity, being higher in Whites, and the $(TA)_n$ repeat polymorphism in the 5α-reductase SRD5A2 gene (R4, R12, S18). The importance of racial variation in the $(CAG)_n$ and $(GLN)_n$ repeat polymorphism in the androgen receptor gene will be reviewed later, under androgen receptors.

Diet and hormones influence body composition and body composition can influence hormonal status. An example is the decreased serum testosterone and increased serum estrogen found in obesity (R11). Recently, body mass index (BMI) and anthropometric measurements (height and weight) were found to be positively associated with prostate cancer risk and even more strongly associated with mortality (A4). Compared to the reference category of BMI < 22.1 kg/m^2, BMI more than 26.2 kg/m^2 displayed a statistically significant higher mortality rate in prostate cancer. The rate ratio (RR) was 1.4, and the 95% confidence interval (CI) was 1.09–1.81. Furthermore, a slight increase in upper body skeleton was also associated with increased risk of prostate cancer (D15). Obesity and weight gain in later life, though not yet established conclusively, have been found to increase risk of prostate cancer, and tend to enhance progression from stage B1 to stage D1 (C7, F12). In another study, however, just the opposite was found, suggesting lower mortality rates and better prognosis in obese men (D7). These conflicting studies may suggest that obesity alone does not increase risk and that more studies are necessary. Total energy intake may increase the risk of prostate cancer, as seen in a case study that was adjusted for age, education, group, and family history. The odds ratio (OD) increased with each quartile of energy intake: 1.00, 1.77, 1.90, and 2.67 (M18). Certain nutrients, such as saturated animal fat (OD = 1.7–2.8), red meats (OD = 1.7–2.6), eggs (OD = 2.1), and dairy products (OD = 1.6–3.1), are associated with increased risk, while vitamin A (OD = 0.4), vitamin E (RR = 0.44), vitamin D (OD = 0.88), and especially lycopene in tomatoes (OD = 0.74) and tomato sauce (OD = 0.66), seem to have a protective effect (C10, G3, G11, G12, G13, H4, H11, W1, W9). Some nutrients, such as essential fatty acids (EFA) and their metabolites, may play a role not only in the prevention of prostate cancer, but also in the reduction of tumor growth (V1). Fatty acids has been implicated in modulation of various receptor activities (N9) and, in unpublished work done by us, certain EFA decreased AR capacity and dissociation constant (K_d), while ER capacity and K_d increased. Therefore, it can be added that a healthy diet is essential for cancer prevention.

Another factor that may be beneficial is physical activity, since it affects the immune function and antioxidant defenses, transit time of digestion, hormones, and body fat, and it improves energy balance. Therefore, it may have a protective effect on prostate cancer and it may even slow progression and metastasis (G14, H8, K7, O2, O3). In a 9-year follow-up study performed by Hartman *et al.,* the relative risk for physical exercise in prostate cancer was compared with sedentary workers and found to be 0.6 (CI = 0.4–1.0), 0.8 (CI = 0.5–1.3), and 1.2 (CI = 0.7–2.0) for occupational workers, walker/lifters, and heavy laborers, respectively. Except for heavy laborers, an inverse association was observed (RR = 0.7, CI = 0.5–0.9) compared to men who were sedentary at work and leisure (H8). However, other studies indicate a positive association between vigorous exercise and prostate cancer (C7), and therefore further study is necessary to provide an activity optimum. Frequency, duration, intensity, type of exercise, and the period during a man's lifetime when exercise may be beneficial, must be investigated (O2, O3).

Mentioned risk factors may cause cancer by genetic alterations or rather a series of genetic alterations affecting genes associated with the cell cycle, cell growth proliferation, and differentiation, such as growth factors, growth factor receptors, regulators of DNA synthesis and repair, regulators of RNA transcription, and modifiers of protein function by phosphorylation. Alterations include gene deletions, loss of alleles, loss of heterozygosity (LOH), point mutations, frameshift mutations, germline mutations, DNA methylation, and gene amplification. Since all receptors are protein molecules that are dependent on genes, genetic alteration is a worthy direction of investigation in this regard.

2. Androgen Receptors

2.1. Androgens

In understanding the role of different receptors in the prostate, it is essential to comprehend the role of their ligands on the prostate. Prostatic growth are controlled by endocrine factors, neuroendocrine factors, paracrine factors, autocrine factors, extracellular matrix factors, and cell–cell interactions. Endocrine factors are produced in distant organs and reach the prostate via serum transport and include steroid hormones such as testosterone and estrogen, as well as peptide hormones such as insulin and prolactin.

Androgens have been considered to play a crucial role in the development and progression of prostate cancer, since men castrated before puberty do not develop prostate cancer (L10). Testosterone is the major steroid hormone that maintains prostate growth, function, and size. Treatment of stage D prostate cancer includes withdrawal of testosterone by surgical or medical castration, which causes atrophy of androgen-dependent cancer cells. Most treatments involve Zoladex (goserelin)

or Lupron (leuprolide acetate) for the testosterone produced by the testes, and Eulexin (flutamide) or Casodex (bicalutamide) for the testosterone produced by the adrenals (B1). Physicians are divided over which of these drugs is superior, but Zoladex and Eulexin are less expensive. The luteinizing hormone-releasing hormone (LH-RH) agonists Zoladex and Lupron are synthetic versions of LH-RH that are up to 60 times as potent as the natural hormone. They are similar in structure and action to the natural LH-RH, but they cause greater release of LH and FSH from the pituitary gland. With prolonged use these drugs deplete the pituitary gland of LH and FSH. Eulexin and Casodex are antiandrogens which inhibit the action of androgen receptors.

Normally, the pituitary gland is stimulated by the hypothalamus as follows: The hypothalamus releases LH-RH, which acts on the pituitary to release luteinizing hormone (LH). The Leydig cells in the testes are then stimulated by LH to produce testosterone from acetate and cholesterol. Testosterone is released into the bloodstream, where 98% binds to a testosterone/steroid binding globulin (T/SeBG). Only 2% of the total serum testosterone are free and available for prostate uptake (D9). In the prostate, testosterone is converted by the 5α-reductase enzyme to DHT, the principal androgenic hormone in the prostate. The DHT binds strongly to the AR and it is this complex that regulates the activity of the androgen-responsive genes that are responsible for prostate growth and function.

The adrenal glands produce androstenedione and dehydroepiandrosterone (DHEA), which contribute about 5–10% of circulating androgens (L16). Adrenal androgens have little effect on normal prostate growth because 5α-conversion of DHEA is slow and androstenedione cannot be directly converted to DHT and is therefore a weak androgen. In the malignant prostate, however, binding specificity of AR for adrenal androgens could increase due to mutations in the AR gene and thus stimulate prostatic neoplasms (B3).

2.2. Molecular Biology

Androgens exert their biological effects via binding to the androgen receptor (AR), a ligand-activated nuclear transcription factor (H1). The AR, a 90-kDa protein, shares a high degree of structural homology with other steroid receptors. It consists of 919 amino acids with three domains: a ligand-binding C-terminal, a highly conserved DNA binding domain that consists of two zinc fingers, and a highly variable polymorphic N-terminal that has glutamine and glycine repeats and acts as a transactivation domain. The 80-kb gene encoding for the AR lies on chromosome Xq 11-12 and consists of 8 exons: exon 1 encodes for the N-terminal, exons 2 and 3 each encodes for a zinc finger, and exons 4–8 encode for the hormone binding domain (Fig. 2).

Within the prostate gland, the steroid hormone testosterone is irreversibly converted to 5α-dihydrotestosterone (DHT), the active androgen in the prostate. Binding of DHT to the AR triggers conformational changes which unmasks the

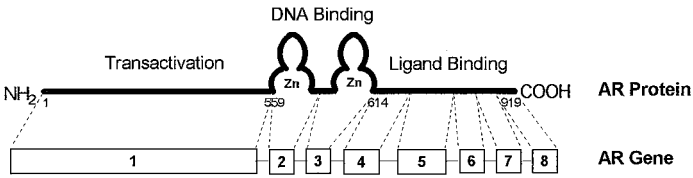

FIG. 2. Diagram of AR protein and gene.

DNA-binding domain of the AR and releases heat shock protein 90 (hsp 90). This facilitates the binding of the DHT–AR complex to the androgen response elements (ARE) and supports dimerization of the DHT–AR complexes on the genome. These complexes provide stability to the process of gene transcription. An androgen-dependent product of AR gene transcription in secretion cells is prostate specific antigen (PSA), since the PSA gene promoter is regulated by androgens (L15).

2.3. BIOMARKER OF PROGNOSIS

Since androgens and the AR play such an important role in development and progression of prostate cancer, the AR has been studied as a biomarker and prognostic factor in the progression of this disease. Prostate tumors can either be AR-positive or AR-negative. Most AR-positive tumors respond to hormonal treatment, but some are resistant to therapy and progress, while androgen receptor-negative tumors usually relapse even though some of them may initially respond to endocrine therapy (P18). In normal prostate epithelium, AR seems to be expressed by the secretory cells and the basal cells in culture (P5). Since secretory cells degenerate during androgen ablation, it has been proposed that the presence of AR is associated with maintenance and survival of these cells (G22). The basal cells are the stem cells for the secretory cells and thus it has been proposed that growth factors could stimulate basal cell differentiation and eventually AR expression to produce secretory cells (L16).

Direct immunohistochemical analysis of prostatic tissue has become very popular since the development of AR antibodies. However, a disadvantage of this technique in quantitative analysis is that the intensity of the immunohistochemical stain is dependent on the intactness of the structure of the AR. Therefore, mutations or alterations in the structure may reduce staining intensity (T5). Biochemical and immunohistochemical studies of AR content in relation to grade or stage of disease, as well as prediction of response to endocrine therapy, has been inconsistent. Nearly all primary prostate cancer specimens positively express AR protein, as determined by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis as well as by immunohistochemical analysis on formalin-fixed, paraffin-embedded primary prostate tissues (D12, H14). In advanced-stage prostate cancer, immunohistochemical techniques has shown that metastases in bone, the

epidural space periosteum, and most of the metastases in pelvic lymph nodes also express AR protein (H14, H15). Only one of 12 pelvic metastases (8%) were completely negative for AR expression (H14). The AR levels in cancerous prostates before and after treatment were observed by employing AR mRNA cytoplasmic dot blot hybridization with autoradiography (M6). The AR levels were higher in endocrine-treated prostate cancer than in untreated and normal prostates ($p < 0.05$). This suggests that endocrine treatment causes an increase in AR content in some cases of prostate cancer and that this may play a role in recurrence, progression, or hormone resistance during therapy. The Gleason score is an indication of the cancer's aggressiveness and relies on the pathologist's interpretation of the cells in a biopsy (G17). The pathologist assigns a single grade from 1 to 5 to the two most prevalent histological components of the tumor, based on the glandular architecture of the area. The sum of these two single grades gives the Gleason score. A score of less than 4 suggests a low-grade cancer, while a score above 7 is probably more aggressive. Comparing the AR expression within the different Jewett stages and Gleason grades, the AR expression increased with higher stages (56% of stage B vs 89% of stage D, $p = 0.001$) and in higher grades (52% of Gleason 3–7 vs 92% Gleason 8–9, $p = 0.015$) after combined androgen blockade (D12).

Although some prostate tumors stain less intensely for AR than normal and BPH prostates, it was noticed that the tumors stain more heterogeneously for AR than normal and BPH prostates, which stain homogeneously (M21). The variability of AR content per nuclear area increases with increasing grade and Gleason score (M3). Prins *et al.* (P18) demonstrated that computer-aided image analysis of AR immunostaining with classification of AR receptograms can predict the outcome of endocrine therapy rather accurately. Receptograms were classified into four different possible types according to the mean optical density distribution, which allowed determination of the percentage of AR-positive and negative nuclei, as well as the frequency of AR-positive subpopulations (type 1 = unimodal/AR positive with well-defined peak; type 2 = bimodal/AR-positive and more than 10% AR-negative subpopulations; type 3 = multimodal AR-positive with well-defined peaks and less than threefold mean optical density concentration range; type 4 = highly skewed, lacking well-defined peaks, and less than threefold mean optical density concentration range). In this study tumors consisting of highly variable or heterogeneous cell subpopulations (type 2 or type 4) were associated with endocrine therapy failure, while more homogenous AR concentrations (type 1 and type 3) were associated with a favorable response to endocrine treatment (P18).

2.4. Androgen Receptor (AR) in Treatment

Treatment of prostate cancer for the various stages is summarized as follows: radiation or radical prostatectomy for stages A and B and radiation with or without hormonal therapy for stage C prostate cancer (G4). Through the years, the method of choice in treatment of advanced stage D prostate cancer has been

hormonal treatment to diminish or deplete testicular and adrenal androgen concentrations in the body (G4). Circulating testicular androgens can be removed by bilateral orchiectomy or the secretion of androgens can be prevented by the administration of the female hormone diethylstilbestrol (DES) or LH-RH agonists. These agonists cause the down-regulation of the production of testosterone by paradoxical suppression. However, long-term androgen ablation may increase AR expression, causing progression of the disease (K14). The action of adrenal androgens in target tissues can be blocked by antiandrogens. A combination of surgical or medical castration with an antiandrogen has been proposed to increase the survival of patients with metastatic prostate cancer (T8). However, this may be a controversial issue, since meta-analysis of available data by the U.S. Department of Health and Human Services suggests that there is no statistically significant difference in survival at 2 years (hazard ratio = 0.871, CI = 0.866–1.087) between monotherapy and combined androgen blockade. Limited data suggested that there is a slight significant difference favoring combined therapy over monotherapy in the survival at 5 years (hazard ratio = 0.871, CI = 0.805–0.942), but more data are needed for a better representation of clinical significance (U1).

Antiandrogens compete with and displace androgens from androgen receptor occupancy and inhibit their action and prevent the secretory epithelium cells from surviving (M5). Two kinds of antiandrogens, steroidal and nonsteroidal antiandrogens, have been used in treatment of various illnesses caused by AR disfunction (K19). The differences between steroidal and nonsteroidal antiandrogens are elucidated next. Nonsteroidal antiandrogens such as bicalutamide, hydroxyflutamide, and nilutamide inhibit androgen receptor action directly. Unfortunately, the cell registers this as an androgen shortage, which causes an increase in LH-RH, LH, and testosterone production. This increase in testosterone could cause a displacement of the antiandrogens from the AR and these are therefore called selective antiandrogens. In contrast, steroidal antiandrogens such as cyproterone acetate, megestrol acetate, medroxyprogesterone acetate, and chlormadinone acetate inhibit AR action both directly and indirectly. Progestational activities of these steroidal antiandrogens explain the indirect action by means of sequential down-regulation of LH-RH, LH, and eventually androgens. These compounds are called nonselective antiandrogens. Side effects of steroidal and nonsteroidal antiandrogens in monotherapy are summarized in Table 1.

2.5. Androgen Receptor Aberrations

Androgen receptor aberrations in structure or content may modify androgen action and in the prostate may lead to an increase in cancer. It is proposed that a disequilibrium in the CAG repeat lengths in the transactivation domain of the AR gene may be involved in the development of prostate cancer (S21). Other aberrations which can be involved in the progression of tumors from an

TABLE 1
SIDE EFFECTS OF ANTIANDROGEN TREATMENT

Name, type	Characteristics	Advantages	Disadvantages[a]
Cyproterone acetate (ANDROCUR) Steroidal	LH decreased T decreased	Monotherapy Few hot flashes	Hepatotoxicity, anemia, myocardial ischemia, edema, thromboembolic disease, infertility, breast tenderness, sedation and depressive mood changes, alterations in hair pattern, skin reaction and weight changes
Flutamide (EULEXIN, EUFLEX) Nonsteroidal	LH increased T increased	Monotherapy Combination therapy Potency spared	Breast tenderness, nausea and vomiting, diarrhea, rectal bleeding, hot flashes, cystitis, increased appetite, sleep disturbances, hepatotoxicity, anemias, hemolysis, headache, dizziness, malaise, blurred vision, anxiety, depression, decreased libido, hypertension, complications in patients with cardiovascular disease
Nilutamide (NILANDRON) Nonsteroidal	LH increased T increased	Combination therapy Potency spared Libido unaffected	Hot flushes, breast tenderness, nausea and vomiting, blurred vision and delayed adaptation to darkness, alcohol intolerance, interstitial pneumonitis
Bicalutamide (CASODEX) Nonsteroidal	LH increased T increased	Combination therapy Potency spared Less diarrhea than with flutamide Libido unaffected	In combination therapy with a LH-RH analog: hot flushes, diarrhea, general pain, constipation, asthenia, hypertension In monotherapy: dizziness, confusion, nausea, vomiting, rash and hepatitis

[a] Micromedex.

androgen-dependent to an androgen-independent state include AR gene amplification, AR point mutations, and aberrant phosphorylation, which activates AR in the absence of a ligand (Fig. 3). A better understanding of AR function in the normal prostate, as well as in hormone-responsive and hormone-unresponsive prostate cancers, may provide a starting point for development of better treatment methods for prostate cancers.

2.5.1. *Polymorphism*

It has been established that the polymorphic $(CAG)_n$ and $(GGN)_n$ regions in the AR gene vary in different racial groups (I2). In Caucasians the most common allele has 21 (CAGs), while the most common allele in African Americans, who

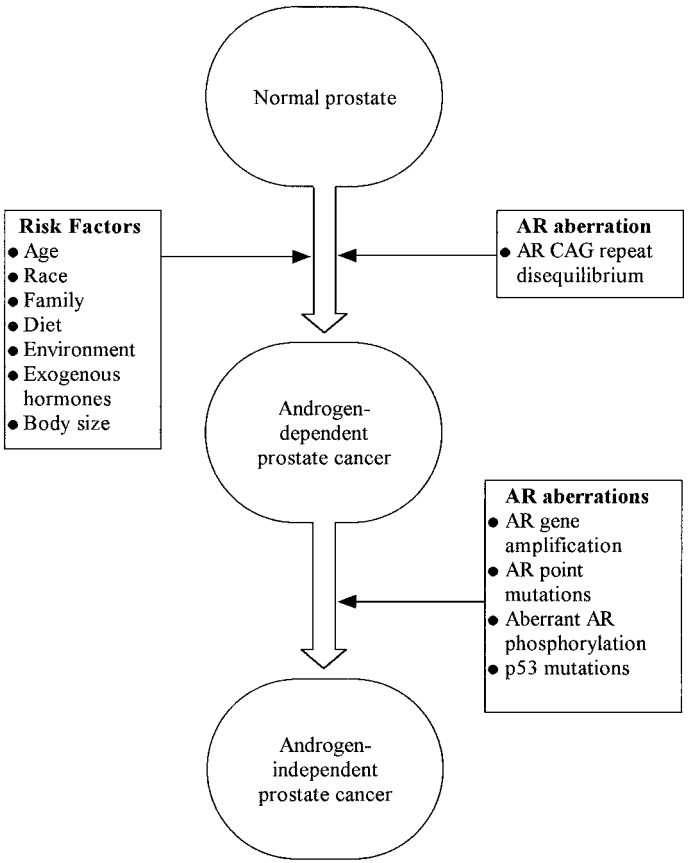

FIG. 3. Risk factors and AR aberrations in progression from androgen-dependent to androgen-independent prostate cancer.

present a higher incidence of cancer, has 18 (CAGs) (E1). In short, an allele is one of a set of alternative forms of the same gene on a chromosomal locus. In diploid organisms such as humans, there are two alleles, one on each chromosome of a homologous pair. Shorter (CAG) repeat lengths is associated not only with the development of prostate cancer at a younger age (H5), but also with increased risk of the development of distant metastatic and fatal prostate cancer (G16, H3). In middle-aged (40–64 years) Caucasian men, the risk of prostate cancer decreases by 3% for each additional (CAG) repeat (S21). This higher risk of prostate cancer may be explained by the fact that the transcriptional activity of AR with shorter repeats is higher than those with longer repeats (C8). Analysis of (GGN) repeats suggests that 16 or fewer repeats is related to higher risk in prostate cancer. When lengths

of both (CAG) and (GGN) repeats were considered jointly, the subgroup with two short repeats [(CAG) < 22, (GGN) < 16] had a twofold elevation in prostate cancer risk relative to those with two long repeats [(CAG) ≥ 22, (GGN) ≥ 16] (S21). In spinal/bulbar muscular atrophy, with elongated (CAG) repeats of AR, an inverse relationship was observed between (CAG) repeat length with AR mRNA and protein levels (C12). Therefore, another question that remains is whether shorter (CAG) repeats may be correlated with increased expression of AR in prostate cancer.

2.5.2. *Androgen Receptor Gene Amplification*

Some androgen-dependent primary tumors initially respond very well to conventional castration therapy or endocrine treatment. The response duration for stage D is usually 12–18 months before recurrence appears, but with earlier diagnosis, androgen suppression can continue for as long as 10–20 years. In recurrent tumors, the average copy number of the AR gene varies substantially, from 2.7 to 28 AR gene copies per cell, with up to 60 copies in some individual tumor cells (K12). No mutations were reported in these cases. However, it was postulated that this increased amplification and expression of a wild-type AR gene enables the cancer cell to utilize the residual low levels of androgens more effectively. After the tumor cells adapt themselves to androgen-deficient levels by AR amplification, cell growth could be sustained in an androgen-independent manner (K13). Therefore, some patients may benefit more from maximal androgen ablation (T8). Recently, it was found that hormone-refractory tumors which exhibit AR gene amplification correlate with an increase in p53 mutation (K11, K18).

2.5.3. *Phosphorylation*

Like other steroid receptors, the human AR has been described as a phosphoprotein (B13, K22). At least 12 potential phosphorylation sites have been elucidated for serine/threonine–proline directed kinase, casein kinase, and double stranded DNA-dependent kinases. Phosphorylation of the AR on serine residues 506, 641, and 653 takes place shortly after synthesis, and this seems to be essential for effective ligand binding (B7). Other functions of AR phosphorylation are regulation of intracellular transport from the cytoplasm to the nucleus, binding to ARE, transcriptional activation, interactions with other proteins, and receptor cycling (Z4). Binding of androgens to AR increases AR phosphorylation on different sites. For example, in LNCaP cells the addition of the synthetic androgen, methyltrienolone, also called R1881 (10 nM), causes a 1.8-fold increase in phosphorylation of AR and therefore it may play a role in activation (K20, V4).

Since the N-terminus plays a major role in transactivation, it has been postulated that phosphorylation of this terminus may modulate transactivation (Z3). Evidence found that a mutation in the ser 650 phosphorylation site decreases transcriptional activity by 30%, endorsing the importance of phosphorylation in the

N-terminus (Z3). Similarly, in LNCaP cells, phosphorylation occurs only in the N-terminus and not in the DNA-binding or ligand-binding domain (K23).

The fact that some tumors are resistant to endocrine therapy and grow well in the absence of androgens may indicate that, in the absence of androgens, the AR could be activated in a different way. This activation can be either by other ligands, or by additional phosphorylation. It has been established that AR can be activated in the absence of androgens by a protein kinase A signaling pathway, by polypeptide growth factors, and by cellular regulators such as IGF-I, KGF, and EGF (C23, N2, R5). Various serine residues have been identified which may be the site of action for this intimate collaboration or cross-talk between androgen-mediated signaling systems and growth factor/receptor tyrosine kinase pathways (G22, M1). A thorough investigation of the mechanism of androgen-independent activation of AR and its function would shed more light on progression of androgen-independent prostate cancer.

2.5.4. *Mutations*

Mutations have been found in primary untreated, local and metastatic hormone-refractory prostate cancer as well as in LNCaP cells. However, mutations seem to be more common in advanced and hormone-refractory prostate cancers (C25, E5, L4, N3, S30, S31, T1, T5). This point is still debated. Transition mutations, where a purine is replaced by a purine or a pyrimidine by a another pyrimidine, seems to be more common in prostate cancer than transversion mutations, where a purine is converted to a pyrimidine or vice versa. It was proposed that this may be due to endogenous carcinogens (H1).

Mutations in the promoter area of the AR gene could lead to loss of AR mRNA and protein, while mutations in the exons could lead to altered structure of the AR protein. Since function is related to structure, these alterations could cause the function of the AR to be enhanced or diminished. Ligand-binding and transactivation properties of the AR could be altered and the AR could also be activated constitutively. Several reports indicated point mutations in the hormone binding domain result in an increase in binding specificity of the AR for other ligands such as estradiol, progestagins, adrenal androgens, and even antiandrogens (C24, C25, C26, E5, G1). Therefore, the AR could even be activated or hyperactivated in the absence of testosterone or DHT during androgen ablation therapy, where residual adrenal androgens, estrogens, or antiandrogens could still activate AR and tumor growth. Antiandrogens, which are used to eliminate the effect of residual adrenal androgens in combined androgen ablation treatment, could themselves cause progression of tumor growth. This could be a reason why the antiandrogen withdrawal syndrome is observed (S30). The antiandrogen withdrawal syndrome occurs when combined hormonal therapy (either orchidectomy or LH-RH agonist combined with an antiandrogen) or antiandrogen monotherapy fails and the administration of pure antiandrogen is discontinued. When hormonal therapy is failing

and PSA levels are rising, antiandrogens is withdrawn from treatment, causing the PSA to drop to undetectable low levels over a few months. In addition to the decreasing PSA levels, some patients may even show signs of clinical improvement, but in others the disease may progress despite the fall in PSA (L12, S5). The antiandrogen withdrawal syndrome was initially detected during treatment with the nonsteroidal antiandrogen flutamide, but since then it has also been detected with other nonsteroidal antiandrogens such as bicalutamide (Casodex), as well as steroidal antiandrogen such as cyproterone and chlormadinone (A1, N5, S16). The mechanism of antiandrogen withdrawal syndrome is still unclear, but it is suggested that antiandrogens have a stimulatory effect on a mutated AR in prostate cancer cells. Recently, a mutation in codon 887 of AR was associated with antiandrogen withdrawal (S30). The effect of antiandrogen withdrawal is always palliative and temporary, but it can prolong the effectiveness of orchidectomy or LH-RH agonists by many weeks or months before PSA begins to rise again.

Increased binding affinity for ligands other than androgens could be established in the absence of mutations with AR coactivators such as ARA_{70}, ARA_{55}, and retinoblastoma. Recently it was demonstrated that ARA_{70} could stimulate AR transcriptional activity more than 30-fold in the presence of estradiol (E_2) (Y2). The ARA_{55} could stimulate AR transcriptional activity in the presence of DHT, E_2, and hydroxyflutamide (F11). Retinoblastoma, a tumor suppressor, increases AR transcriptional activity fourfold in the presence of DHT and up to 13-fold when coexpressed with ARA_{70} (Y3, Y4). Mutations in the DNA binding domain have been reported by various authors. For example, alanine-596 was converted to threonine and this alteration affected receptor dimerisation, which causes an alteration in transactivation (K9). Table 2 summarizes some of the mutations found in humans.

3. Estrogen Receptors and Other Cytoplasmic/ Nuclear Receptors

Estrogens are believed to be "female" hormones, but they are also present in the male, although in much lower concentrations than androgens. Estrone and estradiol are derived from conversion of androstenedione and testosterone by the aromatase enzyme. In the male, this aromatization takes place in various tissues, which include the prostate (S25) (Fig. 4). Binding of estrogens to the estrogen receptor causes a biphasic effect on cell growth (L9, O4). Estrogen receptors (ER) are known to be present in the male reproductive tract, where they are essential for normal growth and function (C19). Hess *et al.* (H12) reported that estrogen may be important in retaining fertility, since estrogen and ER regulate the reabsorption of luminal fluid in the head of the epididymis. The exact function of estrogens in the prostate is unclear, but it is known that total plasma estradiol levels in men

TABLE 2
POINT MUTATIONS OF AR IN PROSTATE CANCER

Substitution	Domain	Activity change	Reference
877 thr→ala	Hormone binding	Binding specificity increase for estrogens, progestagins, antiandrogens	G1
868 thr→ala	Hormone binding	Binding specificity and transactivation increase for adrenal androgens, estrogens, progestagins and antiandrogens	V6
730 val→met	Hormone binding	Growth advantage	N3
715 val→met	Hormone binding	Transactivation increase by adrenal androgens and progestagins	C25
650 ser→ala	DNA binding	Decrease in transactivation	Z3
596 ala→thr	Phosphorylation site	Dimerization	K9

older than 50 are increased. However, plasma levels of free estradiol stay the same, due to an age-related increase in the testosterone estradiol binding globulin (TEBG). In contrast, plasma levels of free testosterone decrease. This causes a 40% increase in the ratio of free estradiol/free testosterone, which could be important in development of BPH and prostate cancer (W8, W11).

3.1. ESTROGENS IN TREATMENT

Estrogens such as diethylstilbestrol (DES) and diethylstilbestrone diphosphate (DESdP) are being used in endocrine therapy in advanced or aggressive prostate cancer to induce regression of tumors (B5, H10, K8, M15). However, the mechanism of action of DES was believed to be ER independent and via a negative feedback inhibition on the pituitary–hypothalamus axis, which leads to lower LH and testosterone levels. Recently there has been evidence that DES could have a direct effect on prostate and prostate tumor cells via inhibition of cell proliferation, cell cycle arrest, and apoptosis (R9). In this study, the average dose of DES at which 50% of prostatic cell lines 1-LN, DU-145, PC-3, and LNCaP were no longer viable (LD_{50}) after 72 h was 20.9 ± 0.5 μM, while the average for DESdP

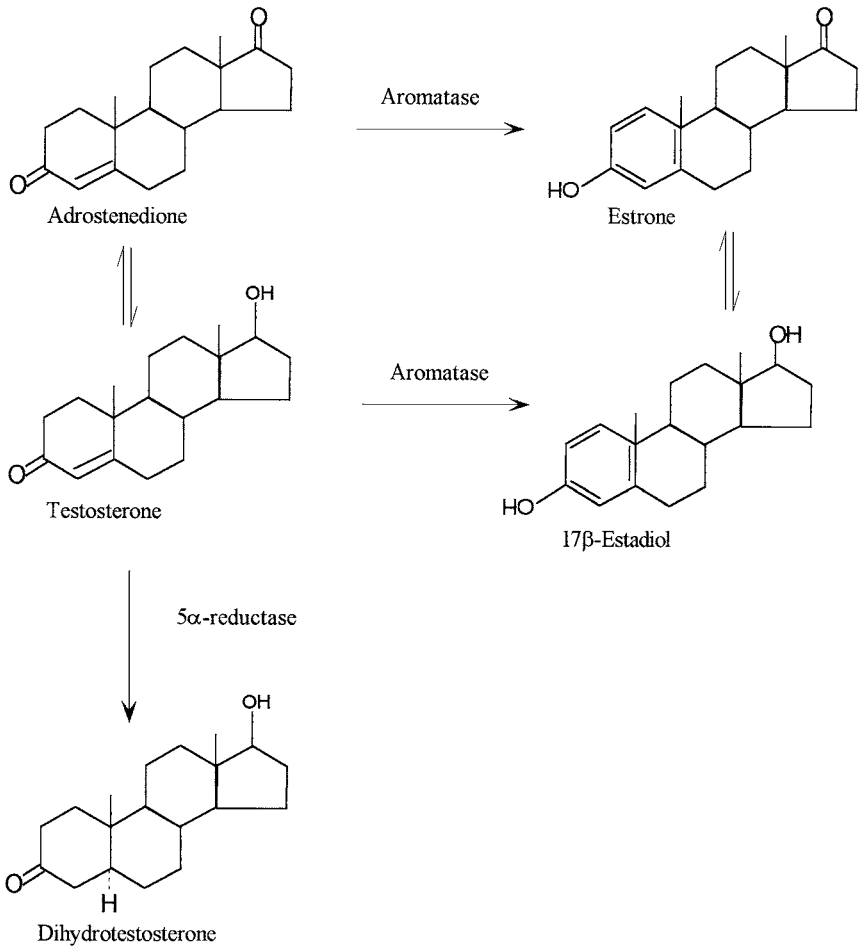

FIG. 4. Conversion of androgens to estrogens in the prostate.

was 23 ± 1.0 μM. Apoptosis was suggested by DNA degradation in PC-3 cells with DES treatment. Cell cycle arrest, as determined by DNA fluorescence flow cytometry, showed that the average percent fluorescence at 30 μM for androgen-independent cell lines 1-LN, DU-145, and PC-3 were increased for hypodiploid nuclei from 3.7% to 19.6% (5.3 times). The amount of cells in the G1 and S phases decreased from 58.2% to 8.3% (7.0 times) and from 21.0% to 5.3% (4.0 times), respectively. Cells in the G_2/M phase increased from 17.0% to 57.2% to (3.4 times). In contrast, the androgen-insensitive cell line LNCaP showed less than 2 times differences after treatment with DES, but treatment of the LNCaP cell with

DESdP showed a notable increase from 9.0% to 30.5% (3.4 times) in hypodiploid nuclei and a decrease in the G_2/M phase from 11.4% to 3.7% (3.1 times).

High doses of DES are no longer in use, since blood clotting caused cardiovascular problems. This disadvantage may be abolished by additional agents such as aspirin (C9). The clotting problem may be part of inherited coagulation problems such as the factor V Leiden mutation, which is a single-base-pair substitution in the coding sequence for factor V that renders it unable to be inactivated (G18). The inability to inactivate clotting factors causes thrombophilia in the presence of high concentrations of estrogens and selective estrogen receptor modulators such as tamoxifen or oral contraceptives (G2, L6). Nevertheless, lower doses of DES are still a good alternative to bilateral orchidectomy (C21). The first case of a similar phenomenon to the antiandrogen withdrawal syndrome has been reported during treatment with DES (B6). In this case the tumor became refractory to DES treatment. Discontinuance of DES administration resolved into complete remission and, after 3 years of follow-up, no clinical or biological evidence of recurrence was documented. This may be explained by the fact that long-term treatment with estrogens causes irreversible impairment of Leydig cell function and consequently reduces testosterone secretion after cessation of estrogen treatment (T7). Other estrogens are also being used in neoplastic treatment. In a 15-year study of estrogen and estramustine phosphate therapy, patients with well-differentiated cancer did best on early polyestradiol phosphate plus ethinylestradiol treatment (L17). Furthermore, epithelium, as well as stromal cell growth, are inhibited by administration of estradiol, even in combination with testosterone (D5).

Biologically active plant compounds with estrogenic and antiestrogenic properties, phytoestrogens, have recently been found to cause prominent apoptosis of a prostate tumor without any side effects (S23). In this a case a 66-year-old physician, on his own initiative, took a concentrated phytoestrogen based on red clover (Promensil tablets: 4×40 mg/day) for only 1 week. Promensil treatment resulted in regression of the tumor before he underwent radical prostatectomy. After the operation, the tumor was compared with the low-grade adenocarcinoma biopsy which was taken before the Promensil treatment. In contrast with the biopsy, the tumor tissue showed a high degree of apoptosis, resembling high-dose estrogen therapy, but with an added advantage of no adverse effects (S23). However, this phenomenon may be different over a longer treatment period.

Phytoestrogens consists of three major groups, isoflavonoids, flavonoids, and lignans. It has been suggested that some of these compounds have anticancer properties, since Asians, who consume a diet rich in phytoestrogen, have reduced prostate cancer risk (D16). Soya and legumes are the major sources of the isoflavonoids genistein and daidzein (D16, M17). Examples of other isoflavonoids are biochanin, equol, and formononetin. Flavonoids such as apigenin and kaempferol are present in high concentrations in many fruits, vegetables, and crop species, such as apples, onions, and tea leaves (D16). Lignans, such as enterolactone and

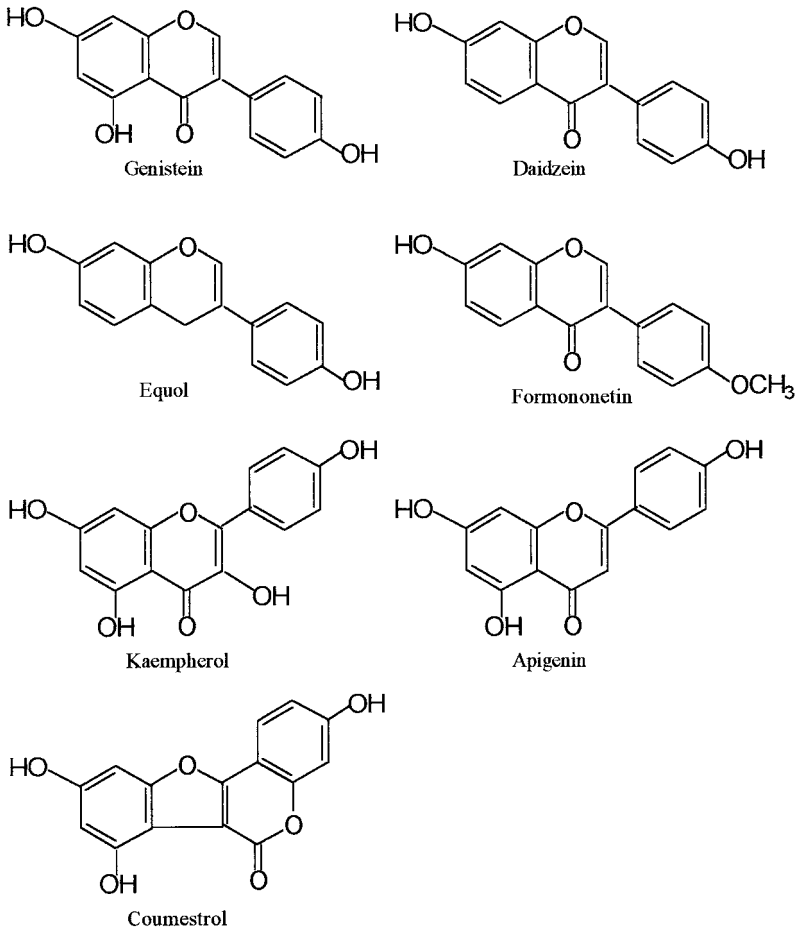

FIG. 5. Structures of some phytoestrogens.

enterodiol, are present in many cereals, grains, fruits, and vegetables and are especially abundant in linseed (flaxseed) and oilseeds (D16, M28). A few structures of phytoestrogens are shown in Fig. 5.

Phytoestrogens can act as both nonsteroidal estrogens and antiestrogens, but their estrogenic effects are much weaker than those of estrogens (D16). Since phytoestrogens compete for estrogenbinding sites, they can attach to these sites when estrogen levels are low and induce an estrogenic effect or, in the presence of high estrogen levels, phytoestrogens can compete with estrogens and blunt the effect of estrogens and in this way reduce cancer risk. In a study to assess

the value of phytoestrogens in prostate cancer, LNCaP cells were inoculated into severe immunodeficient mice which were fed soybean products (Z2). Soybean protein reduced tumor growth by 11% ($p = 0.45$), 0.2% soy phytochemical concentrate reduced tumor growth by 19% ($p = 0.17$), soy protein plus 0.2% soy phytochemical concentrate reduced tumor growth by 28% ($p \leq 0.05$), 1.0% soy phytochemical concentrate reduced tumor growth by 30% ($p < 0.005$), and soy protein plus 1.0% soy phytochemical concentrate reduced tumor growth by 40% ($p < 0.005\%$) (Z2). This anticancer phenomenon was also seen *in vitro,* where soy phytochemical concentrate, genistein and daidzein, reduced proliferation of prostatic cell lines DU-145, LNCaP, and to a greater extent PC-3 cells, which are more invasive (S3, Z2). In a case control study in Caucasian men, prostate cancer risk was decreased in men consuming large quantities of coumestrol ($p = 0.03$) and daidzein ($p = 0.07$), while genistein showed a slight protective effect ($p = 0.27$) (S27). Although phytoestrogens display estrogenic action, interaction with estrogen-binding sites may not be the only way by which malignancy is reduced, since they are also potent enzyme inhibitors. Enzymes that are inhibited by phytoestrogens include aromatase, 5α-reductase, and 17β-hydroxysteroid dehydrogenase, tyrosine kinases, and topoisomerase I and II (G21). Further research on these compounds may therefore be beneficial.

An expensive but over-the-counter Chinese herbal mixture, PC-SPES, which has been commercially available since November 1996, is a popular alternative to demonstrated therapies for prostate cancer (D11, G5, M30). The mixture consists of eight different herbs: chrysanthemum, isatis, licorice, *Ganoderma lucidum,* Panax pseudo-ginseng, *Rabdosia rubescens,* saw palmetto, and scutellaria (skullcap). Although PC-SPES is used as a nonestrogenic supplement, it may contain estrogenic organic compounds that are distinct from DES, estrone, and estradiol, as demonstrated with HPLC (D18). PC-SPES administration may reduce serum testosterone levels (D18,G5), and in a patient with clinically localized prostate cancer (T1c), administration of nine capsules of PC-SPES a day markedly decreased PSA, from 8.8 to 1.4 ng/ml after 3 weeks and to 0.1 ng/ml after 8 weeks (M30). However, adverse affects that were coupled with PC-SPES administration included loss of libido, erectile dysfunction, extreme breast enlargement and tenderness, reduction in overall body hair, pitting edema, and a significant drop in lipoprotein (M30). In another study, administration of three capsules a day also reduced PSA in most patients during 2–6 months of treatment, while two (6%) of these patients experienced nipple tenderness and two (6%) experienced leg clots (D11). *In vitro,* prostate cancer cell lines DU-145, PC-3, LNCaP, and an apoptosis-resistant derivative, LNCaP-bcl-2, showed a dose-responsive reduction in cellular viability at high concentrations of the PC-SPES extract (4 and 6 μl/ml), while only the hormone-sensitive LNCaP cell line showed a reduction at the lowest concentration (2 μl/ml) (D11). Further research on possible advantages and disadvantages of PC-SPES over prescribed hormonal treatment is essential

in establishing future use in hormone-sensitive and/or in hormone-insensitive prostate cancer.

3.2. Estrogens and Carcinogeneity

Estrogens may be culprits in carcinogeneity. It was proposed that estradiol and the cathecolestradiols could be possible genotoxic carcinogens that act as tumor inducers rather than tumor promotors (V3). In this regard, it is interesting to note that total prostate catecholestrogen concentration correlates with prostate mass, protein, and DNA synthesis and may therefore play a role in BPH and prostate cancer (N7). In the rat, chronic administration of estradiol in combination with low doses of testosterone caused low-grade carcinomas and is therefore the tumor-initiating agent in this system (B9). Environmental toxicants with estrogenic activity cause profound effects in the male reproductive system, such as infertility (D10). Therefore these foreign estrogens may cause prostate cancer by increasing free estrogens and destabilizing the testosterone:estrogen ratio. Combination of DHT with estrogen causes an increase in prostate size, AR, DHT formation from steroid metabolism, collagen, and alterations in cell death (L16). Estrogen effects are visible only in the prostate stroma and can cause florid squamous cell metaplasia that can be offset by androgens (L16).

3.3. Estrogen Receptors (ER)

The mechanism of action of estrogens on prostate cells is still largely unclear, and two proposals of estrogen binding have been investigated recently. These include binding to the prostate cellular AR in the presence of an AR agonist, ARA_{70} (Y2), or binding to a mutated AR that has increased affinity for estrogens and other ligands, as already discussed. The second possibility is the binding of estrogens to a genuine ER in prostate cells (C6). The presence of ER was illustrated with the finding that an increase in growth in LNCaP cells by estradiol was totally abolished by the addition of antiestrogen ICI-182,780 (C6). Addition of a synthetic androgen, methyltrienolone (R1881), increases estradiol binding (C6). A possible explanation may be that the R1881 displaces residual estradiol from the AR. Further, type I and type II binding sites of the ER are present in prostate tissues, as well as in human prostate cell lines, LNCaP, PC-3, and DU-145 (B14, C4, C6, E3, E4, M10, V7). However, results have mostly been conflicting, since not all prostate cancer patients are ER-positive (B14, H7, H13). In comparing normal, BPH, and prostate tumor specimens, differences in results have been obtained. In some cases, ER in prostate tumors were either lower (K15, K27) or higher than in BPH specimens (B15, K1, K2), or not significantly different (E6). Reduced levels or no ER were present in metastases (E4, H16). These conflicting results may indicate that ER expression, like AR expression, may be heterogeneous in prostate cancer specimens. However, some ER-positive prostate tumors are associated with a diploid DNA

pattern, which may be useful in predicting prognosis (N1). If present, ER is mostly present in the prostatic stroma, although basal and secretory epithelium cells may contain some ER (C4, D17, E2, K17, W5). Further, androgen ablation treatment with LH-RH agonists and flutamide, as well as estrogen treatment, up-regulates ER expression in a large number of stromal cells. In contrast, ER expression in epithelium cells were only occasionally up-regulated, but not in carcinoma cells (K17). Therefore, it is concluded that the morphological changes induced by estrogens can be explained by paracrine interaction between stromal and epithelium cells or tumor cells. In the Dunning (R3327H) rat model, which was originally developed from a spontaneously occurring prostatic adenocarcinoma in the male rat, DES treatment causes an increase in nuclear ER, which increases the number of ER that could be used in transcription (D22, M23). Furthermore, the role of the newly discovered ER isoform, ERβ, needs to be investigated, since it has been proposed that ERβ may play a role in defining epithelial heterogeneity in the prostatic duct system, which may be the key element in estrogen-mediated events in the prostate (C11).

The rat prostate was one of the first organs in which ERβ was discovered (K24). The human prostate possesses much less ERβ than the human testis, and a variant of ERβ, ERβ1, is detectable in human PC-3, DU-145, and LNCaP cell lines (E7, H2, M29). The function of ERβ in the prostate is largely unclear, but it has been suggested that ERβ could act as a marker of epithelial differentiation of the rat ventral prostate (P17). This hypothesis is based on the fact that normal ERβ expression is low at birth and increases as epithelial cells differentiate into luminal epithelial cells, and increase even more when with functional differentiation (P17). ERβ expression may be related to androgen levels, since ERβ mRNA levels decrease during castration and can be restored with subsequent testosterone replacement. ERβ expression is not regulated by estrogens (P17, S19).

The impact of the discovery of ERβ has yet to be established, but it may partly explain the complexity of selective estrogen and antiestrogen action in various tissues (K1). It may be beneficial to reevaluate previous research on estrogenic compounds in the light of this novel ERβ. Structurally, the ERβ amino acid sequence is highly homologous to the ERα amino acid sequence and, like other steroid receptors, it possesses conserved functional domains which are necessary for receptor function. The rat and human ERα and ERβ amino acid sequences show a 95–97% homology in the DNA-binding domains, while the ligand binding domains show a 55–60% homology, respectively.

The high homology between ERα and ERβ in the DNA-binding domain suggests that these receptors interact with similar estrogen response elements on the DNA. As suggested, ERβ binds to several EREs that are already known to bind to ERα (H22, K24). Although ERα and ERβ can bind to the same ERE, these receptors display different patterns of affinities for these ERE, which can cause differential activation in the presence of estrogens (H22, P7). Important factors which can determine the activation of particular estrogen responsive genes are

the nature of the ERE and the ratio of ERα and ERβ subtypes in a particular cell or tissue (P7).

Human and rat ERβ can form homodimers or heterodimers with ERα (O1, P1). Therefore, the cell can respond to estrogens in three different ways by forming three different types of dimers: ERα homodimers, ERβ homodimers, and ERα ERβ heterodimers (T9). Further research on the estrogen-mediated activation of different genes by these homo- and heterodimers and their content in different cells, tissues, and organs may be very interesting in illuminating selectivity of estrogen action in different cells and tissues.

The relative high homology of ERα and ERβ in the ligand binding domain imply that they have similar binding affinities for estrogen and estrogen-related compounds. Binding affinities for estrogenic compounds such as estradiol, DES, estrone, and 5α-androstane-3β,17β-diol are similar for ERα and ERβ. Even antiestrogens such as tamoxifen, 4-OH-tamoxifen, and the synthetic antiestrogen ICI-164,384 display similar binding affinities for both receptors. Phytoestrogens such as genistein, coumestrol, and zearalenone have up to 10-fold higher affinities for ERβ than for ERα, as reported in cell transfection studies (K21, K25). *In vivo,* coumestrol acts as an antiestrogen in the rat brain, which may suggest that the actions of phytoestrogens are tissue specific (P2). Ligands that acts as agonists on the one ER subtype and as antagonists on the other are being developed and may play a role in determining the different roles of ERα and ERβ (S29).

The low homology between ERα and ERβ in the N-terminal A/B domain indicates different patterns of gene activation between these two receptors. Construction of ERβ chimeras with ERα A/B domain exhibited an improved transcriptional response to estrogens and antiestrogens and indicated that differences in the N-terminal and contribute to cell- and promotor-specific differences in transcriptional activity of ERα and ERβ (M14).

The ERβ may play an important role in the antioxidant pathway (M25). Reduction of toxic and mutagenic quinones are mediated by the quinone reductase enzyme. Expression of quinone reductase and other detoxification enzymes is regulated by the electrophile/antioxidant response element (EpRE), which can be activated by the antiestrogen/ER complexes. ERβ activates EpRE to a greater extent than ERα, which suggests that ERβ may be important in activating chemoprotective detoxification enzymes (M25). Since ERβ is present in the prostate, it is suggested that ERβ may play a role in protecting the prostate from developing cancer (C11).

3.4. Progesterone Receptors (PgR)

Although progesterone receptors are present in prostatic stromal cells and to a lesser extent in epithelium cells, their actual role in the prostate is still unclear (B15, G26). Comparing PgR expression in BPH and prostate cancer patients, 16 of 19

(84%) stromal and 17 of 19 (89%) epithelial cells expressed PgR in BPH patients, while 20 of 26 (77%) stromal and 12 of 20 (60%) carcinoma cells in prostate cancer expressed PgR (H13). Since PgR expression seems to be much higher in BPH than in prostate cancer, it could be postulated that PgR plays a more important role in the development of BPH than in prostate cancer (B15). A possible explanation for this phenomenon is down-regulation of PgR content during progression of prostate cancer. Progesterone receptors were absent in metastatic lesions as well as prostatic cell cultures of LNCaP, DU-145, and PC-3 (H16). Endocrine therapy, however, could alter the tumor cell and PgR content, since estrogens and the antiandrogen cyproterone acetate up-regulate PgR expression in the carcinomatous prostate, causing differentiation (M22, S1). However, the exact role of this up-regulation of PgR and its possible role in the development of tumors resistant to endocrine therapy is still unknown.

3.5. Vitamin D Receptor (VDR), Retinoic Acid Receptors (RAR), and Retinoid X Receptors (RXR)

Vitamin D production in the body is partly dependent on sunlight and the fact that prostate cancer seems to be increased in low-sunlight areas, indicated a possible negative correlation between vitamin D or its metabolites and prostate cancer risk (H4, P4). Since sunlight intensity and duration vary in different seasons, it has been proposed that seasonal down-regulation of serum levels of vitamin D metabolites could increase prostate cancer risk. Therefore, it was demonstrated that 25-hydroxyvitamin D_3 (25-D), the less active form of vitamin D, increases in prostate cancer cases as well as in controls during summer. In contrast, 1α,25-dihydroxyvitamin D_3 (1,25-D), the more active form, shows no variation in controls, while serum levels are decreased in black and white men with prostate cancer (C20). Insufficient levels of the 1,25-D metabolite is therefore a possible determining factor in this disease. Vitamin D and metabolite levels can be influenced by nutrition, not only by vitamin D intake, but also by calcium and fructose intake. High calcium intake increases prostate cancer and metastatic risk, and this may be due to the fact that calcium suppresses 1,25-D formation from 25-D (Fig. 6). In contrast with calcium, fructose increases the 1,25-D serum level and decreases prostate cancer risk. High fruit consumption would play a beneficial role (G15). In addition to low serum levels of vitamin D metabolites, vitamin D-binding proteins (VDBP), as well as VDBP polymorphic variations are associated with increased risks in prostate cancer (B11).

Since sunlight seems to play an important role in preventing the development of prostate cancer (H4), it leads to the investigation of vitamin D and its metabolites as anticancer agents in prostatic cell cultures and the Dunning rat model. Treatment of prostatic cell lines PC-3 and LNCaP *in vitro* and in the Dunning rat model with the 1,25-D metabolite causes a decrease in proliferation of tumor cells (F7, G9,

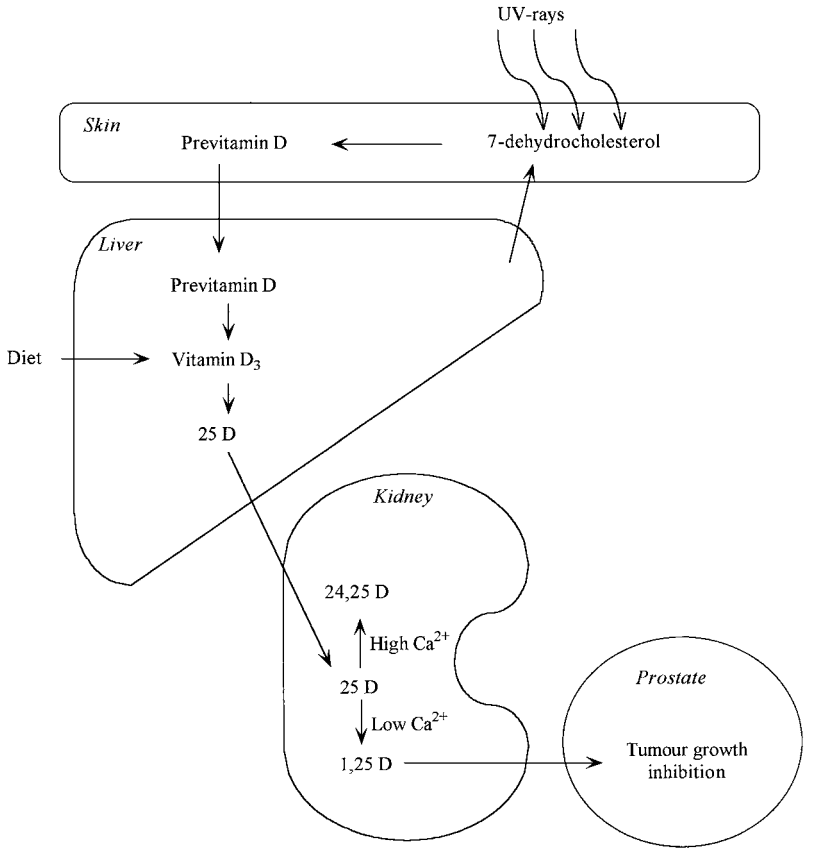

FIG. 6. Vitamin D metabolism in the body.

H9, H20, S12, W2, Z1). Unfortunately, there are limitations to the use of 1,25-D *in vivo,* since 1,25-D induces hypercalcemia (N8). Analogs with antiproliferating effects that eliminate hypercalcemia have been investigated with good results (C1, C2, C3, D13, L13, S12, S20). For example, invasiveness of DU-145 cells was reduced by 59.4% by the noncalcemic vitamin D_3 analog 1,25-dihydroxy-16-ene-23-yne cholecarciferol (16-23-D3) at a concentration of 1×10^{-7}M (S13). The vitamin D_3 analog 1,25(OH)2-16-ene-23-yne-26,27-F6-19-nor-D3 reduced PC-3 cell proliferation by 50% at a concentration of 1×10^{-7}M (C2). When athymic mice were inoculated with PC-3 cells and treated with 1.6 μg of 16-23-D3, tumor volumes were reduced and approximately 15% smaller than in the control groups, with no evidence of hypercalcemia (S12). Although vitamin D_3 analogs show favorable results in *in vitro* cell culture studies and *in vivo* studies in experimental animals, clinical trials in humans are needed to test these compounds.

More than one mechanism, a nongenomic signaling pathway involving a membrane-associated receptor, as well as a genomic pathway involving the nuclear VDR, has been proposed for the antiproliferating effect of 1,25-D (H9, H20). However, the involvement of the mebrane-associated receptor was ruled out by the observation that the addition of antisense VDR inhibited the expression of nuclear VDR and reduced or abolished the effects of 1,25-D in prostate cells (H9, Z5). Further evidence of VDR involvement includes the observation that DU-145 and PC-3 cell lines with low amounts of VDR are insensitive to 1,25-D (Z6).

Another factor that could be involved in 1,25-D sensitivity is androgens, since DHT and 1,25-D inhibit LNCaP cell growth synergistically. Growth inhibition is abolished in the absence of DHT or by the addition of antiandrogens such as Casodex (E8, Z1). This indicates a possible role for AR in 1,25-D sensitivity. In addition to cell differentiation, the 1,25-D metabolite causes a twofold increase in AR and PSA, which is synergistically increased in the presence of DHT (E8, Z1). In the cell, 1,25-D may even promote translocation of AR across the nuclear membrane to the nucleus (H19). Since the AR are increased, this may cause the malignant prostate to respond to androgen ablation therapy in combination with administration of 1,25-D. Although 1,25-D increases PSA production in LNCaP cells, it seems that 1,25-D could also slow down the rate of increasing PSA levels and the recurrence of prostate cancer after primary treatment with radiation or surgery (G23). A role for 1,25-D in preventing metastasis has also been suggested, since 1,25-D and its analogs may selectively decrease the type IV collagenases MMP-2 and MMP-9 (S13).

Normal prostate, prostate carcinoma, and BPH contain indogenous vitamin A (retinol) and retinoic acid (RA). Vitamin A has been found to be twofold elevated in BPH compared to normal prostate and prostate carcinoma. Prostate carcinoma contained five- to eightfold less RA than normal prostate or BPH (P3). A loss of RA in prostate cells could therefore lead to prostate cancer. Administration of RA could be beneficial in the prevention or cure of cancers. Metabolic active metabolites of vitamin A, such as all-*trans*-retinoic acid (atRA), 9-*cis*-retinoic acid (9cRA), and 13-*cis*-retinoic acid, inhibit primary malignant prostatic tissues and cell lines in culture (P4). In nude mice it is involved in cell differentiation (D1, D2, D13, J2). Retinoids often act in concert with vitamin D_3 and its analogs (C3). Clonal growth of LNCaP cells is synergistically or additively inhibited by combinations with vitamin D_3 analogs, such as 1α25(OH)2-16-ene-23-yne-26,27,F6-19nor-D3, and the RAR-selective retinoids (C3). Similarly, synergistic growth inhibition is displayed by a combination of 9cRA and 1,25-D, and this effect is even more inhibitory when the 1,25-D analog EB1089 is used. This synergistic inhibition is mediated through the RXR, since RXR-selective retinoids inhibit this effect (B8).

Retinoids exert their action on the cell by binding to two distinct families of receptors, the RAR and the RXR. Both of these receptors can be divided into three different subtypes, the α, β, and γ subtypes (P6). Not all retinoids bind to both

receptors; for example, atRA and 9cRA bind to RAR, while 9cRA binds to both RAR and RXR (A3, C3, G10, P6). Before transcription can take place, receptor dimers must be formed. The RAR requires heterodimerization with RXR, while RXR can form either heterodimers or homodimers (D13).

Heterodimers of RAR and RXR with VDR may explain the synergistic or additive inhibition observed in prostate cancer cells when combinations of 1,25-D or its analogs and retinoids are administered (B8, C3, C15, P13, S7). Nevertheless, it cannot be excluded that either 1,25-D or retinoids could induce the synthesis of another protein that could enhance the response of the cell to either compound (B8).

4. Growth Factor Receptors

Growth factors and growth factor receptors play an important role in the regulation of normal cell growth and development. They can act as positive or negative regulators of cell activity, causing proliferation and differentiation or apoptosis. Regulation of expression and activation of these proteins is therefore essential in maintaining a normal growth pattern. Growth factors exert their action on the cell by binding to their corresponding cell surface receptors, which initiate an intracellular phosphorylation cascade, which leads to activation of mitogen-activated protein kinases (MAPKs), which activate transcription factors (A2). The genes encoding for growth factors, growth factors, and other factors involved in the growth factor signaling pathway are called protooncogenes. When regulation of protooncogene expression is altered aberrantly, these factors are expressed constitutively, causing uncontrollable cell growth that leads to hyperplasia or neoplasia. Therefore, the expression of growth factors and growth factor receptors have been investigated in the normal, benign, and malignant prostate. The peptide growth factors that play a role in the normal and abnormal prostate are the epidermal growth factors (EGF), transforming growth factors α and β (TGF-α and TGF-β), insulin-like growth factor (IGF-I and IGF-II), fibroblast growth factor (FGF), nerve growth factors (NGF), and certain cytokines such as the interleukins.

4.1. Epidermal Growth Factor Receptors (EGFR)

The EGFR is a transmembrane tyrosine kinase protein of around 1200 amino acid residues (G19). The large extracellular domain is glycosylated and binds several ligands such as EGF, TGF-α, amphiregulin, heparin-binding EGF-like growth factor, and betacullin. Binding of these ligands causes activation of the intracellular tyrosine kinase domain and thus the growth factor signaling pathway. The gene that encodes for EGFR is the c-erbB protooncogene (G19). Mutation of c-erbB produces the c-erbB oncogene, which may result in a deleted extracellular domain

that causes the intracellular domain to be constitutively activated by dimerization. This dimerization allows these domains to cross-phosphorylate or autophosphorylate and induces continuous activation of the signaling pathway (G19, S17).

High- and low-affinity binding sites of EGFR are present in normal, hypertrophic, and carcinomatous prostates (D8). Normal and hyperplastic prostates are usually EGFR-positive, while prostate carcinomas frequently have a lesser and more variable EGFR content (F8, L11, M2, M16, V8). On the other hand, it was reported that EGFR mRNA is much higher in prostate cancer than in BPH (K26, M27). If one assume that this discrepancy between EGFR mRNA and protein is not due to experimental error, it can be hypothesized that translation on EGFR mRNA is to some extent impaired in prostate cancer cells. Another explanation may be that after binding with ligands the receptors accumulate in coated pits and are then degraded in lysosomes. Receptor binding therefore leads to receptor down-regulation and this could also explain the lower levels of EGFR protein compared to mRNA in prostate cancer (G19). In some cases of prostate cancer, well-differentiated tumors express four times more EGFR than poorly differentiated prostate tumors. This means that the expression of EGFR correlates with the histological grade of the cancer and it may be involved in the promotion of prostate cancer (M2). As the tumor progresses, more EGFR are expressed and EGF binding is increased in dedifferentiated specimens (D8, M27). However, other studies reported that there is no significant correlation between tumor grade and EGFR content (H6). Dedifferentiation of tumors indicates a loss of differentiation, as well as a loss of orientation of cells to one another and to their axial framework and blood vessels.

Immunohistochemical studies have shown that the basal cells in BPH and normal prostates stain the strongest for EGFR protein. Malignant cells stain positive in some cases, but much less, and stromal cells do not stain at all (C14, M13, M33). The prominence of EGFR in basal cells compared to secretory/luminal cells can be explained by the fact that basal cells are proliferating cells, which regenerate the epithelium and act as stem cells for the differentiated secretory/luminal cells. In the normal and hyperplastic prostate, as well as in some primary tumors, the main ligand for EGFR, TGF-α, is produced by stromal cells, which do not express EGFR. This suggests a paracrine or juxtacrine loop as the ligand is produced in the stroma, while it exerts its effects on the epithelium via the EGFR receptor (Fig. 7). It was suggested that this paracrine loop may be up-regulated in BPH by increased expression of both TGF-α and EGFR in the stroma and epithelium, respectively (L5). Malignant and metastatic tumors, however, express both the EGFR and the ligand TGF-α or EGF. This autocrine control of cell proliferation results in a growth advantage for these cells above their counterparts, which are dependent on more distant stromal cells (C14, L5, M13, M33, S6).

Cultured prostate cancer cells LNCaP, PC-3, and DU-145 express the EGFR protein, and the PC-3 and DU-145 cells exhibit increased EGFR mRNA (C16,

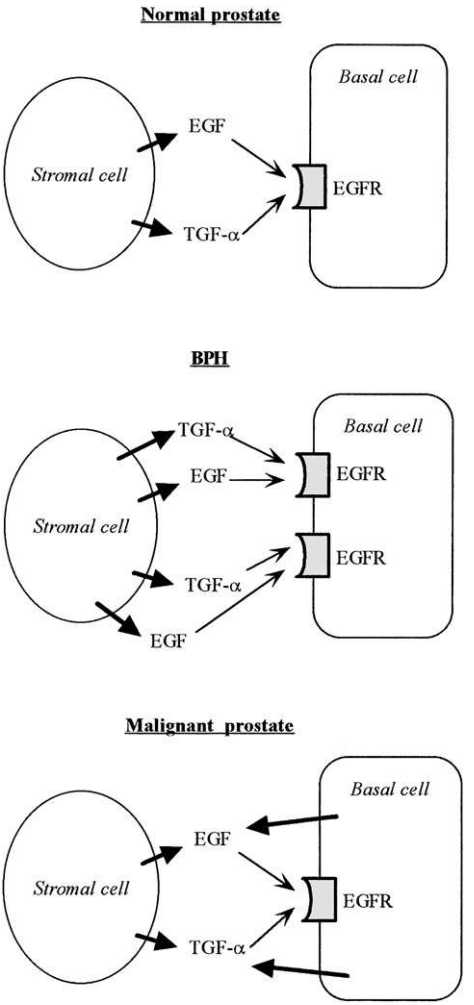

FIG. 7. Paracrine and autocrine interactions of EGFR in the normal, hyperplastic, and malignant prostate.

M27, S11). Cell density can be a crucial factor in determining EGFR content, since lower EGFR are expressed in confluent DU-145 cultures than in subconfluent cultures (C16, T6). This is also true in PC-3 cells, where TGFα, a ligand for EGFR, stimulates only low-density culture cells (H17).

Androgens may not act on the prostate cells directly, but stimulate them to produce growth factors in stromal cells and EGFR in basal cells to cause cell

proliferation via a paracrine effect in the normal and hyperplastic prostate, and an autocrine effect in cancer (C17, L14, S17). Androgens such as DHT and R1881 stimulate hormone-dependent cell proliferation as well as EGFR expression in LNCaP and ALVA101 cultured cells (L14, M31, S8, S9). The EGF causes an increase in LNCaP proliferation and, in combination with the synthetic androgen R1881, it has a synergistic effect (S8). In PC-3 cells that are transfected with human AR, cell proliferation was increased with the addition of either DHT or EGF. Together they exhibited a synergistic effect (B10). In addition to these effects, DHT causes an increase in EGFR mRNA and EGFR-binding affinity of more than twofold. This correlates with an increase in EGF binding and mitogenic response to EGF (B10). Expression of the EGFR ligands EGF or TGF-α in LNCaP cells and amphiregulin in both LNCaP and ALVA101 cells is up-regulated by the addition of androgens (L14, R2, S15). The increase in EGF by the androgen R1881 in LNCaP cells could be blocked by hydroxy-flutamide (R2). In another study, DU-145 cells were grown together with LNCaP cells and DHT, which resulted in cell growth stimulation only of the DU-145 cells (K10). In this case, DHT stimulated LNCaP cells to produce growth factor, which could be utilized by hormone-independent DU-145 cells. This evidence support the idea that there exists cross-talk between the signaling pathways of the AR and the EGFR. Furthermore, estradiol, progesterone, and antiandrogens, such as cyproterone acetate and RU 23908, cause an increase in LNCaP cell growth as well as in EGFR expression (S10, S11). The increased cell growth and EGFR expression paralleled the affinity of the hormone for the AR. Therefore, it may be concluded that the mutant AR is responsible for the up-regulation of EGFR expression in LNCaP cells.

It has been hypothesized that EGFR and the chemoattractant activity of EGF play an important role in invasiveness of prostatic cancers (T13, X1, Z8). In a study, DU-145 human prostatic carcinoma cells were transduced with a full-length, wild-type EGFR or with a mitogenically active but motility-deficient truncated (c′973) EGFR (X1). Migration through a human amniotic basement membrane matrix demonstrated that the DU-145 cells with the full-length wild-type EGFR migrated the most, followed by the parental DU-145 cells and lastly the cells with the truncated EGFR. An antibody which prevented ligand-induced activation of EGFR restricts movement of the full-length wild-type EGFR and parental DU-145 cells to the level of the truncated DU-145 cells (X1). When these three cell types were inoculated into athymic mice, DU-145 cells which overexpressed the wild-type EGFR were the most invasive, and those expressing the truncated EGFR showed no metastasis. Administration of U73122, a pharmacological agent which blocks EGFR-mediated cell motility but not mitogenity, decreases invasiveness (T13). Furthermore, blocking of the EGF-mediated phosphorylation of EGFR inhibits chemomigration of TSU-pr1 prostatic cancer cells *in vivo* (Z8). Since EGFR affects migration and invasiveness of prostate cancer cells *in vitro* as well as *in vivo,* EGFR antagonists may be considered in treatment of metastatic prostate cancer.

Selective blockage of EGFR inhibits the action of EGF as well as IGF-I signaling (P19). Therefore, a variety of possible therapeutic agents that target the EGFR pathway have been investigated. Certolix, a LH-RH antagonist, caused down-regulation of EGFR in human prostate cancer cells *in vitro* and *in vivo,* as did bombesin antagonists RC-3940-II and RC-3950-II (J3, J4, L1, M8). A flavonoid antioxidant isolated from milk thistle, silymarin, causes inhibition of TGF-α, or constitutive activation of EGFR or cell cycle arrest in the G1 phase (Z7). This could be due to an impairment of the EGFR pathway or via up-regulation of the cyclin-dependent kinase inhibitors Cip1/p21 and Kip1/p27 (Z7). A diet rich in soy may be beneficial, since a phytoestrogen, genistein, reduces EGFR and c-erbB2/Neu expression and phosphorylation in the rat dorsolateral prostate (D4). Another potential target in the attempt to limit tumor progression and metastasis is phospholipase c γ, which is involved in the EGFR signaling pathway (T12). Artificially synthesized oligonucleotides that are complementary to specific sequences in mRNA are called antisense oligonucleotides (A2). When these molecules bind to the sequences in mRNA, they inhibit translation of these mRNAs and the expression of its proteins. Antisense TGF-α and EGFR cause inhibition in prostate cancer cells *in vitro* and *in vivo* in athymic mice due to a decrease in expression of these proteins. Therefore, these antisense oligonucleotides may be beneficial in treatment of prostate cancer (R14).

Another cell membrane tyrosine kinase of the EGFR family, c-erbB2/HER2/Neu, is the transcription product of the c-erb-B-neu oncogene, which is overexpressed via gene amplification in breast and prostate cancer (A7). This protein is elevated in serum of patients with advanced prostate cancer, and these patients had a shorter interval before progression occurred (A7). Recently, it was found that c-erbB2/HER2/Neu could induce PSA at low levels of androgen by stimulating transactivation of the AR by promoting the interaction of AR with AR coactivators such as ARA_{70}. This could possibly lead to hormone resistance during androgen ablation treatment, since hydroxyflutamide could not completely inhibit PSA production (Y3).

4.2. Transforming Growth Factor β Receptors (TβR)

Transforming growth factor β-1 (TGF β-1) is a potent negative regulator of cell growth as well as a potential regulator of prostate cancer cell growth and metastasis (S22). Transforming growth factor β receptor types I, II, and III (TβRI, TβRII, and TβRIII) are receptors for TGF β, and only two of these three major classes of TGF β receptors, TβRI and TβRII, have been investigated in prostate cancer. They are not tyrosine kinases like EGFR, but are serine/threonine protein kinases that contain an extracellular ligand-binding domain, a single transmembrane domain, and a cytoplasmic serine-threonine kinase domain (B2). These two receptors share only 40% amino acid homology and have various differences. The TβRI has a

domain that is rich in glycine, serine, and threonine (GS domain), which immediately precedes the kinase domain and is phosphorylated during receptor binding (B2). The TβRI has a shorter C-terminal at the end of the kinase domain as well as a shorter N-terminal and differs from the TβRII in the distribution of conserved cysteines (B2). Before a signal can be obtained, the ligand TGF β must bind to TβRII, whereafter TβRI forms a heterodimer with this complex (B2). The TβRII phosphorylates this complex in the GS domain of TβRI, thereby activating the kinase activity of TβRI and the subsequent signaling cascade. Different substrates may be phosphorylated by TβRI kinase activity, initiating different signaling pathways which cause different responses to TGF β-1 (B2).

In the normal prostate, TβRI is present only in the basal cells of the epithelium (G24, R13). In contrast to TβRI, TβRII is present in the basal cells, as well as in some secretory cells (G24, R13). The ligand, TGF β-1 is mostly secreted by the basal cells, although some cells in the connective tissue in the stroma also produce TGF β-1 precursors (R13). In BPH, TβRI and TβRII are expressed at high levels in both basal and secretory epithelial cells and at lower levels in some stromal cells, while TGF β-1 is produced by both basal and secretory epithelial cells (K5, R13, W10). In prostate cancer, the TβRI and TβRII contents are low and heterogeneous, indicating that some expression occurs, while in well-differentiated specimens, they are overexpressed (G7, G25, I1, K3, K5, W10). Loss of TβRII is associated with increased Gleason score and histological grade (W10). Decreased levels of TβRI are generally associated not only with Gleason score, but also with clinical tumor stage, survival rate, and serological recurrence after radical prostatectomy and could therefore be applied as a possible prognostic marker (K3, W7). As some prostate tumors progress, TGF β-1 expression increases or becomes more heterogeneous (G7, R13, W7). Since TGF β-1 usually causes cell growth inhibition, prostate tumor cells could be in some way resistant to the action of TGF β-1, and this has yet to be cleared up.

Castration seems to influence these receptor and ligand levels, since patients who responded favorably to castration presented apoptosis, as well as increased levels of TGF β-1, TβRI, and TβRII mRNA (L3, W6). In castrated rats that were transplanted with the androgen-insensitive Dunning R3327 cancer, TGF β-1 increased in basal epithelial cells, as well as in TβRI and TβRII levels, and this effect could be enhanced by estrogen treatment (L3, W6). Cell lines DU-145 and PC-3 are sensitive to the anitproliferating effect of TGF β-1, and this can be partly explained by the fact that they express both TβRI and TβRII (K5). Anticancer agents such as fenretinide causes apoptosis, which is accompanied by up-regulation of TGF β-1 secretion as well as an increase in TβRI and TβRII expression in PC-3 cells. An antibody against TGF β-1 cancels this effect, and these results show the importance of the inhibiting effect of TGF β-1 (R8).

In contrast to the androgen-independent DU-145 and PC-3 cells, the LNCaP cell line is resistant to the inhibiting effect of TGF β-1, since it lacks either the TβRI

or TβRII or both of these receptors (K5). When each of these receptors was transfected into genetically changed LNCaP cells lacking TβRI, they became sensitive to the growth inhibitory effect of TGF β-1 (G24, J1). Unchanged LNCaP cells respond to TGFβ-1, but only in the presence of androgens (K4). In another highly aggressive prostate cancer cell line, TSU-Pr1, with TβRI and TβRII, TGFβ-1 stimulates growth (L2).

A role in metastasis has been proposed for TGFβ-1. For example, prostate tumor cell lines derived from focal pulmonary metastasis secrete high levels of TGFβ-1 and do not respond by growth inhibition, but by inducing the type IV collagenase matrix metalloproteinase-9 enzyme. This proteolytic enzyme degrades the basement membrane to facilitate invasion (S14).

4.3. Insulin-like Growth Factor Receptors (IGFR)

The insulin-like growth factor receptor type I (IGFRI) has a similar structure to the insulin receptor. It consists of two disulfide-linked α chains, which bind the insulin-like growth factors (IGF-I and IGF-II), and two β chains that have tyrosine kinase activity for signaling (N6). The IGF type II receptor (IGFRII), also called IGF-II-mannose-6-phosphate receptor, is a single polypeptide chain that lacks tyrosine kinase activity and therefore does not convey signals. This receptor may be involved in internalization and degradation of IGF-II by targeting lysosomal enzymes from the Golgi apparatus or the plasma membrane to the lysosomes (N6). In terms of binding affinity, the IGFRI and IGFRII receptors bind IGF-I and IGF-II to a greater extent, respectively. Insulin binds with lower affinity to IGFRI, but not at all to IGFRII (R3).

The cellular action of IGF is dependent not only on the presence of IGF receptors, but also on the six different available IGF binding proteins (IGFBPs) (C17, F5, H23). Overexpression or limited proteolysis of some of these binding proteins is known to occur, which may act as a regulatory mechanism for IGF action by interfering with binding to IGFRI (A5, A6, D6, F6). Proteases which could be involved in this mechanism include cathepsin D, tissue-specific plasminogen activator (tPA), urokinase-specific plasminogen activator (uPA), PSA, and metalloproteinase-9 (B16, C18, M7). Other growth factor autocrine loops could also interfere with IGFRI function. For example, the secretion of IGFBP-1 is dependent on the EGF autocrine loop, and when this loop is interrupted, IGFBP-1 secretion is inhibited, causing inhibition of IGF-I and IGF-II action (C17). Alteration in IGFBP-1 and -3 levels, in the elderly, could be a causative agent in the development of prostate cancer with age (B4). Cross-talk between the IGFR and AR signaling pathways can occur, since the IGFBP-5 is up-regulated by androgens in the CWR2 human prostate cancer xenograft (G20).

High serum levels of IGF-I have been associated with increased risk of prostate cancer (W12) and since bone are high in IGF-I, it has been suggested that this could

be a reason why prostate cancer frequently metastasizes to bone (B16). Expression of IGF-II (7.5 kDa) in prostate cancer reveals that an incomplete processed form of IGF-II (15 kDa) with higher mitogenic properties is present in cancerous cells (L8).

In normal and BPH prostates, IGF-I and IGFRI are present in stromal and epithelial cells, respectively. In contrast to this paracrinic role in normal and BPH prostates, an autocrine role has been suggested in prostatic adenocarcinoma, since IGF-I and IGFRI are both expressed in epithelial cells (W3). Compared to BPH, cancerous prostates have reduced IGFRI mRNA and increased IGF-II mRNA (T2). This reduction in IGFRI is confirmed *in vitro* when prostate epithelium cells are transformed from a poorly tumorigenic to a malignant phenotype (P16). Although reduced, the IGFRI plays an important role in tumor growth and metastasis, since antisense IGFRI causes further reduction of IGFRI and suppresses tumor growth and metastasis in malignant tumors. Reduced metastasis probably occurs by decreased uPA and tPA secretion (B16).

In cultured prostate LNCaP, PC-3, and DU-145 cells, IGFRI and IGFRII are expressed, as well as IGF-II, while trace amounts of IGF-I are expressed only in the androgen-dependent LNCaP cell line (K6). Another difference between the androgen-dependent and -independent cell lines is the report that IGF-I stimulates DNA synthesis in DU-145 and PC-3 cells, but not in LNCaP cells (I4). Addition of DHT is necessary to cause an increase in DNA synthesis of the LNCaP cells (I4). This may imply a different mechanism for androgen-dependent and androgen-independent tumors and probably interaction or "cross-talk" between the AR and IGFRI signaling pathways. Autophosphorylation has also been reported in these three cell lines (P12).

A reduction in IGF levels could be beneficial in prostate cancer treatment. Administration of the LH-RH antagonist, Cetrorelix, caused a reduction in IGF-I serum levels and tumor IGF-II levels in nude mice transfected with PC-3 cells (L1). Zoladex, a LH-RH agonist with antimitogenic activity, caused a decrease in IGF-I as well as IGFRI levels in DU-145 cells (M9). In the latter case, phosphorylation of IGFRI was prevented, causing a further inhibitory role of IGFRI action.

4.4. Fibroblast Growth Factor Receptors (FGFR)

Fibroblast growth factor receptors (FGFR) are divided into four major forms, designated FGFR1–4 and subtypes, designated by IIIa, IIIb, or IIIc (S26). Like most of the other growth factor receptors, the FGFR span the membrane, with an extracellular fibroblast growth factor (FGF)-binding domain and an intracellular tyrosine kinase domain. The extracellular domain contains three immunoglobulin-like loops that differ due to alternative mRNA splicing. Therefore, different forms of each receptor are produced that differ in extracellular domain and ligand-binding specifities. The FGFR contain high-affinity binding sites for mitogenic FGF. These growth factors consist of a family of nine structurally related peptides and are cell

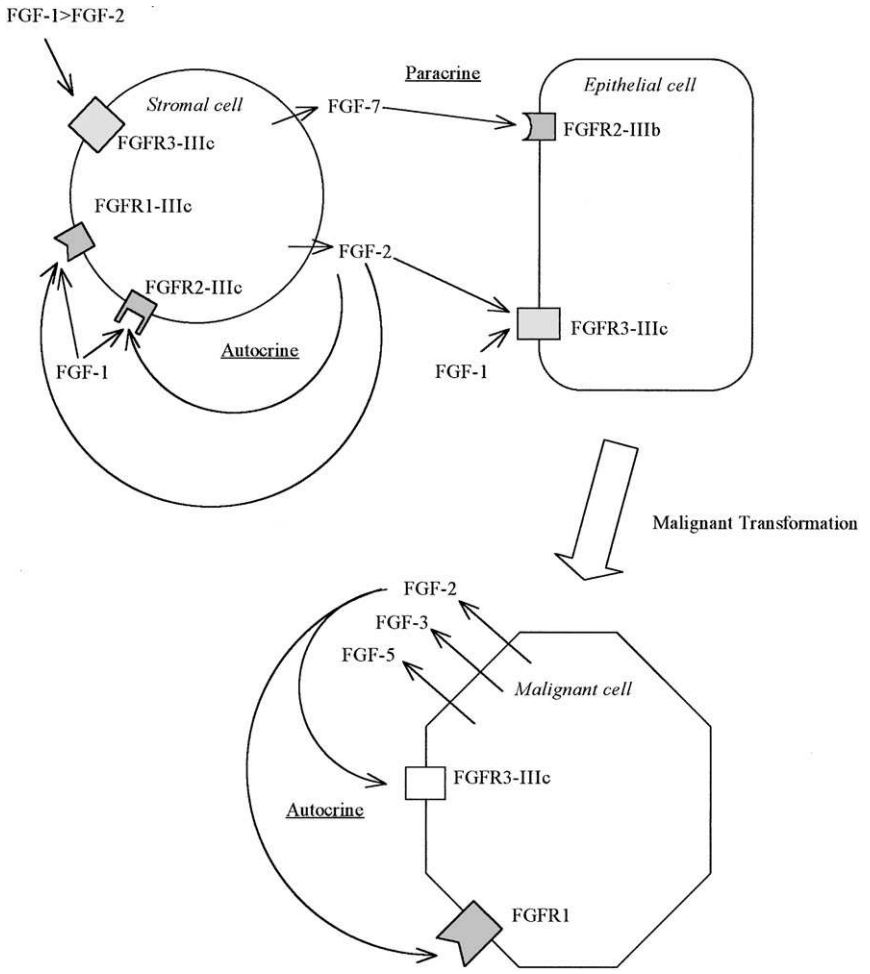

FIG. 8. Paracrine and autocrine interactions of FGFR in prostatic cells.

specific. For example, FGF1–6 are mitogenic for mesodermal and neuroectodermal cells, while FGF7, also called the keratinocyte growth factor (KGF), is mitogenic for epidermal cells (S26).

With the exception of FGFR4, mRNA for all other three receptors have been reported to be present in the normal human prostate (I3). In contrast to the prostatic stromal cells, which mainly secrete FGF7 and to a lesser extent FGF2 and FGF1, the epithelium cells do not produce any FGF. However, both stromal and epithelial cells express FGFR isoforms (Fig. 8) and therefore it can be proposed that the

stromal and the epithelial cells are subject to autocrine and paracrine control, respectively (I3, S26).

In prostate cancer, epithelial cell growth becomes independent from paracrine control of the stromal cells and this effect could be explained by loss of the FGF7-binding receptor, FGFR2-IIIb. Loss of FGFR2-IIIb correlates with androgen insensitivity in human prostate cancer models, which suggests cross-talk between the androgen and FGF signaling pathways in androgen-sensitive prostates (C5). Reexpression of FGFR2-IIIb in malignant cells restores paracrine responsiveness to stromal cells and differentiation of epithelial cells (F4, M12). Therefore it may be proposed that the potential for restoring the FGFR2 in malignant tumors may be investigated as gene therapy in androgen-insensitive prostate cancer.

Independence of malignant cells from stromal cell control is accompanied by initiating the production of FGF2, FGF3, FGF5, and FGFR, which strongly suggests autocrine control of the FGFR signaling pathway in prostate cancer (Y1).

4.5. Nerve Growth Factor Receptors (NGFR)

Neutrophins, such as nerve growth factor (NGF), brain-derived neurotrophic (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) factor and their respective receptors, tropomyosin receptor kinase A (trkA), trkB, and trk C, are present not only in the nervous system, but throughout the body and in the prostate (D3). Prostatic smooth muscle cells express NGF, BDNF, and trkC, while prostatic epithelial cell lines express only the receptors trkA, trkB, and trkC, suggesting a paracrine mechanism. A switch from paracrine control to autocrine control in androgen-sensitive and androgen-insensitive prostate cancer cell lines was reported, since LNCaP expressed no neutrophins, while the androgen-insensitive cell line TSU-pr1 expressed NGF, BDNF, and NT-4/5 (D3). Various trk inhibitors, such as K252a alone or CEP-751 and CEP-701 together with androgen ablation have been investigated as potential anti-prostate cancer drugs (D14, D19, G6).

Loss of another nerve growth receptor, the low-affinity nerve growth factor receptor (LNGFR)/(p75)/(75NTR), occurs in benign and malignant prostate tissues and is associated with an increase in Gleason score and PSA levels in patients with well-differentiated cancerous epithelial cells (P9, P11). No LNGFR is present in prostate cancer cell lines LNCaP, PC-3, TSU-pr1, and DU-145 (P11). Binding of NGF to LNGFR causes growth inhibition in LNGFR-transfected TSU-pr1 cells, and therefore the loss of this receptor could contribute to the uncontrolled growth of prostatic neoplasms (P10).

4.6. Platelet-Derived Growth Factor Receptor (PDGFR)

Two homologous PDFGR, PDGFRα and PDGFRβ, are both tyrosine receptor kinases. They bind the PDGF A and B and induce migration, proliferation, and

differentiation in cells (F9, F10). Receptor dimerization occurs to form homodimers and heterodimers and, similar to other growth factors, binding of the ligand PDGF causes activation of the tyrosine kinase and autophosphorylation (F9, F10). Little research has been done on PDGFR in prostate cancer. However, prostatic adenocarcinomas express PDGFRα and PDGF A in both stromal and epithelial cells, but not PDGFRβ and PDGF B. This suggests an autocrine loop for PDGFRα and PDGF A. Furthermore, a decrease in staining intensity correlates with an increase in Gleason score and vice versa (F9, F10).

4.7. Cytokine Receptors

Several cytokine receptors are present in prostate tumors and in prostate cancer cell lines. These include the granulocyte-macrophage colony-stimulating factor receptors (GM-CSF-R) and macrophage colony-stimulating factor receptor (M-CSF-R) (R7, R10, S4). The presence of interleukin receptors in the prostate tumor which bind interleukins such as IL-2, IL-3, and IL-6 may play a role in hormone-dependent as well as in hormone-refractory prostate cancer tumors (C13, R6). However, the importance of these receptors needs to be illuminated.

5. Other Membrane Receptors

5.1. Adrenergic Receptors

Other cell surface receptors that may play a role in prostate cancer include the adrenergic receptors. The predominant adrenergic receptor in the human prostate seems to be the α-1C-adrenoceptor, which is involved in mediating the contraction of prostatic smooth muscle cells (F1, L7). It is suggested that these receptors may link to a proposed neuroendocrine differentiation in androgen-independent and metastatic prostate tumors, since they occur only in prostate glandular and stromal cells of the tumors (D20, T10). Cross-talk of the β-adrenergic and neurotensin signaling pathways with the androgen receptor pathway may occur (M19).

5.2. Gastrin-Releasing Peptide Receptors (GRPR)

Gastrin-releasing peptide (GRP) and bombesin stimulate tumor growth via an autocrine mechanism through the GRP receptors (M11). In contrast to poorly differentiated prostate tumors, well-differentiated tumors have increased GRPR levels. Therefore, it has been postulated that overexpression of GRPR occurs in tumors that are being transformed or are already transformed to malignant state (M11).

5.3. Urokinase-Type Plasminogen Activator Receptors (uPAR)

In the process of invasion, tumor cells have to attach themselves to the target tissue and then invade or penetrate the matrix by proteolysis of the matrix components before they can migrate through the basement membrane (V2). Therefore, proteolytic enzymes, such as urokinase-type plasminogen activator (uPA), play an important role in successful tumor metastasis. Essential fatty acids were shown to decrease production of uPA in DU-145 cells and could therefore reduce prostate tumor metastasis (VI). Successful invasion is dependent on binding of uPA to the uPA receptor (uPAR) on the cell surface to localize the proteolytic activity. However, binding may be prevented by uPAR antagonists and thus inhibit invasion (C22, E9, H18). The importance of uPA and uPAR in metastasis is further demonstrated by the fact that both of them are increased in prostate cancer and various other cancers (M20, M24, M32, R1, S24, V9). In prostate cancer, uPA and uPAR could be used as predictors of progression, since both uPA and uPAR serum levels are increased in patients with advanced diseases (M20). Androgen-insensitive tumor cells that exhibit a high metastatic potential, such as DU-145 and PC-3 cells, secrete uPA and express uPAR on the cell surface. The LNCaP cells, which are androgen sensitive and with a low metastatic potential, do not produce uPA or uPAR (H18). However, when the extracellular environment for LNCaP cells is altered by coating culture flasks with fibronectin, DHT induces uPA and uPAR expression. This could suggest that the extracellular matrix plays a role in regulation of uPA and uPAR expression and thus controls metastasis (P8). Another important factor is the uPA inhibitor type-1 (PAI-1), which is increased in tumors, especially in tumors with high levels of vascularization and therefore higher invasive and metastatic potential (F2). However, the level of PAI-1 alone cannot be used as prognostic factor in metastases, since the levels of uPA and uPAR must also be considered. For example, in prostate tumors, diploid tumors have an increase in uPA, uPAR, and PAI-1 levels, while aneuploid tumors, which have a higher metastatic potential than diploid tumors, have decreased uPAR, PAI-1, and uPA levels (P15). These stress the importance of the uPAR to localize and control proteolytic activity during metastases.

6. Conclusion

The main purpose in receptor research is, first, to elucidate the structure and function of various receptors, ligands, agonists, antagonists, and signaling pathways, and second, to elucidate the interaction of them with other factors and with each other in the normal and diseased body. In prostate cancer the most important steroid receptor is the androgen receptor, since androgens seem to play an important role in the development and progression of this disease. Androgen

receptor aberrations in prostate cancer include reduced (CAG) repeat lengths in the transactivation domain of the AR gene, which causes an overactive AR. Other aberrations may be caused by certain prostate cancer treatments. For example, in the androgen-deficient prostate, AR aberrations may occur such as excessive AR expression, phosphorylation activation in the absence of a ligand, or point mutations in the different domains of the AR genes. These point mutations could alter ligand-binding specificity of the AR. Under these conditions, the AR may behave more like an ER, since their affinity for estrogens may increase. However, in various studies performed on prostate cancer, the considerable role of ER has been stressed. Phytoestrogens found in soy and Chinese herbs reduced the risk of prostate cancer and may be used in future treatments, since no adverse effects were found with some phytoestrogen preparations. It is interesting to note that phytoestrogens have a higher binding affinity for ERβ than ERα. This could only benefit the human male population worldwide. Binding of vitamin D_3 and its analogs to VDR reduces prostatic cell growth *in vitro* and *in vivo,* and the analogs have the added advantage of not developing hypercalcemia. In contrast, binding of uPA with uPAR leads to successful invasion of metastases and can be prevented by uPAR antagonists.

Most investigators agree that changes in expression of growth factors and their receptors, such as EGFR, TβR, IGFR, FGFR, NGFR, and PDGFR, occur along with malignant transformation of normal prostatic epithelial cells, but the significance thereof remains controversial (W4). It is hypothesized that no receptor–ligand system in the body is completely isolated from another, and that the disruption of the normal prostate with its intertwined system of different ligands and receptors, leads to prostatic cancer. To solve this complexity, the need for application of computational techniques is imperative.

Computational techniques has already been used in database construction, to assign patient symptoms to disease category and to predict medical outcome of treatment (N4). In prostate cancer, these artifical neural networks are computer-based statistical models and have been used in diagnosis, predicting response to therapy and recurrence (D21). The sensitivity of the network can be 81–100% (T4). Computer programs exist that can simulate multiple interactions between cells to produce a visual interpretation that represents tissue growth and differentiation (V5). These programs are user-friendly and enable researchers with almost no computer experience to design complex models of cell interactions on the World Wide Web, and they can also be an excellent resource for teaching (V5).

More laboratory research, clinical trials, and computer programs would bring us closer to winning the battle against diseases like prostate cancer, but since cancer is increasing in this modern, restless, and hurried society, man also needs to reevaluate and alter his lifestyle. These changes may be small and may even be cheap, but could create an immense difference in the quality of life.

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OXIDATIVE MODIFICATIONS OF PROTEIN STRUCTURES

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1. Sources of Reactive Oxygen Species

1.1. Superoxide Anion Radical

The primary source of reactive oxygen species (ROS) is the superoxide radical anion $O_2^{-\bullet}$, formed in biological systems in many enzymatic and nonenzymatic reactions. Considerable amounts of $O_2^{-\bullet}$ are produced and released into extracellular space by mono- and polymorphonuclear phagocytes. In nonphagocytizing cells the main source of $O_2^{-\bullet}$ is the so-called one-electron leak of the respiratory chain. It has been estimated that due to the electron leak, 1–5% of oxygen reduced in the respiratory chain is not processed to H_2O through four-electron reduction but forms superoxide (B22, T21). $O_2^{-\bullet}$ is also formed by various enzymes in microsomal and peroxisomal electron transport chains (Table 1) and by one-electron autoxidation of reduced forms of flavines, pteridines, catecholamines, aldehydes, thiol compounds, transition metals, and metalloproteins. Autoxidation of oxyhemoglobin to methemoglobin effecting the release of $O_2^{-\bullet}$ (instead of O_2) is an important source of superoxide in erythrocytes. This reaction corresponds to oxidation of 3% of hemoglobin daily (fortunately, the methemoglobin formed is reduced back to hemoglobin by methemoglobin reductase) (C4). Other sources of $O_2^{-\bullet}$ and subsequent free radicals are xenobiotics redox cycles, ionizing radiation, and ultrasounds (H4). Two mechanisms of $O_2^{-\bullet}$ formation are considered particularly important in pathology: the NAD(P)H oxidase system of phagocytic cells and xanthine oxidase.

In the early 1960s it was discovered that certain biological functions related to immunodefence, such as bacteria killing and host-self cell and tissue destruction, require the ability to synthesize certain compounds produced from the oxygen molecule. This process is dependent on oxidation of glucose in the hexose monophosphate shunt (HMPS) in phagocytizing cells. This effects a remarkable increase in cellular oxygen uptake and its metabolism in the "cyanide-resistant" pathway, known as a "respiratory burst." The chemical basis for respiratory burst

TABLE 1
ENZYMES ABLE TO GENERATE THE SUPEROXIDE ANION

Enzyme	Classification number
Xanthine oxidase	EC 1.2.3.2
Aldehyde dehydrogenase	EC 1.2.3.1
Dihydroorotate dehydrogenase	EC 1.3.3.1
Cellobiose oxidase	
Diamine oxidase	EC 1.4.3.6
Galactose oxidase	EC 1.1.3.9
Nitropropane oxidase	
Tryptophan dioxygenase	EC 1.13.11.11
Cytochrome P450 reductase	EC 1.6.2.4
Glutatione reductase	EC 1.6.4.2
Peroxidases (including myeloperoxidase)	EC 1.11.1.7
Tryptophan pyrolase	EC 1.13.1.12
NADPH oxidase (phagocytes)	
NADPH-cytochrom c oxidoreductase	EC 1.6.2.4
m-Hydroxybenzoate 4-hydroxylase	EC 1.14.99.13
Anthranilate hydroxylase	EC 1.14.16.3
Omega fatty acids hydroxylases	
Prostaglandin hydroperoxidase	

After Bartosz (B6).

is an activation of a membrane-bound enzyme, NADPH oxidase, which catalyzes the monoelectronic reduction of the O_2 molecule to the superoxide anion:

$$2O_2 + \text{NADPH} \xrightarrow{\text{NADPH oxidase}} 2O_2^{-\bullet} + \text{NADP}^+ + \text{H}^+$$

The NADPH oxidase consists of a complex of several functional subunits built into the structure of the neutrophile plasma membrane. The transmembrane-located cytochrome b_{558} binds the oxygen molecule (O_2) to its outer part and reduces it to the superoxide radical anion, which is released to the extracellular space. The electron donor is the NADPH dehydrogenase, which produces one electron and $NADP^+$. The NADPH dehydrogenase active center is oriented to the inner side of plasma membrane, and $NADP^+$ produced by reduction of O_2 is then utilized as a substrate for glucose 6-phosphate dehydrogenase present in the cytoplasm. The whole NADPH oxidase complex in neutrophils consists of a transmembrane-located 65-kDa subunit of NADPH dehydrogenase, two subunits (91 kDa and 22 kDa) of cytochrome b_{558}, and an inner side membrane located in the 45-kDa diphenylene iodonium-binding protein (L2).

The synthesis of $O_2^{-\bullet}$ makes possible acceleration of glucose metabolism in the hexose monophosphate shunt, yielding $NADP^+$, which is then used for dehydrogenation of glucose 6-phosphate to 6-phosphogluconic aldehyde (K15).

An alternative source of the superoxide radical anion is leukocytic xanthine oxidase, acting in polymorphonuclear leukocytes of individuals with impairment of the NADPH oxidase system. Superoxide is one of the major metabolites produced in stimulated neutrophiles. Various estimations of $O_2^{-\bullet}$ production yielded values ranging from 850 to 1350 μmol/h/10^{10} cells (B1, K16).

Another important pathomechanism involving superoxide and other ROS is ischemia-reperfusion injury. Paradoxically, tissue damage in anoxia due to ischemia is increased in an effect of reperfusion, due to the extensive generation of $O_2^{-\bullet}$ and its derivatives. This effect is caused mainly by the conversion of xanthine dehydrogenase (reducing NAD^+) into xanthine oxidase, capable of reducing O_2 and producing $O_2^{-\bullet}$ (which results in further H_2O_2 synthesis) under ischemic conditions. Energy depletion and degradation of adenine nucleotides to xanthine supplies substrate for the xanthine oxidase $O_2^{-\bullet}$-generation pathway. Other factors contributing to ROS synthesis and ischemia-reperfusion injury are hypoxic release of iron from myoglobin and alterations in function of mitochondria (B32, M12, P8).

Superoxide is in equilibrium with its protonated form, the hydroperoxyl radical $HO_2^\bullet$:

$$O_2^\bullet + H^+ \leftrightarrow HO_2^\bullet$$
$$\underset{\text{Hydroperoxyl radical}}{O_2^{-\bullet} + H^+ \leftrightarrow HO_2^\bullet}$$

The pK value of this reaction is about 4.8. Therefore, at physiological pH most of the $O_2^{-\bullet}/HO_2^\bullet$ radical couple exists in the dissociated form as the superoxide radical anion. However, the hydroperoxyl radical is more reactive than the $O_2^{-\bullet}$ dissociated form (Table 2). Moreover, $HO_2^\bullet$ can easily diffuse through biological membranes, while membrane penetration by the anionic $O_2^{-\bullet}$ is much slower (G30). Therefore, even a small fraction of nondissociated $HO_2^\bullet$ present in cells seems to play an important role in reactions of the $O_2^{-\bullet}/HO_2^\bullet$ couple.

1.2. Hydrogen Peroxide

The $O_2^{-\bullet}$ is transformed to H_2O_2 by the superoxide dismutase (SOD), the enzyme present in the cytoplasm of most mammalian cells and in the extracellular fluids.

$$\underset{\text{Superoxide}}{2O_2^{-\bullet} + 2H^+} \xrightarrow{\text{SOD}} \underset{\text{Hydrogen peroxide}}{H_2O_2 + O_2}$$

Dismutation is a further one-electron reduction of superoxide yielding hydrogen peroxide (H_2O_2). Therefore H_2O_2 formation is a normal subsequent reaction step wherever $O_2^{-\bullet}$ is formed. Hydrogen peroxide is not a free radical; it is less reactive than the superoxide, but more reactive than ground-state molecular oxygen (O_2). Thus H_2O_2 is considered a reactive oxygen species (ROS) (Table 3). Actually,

TABLE 2
REACTION RATE CONSTANTS OF SUPEROXIDE RADICAL ANION/PERHYDROXYL RADICAL WITH AMINO ACIDS AND SELECTED ANTIOXIDANTS[a]

Amino acid	Reacting species/pH	k ($M^{-1}s^{-1}$)	Amino acid	Reacting species/pH	k ($M^{-1}s^{-1}$)
Alanine	$HO_2^{\bullet}$/1.6	<44	Leucine	$HO_2^{\bullet}$/1.4	<23.0
Alanine	$O_2^{-\bullet}$/10	<0.06	Leucine	$O_2^{-\bullet}$/9.9	<0.21
Arginine	$HO_2^{\bullet}$/1.6	<63	Lysine	$HO_2^{\bullet}$/1.4	<13.3
Arginine	$O_2^{-\bullet}$/10.1	<0.13	Lysine	$O_2^{-\bullet}$/8.5	<3.30
Asparagine	$HO_2^{\bullet}$/1.4	4.9×10^7	Methionine	$HO_2^{\bullet}$/1.5	<48.8
Asparagine	$O_2^{-\bullet}$/10.1	<0.16	Methionine	$O_2^{-\bullet}$/8.3	<0.33
Aspartic acid	$HO_2^{\bullet}$/1.5	<12.0	Phenylalanine	$HO_2^{\bullet}$/1.3	<180.0
Aspartate anion	$O_2^{-\bullet}$/10.0	<0.18	Phenylalanine	$O_2^{-\bullet}$/10.1	<0.36
Cysteine	$HO_2^{\bullet}$/1.4	<601	Proline	$HO_2^{\bullet}$/1.4	<17.3
Cysteine	$HO_2^{\bullet}/O_2^{-\bullet}$/7	$\sim 1.8 \times 10^4$	Proline	$O_2^{-\bullet}$/10.0	<0.16
Cysteine	$O_2^{-\bullet}$/10.9	<15	Serine	$HO_2^{\bullet}$/1.2	<54.6
Cystine	$O_2^{-\bullet}$/10.0	<0.40	Serine	$O_2^{-\bullet}$/9.0	<0.53
Glutamic acid	$HO_2^{\bullet}$/1.6	<30.0	Threonine	$HO_2^{\bullet}$/1.4	<12.5
Glutamic acid	$O_2^{-\bullet}$/8.7	<0.39	Threonine	$O_2^{-\bullet}$/10.1	<0.21
Glutamine	$HO_2^{\bullet}$/1.5	<23.0	Tryptophan	$O_2^{-\bullet}$/10.6	<24.0
Glutamine	$O_2^{-\bullet}$/10.0	<0.25	Tyrosine	$O_2^{-\bullet}$/10.8	<10.00
Glycine	$HO_2^{\bullet}$/1.5	<48.6	Valine	$HO_2^{\bullet}$/1.5	<10.5
Glycine	$HO_2^{\bullet}$/1.5	<48.6	Valine	$O_2^{-\bullet}$/10.1	<0.18
Histidine	$HO_2^{\bullet}$/1.8	≤95.0	Ascorbate anion	$HO_2^{\bullet}/O_2^{-\bullet}$/7.4	2.7×10^5
Histidine	$O_2^{-\bullet}$/10.0	<1.00	Bilirubin	$HO_2^{\bullet}/O_2^{-\bullet}$/8.3	2.3×10^4
Isoleucine	$HO_2^{\bullet}$/1.4	<38.9	Glutathione	$HO_2^{\bullet}/O_2^{-\bullet}$/7.8	6.7×10^5
Isoleucine	$O_2^{-\bullet}$/8.0	<2.00			

[a]Reaction rate v between two substances a and b is the product of rate constant k and concentrations of these agents: $v = k[a][b]$. Therefore, comparison of rate constants compares rates of reactions provided the concentrations of reactants are the same in cases compared.

Modified after Bielski (B18).

TABLE 3
REACTION RATE CONSTANTS OF HYDROGEN PEROXIDE WITH SELECTED COMPOUNDS

Compound	Conditions	k ($M^{-1}s^{-1}$)	Reference
N-Acetylcysteine	pH 7.4, 25°C	0.85	(A18)
Cysteine	pH 10, 37°C	17.1	(R2)
–SH of BSA	pH 10, 37°C	5.6	(R2)
Oxymyoglobin	pH 5.5–10.2, 25°C	20.8	(Y9)
Myeloperoxidase-Fe^{3+}	pH 7.4, 25°C	3.1×10^7	(K9)
Cu,Zn SOD	pH 7.3–10.0, 25°C	3.0	(J1)

TABLE 4
STANDARD REDOX POTENTIALS OF SELECTED REACTIVE OXYGEN SPECIES

Reaction system	$E^{0\prime}$ (V)
$HO_2^{\bullet}/, H^+, O_2$	−0.46
$O_2^{-\bullet}/O_2$	−0.33
$H_2O_2/O_2^{-\bullet}, 2\ H^+$	0.94
$H_2O_2/HO_2^{-\bullet}, {}^{\bullet}H^+$	1.06
$RO_2^{\bullet}, H^+/ROOH$	~0.77–1.44
$ONOO^-, 2\ H^+/NO_2^-, H_2O$	1.2 V
$ONOO^-, 2\ H^+/NO_2^{\bullet}, H_2O$	1.4 V
$RO^{\bullet}, H^+/ROH$	~1.60
$HO^{\bullet}, H^+/H_2O$	2.31

Modified from Halliwell (H4).

H_2O_2 has higher redox potential than $O_2^{-\bullet}$ and therefore is a stronger oxidant than the superoxide (Table 4).

The respiratory burst and the subsequent $O_2^{-\bullet}$ and H_2O_2 synthesis is the main function of stimulated phagocytes. The H_2O_2 production utilizes approximately 25% of the oxygen taken up by activated neutrophiles. The H_2O_2 synthesis is undertaken by neutrophilic leukocytes due to stimulation by opsonized bacteria or mediators of the inflammatory reaction. Neutrophiles are also stimulated by certain nonspecific factors, such as tissue ischemia or acidification of extracellular fluids. All these factors induce neutrophilic leukocytes to synthesize remarkable amounts of $O_2^{-\bullet}$ and H_2O_2 when entering inflammed, ischemic, or necrotic tissues.

Hydrogen peroxide in stimulated leukocytes is utilized as a substrate for myeloperoxidase (MPO), an enzyme which is one of the major components of the bacteria killing system. Certain amounts of $O_2^{-\bullet}$ and H_2O_2 are released into the extracellular fluid, where they become substrates for secondary uncontrolled reactions yielding a number of highly toxic ROS. The pathogenetic implication of this mechanism is qualified as a "bystander effect" consisting of damage to the host's own macromolecules by reactive species generated from the $O_2^{-\bullet}$ and H_2O_2 (R14, W12).

1.3. HYDROXYL RADICAL

The most reactive free radical (and the most reactive chemical compound formed in organisms) is the hydroxyl radical, $^{\bullet}OH$. It has the highest redox potential of all substances produced in biological systems (Table 4), and it reacts very quickly with all major components of the cell: proteins, hydrocarbons, nucleic acids, and lipids

TABLE 5
REACTION RATE CONSTANTS OF HYDROXYL RADICALS WITH AMINO ACIDS AND SELECTED ANTIOXIDANTS

Amino acid	pH	k ($M^{-1}s^{-1}$)
Alanine	5.5–6.0	7.7×10^7
Arginine	6.5–7.5	3.5×10^9
Asparagine	6.6	4.9×10^7
Aspartate monoanion	6.8–7.0	7.5×10^7
Cysteine	7	$(3.5–4.7) \times 10^{10}$
Cystine	6.5	2.1×10^9
Glutamate ion	6.5	2.3×10^8
Glutamine	6.0	5.4×10^8
Glycine	5.8–6.0	1.7×10^7
Histidine	6–7	5.0×10^9
Hydroxyproline	6.8	3.2×10^8
Isoleucine	6.6	1.8×10^9
Leucine	5.5–6.0	1.7×10^9
Lysine	6.6	3.5×10^8
Methionine	6–7	8.5×10^9
Norleucine	6.4–6.9	2.5×10^9
Norvaline	6.4–6.9	1.6×10^9
Phenylalanine	7–8	6.9×10^9
Proline	6.8–6.9	4.8×10^8
Selenomethionine	7	1.2×10^{10}
Serine	5.5–6.0	3.2×10^8
Threonine	6.6	5.1×10^8
Tryptophan	6.5–8.5	1.3×10^{10}
Tyrosine	7	1.3×10^{10}
Valine	6.9	8.5×10^8
Ascorbate anion	7	1.2×10^{10}
Bilirubin dianion	10.9	1.3×10^9
Glutathione	5.5	1.3×10^{10}
Uric acid	6–7	7.2×10^9

Modified from Buxton (B34).

(Table 5). Hydroxyl radical, once formed most, probably will react with the first molecule it encounters. The postulated source of hydroxyl radical is a Haber-Weiss reaction between hydrogen peroxide and the superoxide radical anion:

$$H_2O_2 + O_2^{-\bullet} \rightarrow \underset{\substack{\text{Hydroxyl}\\\text{radical}}}{{}^{\bullet}OH} + \underset{\substack{\text{Hydroxyl}\\\text{ion}}}{OH^-} + O_2$$

This reaction can occur spontaneously, but its rate is not high enough to produce a substantial amount of the hydroxyl radical [the estimated reaction rate constant is $0.13\,M^{-1}\,s^{-1}$ (F1) or $2.25\,M^{-1}\,s^{-1}$ (H16)]. However, in the presence of bivalent iron

(also copper, nickel, manganium, and chromium) ions, the reaction is accelerated by two orders of magnitude ($k > 10^2$ M^{-1} s^{-1}). The acceleration of the Haber-Weiss reaction occurs due to a metal-mediated catalysis employing a so-called Fenton reaction,

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$$

and a reaction of ferrous ions regeneration by superoxide,

$$Fe^{3+} + O_2^{-\bullet} \rightarrow Fe^{2+} + O_2$$

Hydrogen peroxide diffuses freely through biological membranes, while ferrous atoms are tightly bound to specific carriers. Therefore, the site of ${}^{\bullet}OH$ formation usually corresponds to the present site of ferrous ions (site specificity of ${}^{\bullet}OH$ formation"). Thus, concentration of available ferrous ions is considered a limiting factor for the hydroxyl radical formation *in vivo*.

Apart from the Fenton reaction, other reactions effecting ${}^{\bullet}OH$ formation have been also postulated. One of them is the reaction between hypochlorous acid and superoxide, characterized by a high rate constant ($k = 7.5 \times 10^6$ M^{-1} s^{-1}) (F11):

$$\underset{\substack{\text{Hypochlorous}\\ \text{acid}}}{HOCl} + O_2^{-\bullet} \rightarrow O_2 + Cl^- + {}^{\bullet}OH$$

Another ${}^{\bullet}OH$ generating reaction is peroxynitrite decomposition to hydroxyl radical and nitrogen dioxide (see sect. 1.12). Also, actions of ionizing radiation and ultrasounds yielding water molecule decomposition are additional sources of ${}^{\bullet}OH$. The hydroxyl radical is probably the main reactive species in the so-called metal-catalyzed oxidation systems (or mixed-function oxidation systems) used in experiments where ascorbate and metal ions are employed (though formation of other products such as high-valency iron species ferryl Fe^{+4} or perferryl Fe^{+5} forms cannot be excluded) (N2, S50).

1.4. Hydroxyl Radical Reactions

Hydroxyl radical reacts very quickly with most organic substances, including free amino acids and amino acid residues in proteins. These reactions are diffusion-controlled and their rate constants are very high (Table 5). Hydroxyl radical reacts with low specificity with all amino acid residues, although tryptophan, tyrosine, histidine, and cysteine are particularly vulnerable.

One of the main reactions of ${}^{\bullet}OH$ is the abstraction of the hydrogen from the α-carbon of an amino acid residue to form a carbon-centered radical. Addition to this radical of the oxygen molecule produces a peroxyl radical. Its reduction to an anion by intramolecular electron transfer or by superoxide yields a hydroperoxide anion, which binds a proton to form an amino acid hydroperoxide. Hydroperoxides of some amino acids are semistable, and some decompose spontaneously to

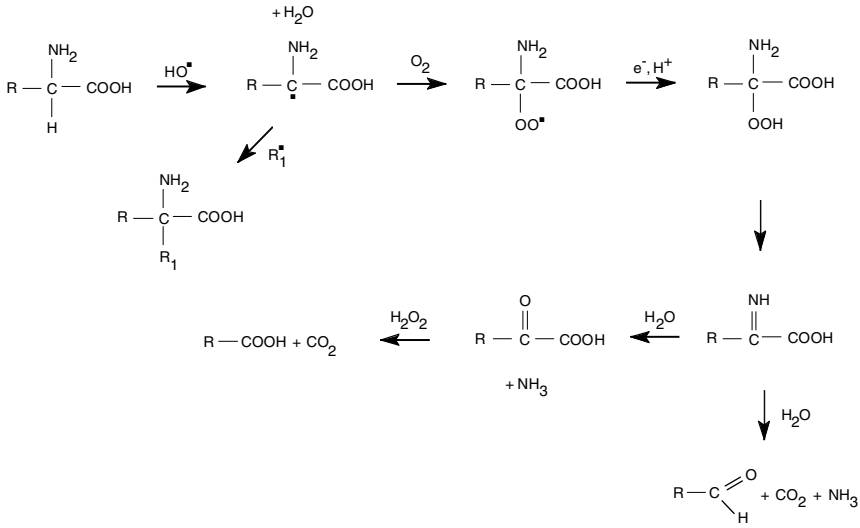

FIG. 1. α-Oxidation of amino acids. Hydroxyl radical (or other reactive radical) abstracts hydrogen atom from the α-carbon. The C-centered free radical formed may react with other amino acid residues or dimerize in the absence of oxygen, which leads to protein aggregation. In the presence of oxygen the carbon-centered radical forms peroxyl radical. Reduction of peroxyl radical leads to protein hydroperoxide. Decomposition of hydroperoxide leads to formation of carbonyl compounds via either oxidative deamination or oxidative decarboxylation. Oxidation of the new carbonyl group forms a carboxyl group.

hydrogen peroxide and an imino derivative which subsequently hydrolyzes to yield either NH_4^+, CO_2, and an aldehyde, or NH_4^+ and an α-ketoacid. The α-ketoacid may be oxidized (e.g., by H_2O_2) to form CO_2 and a carboxylic acid (Fig. 1). This mechanism explains the effect of •OH action on simple amino acids such as glycine and alanine. Amino acids with a more complex structure have more loci vulnerable for •OH attack, and this scheme of the reaction, although possible, may be less probable. With oxygen deficiency, peroxide formation is limited and carbon-centered radicals are produced. These radicals may react through an addition mechanism with other amino acid residues or may recombine to produce covalent aggregation products (S51).

Leucine oxidized with •OH produces a 5′-hydroperoxy derivative which is subjected to chemical reduction to yield (2S)-γ-hydroxyleucine, (2S,4S)-δ-hydroxyleucine, (2S,4R)-δ-hydroxyleucine. The 3′-hydroxyleucines have been confirmed to be the reduction products of the corresponding hydroperoxyleucines. 5′-Hydroxylysines are natural products formed by lysyl oxidase and therefore are not useful markers of radical-mediated oxidation. The other hydroxylysines are useful markers, however, with HPLC analysis of 9-fluorenylmethyl chloroformate (FMOC)

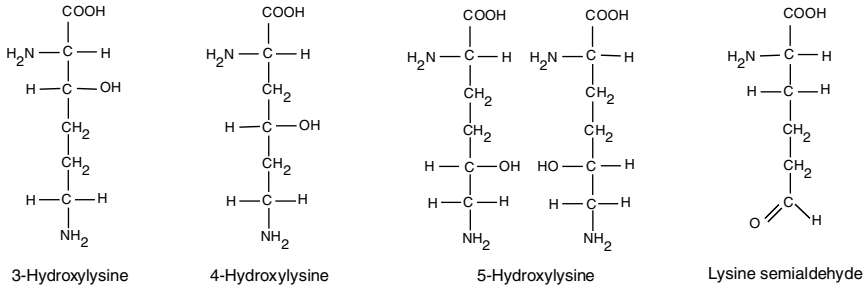

FIG. 2. Oxidation products of lysine.

derivatives providing a sensitive and accurate method for quantitative measurement (Fig. 2).

Proline analogs, (2S,4R)-4-methylproline (*trans*-4-methyl-l-proline) and (2S, 4S)-4-methylproline (*cis*-4-methyl-l-proline), derive from reduction of their corresponding cyclic Schiff bases (F22) (Fig. 3).

Another hydroxylated product of •OH modification of proline is 5-hydroxy-2-aminovaleric acid, arising from oxidation of proline and arginine residues (A20) (Fig. 4).

Oxidation of proline and arginine residues leads also to formation of glutamate semialdehyde and, upon its further oxidation, to pyroglutamic acid (A9). Oxidized proline produces also 2-pyrrolidone (K6) (Fig. 5).

Oxidation of histidine yields 2-oxohistidine and its isomer 2-imidazolone, among other products (U4, U5) (Fig. 6). 2-Oxo-histidine was proposed to be a useful biological marker for assessing protein modifications under oxidative stress (U5).

Oxidation of sulfhydryl groups leads to formation of intra- and intermolecular disulfide bridges but may also go further, with formation of sulfoxides and sulfones

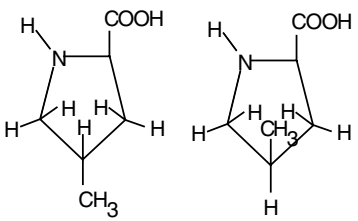

FIG. 3. 4-Methylproline isomers.

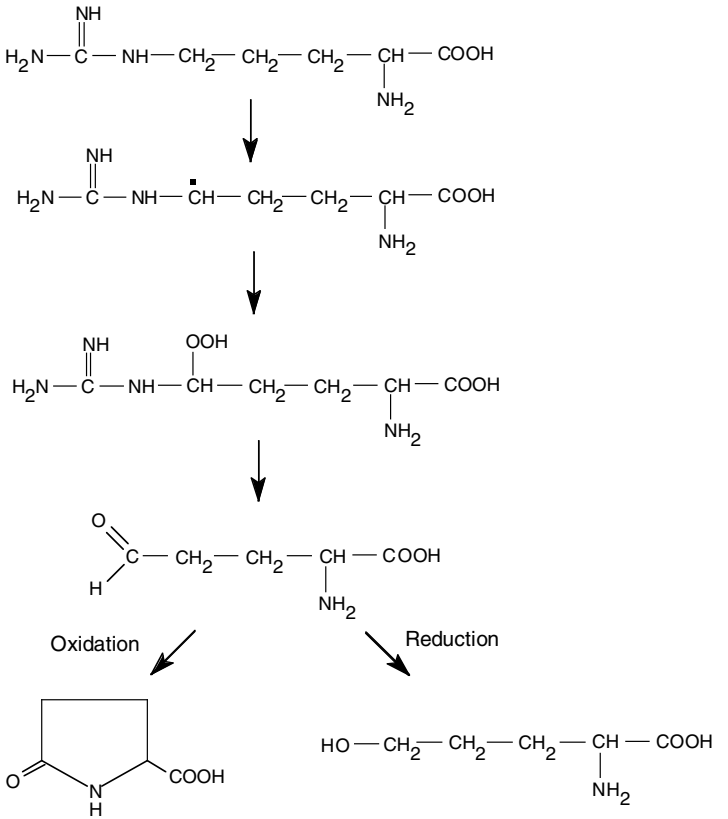

FIG. 4. Oxidation of arginine. 5-Hydroxy-2-aminovaleric acid, which is the arginine oxidation end product, may also be produced during oxidation of proline.

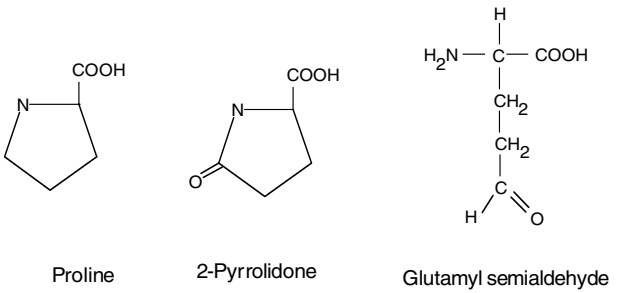

FIG. 5. Proline oxidation products.

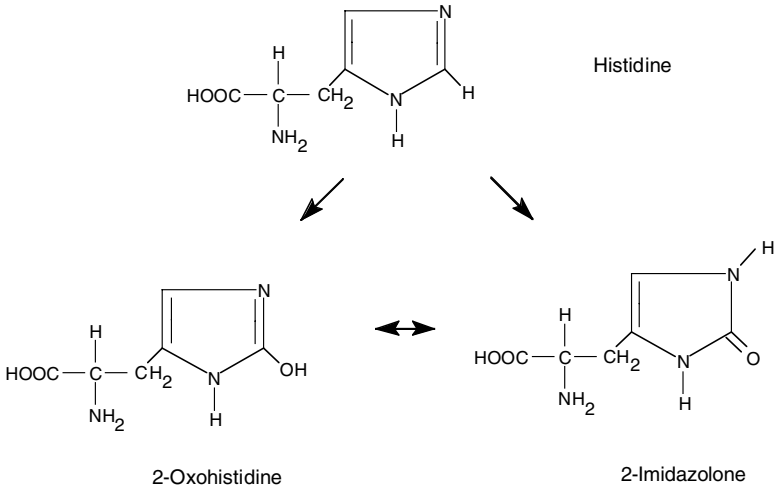

FIG. 6. Oxidation of histidine yields 2-oxohistidine and 2-imidazolone.

(see sect. 1.8). Oxidation of methionine leads to formation of methionine sulfoxide but may go further to methionine sulfone (V12) (see sect. 1.8). In aromatic amino acid residues, the indole ring of tryptophan or the phenol ring of tyrosine are the primary targets of hydroxyl radical attack, while α-hydrogen abstraction and deamination are relatively less intense (S51). Oxidation of tryptophan can lead to several products, among them *N*-formylkynurenine and kynurenine (see sect. 2.1). Both these compounds are formed after breaking the indole ring and show characteristic fluorescence. Aromatic phenylalanine residues react with hydroxyl radical, generating *ortho-, meta-* and *para*-tyrosines. As only *para*-tyrosine is a natural protein component, the two other forms may suggest occurrence of reactions emloying the hydroxyl radical (see sect. 2.6).

Hydroxyl radical may hydroxylate tyrosine to 3,4-dihydroxyphenylalanine (DOPA). DOPAs are the main residues corresponding to "protein-bound reducing moieties" able to reduce cytochrome *c*, metal ions, nitro tetrazolium, blue and other substrates (S32). Reduction of metal ions and metalloproteins by protein-bound DOPA may propagate radical reactions by redox cycling of iron and copper ions which may participate in the Fenton reaction (G9). Abstraction of electron (by $^{\bullet}OH$ or peroxyl or alkoxyl radicals) leads to the formation of the tyrosyl radical, which is relatively stable due to the resonance effect (interconversion among several equivalent resonant structures). Reaction between two protein-bound tyrosyl radicals may lead to formation of a bityrosine residue which can cross-link proteins. The tyrosyl radical may also react with superoxide, forming tyrosine peroxide (W13) (see sect. 2.6).

1.5. Singlet Oxygen

Excitation of ground-state oxygen leads to formation of singlet oxygen, which is also a ROS species. Singlet oxygen is formed mainly in photodynamic reactions; however, it has also been suggested to be generated in some biochemical reactions without the participation of light (S27). Singlet oxygen readily reacts with histidine and tryptophan residues. Reaction of singlet oxygen with tryptophan yields dioxetane (an adduct of —O—O to the double bond in the 2,3-position of the indole ring. The adduct then decomposes to *N*-formylkynurenine (H4).

1.6. Hypochlorite, Myeloperoxidase Hydrogen Peroxide-Chloride System of Neutrophilic Leukocytes

Myeloperoxidase (MPO), donor: hydrogen peroxide oxidoreductase(EC 1.11. 1.7) is a heme-containing glycoprotein produced by neutrophilic granulocytes. The enzyme was first described in 1941 by Agner (A2) and then was extensively studied by others in the 1970s and 1980s due to its function in neutrophil bacteria killing mechanisms. Review of these studies is given in a monography by Klebanoff and Clark (K16), and publications of Zgliczyñski (Z5) and others (H5). Myeloperoxidase is unique for polymorphonuclear neutrophilic granulocytes and monocytes of mammals (K16). In granulocytes the enzyme is present in primary granules and is the single most abundant protein of these cells, consisting of 2–5% of their whole protein content. Functional stimulation of neutrophilic granulocytes effects the hydrogen peroxide production, which along with cellular water readily diffuses through cellular membranes. H_2O_2 is one of the compounds triggering MPO discharge from primary granules to the phagocytic vacuoles, subsequently forming the H_2O_2-MPO enzyme system which is able to oxidize various substrates present in biological systems.

The MPO molecule is composed of two small and two large protein subunits ($\alpha_2\beta_2$) with molecular weights 10,500 and 57,000, respectively (H8). The subunits are organized in into $\alpha\beta$ functional units, each having an independent prosthetic group, which is a formyl-bearing chlorine type, iron containing heme group covalently bound to the heavy subunit (B20, H23). A distal histidine and arginine residues located in the vicinity of the heme are present in the MPO active center. By reducing disulfide bonds, the native MPO molecule can be split into $\alpha\beta$ subunits which retain their original enzyme activity. Also, some proteolytic modifications of MPO protein moiety as one found in enzyme isolated from cells of chronic myelocytic leukemia, in effect of partial trypsin digestion or the other produced, do not affect the MPO enzyme activity (A11).

The mechanism of enzyme catalysis mediated by MPO is similar to other heme-containing peroxidases: MPO reacts with hydrogen peroxide to yield several spectroscopically distinct forms of the enzyme. MPO-compound I is a short-lived

peroxide intermediate containing two oxidizing equivalents versus the enzyme native state. Formation of MPO-compound I is dependent on pH, and protonation of a group with a pK_a of about 4.3 limits binding of hydrogen peroxide (B19). The apparent second-order reaction rate constant for MPO-compound I formation amounts to $2.3 \times 10^7\ M^{-1}s^{-1}$. In the presence of some reductants MPO compound I can be reduced via one-electron transfer to MPO-compound II, with ferryl (IV) heme iron. This reaction is also pH-dependent and is inhibited by superoxide dismutase, which suggests that superoxide anion $O_2^{-\bullet}$ is generated as a by-product. MPO-compound II can be reduced to native MPO using ascorbate or 5-aminosalicylic acid (Z6). The oxidation of MPO yields MPO-compound III (H23). Under some conditions MPO also possesses catalatic activity, effecting reduction of H_2O_2 to H_2O and O_2 (B19). The formation of $O_2^{-\bullet}$ is slow, and the mechanism of this reaction is not quite clear yet. Oxidized forms of MPO are unstable, and rapid inactivation of the enzyme occurs if no acceptor for oxidants is present (N3).

In neutral and slightly alkaline media, MPO-compound I can react directly with iodides, bromides, chlorides (K16), thiocyanates, *N*-acetylmethionine, cysteine, pyridine nucleotides (S20), and phenols (K16), including tyrosine (H14) and thyroid hormones. Some of these reactions have certain biological importance. In extensive studies, Klebanoff *et al*. investigated the potential function of MPO as an iodide-oxidizing enzyme (K16). It was found that iodide is rapidly oxidized, forming a bactericidal derivative which produces a fall in the number of viable *Escherichia coli* 10 times more effectively than bromide and 100 times more effectively than chloride, if used as MPO substrates. Extremely low concentrations of iodides and bromides in leukocytes and blood plasma, however, seem to limit the importance of iodide oxidation in bacteria killing mechanisms.

The unique property of MPO among the mammalian oxidizing enzymes is that MPO-compound I possesses a high enough oxidative potential to oxidize the chloride ion (Cl^-) to hypochlorous acid (HOCl) with re-formation of the native enzyme (A3, H9, S54, Z3):

$$\underset{\substack{\text{Hydrogen}\\\text{peroxide}}}{H_2O_2} + H^+ + Cl^- \xrightarrow{\text{MPO}} \underset{\text{Hypochlorite}}{HOCl^-} + H_2O$$

Bolscher and Wever proposed a more detailed scheme for the oxidation of Cl^- ion by MPO in 1984 (B19):

$$\begin{array}{ccccc} MPO^{FeIII} + H_2O_2 & \rightarrow & \underset{\text{MPO-compound I}}{MPO^{FeV}O^{2-}} + H^+ + Cl^- & \rightarrow & MPO^{FeIII} + HOCl \\ + H^+ \downarrow & & \downarrow & & \\ MPO^{FeIII}H^+ & & \underset{\text{MPO-compound II}}{MPO^{FeV}} & & \\ + Cl^- \downarrow & & & & \\ MPO^{FeIII} \cdot H^+\ Cl^- & & & & \end{array}$$

Hypochlorite, which seems to be the main biological product of the MPO-H_2O_2 system, is itself a strong oxidant, which reacts further with many electron donors present in inflammatory sites and oxidizing some compounds while chlorinating others. Thus, in stimulated leukocytes MPO acts indirectly, producing the highly reactive HOCl (A3, H9, S54, Z3).

MPO, like other hemoproteins, forms complexes with cyanide, azide, thiocyanate, and halide ions (F^-, Cl^-, Br^-, and I^-) having a ligand-specific spectrum (B3). Halides probably bind to MPO at two different sites: one at the proximity of heme iron which acts as a competitive inhibitor of the reaction with hydrogen peroxide (B19, Z1), and the other one yielding well-detectable change in the MPO spectrum. Reactions of complex formation depend on pH. Interactions of chlorides, hydrogen peroxide, and pH kinetics of MPO-catalyzed oxidation of Cl^- is complex; cooperation of all three mentioned factors (concentrations of Cl^-, H_2O_2, and pH) implies a dependence of MPO optimal pH value on the Cl^--to-H_2O_2 concentrations ratio as a logarithmic function (B3, Z1). This property allows MPO to oxidize chloride ion in a broad range of concentrations of H_2O_2 and at various pH values. This may express an adaptation of MPO-mediated hypochlorous acid formation to changing conditions inside the phagocytic vacuole at various periods of neutrophile functional stimulation. At the beginning of the phagocytosis milieu, phagocytic vacuoles inside are neutral and concentrations of H_2O_2 available for MPO mediated reaction are rather low. These conditions confer with the highest affinity of MPO to H_2O_2, which can form the MPO-compound I and subsequently produce HOCl as a bacteria-killing agent. On the other hand, at later stages of neutrophile stimulation the pH inside phagocytic vacuoles drops even to 4.5 but production of H_2O_2 increases by severalfold and this increase supports efficient HOCl production, despite changes occurring in the milieu of the phagocytizing cells.

Despite that the MPO-catalyzed oxidation of halides seems to employ the same mechanisms active in primary production of the respective hypohalous acids, the final products of peptide and protein oxidation vary, depending on the halide ion employed: iodides and bromides when used as substrates yield stable bromo- and iodotyrosine derivatives, whereas direct chlorination of the available free amino moieties and semistable chloramines formation predominate when Cl^- is oxidized as the substrate (S54, Z3). The chlorination is a unique function of polymorphonuclear neutrophilic leukocytes (W6, Z2).

1.7. Reactivity of HOCl with Amino Acids and Other Selected Compounds

Hypochlorous acid, whose pK_a value is close to 7, reacts with various compounds as a one- or two-electron oxidant, or as the chlorinating agent. Due to the presence of multiple potential reductants, HOCl/OCl^- lifetime in biological systems is very short. Thiol and thioether moieties of cysteine and methionine (F11),

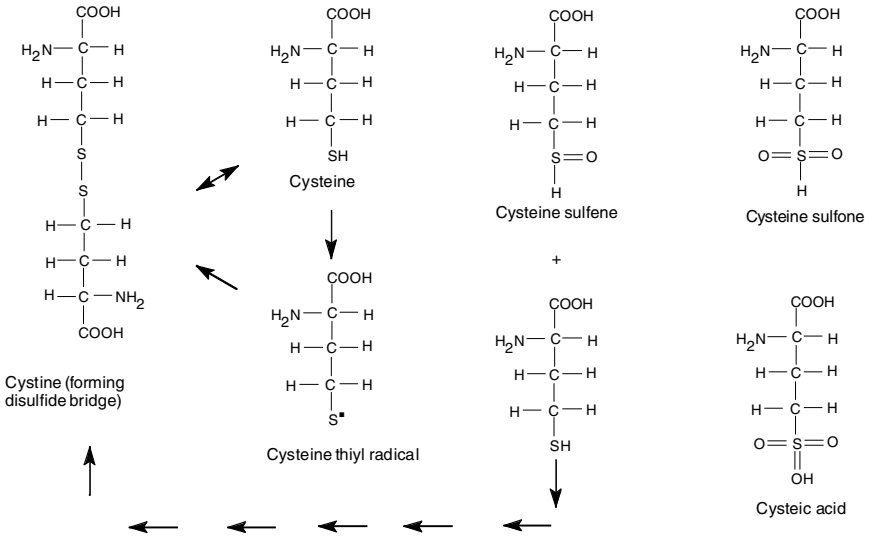

FIG. 7. Cysteine oxidation products.

tryptophan (A10), disulfides (D20), phenol moieties of tyrosine (H15, L27), and the other potential electron donors react readily with HOCl. Among a number of substances readily oxidized with HOCl one should also mention ascorbate (F11, M7, M8), nitrite (NO_2^-) (E2), superoxide anion (F11), and unsaturated fatty acids (P6). All these compounds differ in proneness for oxidation (D18).

Thiol compounds such as cysteine, methionine, and glutathione are strong reductants with a high affinity to HOCl, which they reduce back to Cl^- (F11). Thiol reducing capacity toward HOCl is more than 100 times higher than that of other amino acid residues, so these compounds are considered scavengers of oxidized chlorine species, effectively protecting other potential reductants from oxidation or chlorination (D18, D19, H11, Y4). The primary HOCl/OCl^- reaction product with a thiol moiety is the –SCl group, which rapidly rearranges its structure to –SS– or hydrolyzes to sulfone ($–SO_3^-$) (P13) (Fig. 7).

At low HOCl concentrations which do not exceed the thiol group concentration, the oxidation product could be a disulfide bond. At higher HOCl concentrations disulfides are oxidized and sulfone formation occurs (S10). Acting aither directly by HOCl or using the MPO-H_2O_2-Cl-system, the reaction of both methionine and cysteine oxidation to sulfoxide and sulfone terminates at H_2O_2 or HOCl-to-thiol/thioether molar ratio 1.25:1 (D20). In experimental conditions *N*-acetylcysteine (which, due to binding of its α-alpha amino group by acetylation, can mimic reactivity of cysteine residues in peptides), reacts with the MPO-H_2O_2-Cl^- system or with HOCl to yield cysteic acid at pH ranging from 4.0 to

7.0. Oxidation of *N*-acetylmethionine in the same conditions yields methionine sulfoxide:

$$\mathrm{HOCl} + \underset{N\text{-acetylmethionine}}{\mathrm{CH_3{-}S{-}CH_2CH(NH{-}Act)COOH}} \rightarrow \underset{N\text{-acetylmethionine sulfoxide}}{\mathrm{CH_3{-}SO{-}CH_2CH(NH{-}Act){-}COOH}} + \mathrm{HCl}$$

The other moiety readily reacting with HOCl is tryptophanyl residue, which upon oxidation yields the 2-oxoindole as the reaction product (A10). Thiol compounds prevent tryptophan from oxidation. Tryptophan is not protected from oxidation with HOCl in the presence of taurine or other compounds employing HOCl for chloramine formation. Production of oxoindole effects an increase in light absorption at 250 nm and a decrease at 280 nm. To oxidize one tryptophan residue bound in the N-acetylglycyltryptophan, 3.2 mol of HOCl are needed; whereas 1.3 mol of hydrogen peroxide was necessary when the MPO-H_2O_2-Cl^- system was used. Therefore the stoichiometry of HOCl oxidation is different from that with MPO, which can be explained by the simultaneous action of MPO oxidase-type activity (D20) and thiosulfate. On the other hand, the presence of disulfides does not protect.

The other amino acid reacting with HOCl is tyrosine. At pH ranging from 3.5 to 6.0, HOCl reacts with *N*-acetyltyrosine to produce 3′-chloro derivative (Fig. 8). If the HOCl/N-acetyltyrosine molar ratio exceeds 10, 3′5′-dichlorotyrosine formation is observed. The chlorotyrosine formation is slow compared to chloramine formation or tryptophan oxidation reaction rate. The optimal conditions for tyrosine

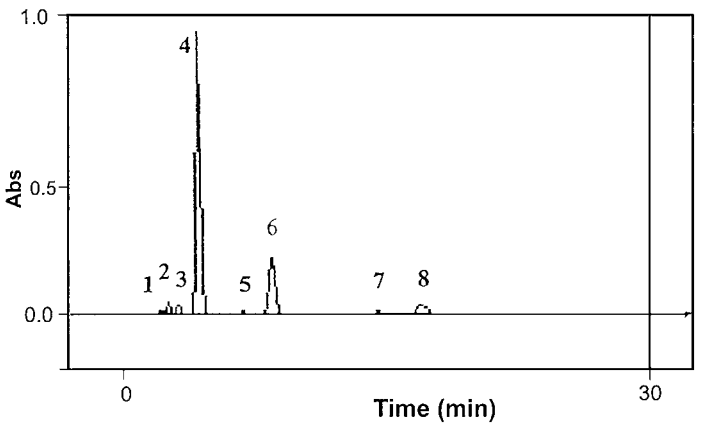

FIG. 8. Products of *N*-acetyl-L-tyrosine treatment with NaOC1 studied by HPLC method. Peak denoted 4 represents *N*-acetyltyrosine, peak denoted 6 is *N*-acetyl 3-chlorotyrosine. Minute peak denoted 8 represents 3,5-dichlorotyrosine. Graph respresents column eluate light absorption at 280 nm plotted versus column retention time. (From Drabik and Naskalski, unpublished results.)

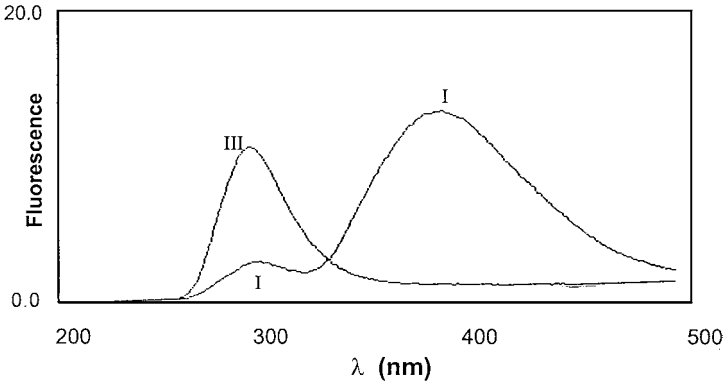

FIG. 9. Fluorescence spectra of myeloperoxidase-mediated products of tyrosine coupling. Curve I represents tyrosine fluorescence (peak at 400 nm) at the initial phase of the reaction. Curve III represents dityrosine fluorescence (peak at 300 nm) after 20 min of reaction flow. The substantial increase in dityrosine fluorescence at 300 nm indicates dityrosine formation. Light of wavelength 222 nm was employed for tyrosine and ditytosine fluorescence excitation. (From Drabik and Naskalski, unpublished results.)

residue chlorination were pH 4.5-5.0 at tyrosine/HOCl molar ratio 1:4 (Drabik and Naskalski, unpublished results). Direct action of HOCl/OCl^- in an alkaline medium can also oxidize tyrosyl residues to dityrosine (O1, V10). The reaction is unspecific for HOCl since dityrosine formation from UV light, ozone, and hydroxyl radical action was also observed (B3, L14, V5).

More recently, MPO-mediated oxidation of tyrosine to dityrosine (*o′o′*-dityrosine, or 3,3′-dityrosine) focused attention as a marker reaction of neutrophile-dependent oxidative damage of proteins and peptides (G11, H14, S3). The reaction occurs both with free tyrosine as well as with tyrosyl residues incorporated into polypeptide structures. The mechanism of dityrosine formation utilizes a relatively long-lived phenoxyl radical that cross-links to dimeric and polymeric structures by formation of carbon–carbon bonds between the aromatic moieties of phenolic tyrosine residues (H14) (Fig. 9).

Different susceptibility of *L*-tyrosine and *D*-tyrosine during oxidation may suggest a MPO–tyrosine complex formation as an intermediate of the reaction (B11). The competitive inhibition of dityrosine formation by Cl^- also suggests transient presence of a MPO-tyrosine complex. The reaction occurs in neutral and slightly alkaline media despite the presence of Cl^- and depends only on H_2O_2 and MPO availability (H14). Dimerization of tyrosine-containing peptides occurs in stimulated polymorphonuclear leukocytes (S3). Though in mammalian tissues MPO is the most probable mediator of dityrosine synthesis, this reaction is not MPO-specific. *In vitro* dityrosine was also obtained using horseradish peroxidase– (G26, T17) and lactoperoxise–H_2O_2 systems (B11, R13). One can

detect dityrosine by its intense fluorescence, excited by a 325-nm wavelength light and emitted at 410 nm. It is generally accepted that protein fluorescence observed in various pathological conditions is due to the presence of dityrosines produced under circumstances favoring uncontrolled action of strong oxidants on tissue proteins.

HOCl also reacts readily with nitrite (NO_2^-), which is the autoxidation product of nitric oxide (NO). HOCl and NO_2^- react to form reactive intermediates Cl-NO_2, and/or Cl^--ONO, which are capable of nitrating, chlorinating, and dimerizing tyrosine residues.

1.8. Chloramines

HOCl chlorinating activity appears in reactions with free amino moieties to produce the respective chloramines (S36, S53):

$$RNH_2 + HOCl \rightarrow \underset{\text{Chloramine}}{RNHCl} + H_2O$$

Chloramines are considered as main products secondary to hypochlorite formation. Taurine is one of the most abundant sources of free amino groups in polymorphonuclear neutrophilic leukocytes, representing approximately 50% of all low-molecular-weight amines released by PMNs (G24, Z5), and taurine chloramine is formed immediately along with MPO-mediated HOCl synthesis:

$$\underset{\text{Taurine}}{HOSO_2—(CH_2)_2—NH_2} + H_2O_2 + Cl^- \xrightarrow{\text{MPO}} \underset{\text{Chloramine taurine}}{HOSO_2—(CH_2)_2—NHCl} + H_2O$$

Formation of taurine chloramines and of other small-molecular-weight amines accounts for 90% of all chloramine output in stimulated PMNs (G24). Chloramine synthesis efficiency approaches 100% of the stoichiometic amount of HOCl employed (W5). Though HOCl reacts spontaneously, with taurine as a chlorine donor, taurine chloramine production in the presence of MPO is one order of magnitude faster than the spontaneous one ($k_i = 4.4 \times 10^5$ versus $k_i = 2.8 \times 10^6$ $M^{-1}s^{-1}$). This indicates that the MPO-mediated taurine chlorination reaction may involve an enzyme intermediate species rather then free HOCl.

Chloramines are themselves mild oxidants capable of reacting with —SH groups of glutathione (GSH), cysteine, thioether groups of methionine, and other thiol compounds, including thiosulfate, which reduce chloramines to Cl^- ion and reconstitute the amino moiety:

$$\underset{\text{Chloramine}}{R—NH—Cl} + 2\,\underset{\text{Thiosulfate}}{S_2O_3^{-2}} \rightarrow \underset{\text{Tetrationate}}{S_4O_6^{-2}} + \underset{\text{Amine}}{R—NH_2} + \underset{\text{Chloride}}{Cl^-}$$

Choramines differ with respect to their oxidative and chlorinating properties. Taurine chloramine is quite stable, and due to its relatively long lifetime in biological milieu, can diffuse to places distant from its production site (T4, T5). On the

other hand, unstable chloramines readily decompose, effecting chloride, ammonia, and carbonyl moiety formation:

$$\mathrm{R{-}CH_2{-}NHCl} \rightarrow \mathrm{R{-}CH_2{-}NH_2^+{-}Cl} \rightarrow \mathrm{R{-}CH{=}NH} + \mathrm{HCl} + \mathrm{H^+}$$

$$\mathrm{R{-}CH{=}NH} + \mathrm{H_2O} \rightarrow \underset{\text{Aldehyde}}{\mathrm{R{-}CH{=}O}} + \mathrm{NH_3}$$

Recently it was found that the reaction employs intermediary nitrogen-centered free radical formation via thermal homolysis of the N—Cl bond (H11). The stable chloramine T was effectively used for relatively specific oxidation of cysteinyl residues exposed to the surface of the protein molecule (S25).

In acidic media in the presence of an excess of Cl^-, chloramines disproportionate, generating active chlorine Cl^0 which readily substitutes into tyrosine aromatic rings, acting therefore as a chlorinating agent:

$$\mathrm{R{-}CH_2{-}NHCl} + \mathrm{Cl^-} + \mathrm{H^+} \rightarrow \mathrm{R{-}CH_2{-}NH_2} + \mathrm{Cl^0}$$

Free amino acids readily form chloramines in reactions with HOCl. However, the reaction can be postponed by the presence of amino acids whose side groups have reducing properties (as is the case with cysteine, methionine, and tryptophan). Amino acid chloramines located at the α-carbon are unstable, decomposing by deamination and decarboxylation. The final stable products are ammonia, chloride, and the aldehyde respective to the amino acid carbon backbone (H12, Z4):

$$\underset{\text{Amino acid}}{\mathrm{R{-}CH{-}(NH_2){-}COOH}} + \mathrm{HOCl} \rightarrow \underset{\text{Aldehyde}}{\mathrm{RCHO}} + \mathrm{CO_2} + \mathrm{HCl}$$

The exceptions are chloramines of valine, leucine, and isoleucine, which form semistable chloramines that remain in the reaction medium for several hours (Z4). The mechanism of aldehyde formation from the intermediary amino acid α-chloramines produced either by HOCl treatment or by the MPO-H_2O_2-Cl^- system was recently verified with use of NMR spectra. The study made possible the identification of short-lived products of the reaction, and confirmed the role of the unstable monochloramine of the α-amino group as the intermediate (H12).

Amino acids incorporated into peptides do not posses free α-amino groups, except for N-terminal amino acid residues. Therefore, treatment of peptides and proteins with HOCl only produces chloramines of α-amino groups of lysine and arginine and N-terminal amino acids (S53, S55). Chloramines of N-terminal amino groups of some peptides decompose specifically, yielding sets of aldehyde and oxo derivatives (S55). Some oligopeptides, such as Gly-Gly-Tyr-Arg, can play an important function as intermediates of chlorination of tyrosyl moieties. Domingan *et al*. have shown that Gly-Gly-Tyr-Arg tetrapeptide chlorinated at the terminal amino group transforms into the 3′-chlorotyrosine derivative, with reconstitution of the free amino group, in a delayed secondary reaction (D13). The reaction was specific for chloramine of the Gly-Gly-Tyr-Arg tetrapeptide and was not observed if

chloramines of taurine, glycine, and lysine were employed. Effective tyrosine chlorination in Gly-Gly-Tyr-Arg occurred also when purified a MPO-H_2O_2-Cl^- system and phorbol myristate acetate-stimulated neutrophilic leukocytes were employed. Polymorphonuclear leukocytes chlorinated tetrapeptide-bound tyrosine with a surprisingly high yield, equivalent to about 10% of H_2O_2 generated. The reaction was dependent on MPO, since leukocytes of MPO-deficient persons were not able to carry out the reaction. Sterical modeling of the Gly-Gly-Tyr-Arg molecule has shown that favorable conditions for tyrosine chlorination occur when a susceptible location of tyrosyl residue gets into steric proximity (a distance of about 30 nm) of an —NHCl group, which acts as the chlorine donor. Such superposition makes possible homolysis of the nitrogen—chlorine bond of terminal chloramine glycine and addition of chlorine to a juxtaposed tyrosine ring. Another example of a tyrosine residue-specific chlorination is D-amino acid oxidase chlorination only by chloramines of *D*-leucine, *D*-isoleucine, and *D*-norvaline, whereas HOCl and other D-amino acid chloramines are not able to carry out the reaction (R15). This suggests the occurrence of such sterical interactions in chlorination of tyrosyl residues in biological systems.

1.9. Targetting of MPO-Synthesized Oxidants and Biological Functions of Chloramines

Chemical properties of hypochlorite, which attacks all available reductants, raise questions about targeting mechanisms of MPO-synthesized HOCl. One such mechanism may employ a basic characteristic of the MPO molecule itself, whose P_i value amounts to 10.5 (Z3) and which tends to aggregate with structures containing acidic residues on its surfaces (F2). This decreases the distance between the site of generation of HOCl and its target substrate. Increasing the distance between the HOCl and the reactive protein causes an unevitable HOCl loss in reactions with other reductants. The slightly acidic molecules albumin and immunoglobin (IgG) are more susceptible to MPO-mediated chlorination than to direct HOCl treatment. This may reflect a hypothetical adaptation of the mentioned proteins in their biological functions: albumin as an oxidant scavenger responsible for thiol and thioether group protection in plasma (Y4), and immunoglobulins (in antigen–antibody comples form) as proteins designated for specific processing in phagocytic vacuoles. Acidic residues binding to the MPO molecules are also present in the bacterial cell wall (N4). This suggestion is in accord with observations that pretreatment of living bacteria, *Staphylococcus epidermis,* with MPO increases chlorination and killing considerably, compared to the same amount of MPO, H_2O_2, and Cl^- (but without MPO pretreatment) (K15). Binding of MPO to some cells may also produce host tissue injury (N5, S7).

The other mechanism proposed consists of attenuation of HOCl reactivity by transforming it into considerably more stable chloramines (S25, T5, Z2, Z3).

Sources of free amino groups for chloramine formation are taurine (W6), free amino acids accumulating in stimulated PMNs (R4), various peptides, proteins, and ammonia produced during neutrophil metabolism (G25). The taurine–MPO intermediate prevents indiscriminate oxidative reactions in leukocytes (K15). Therefore, chloramine taurine, whose concentration in pericellular spaces of phorbol mirystate acetate-stimulated PMNs can reach of 0.1 mM (G24), seems to be the other MPO-dependent biological oxidant. On the other hand, taurine in the neutrophil protects certain targets from HOCl attack, including MPO itself (N3, W5).

Chloramine taurine can damage cellular membranes through oxidation of the available thiol groups. Cysteinyl residues exposed to chloramines are about five times more reactive then methionyl residues. On the other hand, cysteinyl and methionyl residues which are buried within the three-dimensional structure of the molecule, such as three methionyl residues of bovine ribonuclease A or Met_{192} in α-chymotrypsin molecules, were resistant to chloramine oxidation (S25). Chloramines also readily oxidize hemoglobin to methemoglobin. Oxidation occurs both with heme iron and with some exposed thiol groups of the globin moieties (T5). In slightly acidic media (in stoichiometric excess), chloramines also can oxidize the tryptophanyl residues (chloramine/tryptophan concentrations ratio about 10:1) (S25) and disulfide bonds. Therefore oxidative action of chloramines can damage cells directly and can promote indirect damage by activation of some proteases and inactivation of alpha-l protease inhibitor (T5).

At pH 5.0, which occurs temporarily in phagocytic vacuoles, N-dichloramines ($R\text{-}NCl_2$) are produced. When compared on the basis of Cl^- content, the dichloramine of taurine is similar to the chloramine of taurine in its ability to oxidize erythrocytes and GSH. On the other hand, the dichloramine of taurine is about 100 times more effective as an erythrocyte-lytic agent (T5). Dichloramines are active chlorinating agents, yielding nitrogen–chlorine bonds and carbon–Cl derivatives in aromatic rings:

$$\underset{\text{Dichloramine}}{RNCl_2} + R'NH_3 \rightarrow \underset{\text{Monochloramines}}{RNHCl} + R'NHCl + H_2O$$

N-dichloramines can also incorporate themselves into histidyl, tyrosyl, and lysyl residues (T6). N-terminal dichloramines of peptides and proteins decompose into nitriles, depending on the N-terminal amino acid of the peptide (S55). The production of chlorocyan (ClCN) by the MPO-Cl—H_2O_2 system in phagocytizing neutrophils also suggests the possibility of CN moiety substitution into the protein structure. The most probable reaction sites for cyanation are cysteine —SH side groups, yielding thiocyano residues (S53). There are no direct observations of dichloramine formation in stimulated neutrophils, but they are always formed in some proportion to monochloramines depending on the pH of the reaction medium. Some formation of dichloramines derivatives with polyamines and

glucosamine was also noted (T5). Tertiary amines react rapidly with HOCl/OCl^-, yielding chloramines which are effective chlorinating agents, acting with phenols, alkenes, mono and polynucleotides and other compounds (P14).

In the presence of reducing agents, protein chloramines are reversed to native proteins, while in the presence of Cl^-, chloramines disproportionate to yield elementary chlorine (Cl^0), which readily substitutes for phenol moieties of tyrosines to yield stable 3′-chloro and 3′5′- dichloro derivatives. The chlorination of tyrosines retentively marks the protein molecule (R15).

HOCl also readily reacts with nitrite (NO_2^-) (E2). HOCl and NO_2^- react to form reactive intermediates Cl-NO_2, and/or Cl^-ONO, which are capable of nitrating, chlorinating and dimerizing tyrosine residues (E2).

1.10. Peroxynitrite

It has been shown in recent years that nitric oxide ($NO^\bullet$) is a biological mediator synthesized in endothelial cells, monocytes, macrophages, and neural cells. $NO^\bullet$ is produced from *L*-arginine by enzyme *NO*-syntase (NOS), which has two isoforms: the constitutive isoform continously maintaining $NO^\bullet$ basal levels and the inducible isoform responsible for stimulated $NO^\bullet$ synthesis. $NO^\bullet$ is a small lipophilic molecule that readily migrates through biological membranes by diffusion. Due to the presence of an unpaired electron, the $NO^\bullet$ is a free radical with a high redox potential. The high reactivity of $NO^\bullet$ limits its half-life to only a few seconds. $NO^\bullet$ reacts with oxygen, producing nitrite (NO_2^-) and nitrate (NO_3^-). $NO^\bullet$ also has a high affinity to superoxide radical anion, yielding the peroxynitrite [oxoperoxonitrate(1-)] $ONOO^-$:

$$O_2^{-\bullet} + NO^\bullet \rightarrow \underset{\text{Peroxynitrite anion}}{OONO^-}$$

The reaction between nitric oxide and $O_2^{-\bullet}$ is fast, with a rate constant ($k = 4$–$7 \times 10^9\ M^{-1}\ s^{-1}$) which is higher than that of the $O_2^{-\bullet}$-superoxide dismutase reaction ($2.3 \times 10^9\ M^{-1}\ s^{-1}$) (G14, H27). Therefore, in normal conditions, in the cytoplasm, at low $NO^\bullet$ concentration and SOD concentration higher than the $NO^\bullet$, most $O_2^{-\bullet}$ is decomposed in reaction with SOD. However, at high $NO^\bullet$ production, in the neighborhood of activated macrophages, most $O_2^{-\bullet}$ may react with $NO^\bullet$, producing peroxynitrite (B7, K22, M29). The rate of peroxynitrite synthesis in stimulated macrophages may reach even 0.11 nmol $\times 10^{-6}$ cells $\times$ min^{-1} (I4). Interestingly, under conditions of arginine depletion, nitric oxide synthase can simultaneously generate nitric oxide and release superoxide, thus acting as a peroxynitrite synthase (X1, V4). An important source of nitric oxide and peroxynitrite in the lungs of smokers is cigarette smoke (H26). Peroxynitrite is the anion of the weak peroxynitrous acid, whose pK value at 37°C is about 6.75. While the peroxynitrite anion form is relatively stable in alkaline solutions, peroxynitrous

acid is rather unstable and isomerizes to nitrate:

$$ONOO^- + H^+ \leftrightarrow \underset{\text{Peroxynitrous acid}}{ONOOH} \rightarrow H^+ + NO_3^-$$

The first-order reaction rate constant for the isomerization of peroxynitrous acid to nitrate is 4.5 s^{-1} at 37°C; therefore, at pH 7.4 and at 37°C the half-life of the peroxynitrite/peroxynitrous acid couple (let both these species be referred to as peroxynitrite for the sake of brevity) is less than 1 s. The reaction mechanism of peroxynitrite decomposition was a subject of controversy. Primarily proposed was that peroxynitrous acid decomposes by homolysis, producing two strong oxidants: hydroxyl radical and nitrous dioxide (B15):

$$ONOOH \rightarrow {}^{\bullet}OH + NO_2^{\bullet}$$

The other proposed mechanism postulated that ONOOH decays to nitrite via formation of a semistable intermediate, an activated form of peroxynitrous acid (ONOOH*) (G15, P16). Recently, the concept of ONOOH homolysis yielding $^{\bullet}OH$ and $NO_2^{\bullet}$ has been revived; it has been estimated that one-third of the peroxynitrite may decay employing the homolytic mechanism (M15). Regardless of the detailed reaction mechanism, various studies have shown different molecular species, such as peroxynitrite anion, ground-state peroxynitrous acid and the activated intermediate, or hydroxyl radical and nitric dioxide, involved in reactions of peroxynitrite. While the peroxynitrite and ground-state peroxynitrous acid reactions are of second order (first order in peroxynitrite and first order in the substrate), the reactions of the activated intermediate are first order (first order in peroxynitrite and zero order in the substrate). Ground-state peroxynitrous acid and peroxynitrite may react via one-electron and two-electron mechanisms. Both reaction pathways, as evidenced by their high redox potentials, have strong oxidative properties:

$$ONOO^- + 2H^+ + e^- \rightarrow NO_2^{\bullet} + H_2O \qquad E_0' = 1.4\ V$$
$$ONOO^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O \qquad E_0' = 1.2\ V$$

The hypothetical activated intermediate, with a very high redox potential ($E_0' =$ 2.1 V), would be an even stronger oxidant, yielding reaction products similar to those of $^{\bullet}OH$ (K23).

1.11. Reactivity of Peroxynitrite with Biological Compounds

Peroxynitrite is a nonspecific oxidant that reacts with all classes of biomolecules: depleting low-molecular-weight antioxidants, initiating lipid peroxidation, damaging nucleic acids and proteins. Its reactions are much slower than those of the hydroxyl radical but are faster than those of hydrogen peroxide. Comparison of peroxynitrite reactivity with various amino acid residues of human serum albumin have shown that cysteine, methionine, and tryptophan are the most reactive

TABLE 6
SECOND-ORDER RATE CONSTANTS FOR THE REACTION OF PEROXYNITRITE WITH VARIOUS PROTEINS AND THEIR CONSTITUENTS

Compound	k ($M^{-1}s^{-1}$)	Reference
Albumin (human serum—HSA)	9.7×10^3	(A7)
Ascorbate	236 (25°C)	(S49)
Carbon dioxide	5.8×10^4	(D12)
Creatine kinase	8.9×10^5	(K20)
Cysteine	5.9×10^3	(R2)
Cytochrome *c*—Fe^{2+}	2.3×10^5	(T11)
Glutathione peroxidase (GPX)	1.8×10^5 per tetramer (pH 7.1)	(P5)
GPX—oxidized	7×10^5 per tetramer (25°C)	(B23)
GPX—reduced	8.0×10^6 per tetramer (25°C)	(B23)
Glyceraldehyde 3-phosphate dehydrogenase	2.5×10^5 (25°C)	(S47)
Hemoglobin	$2–3 \times 10^4$	(A5)
Lactoperoxidase (for formation of Compound II)	3.3×10^5, (pH 7.4, 12°C)	(F10)
Mitochondrial aconitase (inactivation rate constant)	1.4×10^5 (pH 7.6, 25°C;)	(C5)
Myeloperoxidase (formation of Compound II)	6.2×10^6 (pH 7.2, 12°C)	(F10)
Myeloperoxidase (for reaction with ONOOH)	2.0×10^7 (independent of pH, 12°C)	(F10)
Pyruvate	88	(V3)
Thiol group in BSA	$(2.6–2.8) \times 10^3$	(R2)
Thiol group of HSA	3.8×10^3	(A7)
Tryptophan	77 (35°C, pH 7.1)	(P1)

[a] At pH 7.4 and 37°C unless indicated otherwise.

entities (A7). Peroxynitrite reactions with thiols are rather fast, with rate constants three orders of magnitude greater than the corresponding rate constants for the reaction of hydrogen peroxide with sulfhydryls at pH 7.4 (R2) (see Table 6). At higher pH, two-electron thiol oxidation predominates, first yielding unstable sulfenic acid derivatives R—S—OH, which next react with another thiol, producing the disulfide:

$$\underset{\text{Sulfenic acid}}{R_1\text{—S—OH}} + R_2\text{—S—H} \rightarrow \underset{\text{Disulfide}}{R_1\text{—S—S—}R_2} + H_2O$$

At low pH, one-electron oxidation predominates, yielding thiyl radicals R—$S^{\bullet}$. At physiological values of pH, both pathways coexist; however, two-electron oxidation predominates (G5, Q1, S12). The peroxynitrite inactivation of the sarcoplasmic reticulum Ca^{2+}-ATPase (at a low concentration of $ONOO^-$) mainly accounts for the thiol group oxidation, and can be reversed by thiol reducing agents (V8).

Peroxynitrite can also react with thiols to form *S*-nitrosothiols, R—S—NO, although the yield of this reaction is about two orders of magnitude lower than with thiol oxidation by peroxynitrite. The $ONOO^-$ may also nitrosate proteins, though the efficiency of this reaction is much lower. Nitrosothiols are unstable and decompose to form $NO^\bullet$ and thiyl radicals $—S^\bullet$, thus being a secondary source of nitric oxide. In this way peroxynitrite may generate nitric oxide. The thiyl radicals formed can react with glutathione, yielding protein–glutathione mixed disulfides (protein glutathionylation) (M21, M22). The formation of *S*-nitrosothiols has been postulated to explain a paradox effect of peroxynitrite-induced vasorelaxation: injection of about 10 μg/kg of peroxynitrite produces a substantial fall in blood arterial pressure associated with reduction of vascular resistance in anesthetized rats (K21). However, it seems that the vasodilatation induced by peroxynitrite may also occur due to the presence of other intermediates, formed during the interaction of $ONOO^-$ with blood constituents (G23). Peroxynitrite anion reacts rapidly with carbon dioxide, forming nitrosoperoxycarbonate anion, which can rearrange to form further reactive species:

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \rightarrow \text{other reactive species}$$

As a result, the presence of CO_2 or bicarbonate attenuates the oxidation and hydroxylation reactions of peroxynitrite; on the other hand, nitration reactions are enhanced. This feature of peroxynitrite reactions may have profound significance for *in vivo* situation (D12, L15, L28, L29).

Peroxynitrite has the ability to nitrate aromatic moieties of tyrosine, producing 3-nitrotyrosine; tryptophan, producing 6-nitrotryptophan and 5-nitrotryptophan (A8, P4); and phenylalanine, producing 3-nitrophenylalanine (V2) (see Fig. 10, sect. 2.1). There exists a controversy concerning the nitration mechanism by peroxynitrite. The commonly accepted concept postulates an intermediary role of nitrogen dioxide, NO_2, or another "NO_2-like product" formed by peroxynitrite decomposition (L13); the other mechanism postulates nitration via the nitronium ion NO_2^+ (B7, M29). The nitration reaction of phenolic groups is catalyzed by transition metal ions and metalloenzymes, especially SOD (B17, C18), catalase, or horseradish peroxidase, but not hemoglobin, myoglobin, or cytochrome *c* (K19).

Another pathway of peroxynitrite-mediated modification of aromatic amino acid residues is hydroxylation. Products of peroxynitrite reaction with phenylalanine include *p*-, *m*-, and *o*-tyrosine. Peroxynitrite also forms dityrosine from tyrosine (V2). Major products of oxidative modifications of tryptophan by peroxynitrite include hydropyrroloindole, oxindole, and *N*-formylkynurenine (K4).

Peroxynitrite reacts rapidly with glutathione peroxidase and other seleno compounds [(B23), see Table 6]. A protective role of selenoproteins against peroxynitrite has been proposed, as glutathione proxidase in the presence of glutathione

is apparently resistant to inactivation by low concentrations of peroxynitrite. This concept implies that glutathione peroxidase also acts as a peroxynitrite reductase. The postulated reaction mechanism, based on studies of ebselen reaction (a seleno-containing organic compound), suggests oxidation by peroxynitrite of the selenomethionine to selenoxide in the active site of the enzyme. The reaction effects reduction of peroxynitrite to nitrite and further two-step reduction of selenoxide by glutathione, recovering active selenium (A16, S30):

$$\begin{aligned} ONOO^- + \text{enzyme-Se}^- + H^+ &\rightarrow \text{enzyme-SeOH} + ONO^- \\ \text{Enzyme-SeOH} + GSH &\rightarrow \text{enzyme-Se-SG} + H_2O \\ \text{Enzyme-Se-SG} + GSH &\rightarrow \text{enzyme-Se}^- + GSSG + H^+ \end{aligned}$$

Actually, glutathione peroxidase protects from peroxynitrite-mediated oxidation and nitration reactions in model systems. Selenoprotein P from human plasma exhibits a similar property (A16, A17). A similar function of "peroxynitritase" has been ascribed to bacterial catalase/peroxidase (W10).

At physiological pH the protonated form of $ONOO^-$, the peroxynitrous acid (ONOOH), is unstable and decomposes to nitrate (NO_3^-). ONOOH can also react directly with reductants or can decompose by homolytic dissociation to form nitrogen dioxide (NO_2) and hydroxyl radical ($OH^\bullet$), or can dissociate by a heterolytic mechanism to yield nitryl cation (NO_2^+), which reacts with thiol, methionyl, tyrosyl, and tryptophanyl residues in proteins.

1.12. Nitrogen Radical and Other Free Radicals Produced in Biological Systems

Reactions of the hydroxyl radical and other reactive oxygen and nitrogen species (RONS) generate secondary radicals such as alkoxyl ($—RO^\bullet$), peroxyl ($—ROO^\bullet$), thiyl ($—RS^\bullet$), and carbon-centered ($—R_1—C^\bullet—R_2$) radicals. These radicals may also attack lipids, carbohydrates, and proteins dissolved in tissue fluids. Some nitrogen-centered and carbon-centered radicals are temporarily formed in enzymatic proteins. For instance, a tyrosyl radical (S57) and a thiyl radical (L22) are formed in the catalytic cycle of ribonucleotide reductase. Lipoxygenases, cytochromes P_{450}, and peroxidases may generate radical species during interaction with substrates (G1). Lipid peroxidation is an important source of free radicals, which in biological systems may attack membrane proteins. Also, the reactive aldehydes which are diffusible can cause secondary damage by reactions with proteins (C16, G10). It is currently thought that although the hydroxyl radical is formed in small amounts *in vivo,* its extreme reactivity accounts for most of the damage produced by ROS in biological systems.

2. Oxidative Modifications of Protein Structures

2.1. Proteins as Targets for Action of Oxidants

Proteins, due to the complexity of their chemical structures, undergo oxidative modifications in subsequent stages which depend both on the presence of oxidation-susceptible groups and on steric availability of these groups for oxidant attacks (S25). Some oxidative structural modifications produced in proteins are common in various oxidants. Some modifications, such as chlorinated and nitrated protein derivatives produced in reactions with hypochlorite, peroxynitrite, and nitric dioxide, are specific for the oxidants employed. Certain oxidative protein modifications, such as interchain or intrachain disulfide bond formation or thiolation, are reversible and may be reduced back to the protein native form when oxidative stress is over (D1). Other changes, such as sulfone formation, chlorination, and nitration, are irreversible and effect protein denaturation and promote its subsequent degradation.

Among proteins, most susceptible for oxidation are thiol groups of cysteinyl residues and thioether groups of methionyl residues (S59). As most cysteinyl residues in proteins are used for disulfide bridges stabilizing protein tertiary structures, only limited amounts of free —SH groups are available (F8). Still, studies on protein oxidation by phorbol myristate acetate-stimulated neutrophils showed that thiol residues first react with the oxidants produced in the respiratory burst. This effects prompt oxidation, 22% of all protein-bound thiol content of these cells (F8). Similar treatment of albumin with HOCl first effects oxidation of all exposed —SH groups (S10). Oxidation of thiol groups may effect rearrangement of disulfide bridges, inducing conformational changes in the protein molecule.

The methionyl residues, except for those buried inside the hydrophobic cores of protein molecules, are exposed to oxidants. This may effect a preferential spontaneous oxidation of methionyl residues, occurring both *in vitro* and *in vivo* (S59) (Fig. 10).

Oxidation of methionyl residues in certain proteins does not impair their biological function (Table 7). Such proteins usually possess oxidized and reduced thiol- or thioether groups in certain proportions (V13). An example is the apolipoprotein-A1 (Apo-A1) which in the presence of atmospheric oxygen is subjected to spontaneous oxidation of methionines in positions 112 and 148 to methionine sulfoxide (V13). The methionine sulfoxides contribute to Apo-A1 heterogeneity in human plasma without affecting its biological function. It was also found that some thiol groups of erythrocyte plasma membranes do not reduce dithionitrophenyl benzoic acid (DNPB), which suggests that some thiol groups of exposed membrane proteins are in the oxidized form (S10). Reversible oxidation of —SH groups may also activate some proteins: membrane-bound GST transferase (GSTm) present in hepatic microsomal and plasma membranes, as well as in outer mitochondrial membranes,

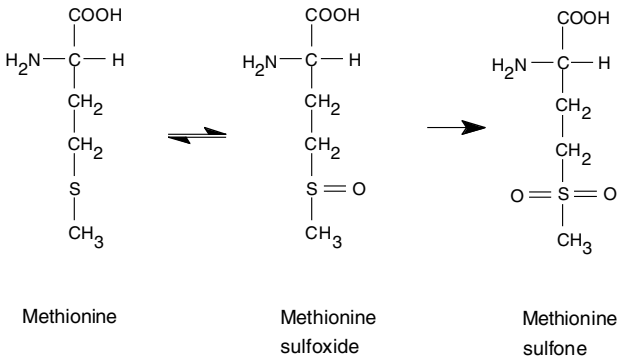

FIG. 10. The first step of methionine oxidation to methionine sulfoxide is reversible owing to methionine sulfoxide; furtehr oxidation to methionine sulfone is irreversible.

is activated about fivefold by thiolation (S29). Also, oxidation of methionine in the human complement C5 component activates it and affects release of "anaphylatoxin" activity (V12).

There is also a large body of data concerning the deleterious effects of cysteinyl and methionyl residue oxidation on protein function. A few selected examples of such effects are discussed here. The oxidative inactivation of α_1-proteinase inhibitor (α_1PI) (formerly $\alpha_1$1-antitrypsin), due to its possible involvement in human pathology, was extensively studied. The α_1PI is one of the proteinase inhibitors present in blood plasma at a concentration of 108–240 mg/dl. *In vitro* and *in vivo,* α_1PI binds to various serine proteinases, but its most important physiological substrate is the neutrophil elastase (T16). The α_1PI contains one methionyl residue at position 358 in the polypeptide chain built of 394 amino acids. Oxidation of this methionine residue produces loss of α_1PI-binding ability to trypsin (D11) and other proteinases (M11). The other possible oxidants acting on α_1API in biological fluids are superoxide anion, singlet oxygen, hydrogen peroxide, hypochlorite, and chloramines as products of neutrophile respiratory burst (S59). Moreover, cigarette smoke as a promoter of α_1PI oxidation due to proxynitrite formation should be mentioned (S56). Similar effects were observed for other proteins exposed to oxidant action. The other protein involved in control of proteolytic activity of blood plasma is the plasminogen activator-inhibitor (PAI), whose function is abolished in oxidation of a single methionine residue. This protein, involved in fibrynolysis *in vivo* in the presence of H_2O_2, chloramine of taurine, and N-chlorosuccinimide, is transformed into the oxidized form and is then unable to bind the plasminogen activator (S56). Oxidation of methionins at positions 9 and 60 in apoliporoteins CII and C-III, impair Apo-C function as a lipid carrier (V13). Oxidation of methionine residues located in the active centers of chymotrypsin, phosphoglucomutase, and ribonuclease inactivates these enzymes (S59). Oxidation of vitamin B_{12}-binding

TABLE 7
EXAMPLES OF SITE-LOCATED SPECIFICITY IN OXIDATION OF THIOL AND THIETHER RESIDUES

Protein	Thiols susceptible for oxidation	Biological effect	References
Growth horomone (human)	Met_{64} and met_{179}	Abolition of binding ability to hGH receptor (Nb2 rat lymphoma cells)	(T1)
Enkefalin	Meth residue oxidation	Degradation of peptide structure	(T20)
Hemoglobin (β-globin chain)	Oxidation of Meth-$\beta_{55}D_6$	Increase in in affinity to oxygen; abolition of Bohr effect	(A10)
Hemoglobin (β-globin chain)	Oxidation of Meths $\alpha_{32}\beta_{13}D$, $\alpha_{76}EF_5$, $\beta_{55}D_6$	Loss oxygen-binding properties	(A10)
Apolipoprotein AI (wild type)	$Meth_{112}$ and $Meth_{148}$	Neutral	(V13)
Apolipoprotein AI—variant	Mets substitutuded for Lys_{107}—Meth, and Glu_{139}—Gly	Neutral (?), change in ratio of Meth-SO_{112}/Meth-SO_{148}	(V13)
Apolipoproteins C-II and C-III	$Meth_9$ and $Meth_{60}$	Loss of lipid-carrier properties	(M11)
α_1-Antiproteinase (α_1-PI)	$Meth_{358}$	Loss of binding ability to elastase and trypsin; decay of α_1-PI–proteinase complexes	(D11, D18, M11)
α_2-Macroglobulin (α_2-M) (human)	Meth residues	Abolition of binding proteinases, inhibition of "fast-form" formation	(S59)
Chymotrypsin	$Meth_{180}$ and further two Meths located in active center	Loss of proteolytic function	(S25, S59)
Lysozyme	Meth residue susceptible for photo oxidation	Loss of enzyme function	(J4)
Pepsin (pancreative, porcine and bovine	Oxidation of methionine residues	Loss of enzyme properties	(K13)
Phosphoglycomutase (bacterial)	One Meth residue in ezyme active center	Enzyme inactivitation, abolition of glucose glycolytic metabolism	(S59)
Serum albumin (bovine)	Exposed thiol/thioether groups (generally)	Reduction of HOC1 and chloramines; oxidant-scavenging function suggested	(H25, Y4)

protein by neutrophile respiratory burst products abolishes its ability as a plasma vitamin B_{12} carrier (C12). Also, oxidation of catalytically essential —SH groups inactivates (Na^+K^+)-ATPase or the sodium–potassium pump (a membrane protein involved in the maintenance of the cation concentration gradient across the plasma membrane) (C11) and causes the loss of ability of calmodulin to activate Ca^{2+}-ATPase (S58). Oxidation of the methionine residue in the peptide Met-Leu-Phe inactivates its chemotactic activity (B24), while oxidation of Met_{64} and Met_{179} in human chorionic somatomammotropin leads to loss of biological activity as a hormone (T1).

In tertiary structures of proteins, methionine residues differ with respect to their exposure to oxidizing agents and the protective effect of their intramolecular

environment. The site-related specificity in oxidation of methionine residues has been observed in human Apo-A_1 lipoprotein, in which methionine located at position 86 remains in the reduced state, even if both subsubsequently located methionines, Met_{112} and Met_{148}, are oxidized. In normal Apo-A_1 the amounts of Met_{112} and Met_{148} are highly variable, but the ratio of Met-SO_{112}/Met-SO_{148} is constant (V13). Other examples of different oxidation susceptibility various methione residues are chorionic somatomammoprotein, human growth hormone (T1), and the beta-globulin chain of human hemoglobin (A10). Water-soluble strong oxidants, such as hypochlorite, chloramines, or peroxynitrite, may not oxidize potential electron donors if they are buried in the hydrophobic core of protein molecules. One example of such an effect is protection of oxidizable histidine and cysteine residues in the vicinity of the active site of MPO (B20) or histidine residue of *E. coli* glutamine synthetase, which is resistant to sole HOCl/OCl^- treatment or even to oxidation with a HOCl and H_2O_2 mixture (L9).

Methionine sulfoxide formation may occur without noticeable changes in physical or immunochemical properties of the protein. Thus reduction of sulfoxide to thioether often completely restores the lost protein function. Many cells, including human polymoprhonuclear neutrophilic leukocytes, contain enzyme methionine sulfoxide reductase, which is able to convert methionine sulfoxide to the reduced methione form in a variety of proteins (B25, F8). Methionine reacting with a strong oxidant effects methionine sulfone production, which *in vivo* is not reduced back to methionine.

Another oxidative reversible protein modification is thiolation, i.e., formation of mixed disulfides with low-molecular-weight thiol compounds (S22). In intracellular milieu, glutathione is the most abundant low-molecular thiol-binding to proteins; others are γ-glutamylcysteine and cysteine. Thiolation occurring after oxidative burst of human monocytes inactivates cellular glyceraldehyde 3-phosphate dehydrogenase (R5). In rat liver, one of the main proteins thiolated by oxidative stress is carbonic anhydrase III (C6). Thiolation of creatine kinase inactivates the enzyme, while thiolation of glycogen phosphorylase *b* has no observable effect on any activity parameter, but it effectively prevents binding of phosphorylase to high-molecular-weight glycogen, probably at the glycogen storage site of phosphorylase (M17). Thiolation of rat liver fatty acid-binding protein by glutathione decreases the affinity of the protein for unsaturated fatty acids without changing the equimolar maximum binding (H22). It was proposed that thiolation is employed in control of protein irreversible inactivation (D8) or has a protective role against irreversible oxidation of cysteine residues. Binding of protein —SH groups in mixed disulfides with glutathione (protein *S*-thiolation) can prevent their oxidation to sulfone residues (G21, T9).

Spontaneous oxidation of proteins, functional thiol, and thioether groups is inhibited by a reducing potential of plasma and extracellular fluids (A2). The active extracellular reductant is ascorbate, which in reduced form is in certain proportion

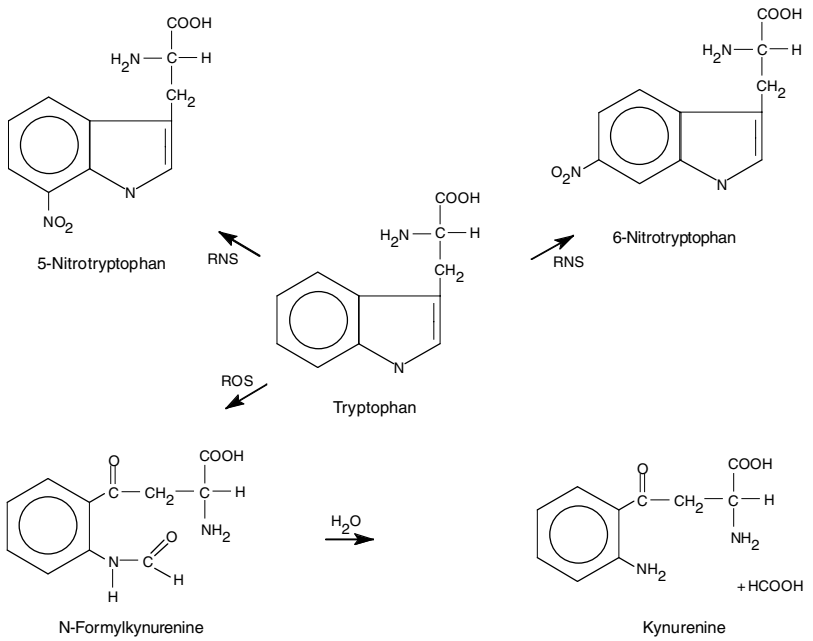

FIG. 11. Modification of tryptophan by reactive oxygen species and reactive nitrogen species.

to the concentration of free —SH groups in plasma. The other reductants are uric acid, billirubin, and carotenoids (H25). Plasma albumin, which is rich in thiol groups, also reduces oxidants (including hypochlorate and chloramines) (H25). Antioxidant potential of blood plasma and the extracellular fluids, also called "antioxidant defense," has an important role in limiting toxic effects of respiratory burst products released in areas of inflammatory reactions (see sect. 3.5).

The other amino acid residue present in proteins that is susceptible to oxidation is the indole moiety of tryptophan (Fig. 11). The reducing potential of tryptophan is considerably less than that of cysteine and methionine, so oxidation of tryptophanyl residues usually does not occur until all exposed thiol residues are oxidized. Also, the spontaneous oxidation of tryptophanyl residues in proteins is much less probable than that of cysteinyl and methionyl residues. Tryptophan residues are the only chromophoric moieties in proteins which can be photooxidized to tryptophanyl radicals by solar UV radiation, even by wavelengths as long as 305 nm (B12). Tryptophanyl residues readily react with all reactive oxygen species, hypochlorite, peroxynitrite, and chloramines. Oxidative modifications of other amino acid residues require use of strong oxidants, which eventually are produced in the cells. Detailed mechanisms of action of these oxidants is described in subsequent sections of this chapter.

Among protein oxidation products, formation of protein peroxides should be mentioned. Protein peroxidation occurs as a reaction secondary to free-radical attack on amino acid side groups, effecting a carbon-centered free radical of amino acid formation. Such a radical reacts with the oxygen molecule and produces a hydroperoxide radical:

$$\text{Protein}^{\bullet} + O_2 \rightarrow \text{Protein–OO}^{\bullet}$$

The reaction is irreversible. Peroxyl radicals are easily reduced to hydroperoxide anions,

$$\text{Protein–OO}^{\bullet} + e^{-} \rightarrow \text{Protein–OO}^{-}$$

which binds a proton to form protein hydroperoxide [G6]:

$$\text{Protein–OO}^{-} + \text{H}^{+} \rightarrow \text{Protein–OOH}$$

(G6). Peroxides of some amino acid residues are unstable *in vitro,* while in other cases they are semistable enieties. The yield of relatively stable hydroperoxides varies depending on the amino acid residues. Residues of Ile, Leu, Val, Glu, Pro, and Lys (having a tertiary carbon and at least two adjacent methylene groups) are most susceptible to form stable hydroperoxides. Other hydroperoxide-forming residues are those of Ala, Arg, and Tyr, while residues of Asn, Asp, Cys, Met, Gly, His, HO-Pro, Phe, Ser, Thr, and Tyr do not form, or form stable hydroperoxides with a negligible yield (G7). In pure protein preparations at 20°C, 40% of bovine serum albumin (BSA) hydroperoxides and 50% of lysozyme hydroperoxides decay spontaneously during 24 h (G7); however, in the reducing intracellular environment, protein peroxides are short-lived agents, rapidly reduced by most biological reductants, such as ascorbate and glutathione (S33). Reduction of hydroperoxides can be remarkably accelerated by the enzyme glutathione peroxidase. The reduction products are corresponding hydroxides (F20, G6). Transition metal ions decompose hydroperoxides, forming secondary free radicals (G6).

In effecting oxidative modifications, proteins lose their native conformations (i.e., protein denaturation occurs), which is a direct cause of the abolishment of protein functional properties. Oxidative denaturation usually produces protein unfolding and increases surface hydrophobicity (C7). Increased hydrophobicity of oxidatively denatured proteins seems to promote proteolysis. The proteasome complex recognizes hydrophobic amino acid residues, aromatic residues, and bulky aliphatic residues which are exposed during the oxidative rearrangement of secondary and tertiary protein structures. Though it may be a simple effect of protein unfolding, increased surface hydrophobicity is a common feature of all oxidized proteins studied so far (G28, P1). Some proteins extensively oxidized appeared to be more resistant to proteolysis then the native ones (D9).

2.2. Reactions of the Hydroxyl Radical

The hydroxyl radical reacts with all amino acid residues in proteins. Due to its high activity, the $^{\bullet}OH$ may react with the first entity encountered. However, amino acid residues having reducing properties, such as thiol, thioether, methionyl, and aromatic and heterocyclic moieties, are more vulnerable to $^{\bullet}OH$ attack. The reaction begins with cleavage of chemical bonds and the production of the amino acid radical. In proteins, such radicals may interact with neighboring amino acid residues, thus effecting a free-electron migration within the molecule. Therefore, the final reaction product does not have to correspond to the site of primary attack (H4). As mentioned before (sect. 1.3), amino acids interacting with $^{\bullet}OH$ may undergo a profound structural transformation. The hydroxyl radical acting on bovine serum albumin (BSA) produces destruction of amino acids, which at the $^{\bullet}OH$/BSA molar ratio (nmol of radicals/nmol of BSA) about 10, results in an amino acid loss amounting to 9–10%, whereas at the $^{\bullet}OH$/BSA ratio 100, the average amino acid loss is 45% (D6). The transient radicals generated by $^{\bullet}OH$ in amino acid backbones tend to create new chemical bonds, producing aggregation of protein molecules. An important part of the protein intermolecular coupling is new —SS— bond formation and dityrosine bridge formation. The $^{\bullet}OH$ interaction with proteins in the presence of oxygen and $O_2^{-\bullet}$ produces polypeptide fragmentation rather than aggregation; while in the absence of oxygen, aggregation predominates (D5).

Fragmentation of model peptides (U3) and of proteins such as bovine serum albumin (S11), myoglobin (S11), and hemoglobin (P17) by $^{\bullet}OH$ generated by water radiolysis is not at random, and produces fragments corresponding to breakage of polypeptides at the aminoacyl–proline peptide bond. Such a polypeptide fragmentation pattern can be explained by the ease of electron transfer within the protein molecule and the relative stability of the α-carbon radical formed from proline. However, another explanation of the polypeptide breakage pattern is also possible: the molecular weights of the fragments suggest cleavage at glycine. The α-carbon radical of glycine is also very stable (D9). Specificity of protein–polypeptide cleavage by metal ion-containing systems is often different from that which is described above. The breakage points correspond to site-specific formation of hydroxyl radicals in the reaction of hydrogen peroxide with protein-bound metals.

As already mentioned, one of the products of action of hydroxyl radicals on proteins is protein hydroperoxides (G6). Valine and lysine residues are particularly susceptible to hydroperoxide formation. Reduction of hydroperoxides produces respective hydroxy derivatives of amino acids. Three valine hydroxides derived from hydroperoxides of this amino acid have been characterized structurally as β-hydroxyvaline [(2S)-2-amino-3-hydroxy-3-methyl-butanoic acid], (2S,3S)-γ-hydroxyvaline [(2S,3S)-2-amino-3-hydroxymethyl-butanoic acid], and (2S,3R)-γ-hydroxyvaline [(2S,3R)-2-amino-3-hydroxymethyl-butanoic acid (Fig. 12). They are suggested to be possible markers of protein peroxidation (F21).

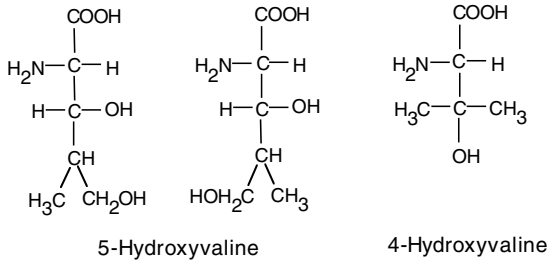

FIG. 12. Hydroxyl radical oxidation products of valine residues.

Comparing yields of hydroxylysine, hydroxyvaline, and hydroxyleucine production supports the concept that surface-exposed residues, which are expected to be the first targets for free-radical attack, are more readily oxidized than the hydrophobic amino acids (such as leucine and valine). Hydroxylysines, and particularly 3-hydroxylysine, may therefore be sensitive and useful markers of free-radical protein oxidation in biological systems (at least for globular proteins such as BSA) (M25). Other products of lysine modification include lysine semialdehyde or 6-oxo-2-aminohexanoic acid (K3). Oxidation of basic amino groups in lysyl residues producing uncharged aldehydic groups causes a net loss of positive charge of the whole protein molecule. This effect was observed upon oxidation of the protein components of low-density lipoprotein (LDL) (S48).

2.3. Reactions of Superoxide Anion Radical and Hydrogen Peroxide

Hydrogen peroxide and superoxide radical anion are relatively nonreactive oxygen species. The superoxide anion radical reacts with proteins at physiological conditions much more slowly than the hydroxyl radical and is not considered critical in protein damage *in vivo*. This is evidenced by the respective rate constant values (Tables 2–6). However, there are some exceptions: superoxide radical reacts rapidly with iron–sulfur clusters and inactivates proteins containing these clusters. Reaction rate constants for the inactivation of cytosolic and mitochondrial aconitase by $O_2^{-\bullet}$ is high (3×10^7 and 3.5×10^6 M^{-1} s^{-1}, respectively) (C5, H10). Also, other Fe-S proteins (as dihydroxy-acid dehydratase, fumarase A, and fumarase B) are inactivated by $O_2^{-\bullet}$ with rate constants of 10^6–10^7 M^{-1} s^{-1}, while the rate constant for the inactivation of mammalian aconitase is only about 10^3 M^{-1} s^{-1} (F7). The superoxide anion can oxidize reactive –SH groups, abolishing biological function of some proteins, such as glyceraldehyde 3-phosphate dehydrogenase and ribonuclease inhibitor.

Reactions of hydrogen peroxide with proteins are generally slower than those with superoxide anion. H_2O_2 reacts with heme proteins, producing the Fe(IV)-oxo

species and protein-derived free radicals. In the case of myoglobin, there are at least two sites of formation of protein (globin)-derived free radicals, located on tyrosine and on tryptophan residues (I1). Many reactions of H_2O_2 with proteins are mediated by the hydroxyl radical. For instance, superoxide dismutase treated with H_2O_2 produce the 2-oxohistidine from histidine-118 located in the active site of the enzyme (U6). The reaction occurs due to the site-specific generation of $^{\bullet}OH$ in the reaction of H_2O_2 with copper located at the active site of the enzyme. However, it seems that at higher concentrations of H_2O_2 the methionyl residues are oxidized directly, without generation of the hydroxyl radical intermediate (V12).

2.4. Hypochlorite

Hypochlorite is another strong oxidant produced in stimulated neutrophils. Since its oxidizing potential is much less than that of the hydroxyl radical, the HOCl/OCl^- lifetime in biological systems is long enough for it to react selectively with preferable electron donors. Such donors are thiol, thioether, and disulfide and tryptophanyl indole moieties (with lower affinity) (see sect. 1.4). These particular reductants compete for hypochlorite and act as HOCl scavengers, protecting from oxidation less active reductants built into the protein molecule. The protective action of thiol groups in the same polypeptide chain was observed in the reaction of Leu-Trp-Met-Arg-Phe-COOH pentapeptide with HOCl, showing that oxidation of less active typtophanyl residues proceeds only after oxidation of all available methionyl residues (D20). Similar treatment of albumin with HOCl first effects oxidation of all available —SH groups (S10). The α_1-proteinase inhibitor (α1PI) has one methionyl in its active center. Using the HOCl-generating MPO-H_2O_2-Cl^- system for oxidation of α_1PI-methionine residue, the H_2O_2 to α_1PI molar ratio amounts to 1.2:1 (D18). The α_1PI–trypsin complex, treated with HOCl or with the MPO-oxidizing system, decays, releasing free trypsin and the oxidized, inactivated α_1PI. This indicates that binding of the α_1PI-proteinase in a complex with trypsin does not restrict the HOCl access to the critical methionine residue in the α_1PI molecule (D18). The activator-inhibitor (PAI) is another blood plasma proteinase inhibitor which loses its substrate binding ability (the plasminogen activator) because of oxidation of a single methionine residue (S56).

As already mentioned, a disulfide bond forms —S—S— bridges linking particular fragments of polypeptide chains in the protein molecule, and immunoglobulins IgG, IgM, and IgA are examples of proteins whose structures depend highly on a disulfide bonds. Some of these —S—S— bonds are readily oxidizable, and immunoglobulins tend to denaturate and precipitate from the solution. Treating carefully IgG with HOCl effects cleavge of its molecule into fragments corresponding to half of the light-chain size, the whole light-chain size, and the heavy-chain size. IgA treated in the same conditions splits into light chains (molecular weight 2.27 kDa), heavy chains (molecular weight 136 kDa), and a third polypeptide

of size amounting to one-half of the light chain (molecular weight 11.1 kDa). Increase in the HOCl-to-IgA concentration ratio up to 1.6:1 produces both precipitation of protein and denaturation of immunoglobulin structure. Similar treatment of IgM liberates only two fragments, one smaller than the heavy chain, and the other fragment of the light-chain size. Carrying out the reaction by titration of immunoglobulin with HOCl, a relationship between the amount of liberated light chains to the amount of HOCl employed was found. However, upon exceeding IgA-to-HOCl ratio 1:1.5, further additions of HOCl produced profound degradation of immonoglobulin polypeptide chains, yielding yellow-colored products and a number of smaller-sized polypeptides (D16).

Susceptibility to oxidation of disulfides built into proteins is strongly dependent on their location in the protein molecule (G3). Since the disulfides have a crucial role in maintaining protein tertiary structure, oxidation of certain —S—S— bridges may expose further disulfides and cause unfolding of the protein molcule. The final disulfide oxidation is a sulfone residue, which is stable and does not tend to reverse to sulfide. Therefore oxidative breakage of disulfides is irreversible. The spatial location of disulfides inside protein molecules influences their susceptibility to oxidation. The ribonuclease molecule has four —S—S— bonds, and at least three correctly located disulfide bonds are necessary to retain the ribonuclease enzyme properties. The compact ribonuclease molecule is relatively resistant to HOCl oxidation (D18).

The other amino acid residue in proteins that is particularly susceptible to oxidation is the indole moiety of tryptophan. HOCl oxidizes indole residues in pH range 2.0–11, and chloramines readily oxidize tryptophan at pH below 6.5 (A6). Acting with HOCl and chloramines, the reaction product is the 2-oxoindole derivative. This is readily visible by spectral changes at 250 and 280 nm due to oxoindole formation from tryptophan residues. At pH values between 3 and 5, oxoindole residues in peptide structures tend to spontaneously hydrolyze peptide bonds and undergo cyclization to iminolactone, thus effecting oxidative breakage of polypeptide structures (A6). Oxidation of tryptophanyl residues may not occur if they are buried in the matrix of the protein. For example, 40-fold molar excess of HOCl over tryptophan did not affect Trp residues in fibronectin, although extensive chlorination of amino groups and tyrosines was carried out (V10).

Chicken egg white lysozyme (LZM) does not possess exposed methionyl residues, and it has six tryptophan residues, three of which, located at positions 62, 108, and 111 are readily oxidizable with ozone and are built-in the LZM active center (D14). Tryptophanyl residues are also the first reacting moieties upon treatment of LZM with the MPO-Cl^--H_2O_2 system (at pH 4.5). The reaction occurs in several stages. In the first stage, which occurs when 1.4–1.8 mol of H_2O_2 for 1 mol of LZM is used, LZM loses its enzyme activity, but no derivative distinguishable from the native protein on the polyacrylamide gel electrophoresis is formed. The inactivation may be prevented by addition to the reaction medium *N*-acetylcysteine or

N-acetylmethionine in quantities equal to LZM. Inactivation of LZM also may be restricted upon addition of *N*-acetylcystine or *N*-acetyltryptophan. The *N*-acetyltryptophan competes with LZM for the oxidizing agent. Amino compounds binding hypochlorite to relatively stable chloramines, such as leucine, lysine, and taurine, do not protect LZM from MPO-mediated reaction. Oxidation of tryptophan residues in lysozyme molecules treated with HOCl occurs along with a decrease of lysozyme spectral properties at 280 nm.

The second stage of oxidation, which begins at LZM-to-H_2O_2/HOCl molar ratio 1:5, yields a LZM derivative with changed spectral properties and molecular masses equal to multiples of 14.3 kDa (i.e., the native LZM molecular mass), which suggests the oxidative coupling of lysozyme molecules (D19). Oxidation of tryptophan residues in LZM can be restricted by addition of disulfide groups of *N*-acetylcystine. Tryptophan competes for HOCl with disulfides, and a certain equlibrium between the HOCl and nonreacting cystine residues remaining in the reaction medium occurs. The reaction product of *N*-acetylcystine is *N*-acetylcysteic acid. In the third stage, occurring with a large excess of HOCl (1:12), LZM forms oligomeric derivatives and finally polymerizes to water-insoluble products (D17). Lysozyme coupling to oligomeric structures is not specific for HOCl/OCl^- oxidation. Similar loss of LZM tryptophanyl residues increases bityrosine formation and production of di- and trimeric LZM conjugates, which was observed upon treatment of LZM with the hydroxyl radical (F13).

In the absence of reductants, HOCl reacts with free amino moieties, producing semistable chloramines. In an excess of HOCl, stable tyrosine chloroderivatives are also produced. The chloramine formation causes a loss of side amino groups, changing the protein p*I* value. As chloramines are oxidants themselves, a number of secondary reactions producing carbonyl and aldehyde moieties (at the N-terminus of polypeptide chains) from lysine α-carbons also occurs. Another reaction is polypeptide chain cross-linking due to coupling of aldehyde residues with H_2N– groups of the adjacent chains. Beside oxidation of individual amino acid residues, HOCl induces oxidation of some prosthetic groups of enzymes and carbohydrate moieties of glycoproteins. All mentioned modifications profoundly change the properties of the oxidized proteins and have an impact on their catabolism and degradation, both through the proteolytic digestion pathway and through immune mechanisms.

In peptides and proteins, the α-amino groups of lysine residues are major targets for HOCl attack (T4). There are also some free amino groups at the N-terminus of polypeptide chains, but effects of chlorination of these groups in proteins is less known. Chlorination of lysine α-amino groups in bovine serum albumin achieves a plateau level in seconds at a HOCl/albumin molar ratio amounting to about 1/30. Then, there is a slow, temperature-dependent decomposition of chloramine derivatives with subsequent formation of cabonyl groups reacting with 5-thio-2-nitrobenzoic acid (TNB). At room temperature, 3 h are necessary for decay of

about 50% of all initial chloramine residues (H11). After standing for a few hours in room temperature, the presence of aldehyde residues is easily detectable (H11). The aldehydes yield a coupling reaction with a reduced colorless *p*-fuchsine sulfite. The nondialyzable, purple-red color of the albumin solution, with maximum light absorption at 555 nm, indicates formation of the aldehyde. The color precipitate obtained by treatment with trichloroacetic acid suggests the retentive incorporation of a dye into the albumin structure. When an MPO-Cl^--H_2O_2 system (at pH 4.5) for oxidation of albumin was used, the concentration of aldehyde groups in albumin was proportional to the amount of H_2O_2 employed (S61). Deamination of albumin due to chloramine formation influences its electrophoretic mobility. Gradually adding limited concentrations of H_2O_2, a number of albumin derivatives of increasing electrophoretic mobility are obtained. There was also a gradual decrease in albumin affinity to anti-albumin antibodies. The increase in HOCl or H_2O_2 employed in albumin oxidation effects concentration increase of aldehyde residues, and also decrease in albumin ability to develop the color reaction with the Folin-Ciocalteau reagent. This in turn may suggest loss of some aromatic amino acids. With an excess of the HOCl some of the aldehyde groups probably undergo further oxidation to carboxyl residues. At H_2O_2 exceeding molar ratio with albumin 30:1, a precipitation of albumin occurred, presumedly due to new polymeric albumin derivatives formed. The amount of precipitating albumin was proportional to the amount of H_2O_2 added (S61).

The mechanism of albumin oxidative precipitation probably employs reactions of aldehyde groups via aldol condensation or reaction with the remaining amino groups. This renders possible cross-linking of protein molecules to insoluble polymeric albumin derivatives. The amount of precipitated albumin was directly related to the amount of hypochlorite added (D18). At HOCl/albumin molar ratio 70/1, a fragmentation of polypepeptide chain occurs. Since methylation of α-amino groups markedly increases the albumin fragmentation process, the reaction seems to occur by a direct attack of HOCl on some susceptible peptide bonds (H11). Albumin molecule fragmentation may also occur in a short time, and at a much lower HOCl/protein concentration ratio, but subsequent treatment of albumin-chloramine derivatives with methionie is necessary. This effects formation of nitrogen-centered free radical from chloramines. The mentioned observation suggests some role of the nitrogen free radical in oxidative protein fragmentation (H11). Similar stages of denaturation were observed in HOCl treatment of human immunoglobulins and bovine fibrinogen (D16). No denaturation of protamine upon HOCl treatment was observed (O6). Reaction of polypeptide backbone fragmentation may imply chloramide formation from nitrogen in peptide bonds:

$$R—C(O)—NCl—CH—R'—C(O)—R''$$

The albumin chloramines obtained with mild chlorination at low HOCl concentrations or by the MPO system can be stoichiometrically reversed to amines by

treatment with thiosulfate, methionine, cysteine, or other reductants (O6). However, using higher concentrations of HOCl and prolonging the chlorinated albumin storage time leads to the decomposition of chloramines to aldehyde residues (D13). Production of carbonyl moieties causes a decrease in concentration of the TNBS-detectable amino groups recovered after treatment with thiosulfate, which suggests that lysine amino side residues are primary substrates for carbonyl formation (Table 7). Both chloramine and carbonyl formation in the albumin molecule can be prevented by a number of HOCl scavengers, such as glutathione, dihydrolipoic acid, N-acetylcysteine, ascorbic acid, cystine, oxidized glutathione (GSSG), and uric acid (Y4).

Treatment of albumin with HOCl (both direct or in MPO-generated reactions) also effects chlorination of tyrosyl residues to 3′-chlorotyrosine derivatives (K8). The reaction requires a lag time (5–10 min) and then proceeds in the subsequent 50–60 min following the albumin-HOCl treatment. Thus the reaction probably depends on secondary rearrangements of primarily synthesized chloramines. The amount of chlorinated tyrosyl residues depends on the HOCl concentration. Reaction terminates at conversion of about 0.5% of all tyrosil residues present in the albumin molecule, and accounts for 2% of HOCl (or 3% of H_2O_2) employed in albumin chlorination reaction (K8). Also, oxidative inactivation of vitamin B_{12}-binding protein by respiratory burst products requires the initial lag period, presumably related to time necessary for accumulation of sufficient quantities of HOCl/OCl^- or secondary chloramines in the extracellular fluid. Effects observed upon treatment of the several mentioned proteins with the HOCl/OCl^- system are similar to those described for other proteins, such as chicken egg white albumin (O7), fibronectin (V10), Lp(a) (O1), elastin, and insulin (H6), and can be referred to most other proteins.

Changes in the number of free amino residues alter the modified proteins' susceptibility to proteolysis. Albumin chlorination and *N*-chloramine formation decreases susceptibility to trypsin digestion. Removing of chloramine residues by treatment with thiosulfate shows that chlorination alters albumin properties by a biphasic mode: the reversible chlorination and removal of chloramine moieties markedly increases albumin susceptibility to proteolysis, whereas chlorination produces the irreversible loss of amino moieties and carbonyl group formation effects decrease in albumin susceptibility to trypsin digestion. The effect is related to the number of lost amino residues. A similar relationship was observed for IgG. Fibrinogen and protamine, on the other hand, did not show dependence between chlorination and proneness to trypsin proteolysis (O6).

Trypsin attacks the peptide bonds following the basic amino acids arginine and lysine. Formation of chloramines decrease trypsin binding sites, which causes a decrease in protein susceptibility to trypsin digestion. On the other hand, chloramine formation from free amino residues may induce changes in tertiary albumin structure, revealing some normally inaccessible amino residues. Therefore, removal

of chlorine from side amino groups causes an increase in protein susceptibility to trypsin digestion. If the amino groups of lysine and arginine are replaced by carbonyl or carboxyl moieties, trypsin loses its specific grip point and decreases affinity to the modified protein. In contrast to albumin chlorination, fibrinogen treated with HOCl decreases the susceptibility to trypsin proteolysis, whereas chlorination of protamine sulfate does not affect its proneness to trypsin digestion (O6).

Digestion of the chlorinated albumin and human fibrinogen by pancreatic elastase has shown no effect of chlorine removal by thiosulfate treatment on protein susceptibility to elastase digestion. The HOCl-treated proteins showed significantly increased susceptibility to proteolysis and no relationship between the chlorination level and protein susceptibility to digestion similar to that observed for trypsin (O6). Digestion of fibronectin by human neutrophil elastase also has shown a remarkable increase in protein proneness to elastase degradation. The peptide composition generated in HOCl-treated fibronectin-elastase digestion differed from peptides obtained by similar digestion of native elastase, which suggests that HOCl-mediated oxidative modification of protein structure substantially alters protein tertiary structure, exposing new sites susceptible to elastase attack (V10). Chymotrypsin also decomposes HOCl-treated proteins more effectively than native ones (O6).

2.5. Chloramines

Chloramines have a particular affinity to the protein surface-oriented thiol and thioether residues. Oxidation of the particular methionine residues in human hemoglobin with chloramine T have shown that chloramine almost exclusively attacks the exposed methionyl residues (at 1:5 mol/mol ratio), yielding methionine sulfoxide. The first oxidized were three methionyl residues located at positions $\alpha_{32}\beta_{13}$, $\alpha_{76}EF_5$, and $\alpha_{55}D_6$. The buried cysteinyl residues, located at positions $\alpha_{104}G_{11}$ and $\beta_{112}G_{14}$, remained unaffected. Also, methionine $\alpha_{32}B_{13}$ is fully protected, and methionine $\alpha_{76}EF_5$ appears to be oxidized only partially (A10).

2.6. Peroxynitrite and Nitric Oxide and Their Effects on Proteins

Peroxynitrite, like other oxidants, reacts with proteins, first oxidizing cysteine methionine and tryptophan residues (A7). The reaction products are sulfones, carbonyl moieties, and dityrosines (K23, M29). Formation of protein hydroperoxides and protein fragmentation was also observed (B7, G6). Nitric oxide induces oxidation of methionine residues, thus effecting oxidative damage to proteins (C11). It also reacts with Fe-S clusters of aconitase (D15), though in most cases it is difficult to assess whether these effects are produced by the $NO^{\bullet}$ itself, or rather by a more reactive secondary product such as peroxynitrite (C5). At physiological

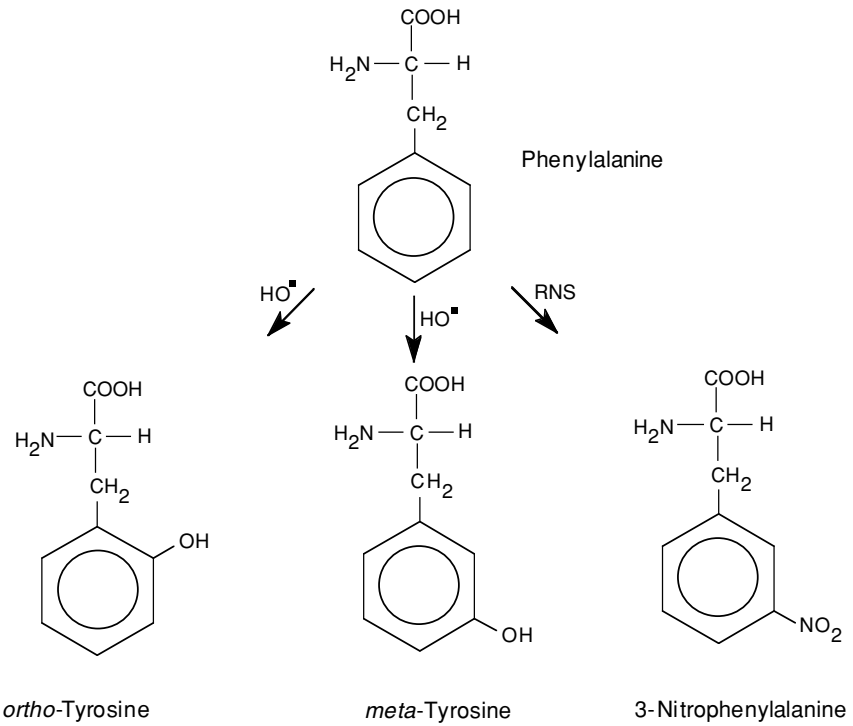

FIG. 13. Oxidation of phenylalanine by reactive oxygen species and reactive nitrogen species.

pH the protonated form of $ONOO^-$, the peroxynitrous acid (ONOOH), is unstable and decomposes to nitrate (NO_3^-). ONOOH reacts directly with reductants or can decompose by homolytic dissociation, producing nitrogen dioxide (NO_2) and hydroxyl radical ($^{\bullet}OH$), or it can dissociate by heterolytic mechanisms, yielding nitryl cation (NO_2^+), which reacts in proteins with thiol, methionyl, tyrosyl, and tryptophan residues. Peroxynitrite-mediated specific protein modifications are 3-nitrotyrosine formation (I2), nitration of tryptophan residues to form 6-nitrotryptophan and 5-nitrotryptophan, respectively (A8, P4), and nitration of phenylalanine residues to 3-nitrophenylalanine (albeit with a lower yield) (V2) (Fig. 13). Protein nitration occurs in PMA-activated neutrophilic leukocytes and in plasma proteins exposed to products of activated neutrophils. The substrate for this reaction is nitrite (NO_2^-), which is formed as the nitric oxide (NO) oxidation product in reaction with HOCl, which acts as an oxidant. The reaction seems to employ an unstable intermediate, nitryl chloride ($Cl\text{-}NO_2$), which rapidly binds to tyrosyl residues of *N*-acetyltyrosine, yielding 3-chlorotyrosine and 3-nitrotyrosine (E2).

Peroxynitrite oxidizes Fe-S clusters, producing inactivation of enzymes containing these clusters (such as the aconitase and other dehydratases) (H10). The other effect of peroxynitrite destruction of Fe-S clusters is release of a significant amount of free iron into the cell (K11). Peroxynitrite reacts rapidly with the prosthetic groups of metalloproteins. It oxidizes ferro-cytochrome *c* (T11) and hemoglobin (but more slowly) (Table 6). Reaction with oxyhemoglobin effects oxidation of iron and methemoglobin formation, whereas with peroxynitrite excess the ferryl derivative (Fe^{4+}) is formed (A5). Peroxynitrite also inhibits the activity of mitochondrial MnSOD and induces formation of both nitrotyrosine and dityrosine along with higher-molecular-mass species. Only three of the nine total tyrosine residues (Y34, Y45, and Y193) in inactivated manganese superoxide dismutase were nitrated by peroxynitrite. Tyr34, which is present in the SOD active site, appeared to be most susceptible to the peroxynitrite-mediated nitration (M2). Peroxynitrite acting on proteins (blood plasma proteins, hemoglobin) also induces tyrosyl radical formation (M18, P12), which may produce protein aggregation through dityrosine bridges (Fig. 14).

It has been suggested that protein nitration, particularly tyrosine residues, is a specific footprint of peroxynitrite presence in biological systems (I2). However, this concept cannot be held in the light of recent data. The gas phase of cigarette

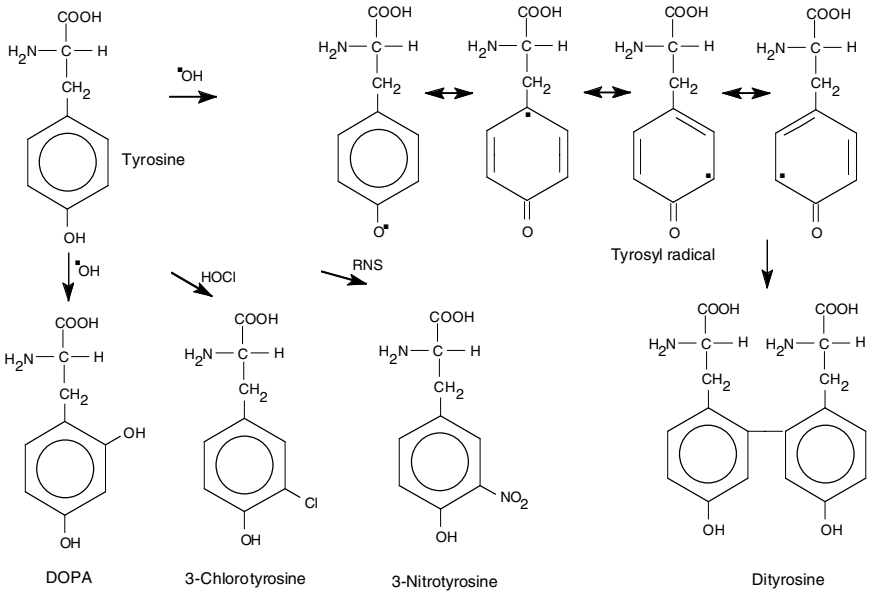

FIG. 14. Modification of tyrosine by reactive oxygen species, reactive nitrogen species, and hypochlorite.

smoke contains nitric oxide, which may autoxidize to nitric dioxide, thus contributing to nitration of tyrosine (E3). Apart from NO_2 itself, there are several other agents which can nitrate tyrosine residues. 3-Nitrotyrosine is formed during oxidation of nitrite, catalyzed by myeloperoxidase and lactoperoxidase (V1), and during reaction of nitrite with hypochloric acid in which nitryl chloride ($Cl\text{-}NO_2$) is generated (E2, H3). Nitrite is a good substrate for myeloperoxidase (MPO) at physiological concentrations, and its oxidation by MPO-H_2O_2 system forms nitrogen dioxide, which is a direct nitrating agent (K10).

2.7. Reactions of Products of Lipid Peroxidation

Radicals generated during peroxidation of lipids and proteins show reactivity similar to that of the hydroxyl radical; however, their oxidative potentials are lower. It is assumed that the reactive alkoxyl radicals rather than the peroxyl radicals play a part in protein fragmentation secondary to lipid peroxidation process, or protein exposure to organic hydroperoxides (D10). Reaction of lipid radicals produces protein–lipid covalent bonds and dityrosyl cross-links. Such cross-links were, for example, found in dimerization of Ca^{2+}-ATPase from skeletal muscle sarcoplasmic reticulum. The reaction was carried out *in vitro* by treatment of sarcoplasmic reticulum membranes with an azo-initiator, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), which generated peroxyl and alkoxyl radicals (V9).

Aldehydes, which are secondary products of lipid peroxidation, consist of other groups of agents involved in oxidative stress and modification of proteins structures. Protein cross-linking can occur *in vitro* employing the malondialdehyde (MDA) Schiff-base-type adducts produced by lysine residues.

$$\text{Amino acid}\underset{\text{MDA adduct}}{\text{—N—CH=CH—CHO}}$$

Protein cross-links may be also produced in reaction of 4-hydroxynonenal with lysine, histidine, serine, and cysteine residues, primarily via Michael addition (J5, R7, U8). These reactions occur spontaneously, but also may be catalyzed by certain glutatione *S*-transferases. The glutathione transferase A4-4, which unlike other alpha-class glutathione transferases, shows high catalytic activity toward lipid peroxidation products such as 4-hydroxynon-2-enal, is the key enzyme for these reactions (B31). Products of protein coupling with aldehydes secondary to lipid peroxidation have a specific fluorescence, which can herald the protein oxidative modification process (C10).

Acrolein, which is a ubiquitous product of lipid peroxidation, binds to proteins, contributing to carbonyl group formation. Such processes employing acroleine binding to lysine residues were observed in human LDL lipoprotein (U2). Acrolein–protein conjugates have been suggested to be a marker of oxidative stress in mammalian cells (U2). A similar reaction, producing a decrease a free amino group

content and an increase in LDL electrophoretic mobility, was observed upon MDA action on LDL particles (C9). The major protein–MDA covalently bound adduct is N^{ε}-(2-propenal)lysine (U7). Binding of aldehyde residues to amino groups decreases the positive charge of protein molecules and affects their affinity to the LDL receptor (K1). Also, addition of aldehyde carbon backbones decreases LDL binding to classical LDL receptor and enhances LDL binding to the macrophage "scavenger receptor," contributing to foam cell formation and further process of atherogenesis (E5, K26).

2.8. Reactions of Other Secondary Radicals

Free radicals formed from amino acid residues of proteins can react with other protein molecules. It has been demonstrated that tryptophan peroxyl radicals and tyrosine phenoxyl radicals (O10) of heme proteins, such as peroxidases and proteins showing pseudo-peroxidase activity (myoglobin, hemoglobin), react with various other proteins to produce secondary radicals, mainly from tyrosyl and (to a lesser extent) from tryptophanyl residues. In proteins, tyrosil residues may be oxidized to tyrosil radicals by peroxidase action. The secondary effect of this reaction is intermolecular dityrosyl bond formation, producing protein polymerization (M16). The horseradish peroxidase/H_2O_2 system *in vitro* produces an LDL apolipoprotein, a long-lived radical. This suggests that also *in vivo* the peroxidases, such as prostaglandin synthase and myeloperoxidase, acting on proteins may promote oxidation of LDL (K1). Free radicals generated from human albumin may react with hemoglobin and produce an albumin–hemoglobin crosslink, modifying functional properties of hemoglobin (P18).

2.9. Effects of Glycoxidation

Although the initial reaction of nonenzymatic protein glycation is just a simple condensation between the glucose aldehyde group and one of the protein free amino groups (Maillard reaction), the reaction product, which is a Schiff base, undergoes further reactions, forming advanced glycosylation end product (AGE) synthesis and protein browning. Reactions involving oxidative modifications of primary adducts are strictly interconnected; and to emphasize this connection, the term "glycoxidation" is often used (B10). Glucose and other α-hydroxyaldehydes as well as dicarbonyl compounds and ketoamines are produced as intermediates of the browning reaction. These compounds may be reversibly transformed to enediols and aminodiols, respectively, easily forming complexes with metal ions. Such complexes may transfer one electron to the oxygen molecule, producing superoxide and, in turn, hydrogen peroxide and other ROS. Development of glycated albumin fluorescence (excitation and emission maxima of about 360 and

454 nm, respectively) contributes to metal-catalyzed oxidations and hydroxyl radicals (L4). α-Dicarbonyl compounds may be formed in a variety of ways, including Fenton reaction-mediated oxidation of sugars, lipids, and proteins. Metal ion-catalyzed oxidation of glucose is considered a more important factor for glycation than the Amadori product formation from glucose itself (J3, W17, W18). The α-dicarbonyl compounds or α-ketoaldehydes are mainly responsible for production of protein inter- and intramolecular cross-links, known as advanced glycation products (AGEs). AGEs accumulate in long-lived proteins such as collagens and lens crystallines.

In the course of glycoxidation, various ROS are formed. Glycation of proteins by methylglyoxal generates protein-bound free radicals which were identified as cation radicals of the cross-linked Schiff base. These radicals are able to reduce ferricytochrome *c* (L5, Y7). Nonenzymatic glycation of reactive amino groups in model proteins increases the rate of free-radical (mainly superoxide) production at physiological pH by nearly 50-fold over nonglycated protein. Both Schiff bases and Amadori glycation products were found to generate free radicals in a ratio of 1:1.5 (J3). Nonenzymatically glycated human serum albumin and glycated poly-lysine (Lys) *in vitro* brought about the reduction of nitroblue tetrazolium and ferricytochrome *c* at pH 9.06 and 7.8, respectively. This reaction was inhibited partially by superoxide dismutase, confirming involvement of the superoxide radical anion. Glycated material was found to function as both a reductant and an oxidant (S1) (Fig. 15). Mixtures of protein and glucose generate nanomolar levels of hydrogen peroxide in the presence of protein under physiological conditions of pH and temperature (J3). AGEs were also demonstrated to generate reactive oxygen species upon exposure to UV radiation, a process which may be relevant to skin damage (M10).

Part of the redox activity of both early products of glycation and AGEs may be due to their binding of redox-active metal ions. Superoxide generation by Amadori compounds is substantially accelerated by Cu^{2+} though not by Fe^{2+} ions (M27). Amadori compounds such as fructose-β-alanine in the presence of copper ions degrade bovine serum albumin (K7). The presence of Cu^{2+} markedly increased the rate of superoxide radical formation at metal concentrations higher than 1 μM, while Fe^{3+} was not effective. Carboxymethyllysine (CML)-rich-poly-L-lysine and bovine serum albumin were found to bind nondialyzable Cu^{2+} and Zn^{2+}. CML-rich poly-L-lysine tail tendons implanted for 25 days into the peritoneal cavity of diabetic rats had a 150% increase in copper content. CML–BSA–copper complexes oxidized ascorbate and depolymerized protein in the presence of H_2O_2. CML-rich proteins immunoprecipitated from serum of uremic patients oxidized four times more ascorbate (S8).

Also, interaction of AGEs with their receptors is thought to generate oxidative stress. Cellular interactions of AGEs are mediated by specific cellular binding proteins, receptors for AGE (RAGE). AGE-albumin or AGEs isolated from plasma of

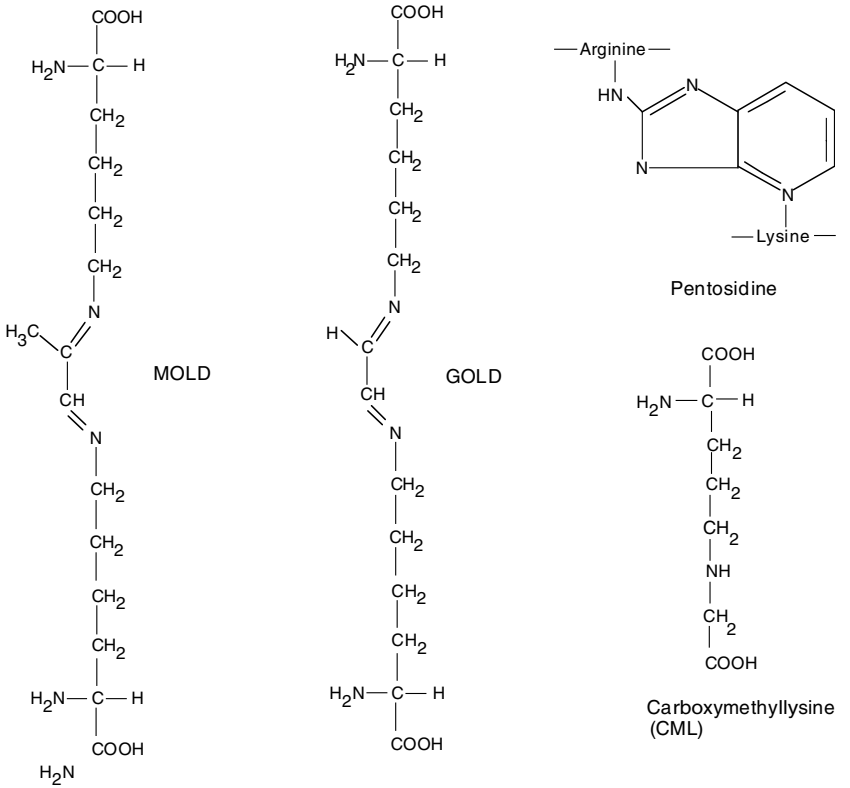

FIG. 15. Some products of protein modification by compounds formed during glycoxidation and lipid peroxidation.

diabetic rats induced endothelial cell (EC) oxidant stress, appearing as generation of thiobarbituric acid reactive substances (TBARS) and resulted in activation of NF-kappa B. Production of these compounds was blocked by antibodies to AGE receptor-polypeptide and by antioxidants. Infusion of AGE-albumin into normal animals effected appearance of MDA adducts in the vessel walls and increased TBARS in the tissues. Moreover, activation of NF-kappa B and induction of mRNA for heme oxygenase was noted. AGE-induced oxidant stress was inhibited by pretreatment of animals with either antibodies to the AGE receptor-binding protein or by antioxidants (Y5). Interaction of AGEs with RAGE triggers a cascade of intracellular signals activated by oxidative stress involving p21(ras) and MAP kinase, culminating in activation of transcription factor (L1).

Long-term incubation of methylglyoxal-glycated bovine serum albumin and cytochrome *c* produces cross-links between these proteins. In the presence of

oxygen, methylglyoxal-glycated bovine serum albumin shows a quasi-enzymatic activity oxidizing ascorbate (L5).

Hydroxyl radicals are also generated during glucose autoxidation and induce protein fragmentation and conformational changes (H28, W16). Proteins subjected to glycation *in vitro* lose tryptophan fluorescence, acquire fluorescence typical of tryptophan degradation products and lose —SH groups (T15). Glycation also enhances malondialdehyde binding to proteins (M23). AGE-collagen produced *in vitro* by incubation with glucose under oxidative conditions was found to contain not only typical glycoxidation products such as CML but also amino acid oxidation products of proteins, including *m*-tyrosine, dityrosine, DOPA, and valine and leucine hydroperoxides (F19).

ROS produced by sugars and glycated protein autoxidation participate in oxidizing of already glycated proteins and affect other proteins (H24). These reactions are catalyzed by metal ions (W17). The presence of metal ions may, moreover, initiate the Fenton reaction and produce hydroxyl radicals. A carbon-centered 1-hydroxyalkyl radical was found during autoxidation of glyceraldehyde (T12).

Free radicals generated by glycated proteins initiate lipid peroxidation (H21) and effect nearly twofold increase (over control) of peroxidation of membrane linoleic/arachidonic acid vesicles. This suggests that the increased protein glycation in diabetes accelerates vascular wall lipid oxidative modification (M28).

It was demonstrated that oxidative stress stimulates synthesis of two AGEs: N^{ε}-(carboxymethyl)-lysine and pentosidine (in contrast to such compounds as imidazolone and pyrraline) (M20). The Amadori products formed in glycated proteins subsequently degrade into α-dicarbonyl compounds, deoxyglucosomes, which are more reactive with protein amino groups than the parent sugars. N^{ε}-fructosyllysine is oxidatively degraded to N^{ε}-(carboxymethyl)lysine, which accumulates in glycated proteins (D24, D25); one oxidizing factor, at least *in vitro,* may be dehydroascorbate (D23). Interestingly, carboxymethyllysine may be a product of not only glycoxidation but also of lipid peroxidation; it is formed, as well, during metal-catalyzed oxidation of polyunsaturated fatty acids in the presence of protein (F16) and from oxidation of L-serine by hypochlorous acid (A12).

An important, highly fluorescent cross-linking compound containing an imidazolo(4,5,6)pyridinium ring was identified initially on aged collagen and called pentosidine. This compound apparently derives from a pentose, arginine, and lysine (S16, S17). Originally pentosidine was thought to be derived from ribose (S18), but subsequent studies have shown that it is also formed from glucose, fructose, and ascorbate in a sequence of transition metal-catalyzed oxidations and decarboxylation reactions, and that arabinose is its main precursor (D26, G20, S18). Fluorophores such as pentosidine do not have to be produced by glycation; similar fluorophores are also formed in oxidation reactions involving trace amounts of

transition metal ions (G31). Pentosidine is specific for amino-carbonyl reactions of sugars or ascorbate with proteins but cannot be formed through a mechanism based on reaction of peroxidizing lipid with protein (F16). Pentosidine formation requires oxidation, so it is referred to as a glycoxidation process (B10).

Glycoxidation may lead to acquisition of carbonyl groups by protein molecules; however, *in vitro* studies have shown that it occurs during incubation of proteins with ascorbic acid and arachidonate but not with glucose, while CML and pentosidine were formed in all cases (M19). Also, protein cross-linking during glycation was observed with ascorbate but not with glucose (L6).

Imidazolone adducts formed by reaction of 3-deoxyglucosone or methylglyoxal with arginine and the imidazolium cross-links formed by the reaction of glyoxal and methylglyoxal with lysine residues in protein are also considered as biomarkers of protein oxidative damage (O8).

3. Biological Effects of Protein Oxidation

3.1. Introduction

Synthesis of the hydroxyl radical, which is the most toxic of the ROS family, is effectively inhibited in living organisms. The particular circumstances that promote hydroxyl radical synthesis are radiolysis of water or eventual combination of constituents of the Fenton reaction system. Conditions favoring occurrence of the Fenton reaction may arise in tissue necrosis with subsequent release of Fe^{2+} from denaturated hemoglobin, mioglobin, and other hemoproteins, or in chemical poisoning. Usually, even in phagocytizing neutrophilic leukocytes, which are the most aboundant source of ROS in organisms of mammals, there is no proof of significant hydroxyl radical production (R14). The most important biological effects of hydroxyl radical generation refer rather to DNA damage than to modification of protein structures.

Biological effects of protein oxidative damage depend on the acting oxidant and presence of readily oxidizable amino acid residues in the protein molecule (Tables 8 and 9). The state of protein oxidation is a result of equilibrium between rates of protein modifications and repair or removal. Repair of protein oxidative damage is possible only with respect to reversible redox reactions. The —SS— bridges or protein–glutathione mixed disulfides, formed due to oxidative stress, can be rereduced either nonenzymatically by excess of reduced glutathione or by enzymatic reactions including thioltransferase (D8), protein disulfide isomerase (U1), thioredoxin, glutaredoxin (C6), and perhaps glutathione *S*-transferase (R3). Methionine sulfoxide may be reduced by methionine sulfoxide reductase (EC 1.8.4.6) (B24, M26, S58). Oxidatively modified proteins are usually preferentially degraded by

TABLE 8
REACTION RATE CONSTANTS FOR HYDROXYL RADICAL WITH SELECTED PROTEINS

Protein	pH	k ($M^{-1}s^{-1}$)
Albumin (human serum)	7	7.8×10^{10}
Carbonic anhydrase (beef blood)	7	6.5×10^{10}
Catalase		1.4×10^{11}
α-Chymotrypsin	6.9	3.7×10^{10}
Collagen		4.0×10^{11}
Cytochrome *c*		1.4×10^{10}
Lactate dehydrogenase	7.2	2.1×10^{11}
Papain	6.4	4.7×10^{10}
Superoxide dismutase	7.2	5.3×10^{10}
Trypsin	7	8.2×10^{10}
Trypsinogen	7.4	1×10^{11}

Modified from Buxton (B34).

proteolytic enzymes; this refers to proteins modified by the hydroxyl radical (D4, D7), hydrogen peroxide (S4), peroxynitrite (G27), and other agents (T13). In most cells, oxidized proteins are cleaved in an ATP-and ubiquitin-independent pathway by the 20 S "core" proteasome (G28). The complex mechanism of this process may restrict detection of oxidatively modified proteins.

The repair of oxidative damage may be incomplete. Only reduction of the D-diastereomer of calmodulin-bound methionine sulfoxide (L-Met-D-SO) by methionine sulfoxide reductase was demonstrated, while in the cells both D- and L-steoreoisomers are formed. Such incomplete, diastereoselective repair by methionine sulfoxide reductase contributes to the accumulation of methionine sulfoxide residues during oxidative stress and aging *in vivo* (S24).

TABLE 9
REACTION RATE CONSTANTS FOR SUPEROXIDE RADICAL ANION/PERHYDROXYL RADICAL WITH SELECTED PROTEINS

Protein	Species/pH	k ($M^{-1}s^{-1}$)
Ceruloplasmin	$HO_2^{\bullet}/O_2^{-\bullet}$/7.8	1.8×10^{6}
Cytochrome *c* (Fe^{3+})	$O_2^{-\bullet}$/7.3	5.8×10^{5}
Methemoglobin	$HO_2^{\bullet}/O_2^{-\bullet}$/7.0	6×10^{3}
Oxyhemoglobin	$HO_2^{\bullet}/O_2^{-\bullet}$/7.0	4×10^{3}
Cu,Zn-superoxide dismutase	$O_2^{-\bullet}$/7.4	2.3×10^{9}

Modified from Bielski (B18).

3.2. Role of Myeloperoxidase-Dependent Protein Oxidation in Killing and Cell Lysis in Inflammatory Sites

Activation of the MPO-H_2O_2-Cl^- system, producing HOCl, is part of the neutrophil-killing mechanisms of: bacteria, parasitic microorganisms (F2, K24), tumor cells (S36), and self-tissue damage (H14, K24). Moreover, there is increasing data suggesting that production of HOCl/OCl^- triggers further biochemical reactions necessary for proper functioning of the mammalian immune system. In particular, a specific role of protein oxidation has been shown in regulation of leukocytic proteinase activity responsible for cell lysis, removal of injured tissue fragments, and antigen processing by T-lymphocytes.

It is now generally accepted that MPO-dependent mechanisms of killing and tissue injury employ diffusible oxidants acting on target cells. The oxidants interact with cellular membranes (D17, S21) as well as penetrate into the cell, inactivating enzymes and lethally disrupting cellular metabolism (L18). The HOCl acting on the plasma membrane of murine macrophage-like tumor cells rapidly impairs K^+ and Ca^{2+} membrane transport, with a subsequent loss of intracellular K^+, inhibition of the cell metabolite uptake process, and ATP depletion (B5, S10). HOCl attack causes a specific oxidation of one particularly vulnerable thiol group (Cys_{149}) in glycerol-3-phosphate dehydrogenase (G-3-PDH), responsible for glycolytic glucose metabolism. The ATP loss is partially reversibile by ascorbate and cysteine (S37). Due to diffusion of HOCl or its derivatives, the ATP depletion is also induced in cells co-cultured with stimulated neutrophils (D2). Similarily, extracellularly administrated HOCl inhibits ATP production in human mononuclear leukocytes. The reaction is carried out in a "dose–effect" manner, and effects inhibition of cell mitogen-induced proliferation (S37). Some inflammatory cytokines such as tumor necrosis factor-α (TNF-α) stimulate neutrophils to release MPO (C13), which subsequently binds to target cells, thus potentiating the direct action of HOCl. Binding of MPO to some cells contributes to host-tissue damage. Presence of MPO was observed in renal glomeruli in extracapillary glomerulonephritis (N5). Endothelial cells pretreated with MPO detach from the substratum and become prone to complement-mediated injury (S7). Oxidation of one methionine in the C5-component of human complement causes a release of "anaphylatoxin" activity . The subsequent lysis of the phagocytized material by lysosomal enzymes makes possible degradation and removal of the damaged cell (A3, F8, H5, H14, M11, S10).

Exposure of cartilage to HOCl causes a rapid damaging process, which then proceeds for two further days after the removal of HOCl. The methionine, which readily reduces chloramines, used in a concentration 100 times above the HOCl concentration, abrogated the cartilage-damaging effects of HOCl (K24). Secondary to HOCl treatment, abolition of vitamin B_{12}-binding protein carrier properties was also described. This protein, located primarily in specific granules of

polymorphonuclear leukocytes, is secreted from stimulated neutrophils into extracellular fluid. Decrease in vitamin B_{12}-binding capacity is delayed in time, beginning a few minutes after leukocyte stimulation and then prolonged in time up to 30 min of the neutrophil stimulation process. Reaction is limited to the extracellular space (C12). The initial lag period necessary for vitamin B_{12}-binding protein is required to accumulate sufficient quantities of hypochlorite or secondary chloramines in the extracellular fluid. This suggests that the MPO—H_2O_2—Cl^- system plays some regulatory function in the interaction of lysosomal enzymes and oxidants with the phagocytized material.

Activated neutrophils themselves interact with HOCl and chloramines and undergo rapid self-destruction. However, activity of stimulated neutrophiles is well organized in time. Oxidative burst and the appearance of azurophilic granules discharge products in the reaction medium, which is secondary to release of the specific granules. At this stage of the reaction, intragranular inactivation of neutrophile lysosozyme, β-glucuronidase, and α-glucosidase occurs (K17), and also several lysosomal hydrolases of neutrophil-specific granules are inactivated (V11). It is probable that protein oxidation is used efficiently in bacteria killing systems. In phorbol myristate acetate-stimulated neutrophils, respiratory burst causes prompt oxidation of thiol residues, which manifests in a loss of 22% of all protein-bound thiol content (F8).

3.3. Action of Chloramines on Living Cells

Chloramines of taurine as well as chloramines of lysine, which are major products secondary to HOCl (being hydrophilic, negatively charged moieties) can be nonspecifically taken up into cells by mechanisms of anion transport (T5). In erythrocytes, chloramine taurine is first reduced by glutathione (GSH) with reconstitution of free taurine, which accumulates in the cell. Protection of the erythrocyte from oxidative damage is ensured as long as intracellular uptake of chloramine taurine is within the range of the intracellular concentration of GSH. Regeneration of G-SH by NADPH-dependent G-SS-G-reductase has to be fast enough to sustain the cellular GSH level:

$$\text{R—HNCl} + 2\text{GSH} \rightarrow \text{RNH}_3^+ + \text{Cl}^- + \text{GSSG}$$
$$\text{G—SS—G} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}$$

When the taurine chloramine uptake rate exceeds the rate of NADPH-dependent regeneration of GSH, there is a net loss of cellular GSH level, causing protein-thiol oxidation, ATP loss, and disruption of cellular metabolism. Heme moieties are the other target of chloramine attack on cellular constituents. Oxidation of hemoglobin to methemoglobin (and other hemoproteins to their oxidized derivatives) occurs at 10-fold excess of chloramine taurine molar concentration compared

to the erythrocyte hemoglobin level (T5). The chloramine toxicity to erythrocytes manifests as GSH depletion, ATP depletion, methemoglobin formation, and a marked decrease in total protein-SH content. Functionally, chloramine-treated erythrocytes become prone to osmotic lysis and decay, releasing their content into the extracellular space (T5). One may conceive that a similar mechanism of chloramine toxicity refers to most other cells. Chloramines, which are long-lived entities, react promptly with methionine residues, prolonging the deleterious action of hypochlorite on target cells. Oxidation of methionine residues causes inactivation of the ball domain of the A-type potassium channel, which slows down the channel activation (C11) and induces loss of calmodulin ability to activate Ca^{2+}-ATPase (S58). Also, the chemotactic peptide Met-Leu-Phe is inactivated by oxidation of the methionine residue (B24). Results of the step-by-step oxidation of human hemoglobin with chloramine-T have shown that selective oxidation of particular methionine residues can affect protein functional properties (A10): oxidizing of the first easily accessible methionine $\beta_{55}D_6$ due to decrease in hemoglobin subunit cooperativity, and destabilization of hemoglobin T-state, effects disturbance of hemoglobin function as an oxygen carrier. The modified hemoglobin abolishes the Bohr effect and increases oxygen affinity. Treatment of hemoglobin with chloramine-T under more drastic conditions (10:1 molar ratio of chloramine to methionyl residues) produces complete oxidation of all methionines, changes in hemoglobin spectrum, and loss of oxygen-binding capacity (A10). Oxidation of Met_{64} and Met_{179} in human chorionic somatomammotropin leads to loss of hormone biological activity (T1).

3.4. Oxidative Regulation of Proteinase Activities—Protein Inhibitors Balance

A large amount of data based on experiments both *in vitro* and *in vivo* demonstrated that oxidative modification of protein structures results in loss of the protein's primary biological function (D17). On the other hand, activation of latent forms of certain enzymes due to HOCl treatment (W7) or the superoxide radical action (B33) on the inactive proenzymes has been described. The activation process requires the presence of oxidized glutathione, which can effect rearrangement of disulfide bridges in the proenzyme structure (T19). Reversible protein *S*-thiolation occurs in endothelial cells exposed to nitric oxide and may be even more significant quantitatively than protein *S*-nitrosation (P3). A specific thiolation process also activates granule-bound proenzymes of collagenase (B33) and elastase (D11).

Methionine oxidation inducing abolition of protein function usually does not produce any changes in chemical and immunochemical properties of the protein. This infers that the oxidized protein can be reactivated again by reduction of sulfoxide residue, or may be catabolized through its regular proteolytic pathway. Facility of methionine oxidation, along with abundant production of various oxidants, enables oxidation of methionine residues. This rises a question concerning

the possible biological function of oxidative protein inactivation via formation of methionine sulfoxide. The answer to this question is formulated as the concept of "oxidative regulation of proteinase activities—protein inhibitor balance" (S59).

The neutrophil elastase is one of the most potent proteinases released from activated neutrophiles into inflammatory sites. In extracellular fluids, elastase is rapidly bound and inactivated by α_1-antiproteinase, which is the main proteinase inhibitor in human plasma (B14, T16) due to elastase-α_1-PI complex formation, which then is stabilized by a covalent bond between the bound subunits (T16). The inhibition equilibrium constant for the reaction of binding elastase to α_1-PI is 10^{-14} mol and is sufficiently low to enable the effective control of the plasma and tissue fluid elastase proteolytic activity. On the other hand, oxidation of one methionine residue abolishes binding of α_1-PI to elastase, and in physiological conditions increases the serum half-life of elastase from milliseconds to 1.3 s (B14). This enables elastase to bind to connective tissue suffering proteolytic injuries and stimulates further inflammatory reaction. The other protein involved in control of proteolytic activity of blood plasma, whose function is abolished due to a single methionine residue oxidation, is the plasminogen activator-inhibitor (PAI). This protein, produced by endothelial cells, is involved in fibrinolysis *in vivo*. In the presence of H_2O_2, chloramine of taurine, and *N*-chlorosuccinimide, PAI is transformed into an oxidized form that is unable to bind the plasminogen activator (S56). Then, the hypothetical function of mild oxidants, such as taurine chloramine and other chloramines, specifically reacting with cysteine thiol groups and thioether methionyl groups, should protect elastase and other neutrophil serine proteases at the inflammatory site. The α_2-macroglobulin and plasminogen-activator-inhibitor are also readily inactivated by oxidative modification of selected methionyl residue. Thus oxidative regulation of proteinase–antiproteinase balance has a more general importance of providing a mechanism of antiproteinase barrier control, enabling the subsequent action of elastase, collagenase, and other leukocyte proteinases in tissues overtaken by the inflammatory process. Due to abolition of the proteinase inhibiting barrier, the PMN oxidative attack, effecting the release of HOCl, would be supplemented by proteolytic degradation of collagen, elastine, and other structural proteins in the peri-inflammatory space. Degradation of collagen contributes substantially to tissue injury in acute inflammation and rheumatoid disease. Studies of Travis and co-workers (W19) showed that α_1-PI is inactivated in rheumatoid synovial fluid and in other sites of inflammatory reaction (S56). All these data support the concept that activated neutrophils oxidatively inactivate the α_1-PI to produce space for undisturbed proteolytic action of lysosomal proteases.

The α_1-PI (along with other serum proteinase inhibitors) can attenuate lymphocyte antigen processing by binding to lymphocyte surface proteinases and subsequently down-regulate lymphocyte proliferative response. Therefore, oxidation of α_1-PI may activate local immune response in sites of bacterial invasion and contribute to the development of the inflammatory process (B8, B9, S59). On the other hand, oxidative inactivation of some neutrophilic lysosomal enzymes can

have a protective effect against the destruction of lymphoid cells in the inflammatory site (V11). The α_1-API is not a specific antiproteinase-type immunomodulator, and similar properties of binding to lymphocyte surface proteinases has the α_2-macroglobulin, whose immunomodulatory function is already long known (G22).

3.5. Role of HOCl and Chloramines in Development of Immune Response

Chlorination and oxidation of proteins, occurring in the phagosome of activated neutrophils, provides a tool for specific biological marking, which attracts further immunologically active cells and stimulates their activity. Comparison of the immunogenicity of a native chicken egg-white albumin with HOCl-treated albumin versus mouse T-hybrydoma cells, specifically sensitized to the egg-white albumin, has shown that chlorinated albumin yielded a considerably stronger hybridoma-cell response manifesting as IL-2 production (M4). As oxidatively modified proteins are more susceptible to proteolytic degradation, it is conceivable that they are in some way specifically prepared for proteolytic processing by T lymphocytes. This process effects formation of short segments of antigen primary peptide chains, binding to the "polypeptide-binding groove" of the major histocompatibility complex molecules. Production of such small polypeptide segments is an important part of the induction phase of immune response. Proteolytic antigen processing is necessary for T-helper cells to recognize the antigens exposed by the antigen-presenting cells (L21). Therefore, protein oxidation may be part of the immune regulation network, enhancing the immunoresponse to proteins marked by the MPO-mediated oxidation and chlorination process.

The hypothesis concerning the involvement of protein oxidation/chlorination in immunological reactions concords with other findings. First, the HOCl-treated ovoalbumin (2.4–7.2 mmol HOCl/1 mg of protein) *in vitro* stimulates IL-2 output of mouse antigen-processing A_{20}-2JB lymphoma-line cells (APC) three times more effectively than the native ovoalbumin (M4). Second, HOCl-treated bovine serum albumin and IgG coupled with trinitrophenol (TNP) stimulate TNP–anti-TNP humoral response 2.5 times more effectively than the native proteins *in vivo* (M5). Both effects are dose-dependent on the HOCl treatment. On the other hand, extensive antigen chlorination decreases its immunogenicity, even below the level of the native protein. Since the anti-TNP secondary response was enhanced only when HOCl-treated primary antigen had the same carrier as the secondary one, it is conceivable that oxidation and chlorination enhances clonal expansion of carrier-specific T-helper cells (Th1/Th2) (M5). These cells release cytokines required for B-cell proliferation and differentiation.

The data presented above support the Levine and Oppenheim hypothesis (L25) that polymorpohonuclear leukocytes play some specific role in the induction of immunoresponse: the MPO-dependent oxidation and chlorination of proteins seems to interface with the neutrophil function with functions of the lymphoid-line cells (M6). Moreover, the HOCl and chloramine taurine, which is its stable

chlorinated derivative, in lower, nontoxic concentrations, decrease generation of neutrophilic inflammatory mediators and inhibit generation of NO, PGE_2, TNF-α, and IL-6 in activated resident tissue macrophages, thus attenuating inflammatory process in tissues adjacent to the inflammatory site (S31). Therefore, taurine chloramine in low concentrations indirectly promotes tissue protection in areas surrounding the inflammatory site from injury by oxidants and lytic enzymes.

3.6. Biological Effects of Peroxynitrite

Peroxynitrite ($ONOO^-$), whose toxicity is similar to other ROS, is considered as an undesired reaction by-product of reactive oxygen and nitrogen species. On the other hand, peroxynitrite in low concentrations has some mediatory properties and is able to stimulate apoptosis. Also, peroxynitrite generated by activated phagocytes may play some part in bacteria killing mechanisms (X1). At higher concentrations peroxynitrite reacts with proteins oxidizing thiol groups of cysteine and glutatione, inducing thiolation and oxidizing the other components of living cells (including nucleic acids). An overproduction of peroxynitrite in the virus-infected host may play a role in viral infection propagation (A4). Peroxynitrite also reacts with tyrosine residues to yield protein nitrous derivatives. Nitrated proteins lose their biological function and change their physicochemical properties.

There are numerous data that peroxynitrite is involved in cell death and tissue injuries in many clinical conditions. An important mechanism underlying peroxynitrite toxicity is the reaction of tyrosine nitration. Tyrosine nitration inactivates certain enzymes, as was postulated for prostacyclin (PGI2) synthase (M14), cytochrome P450 2B1 (R10), tyrosine hydroxylase (A14), and MnSOD (Y1). Moreover, nitration blocks tyrosine phosphorylation, and thus interferes with the tyrosine kinase signaling pathways (K18). The peroxynitrite treatment of rat liver epithelial cells stimulates mitogen-activated protein kinases p38 MAPK, JNK1/2, and ERK1/2; the mechanism of this effect awaits elucidation (S9).

The fate of nitrated tyrosines has been the subject of considerable interest. A hypothetic enzyme system of nitrotyrosine removal has been suggested (G17). Such a "nitrotyrosine denitratase" activity, in the presence of protease inhibitors, effecting loss of the nitrotyrosine epitope without protein degradation and hydrolysis, was detected in dog prostate (K27) and rat spleen and lung (but not liver or kidney) homogenates (K2). The observed activity in spleen extracts was increased about twofold after endotoxin treatment. This suggests that denitrase activity is inducible or regulated (K2). On the other hand, it has been recently found that low concentrations of HOCl ($<30\ \mu M$) cause a rapid loss (<10 min) of free and protein-bound 3-nitrotyrosines. In contrast, no loss of 3-nitrotyrosine was observed with hydrogen peroxide, hydroxyl radical, or superoxide-generating systems (W11). Nitrotyrosine residues can also be reduced by NAD(H)-cytochrome *c* reductase and a corresponding electron donor to corresponding nitro anion radicals. However, this reaction does not go any further and the nitro anion radicals autoxidized

in one-electron reaction with molecular oxygen generate the superoxide radical anion:

$$\text{Protein-NO}_2 + \text{reductor} \rightarrow \text{protein-NO}_2^{-\bullet} + \text{oxidized reductor}$$

$$\text{Protein-NO}_2^{-\bullet} + \text{O}_2 \rightarrow \text{protein-NO}_2 + \text{O}_2^{-\bullet}$$

Therefore, this incomplete reduction may lead to redox cycling, and the presence of nitrated proteins in the cell may be a source of oxidative stress (K25).

Mitochondria, which are the main cellular source of $O_2^{-\bullet}$, are also a significant peroxynitrite source. Nitric oxide, which is a small uncharged molecule, freely penetrates cellular membranes, and there is a high probability of peroxynitrite generating reactions of $O_2^{-\bullet}$ with $NO^{\bullet}$ inside mitochondria or in their close vicinity. Moreover, nitric oxide synthase presence in mitochondria has been also reported (G8, G12). Thus, mitochondria should be considered as the autonomous producer of peroxynitrite. The mitochondria are also particularly susceptible to deleterious $ONOO^-$ action. Peroxynitrite produces irreversible inhibition of mitochondrial respiration chain enzymes and damages a variety of mitochondrial components. Particularly susceptible to peroxynitrite damage are mitochondrial respiratory complexes, due to their contents of iron–sulfur clusters. Indeed, peroxynitrite inhibits or damages mitochondrial complexes I, II, IV, and V, aconitase, creatine kinase, the mitochondrial membrane, and mitochondrial superoxide dismutase (B26). Inhibition of mitochondrial MnSOD increases the amount of superoxide available for reaction with nitric oxide, thus constituting a factor of autocatalytic enhancement of mitochondrial peroxynitrite formation (M1). Peroxynitrite induces mitochondrial swelling, depolarization, calcium release, and permeability transition (B21, B26, P2). Peroxynitrite is a potent stimulant of apoptosis (S2, S5). The opening of the mitochondrial pores (megachannels) be peroxynitrite may contribute to apoptotic cell death.

Peroxynitrite is considered a toxic oxidant involved in pathomechanisms of various diseases (Table 10). In particular, ischemia-reperfusion injury (W4), allograph rejection (M1), systemic hypotension, immune complex-stimulated pulmonary edema, cold-induced brain edema (O11), glutamate-mediated neuronal toxicity, cytokine-induced oxidant lung injury (F15), and myocardial failure (S60) has been implicated. In the organ preservation–transplantation model of rat livers stored in University of Wisconsin solution, after 6 h of storage, the protein-bound nitrotyrosine increased from 9.5 ($\pm$1.1) group to 27.5 ($\pm$0.7) nmol/mol tyrosine (S35). Increase of protein 3-nitrotyrosine content also was found in brain-degeneration areas in patients with Huntington's disease (B27) and in motor neurons of both sporadic and familial amyotrophic lateral sclerosis (ALS) patients. A key role of peroxynitrite in the pathogenesis of ALS has been postulated. Mutant forms of the Cu,Zn-SOD, producing more H_2O_2, which occur in the familial form of ALS, may promote the tyrosine nitration, thus contributing to the motor neuron death typical for this disease (B16). The concept of enhanced nitration is in accord with a decreased affinity of Zn-SOD mutants (C19). Indeed, the spinal cords and

TABLE 10
DISEASES OCCURRING WITH AN IMPAIRMENT OF ANTIOXIDATIVE STATUS

Disease	Factors involved	Impairment of antioxidative defense observed
Ischemia/reperfusion injury	Activation of NADPH oxidase and xanthine oxidase	Not defined
Late diabetic tissue injury (impairment of glomeruli, cataract formation)	Protein glycooxidation	Decrease in vitamin C and GSH concentrations
Parkinson's disease	Oxidation of 6-hydroxy dopamine	Decrease in glutatione pool (both GSH and GSSG)
Alcoholic liver injury	Cytochrome P450	Decrease in vitamin C and GSH, and increase in lipid peroxides formation
Autoimmune diseases (rheumatoid, lupus, Crohn's)	NADPH oxidase	Decrease in vitamin C and GSH concentrations, increase in SOD activity
Cigarette smoking-related tissue injury (emphysema, coronary atherosclerosis, lung cancer)	Tar stimulation of H_2O_2 formation, oxidation of α_1-PI, DNA damage	Decreased vitamin C and GSH concentrations
Ionizing-radiation tissue injury	Fission of H_2O molecule to $^{\bullet}OH$ and $H^{\bullet}$; DNA damage	Decrease in tissue —SH pool; tyrosine, phenylalanine hydroxylation
Hyperoxygenation syndrome	Molecular oxygen	Decrease in vitamin E concentration

cerebrospinal fluid of ALS patients have shown increased levels of free 3-nitrotyrosine (T14). Increased 3-nitrotyrosine has also been shown in brains of patients with cerebral ischemia (B13). On the other hand, no increase in protein-bound nitrotyrosine was found in ALS patients (B29).

Other effects of peroxynitrite on proteins of potential biological importance are related to inhibition of sodium transport in lung alveolar type II cells, due to damage of apically located amiloride-sensitive Na^+ chanels (H26), inactivation of membrane ATPases (S44, V8), of multidrug-resistance-associated protein (MRP), and the glutathione S-conjugate pump (S46). Inhibition of glutamate transporter by peroxynitrite may contribute to the buildup of excitotoxic extracellular glutamate (T18). Peroxynitrite modifies low-density lipoprotein to a form recognizable by the macrophage scavenger receptor (G18) and inhibits α1-antiproteinase (R6) and tissue inhibitor of metalloproteinase-1 (F14). Inactivation of tyrosine hydroxylase inhibiting melanin biosynthesis may have relevance for the development of Parkinson's disease (I3). Peroxynitrite is capable of priming human neutrophils for undertaking the superoxide production by low doses of stimulants, which may

lead to enhancement of local inflammatory response (R11). Peroxynitrite also induces cardiac myocyte injury by decreasing the spontaneous contraction of myocytes, producing disturbances of Ca^{2+} transport systems, increase in cytosolic Ca^{2+} concentration, and impairment of contractile protein function (I5). Peroxynitrite treatment of blood plasma inhibits cholesterol esterification via lecithin: cholesterol acyltransferase (G19). Interestingly, peroxynitrite, though formed under some conditions by nitric oxide synthase, can inhibit all classes of nitric oxide synthases (P10).

3.7. Protein Oxidation and Aging

Protein oxidation is at present considered one of the most important factors in aging, cataract formation, and organ injury by immune reactions. Increased protein oxidation occurs also in patients with premature aging with progeny and Werner's syndrome, whose fibroblasts show accumulations of oxidatively modified proteins equal to 80-year-old individuals. Oxidation of some enzyme proteins, effecting their inactivation, can essentially disturb cellular metabolism (H7). An example of such a process is the age-dependent loss of calcium homeostasis and alterations in function of calcium regulatory proteins in the brains of Fischer 344 rats. The main cause of these effects is the progressive decrease in calmodulin ability to activate by plasma membrane Ca^{2+}-ATPase. This in turn correlates with the oxidative modification of certain methionine residues to methionine sulfoxides in the calmodulin molecule (G2). Oxidative inactivation of certain enzymes also can in part explain a slowing down of the metabolism rate in old individuals, along with aging (E1, L2). Oxidative modifications accelerate proteins' proteolytic degradation, which may effect final decrease in cellular protein content. Continuous replacement of denatured proteins from living cells makes it difficult to show this process explicitly, but in the case of some particular proteins, lens proteins, which are stable and do not undergo fast proteolysis, an increase in oxidized protein content is observed along with age (L26, S51).

The accumulation rate of oxidatively modified proteins is tissue-specific. Lens nuclear color and opalescence intensity correlate with nuclear protein S-thiolation, suggesting that mixed protein–thiol disulfide formation may play an important role in cataractogenesis and development of brunescence in human lenses (L26). Carbonyl contents is elevated in brains of aged gerbils (C3). On the other hand, in old (28–34 months) male F344 rats a remarkable increase in protein carbonyl content was observed in kidneys, but not in the brain, liver, lung, and heart (G16). Protein carbonyl concentration increased from 8 to 27 months of age in most regions of the mouse brain, with the most noticeable increase in the striatum and hippocampus, regions of the brain that are involved in age-associated functional loss. The age-associated decrease in protein sulfhydryl content was more uniform across brain regions and did not occur in the hippocampus (D21). The level

of protein carbonyls increases with aging in mouse brain synaptic mitochondria (M9).

In another study, senescent mice (aged 22 months) were subjected to complex behavioral tests for motor and cognitive function. Oxidative protein damage was assessed by protein carbonyl concentration in various brain regions. The age-related loss of ability to perform a spatial swim maze task was positively correlated with the level of oxidative damage in the cerebral cortex, whereas age-related loss of motor coordination was correlated with oxidative damage within the cerebellum (F12). These results were interpreted as indicative that oxidative stress and protein oxidative damage are causal factors in brain senescence. Carbonyl content also rises exponentially with age in the human brain, at double the rate in the frontal pole compared with the occipital pole (S38). In livers of old rats, elongation factor 2 (EF-2), which in protein biosynthesis is the main protein factor involved in the elongation step, shows higher carbonylation level and lower activity than in young rats (P9). An age-related bidirectional change in protein carbonylation level and 5-hydroxy-2-amino valeric acid (HAVA) concentration in human liver samples, consisting of a significant decrease from age 16 to 40 years and then an increase from 40 to 85 years of age, was observed. Liver proteins may be oxidized in reactions with hydrogen peroxide produced by the cytochrome P450 detoxification system. This suggestion was supported by a significant positive correlation between HAVA concentration and cytochrome P450 content found in 18 humans of various ages (A19). An age-dependent increase in the protein carbonylation level in muscle biopsy specimens collected from humans aged 25 to 93 years was also found. This suggests that protein carbonylation contributes to age-dependent loss of muscle strength and stamina (M13).

Comparison of tissue susceptibility to oxidative protein damage, manifested as protein carbonylation in response to acute oxidative stress induced by exposure to X-rays, has shown that brain homogenates from 22-month-old rats were more susceptible to oxidative stress than those from 3-month-old rats. The brain was more susceptible to oxidative damage than the heart. A comparison of brain and heart homogenates susceptibility to acute oxidative stress in five different species (mouse, rat, rabbit, pig, and pigeon) indicated that maximum life-span potential of the species was related directly to their ability to control oxidative damage (A1).

In the housefly, the protein carbonyl content rather than chronological age was found to be associated with life expectancy. Exposure of flies to sublethal hyperoxia (100% oxygen) irreversibly enhanced the carbonyl content of the flies and decreased their rate of oxygen consumption. Results of this study were suggested to indicate that protein carbonyl content may be a biomarker of aging (S40, S52, Y6). The average life-span potential of several insect species is inversely correlated with the level of protein carbonylation (S43). The white-footed mouse (*Peromyscus leucopus*) has more than a twice as long a life span as the house mouse (*Mus musculus*), and tissues of the white-footed mouse also have lower levels of

protein carbonylations than those of the house mice (S41). In cultured fibroblasts from normal human donors the levels of oxidatively modified proteins increased after the age of 60. The levels of oxidatively modified proteins in fibroblasts from individuals with progeria or Werner's syndrome were significantly higher than in age-matched controls. Protein carbonylation also increases in erythrocytes during aging (O5).

Immunochemical detection of carbonylated proteins demonstrates a high degree of specificity to protein oxidation. In the rat liver, carbonic anhydrase, isozyme III, is the most carbonylated (oxidized) protein (C1). In rat kidney, the main carbonyl-bearing protein is serum albumin (G16). Adenine nucleotide translocase (ANT) was found to be the only protein in the mitochondrial membranes exhibiting a detectable age-associated increase in carbonyls in mitochondria of flight muscles of the housefly (this protein was also the only one showing the presence of 4-hydroxynonenal adducts) (Y3). Another carbonyl-bearing protein was cytosolic arginine kinase, an enzyme that is involved in the energy metabolism of insect muscle cells (Y2).

Glycation and subsequent oxidation of glycation products is thought to underlie aging of collagen and dysfunction of collagenous tissues in old age (B2). Pentosidine formation increases progressively with age, but it is accelerated in diabetes (W15). In individuals with long-standing insulin-dependent diabetes mellitus, pentosidine content in skin correlates with severity of such complications as retinopathy and joint stiffness (S15). Pentosidine content of skin collagen from eight mammalian species was determined as a function of age. The rate of skin pentosidine formation correlated inversely with maximum life span. This suggests that the ability to withstand damage due to glycoxidation and the Maillard reaction may be under genetic control. Dietary restriction, a potent intervention associated with increased life span, markedly inhibited glycoxidation rate in the rodents (S14). In cultured human skin fibroblasts, pentosidine content increased with the number of passages, while in lymphocytes isolated from blood, it increased with the age of the donors (S19). Comparison of the nitrotyrosine content in skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase, isolated from young adult (5 months) and aged (28 months) rats, showed that in old rats the 2a isoform of the enzyme contained approximately four times more nitrotyrosine residues than that in the young rats (V7).

Not all data confirm accumulation of protein oxidation products with age. For example, comparison of 9-month-old and 24-month-old female Long-Evans/Wistar hybrid rats did not demonstrate any age-related increase of *o*-tyrosine and 3-nitrotyrosine in the heart, skeletal muscle, and liver. These observations were interpreted as indications suggesting that proteins damaged by the hydroxyl radical and reactive nitrogen species did not accumulate in these tissues with advancing age (L7). Dityrosine could not be detected in human plasma proteins or haemoglobin (with the detection limit of 1 pmol/mg protein) (D3), but may accumulate in

more long-lived proteins. Recently, carnosine (β-alanyl-L-histidine), a dipeptide present in long-lived cells at high concentrations (up to 20 mM), was found to react with carbonyl groups present on oxidized proteins. The role of this modification is not clear; it may inhibit further reactions of oxidized proteins or assist in recognition and degradation of advanced glycosylation end products (AGEs) (B28).

An "age pigment" referred to as lipofuscin accumulates with age in postmitotic cells, especially neurons and cardiac myocytes. This brown-yellow, electron-dense, autofluorescent material, which avoids enzymatic degradation, is thought to be formed in secondary lysosomes; it contains oxidized proteins (B30, T3). Lipofuscin is the classical age pigment of postmitotic cells, while the material accumulating due to pathological and experimental processes is usually called ceroid. There are good reasons to consider both ceroid and lipofuscin as materials of the same principal origin. The age-related intracellular fluorophores of retinal pigment epithelium (RPE) seem to represent a special class of lipofuscin, which partly contains derivatives of retinoids and carotenoids. Saccharide-originated fluorophores, principally AGEs formed during glycation/Maillard reactions, may be mainly responsible for the extracellular fluorescence of long-lived proteins such as collagen, elastin, and lens crystalline. Although lipofuscin, ceroid, and AGEs can be produced from different types of biological materials due to different side reactions, the cross-linking of carbonyl-amino compounds seems to be a common process during their formation (Y8). Usually lipofuscin is believed to be an innocuous waste material (though it may occupy a considerable portion of cell volume in aged cells), which, however, inhibits lysosomal and proteasomal protein degradation (E4, S34). CML and pentosidine were found in lipofuscin granules of brain tissues in nondemented elderly individuals (K14). Also, dityrosine was detected in lipofuscin in the aged human brain (K5).

3.8. Oxidative Protein Modification as an Oxidative Stress Marker

The level of oxidative protein modifications may indicate oxidative stress in normal and pathological conditions. Increased content of carbonyl groups was found in diffusely thickened intima (neointima) of arterial walls in arteriosclerotic tissues (M19). Comparison of oxidatively modified amino acids content in normal intima and in human carotid plaque samples revealed an increased content of 3-chlorotyrosine, DOPA, *o*-tyrosine, *m*-tyrosine, hydroxyleucine, hydroxyvaline, and especially, dityrosine (F17, H13). These plaques are indicative of the role of protein oxidative damage in the formation of atherosclerotic plaques. Postmortem studies revealed that the content of dityrosine is increased in the stage of advanced lesions. However, the *o*-tyrosine and *m*-tyrosine are not elevated in plaque proteins (H13, L11). The question of 3-nitrotyrosine level in atherosclerotic plaques remains unresolved, the reported data being contradictory (E6, L10, M24).

Similarly, DOPA, *o*-tyrosine, *m*-tyrosine, dityrosine, hydroxyleucine, and hydroxyvaline increase progressively during cataract formation, this increase being even more pronounced than for atherosclerotic plaque (D9, F18, F22). This oxidation does not seem to be due to the direct effect of UV on the lens but rather to reactions of the hydroxyl radical (F17). However, neither *o*-tyrosine nor dityrosine increases significantly during aging of normal human lenses in the absence of cataract formation (W8). Also, methionine sulfoxide accumulates in proteins of cataractous but not normal lenses (G4). *o*-Tyrosine and methionine sulfoxide content increased with age in human skin collagen; interestingly, the age-adjusted levels of these oxidized amino acids in collagen was the same in diabetic and nondiabetic subjects, suggesting that diabetes per se does not cause an increase in oxidative stress or damage to extracellular matrix proteins (W9).

The level of protein carbonylation and protein glycoxidation products increases in brains of patients with Alzheimer's disease (AD) compared with age-matched controls (S38, S39). Dityrosine and 3-nitrotyrosine are elevated in some regions of the human brain that are differentially affected in AD. Dityrosine and 3-nitrotyrosine levels are elevated consistently in the hippocampus and neocortical regions of the AD brain and in ventricular cerebrospinal fluid (VF), reaching quantities five- to eightfold greater than mean concentrations in brain and VF of cognitively normal subjects (H19). However, no increase in pentosidine or CML was observed in frontal cortex specimens of patients with Alzheimer's disease with respect to normal subjects (S13).

Protein carbonyls were found to be elevated in the brain cortex of patients with sporadic amyotrophic lateral sclerosis but not autosomal dominant familial amyotrophic lateral sclerosis (F4). Protein carbonyls were also elevated in the spinal cord tissues of transgenic mice that overexpress the SOD1 mutation [TgN(SOD1-G93A)G1H] in comparison with nontransgenic mice. Such mice represent an animal model of familial amyotrophic lateral sclerosis. One of the most heavily oxidized protein bands (14 kDa) was identified as Cu,Zn-SOD (A13). In humans with both autosomal dominant familial amyotrophic lateral sclerosis and familial amyotrophic lateral sclerosis, malondialdehyde-modified proteins were detected, while in patients with sporadic amyotrophic lateral sclerosis they were absent (F4); 4-hydroxynonenal modified proteins were detected in the lumbar spinal cord. One of the proteins modified was the astrocytic glutamate transporter (P11). The protein carbonyl content was found to be about twofold higher in substantia nigra pars compacta than in other regions of postmortem samples of human brain. This result is in agreement with the view that oxidative stress underlies degenerative processes in dopaminergic neurons in human substantia nigra during normal aging which is accelerated in Parkinson's disease (F9). In stroke-prone, spontaneously hypertensive rats, the diet was demonstrated to affect the protein carbonyl group content. The protein carbonyl content in a lard-fed group was higher in the brain and liver compared to the other dietary groups The peroxidizability of tissue lipids

was positively correlated with the protein carbonyl content in skeletal muscle, but not in the brain, heart, or liver (S6).

Carbonyl content of proteins in the synovial fluid is increased in patients with rheumatoid arthritis (C8). Carbonyl content of blood plasma proteins was higher in hemodialysis patients (1.49 ± 0.05 nmol/mg protein) than in the healthy subjects (1.08 ± 0.03 nmol/mg protein). After hemodialysis, the carbonyl group level was reduced but did not return to the control value, suggesting that an impaired redox status was maintained (O3). Pentosidine levels of plasma proteins are elevated in uremia associated with end-stage renal disease, much more even than in diabetes (23-fold versus 2.5-fold), probably due to reactions of dehydroascorbic acid formation in uremia due to oxidative stress (O2). The level of protein carbonyl groups increases in the lungs of neonates being ventilatied with atmosphere enriched in oxygen (G13).

Rats inhaling cigarette smoke for 30 days, three times a day, showed increased protein thiolation in the lung (P7). A marked increase in protein thiolation was observed after exposure of HT4 cells (a mouse neuronal cell line) or rat primary mesencephalic cultures to Cd^{2+} (25–50 μM) (F5). Massive thiolation was revealed after stimulation of the respiratory burst in mouse macrophages and human neutrophils, reaching maximum 10–20 min after stimulation by opsonized zymosan or phorbol diester. Individual proteins underwent thiolation and dethiolation at different rates (S22). *S*-Thiolation of proteins was suggested to protect phagocytes against the autoxidative damage associated with the respiratory burst (R12). Tyrosine radicals and ditotyrosine were found to be formed on proteins exposed to activated human polymorphonuclear leukocytes (U9).

Exercise was found to cause a 50% increase in *o*-tyrosine, *m*-tyrosine, and dityrosine in mitochondrial proteins but not cytosolic proteins of rat heart muscle. This increase was transient, and levels returned to normal when exercised animals were allowed to rest. There was also a transient increase in the level of *o*,*o*′-dityrosine in the urine of exercised rats (L8). A single bout of exhaustive running or endurance training for 12 weeks significantly increased the level of protein carbonyls in rat skeletal muscles (R8, W14). Extensive running of rats induces a 40% increase in protein carbonyls in the lung (R1).

3.9. Antioxidative Defense and Oxidative Stress

The imbalance between the $O_2^{-\bullet}$ production and the capacity of $O_2^{-\bullet}$ removal and control formation of its derivatives is qualified as "oxidative stress." Individual cells, as well as tissues as a whole, possess several mechanisms to control production of ROS and prevent cells from oxidative damage. The intracellular protection system against ROS employs the system of immediate $O_2^{-\bullet}$ removal by its dismutation to H_2O_2 and O_2. This reaction is mediated by enzymes of the SOD family. Ubiquitous presence of SOD in cells of aerobic organisms, as well as deleterious

effects of SOD deficiency in the affected cells, suggests that this enzyme is a major constituent of cellular antioxidative defense. Removal of $O_2^{-\bullet}$ prevents its spotaneous reactions with cellular substrates and also limits the unwanted ROS formation. In most somatic cells, H_2O_2 is then removed by catalase, the hemoprotein enzyme present in peroxisomes of mammalian cells, decomposing H_2O_2 to water and molecular oxygen. Transgenic fruit flies carrying three copies of genes for superoxide dismutase and catalase, yielding more effective removal of $O_2^{-\bullet}$, showed as much as a one-third life-span extension and a delayed loss in physical performance; they had decreased levels of protein carbonyls and a slower rate of carbonyl accumulation with age (O9).

Other constituents of antioxidative defense are glutathione peroxidase decomposing lipid peroxides and hydrogen peroxide to water and oxidized glutatione (GSSG), the thioredoxin system, thiol-specific antioxidant (thioredoxin peroxidase) and methionine sulfoxide reductase converting methionine sulfoxide to methionine. Moreover, in the extracellular fluids are a number of small molecular reductants such as glutatione, cysteine, ascorbate (vitamin C), tocopherol (vitamin E), uric acid, bilirubin, flavonoids, and carotenoids that react with oxidants, removing them from biological milieu. An addition to the small molecular reductants, extracellular fluids contain factors inhibiting formation of ROS: albumin, which binds Cu^{2+}, lactoferrin, which sequestrates Fe^{2+} (in an acidic medium of the inflammatory sites); and ceruloplasmin, a copper-binding protein acting also as a ferrooxidase, which can control excessive concentrations of ferric ions (Fe^{2+}) by its oxidation to ferrous ions (Fe^{3+}). Ceruloplasmin also oxidizes $O_2^{-\bullet}$ to O_2 (molecular oxygen) at the expense of reduction of Cu^{2+} to Cu^{1+}. Extracellular fluids also contain some amounts of SOD (especially the extracellular SOD) and catalase, decomposing the superoxide anion and hydrogen peroxide, respectively. All these compounds form the antioxidative defense system (S28). The total level of antioxidants is qualified as an "antioxidative status," which can be assessed by evaluation of concentration or activities of all its components or assessement of total capacity to prevent ROS-mediated reactions.

Biological observations show that degree of oxidative stress is dependent on many factors, including environmental ones. In rats, mice, and gerbils, dietary restriction delays the age-related increase in protein carbonyls in different regions of the brain, and results in reversal of some age-associated regional trends in carbonyl and sulfhydryl concentration in the brain. Mice fed a diet that was 40% lower in calories than that of animals fed *ad libitum* exhibited an average life span 43% longer and a 61% prolongation in mortality-rate doubling time. A comparison of *ad libitum*-fed animals and dietary-restricted mice at 9, 17, and 23 months of age indicated that the protein carbonyl content in the brain, heart, and kidney increased with age and was significantly greater in the *ad libitum*-fed animals than in the dietary-restricted group in each organ at each of the three ages (S42). Caloric restriction also decreased age-related oxidative damage to mitochondrial proteins,

measured as amounts of protein carbonyls and loss of protein sulfhydryl content (L3). In mice fed *ad libitum,* levels of dityrosine increased with age in cardiac and skeletal muscle but not in liver or brain. Caloric restriction prevented the increase in dityrosine levels in cardiac and skeletal muscle (L12).

Chronic administration of the spin-trapping compound *N-tert*-butyl-α-phenyl-nitrone (PBN) also caused a decrease in oxidized protein level and an increase in both glutamine synthetase (GS) and neutral protease activity in aged Mongolian gerbil brains (D22). In contrast to aged gerbils, PBN treatment of young adult gerbils had no significant effect on brain oxidized protein content or GS activity. If PBN administration was ceased after 2 weeks, the significantly decreased level of oxidized protein and increased GS and neutral protease activities in old gerbils changed in a monotonic fashion back to the levels observed in aged gerbils prior to PBN administration. Old gerbils make more errors than young animals, and older gerbils treated with PBN made fewer errors in a radial-arm maze test for temporal and spatial memory than the untreated aged controls (C3). These results were interpreted as indicative that oxidative stress and protein oxidative damage are reversible. However, effects of PBN on protein carbonyls are variable depending on tissue and species: a decrease in protein carbonyl content was not observed in gerbils' hearts or mouse brain cortex after PBN treatment. In spite of the positive effect on aged tissues, PBN administration did not extend the life span of houseflies (D22). Administration of N-acetylcysteine prevented the age-related accumulation of protein carbonyls in synaptic mitochondria of female OF-1 mice (B4).

Problems of ROS generation in various pathologies remain in the scope of interest of both basic biomedical researchers and clinicians attempting to modify balance between ROS formation and antioxidative defense systems. Chronic or acute imbalance between the generation of ROS and decrease in effectiveness of antioxidant protection from biochemical mechanisms leads to some diseases effecting tissue injury and progressive organ degeneration (Table 8). More detailed data on various aspects of excessive ROS formation in pathology are the subject of review publications (F3, H7, H16, K16, S31, S52).

4. Detection of Oxidatively Modified Proteins in a Clinical Laboratory

Protein oxidation products are assayed by a number of direct and indirect methods, depending on properties of the chemical group to be detected. The most commonly used is the assay of protein —SH groups concentration, supposedly decreasing in the oxidative stress. The assays are based on the color reaction produced by protein —SH groups with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman reagent) (H1, R9). The Ellman reagent is a disulfide that comes into exchange with protein-bound —SH, producing the yellow-colored 5-thio-2-nitrobenzoate anion,

with light-absorption maximum at 412 nm and a molar absorption coefficient of $\varepsilon = 13600\ M^{-1}\ cm^{-1}$. There is also an interesting assay of thiol groups based on spin label for thiols that employs the imidazolidine biradical disulfide label, where the electron spin resonance (ESR) spectrum changes due to protein thiol-group exchange (K12). This method can be preferably applied to colored and turbid samples.

Oxidation products of methionine and other amino acids are, after protein hydrolysis, measured by HPLC or gas chromatography–mass spectrometry. As protein hydrolysis in the anaerobic environement reduces methionine sulfoxide back to methionine, this method does not detect methionine sulfoxide. To circumvent this problem, hydrolysis in 4 mol/liter NaOH solution was used (methionine sulfoxide is stable under these conditions), or the alkylation of methionine residues was employed (methionine sulfoxide is resistant to alkylation). The other approach to metionine sulfoxide assay consists of protein fragmentation by cyanogen bromide treatment, cyanogen bromide producing peptides containing homocysteine cleaves proteins at methionine residues (methionine sulfoxide is resistant). Hydrolysis of cyanogen bromide peptides in acidic media reduces methionine sulfoxide to methionine, so the amount of homocysteine corresponds to the amount of methionine in the primary samples. The amount of methionine after hydrolysis corresponds to the amount of methionine sulfoxide in the protein (B24). The estimation of thiol groups reacting with Ellman reagent to total thiol protein content ratio may be used as the other protein oxidation index (S45). In membrane proteins the —SH groups are assayed by the maleimide spin label. The 2,2,6,6-tetramethyl-4-maleimido-piperidin-1-oxyl, binding to protein —SH groups, increases a number of strongly immobilized spin label residues. On the other hand, the weakly immobilized residues correspond to oxidatively modified thiols. Protein thiolation is quantified by measurement of release of protein-bound thiols under reducing conditions; alternatively, modification of protein isoelectric point due to thiolation is to be detected (H17, H18, S45). ESR and especially the technique of spin trapping are the methods used for detection of free-radical formation on proteins.

Protein thiolation is quantified by measurement of release of protein-bound thiols under reducing conditions; alternatively, modification of protein isoelectric charge due to thiolation is detected (M17, T7, T8, T10).

Action of various reactive oxygen and nitrogen species on amino acid residues generates products containing carbonyl groups (e.g., *N*-formylkynurenine or 2-oxohistidine). Therefore, the "assay of carbonyls" is considered a general measure of oxidative protein damage. However, binding of some aldehydes, such as malondialdehyde or 4-hydroxynonenal, as well as glycoxidation, may also cause an increase in content of carbonyl groups in proteins. The common carbonyl assay is based on the reaction of 2,4-diphenylhydrazine with proteins, removal of reagent excess, and colorimetric measurement of bound 2,4-dinitrophenyl residues (L17, L19, L20, O5). The applicability of this method to crude tissue extracts has been

questioned, however. As pointed out by Dean *et al.,* the estimated level of protein carbonyls, being of the order of 1 nmol/mg of protein, suggests the presence of one carbonyl group per 3000 amino acid residues. It implies the presence of one carbonyl group in roughly 5% of cellular proteins. This may suggest that the carbonyl assay overestimates protein oxidation levels (D9). Probably, unbound dinitrophenylhydrazine and nucleic acids contribute to the false elevated results (C2). Another approach to protein carbonyl group assay implies the reduction of carbonyl moieties with tritiated borohydride. This measurement is based on subsequent radioactivity counting (after removal of the borohydride excess) (L16). The labeled amino acids may also be separated by HPLC, identified, and quantified (A9). Immunochemical detection of proteins bearing carbonyl resides is becoming increasingly popular; proteins separated by one-dimensional or two-dimensional electrophoresis reacted with antidinitrophenyl antibodies employing the Western blotting technique are used. This method allows for identification of oxidatively modified proteins in complex mixtures (N1, S23, Y2). Reaction of fluorescamine with carbonyl groups of oxidized proteins produces Schiff bases. The subsequent reduction of the Schiff base with cyanoborohydride yields a stable chromophore on the oxidized residues. Acid hydrolysis of a labeled protein can allow for isolation of the derivatized, oxidized residue (C15).

Formation of carbonyl groups causes a decrease in amino group number in protein molecules. Therfore the loss in amino group content is also used as a measure of oxidative protein modification marker. The protein amino group quantitation is most commonly performed employing the fluorescamine assay. Fluorescamine reacts with primary amines to form a fluorescent product (excitation wavelength 390 nm; emission at 440–500 nm) (R9). Older methods, such as employing trinitrobenzenesulfonate (H2), are less sensitive and more troublesome.

Chlorination products (mainly chlorotyrosine) are measured by HPLC (Fig. 8) or gas chromatography–mass spectrometry. Also, 3-nitrotyrosine can be detected in protein hydrolysates by HPLC in combination with various detection systems, including UV and electrochemical detection (C17, C20, L23, L24, O4, S26), gas chromatography, gas chromatography–mass spectrometry (J2), electrospray mass spectrometry, and Western blotting or ELISA using antinitrotyrosine antibodies (H20, T2, V6).

Tryptophan damage is estimated by decrease of its native fluorescence (excitation 279–298 nm, emission at 320–350 nm, depending on the microenvironment) or increase in fluorescence of its oxidation products (R9). *N*-Formylkynurenine emits light with maximum intensity at 454 nm when excited at 360 nm in neutral or acid medium. The fluorescence yield increases about 20-fold when the measurement is performed at pH 10.5 (excitation at 315 nm and measuring emission at 400 nm) (G32).

Dityrosine, due to its specific fluorescence, is the most-often assayed tyrosine oxidation product (excitation 315–325 nm, emission at 410–420 nm) (H4, P15).

Also, HPLC methods with electrochemical or fluorescent detection are used (H19, M3). In proteins, dityrosine can be estimated by immunochemical methods employing dityrosine-specific antibodies (K5). Measurements of *o*,*o*′-dityrosine and *o*-tyrosine levels in rat urine express dityrosine contents in skeletal muscle proteins, and have been proposed as the noninvasive oxidative stress test *in vivo*. One should be aware, however, that *N*-formylkynurenine, also formed in protein oxidation, has similar fluorescence properties as dityrosine (excitation 325 nm, emission at 400–450 nm) (G29). Also, oxidation of mellitin when excited at 325 nm produces an increase in fluorescence at 400–450 nm, despite the fact that mellitin does not contain tyrosine. Oxidation of noncontaining Trp residues ribonuclease A and bovine pancreatic trypsin inhibitor with $^{\bullet}$OH produces loss of tyrosine residues with no increase in fluorescence at 410 nm (S51). There are also methods measuring the increased hydrophobicity of oxidized proteins. Assays are carried out measuring protein binding of a fluorescent probe, 8-anilino-1-naphthalene-sulfonic acid (ANS). Increase in probe binding reflects increased surface hydrophobicity (C7).

Determination of oxidized amino acids in urine is usually performed by isotope dilution gas chromatography–mass spectrometry (L9). DOPA is estimated by HPLC separation of acid protein hydrolysates with fluorescence detection (excitation 280 nm, emission at 320 nm) (A15). Other methods are based on borate–hydrochloric acid difference spectroscopy (this method suffers interference from tyrosine and tryptophan) (W2), derivatization of DOPA with nitrite and subsequent coulometric determination (W3), and fluorometric detection after derivatization with ethylenediamine (A15). 3-Hydroxylysine is quantitated by HPLC with 9-fluorenylmethyl chloroformate precolumn derivatization (M25) of amino acids obtained by gas-phase hydrolysis of proteins (F21). Other general methods to detect amino acid damage are mass spectometry methods applied to protein hydrolysates, such as tandem mass spectrometry (F6).

Pentosidine is determined by HPLC with spectrofluorimetric detection (excitation and emission wavelengths of 335 and 385 nm, respectively) (S14), although immunochemical and ELISA assays for determination of various protein oxidative modification products have become increasingly popular (O8). Protein-aldehyde adducts can be estimated using adduct-specific antibodies (U2, W1). Another approach requires stabilization of adducts, producing derivatives resistant to conditions used in protein acid hydrolysis and quantification of hydrolysis products by gas chromatography–mass spectrometry (R7).

Estimation of oxidatively modified proteins does not have direct importance in clinical laboratory diagnostics yet. On the other hand, there are a large number of publications indicating that increase in protein oxidative damage always accompanies tissue injuries, organ degeneration, and aging. Therefore it is conceivable that in the near future some assays of oxidized proteins will be used as auxiliary tests in assessment of organ damage in hypoxia, tissue necrosis, and diseases occurring with inflammatory process. While estimating products of protein

oxidative damage, one should be aware of artifacts in analysis of oxidatively modified proteins. Some oxidation products may be formed during sample processing and storage; especially difficult is elimination of trace amounts of transition metal ions, commonly present in samples. Therefore, transition metals often interfere, catalyzing extensive oxidation of methionine residues in samples upon storing (C14). Also, a number of secondary derivatives of protein oxidation products can interfere with assays of protein oxidative damage.

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SELECTED MARKERS OF BONE BIOCHEMISTRY

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1. Introduction

Mature cortical and trabecular bone undergo consistently a process called bone remodeling. Bone remodeling is in progress with variable intensity throughout life, and the connection of resorption to subsequent bone formation is essential. Disorders of this connection lead to imbalance between resorption and new formation of bone tissue, and may cause number of pathological conditions. The most serious of these diseases—especially from the point of view of incidence—is osteoporosis. This disease affects patients of all ethnic groups, and has increasing incidence and prevalence. In the developed countries, it affects about 7–8% of the population. Considering the fact that clinical symptoms can usually be found in patients of higher age groups, it occurs more frequently with extending life expectancy of the population. Besides deteriorated quality of life of the affected patients, the increased risk of bone fracture is the principal health care problem of osteoporosis. Currently, lifetime risk of osteoporotic fracture is approximately 40% for females and 13% for males (D6). Main fracture locations are the proximal parts of the femur, vertebrae, and distal forearm. Fractures of the proximal femur are an especially serious problem, because 10–20% of victims die during the first year after the accident, and in the survivors, the quality of life often deteriorates. Up to 50% of survivors are somehow physically or socially handicapped. Qualified estimates still expect substantial increase in the number of osteoporotic fractures. In the year 2000, the expected incidence of fractures of the proximal femur is estimated at 337,400 in the United States, and at about 414,000 in the European Union countries, while in the year 2050, about 650,000 fractures of the proximal femur in the United States, and 972,000 fractures in the European Union can be expected. It is obvious that detailed recognition of causes, pathobiochemistry of bone changes, and the influence of systemic and local agents is of extraordinary importance.

Remodeling takes place in 3–10% of the total mass of the skeleton per year. It is increased by parathormone, thyroxine, growth hormone, and vitamin D and is reduced by calcitonin, estrogen, and glucocorticoids. Microfractures and mechanical stimuli also influence this process. Trabecular bone, which represents about 20% of the total skeleton mass, participates in bone remodeling up to 70% while cortical bone, being a part of the skeleton to 80%, participates in bone remodeling to only 20%. Remodeling consists of several typical phases. First, differentiation and migratory activity of the preosteoclasts occur, and these transform into osteoclasts which are consequently activated—the activation phase. After activation, adhesion of the osteoclasts to the bone not covered by the osteoid starts, and the conditions for resorption are prepared. Osteoclasts that perform the resorption create the so-called resorption front. As a result of their activity, a cutting cone is formed in the cortical bone, as well as so-called Howship's lacunas in the trabecular bone. After the resorption is terminated, the so-called phase of reverse starts; during this phase,

osteoclasts disappear from the resorption plate. In this phase, osteoblasts are differentiated and activated. The formation phase is the last period of remodeling; in it the activity of osteoblasts takes over the principal role. Disorders of the balance between the functions of osteoclasts and osteoblasts during skeleton remodeling may lead to osteoporosis, or, on the other hand, to osteopetrosis. Local factors responsible for regulation of the connection of the functions of osteoclasts and osteoblasts are subject to intensive research, and the number of the factors or mediators mentioned in the literature in connection with this topic is on a permanent rise (K3).

Recent studies on genetically knockout and transgenic mice bring a substantial number of so far only partially assorted data about the regulation of bone modeling during the development and remodeling of the mature bone. Understanding the principles of these activities would undoubtedly explain the essence of the development of numerous bone diseases. For example, fibroblast growth factor receptor-3, parathyroid hormone-related protein, and tartrate-resistant acid phosphatase influence the function of chondrocytes during enchondral ossification. Some ubiquitously expressed genes have a number of totally unexpected functions in various bone cells; for example, M-CSF, C-Phos, PU.1, and NFκB are necessary for the formation of osteoclasts, while c-Src and Mitf (microphtalmia transcription factor) are necessary for further activity of the osteoclasts after their formation. Some proteins and factors act as negative regulators of bone cell function, for example, osteoprotegerin (soluble TNF receptor) in osteoclasts, or osteocalcin, bone sialoprotein, and 5-lipooxygenase in the osteoblasts. Regulation of the survival period of the osteoclasts seems to be one of the possible mechanisms by which estrogens, and therapeutically also bisphosphonates, prevent loss of bone tissue under conditions characterized by increased bone resorption, for example, in postmenopausal osteoporosis (B9). In *in vitro* studies, a number of substances show chemotactic effect for human osteoblasts. For example, TGF-betal, PDGF-BB with a very strong chemotactic effect, as well as PDGF-AA, IGF-I, and IGF-II with substantially lower but also significant positive chemotactic effects for human osteoblasts, may be mentioned. All these results were also confirmed *in vivo* (L3). Also, beta 2 microglobulin (beta2M), a water-soluble light chain of MHC protein class I, has been recently isolated in the supernatant of cell cultures of adult bone cells. It appears that beta2M is probably one of the bone-derived growth factors that influence the function of both steoblasts and osteoclasts, especially under conditions of increased bone turnover (Q1). There are other substances or receptors whose roles are mentioned in the process of bone remodeling, such as receptor tyrosine kinase (RTK or TEK)(S3), calcium sensing receptor (CaR), which probably starts bone resorption but also participates in the regulation of the secretion of the "osteotropic cytokines" (Y2), and gelatinase B (O2). Mechanical factors as well, and the effect of the endogenously induced electromagnetic field, must be at least mentioned. The question remains, however, whether these factors

may act by themselves, or must be mediated by the endless number of mediators (S11). The process of bone remodeling (especially resorption) also includes so-called bacterial porines-proteins present on the external surface of the cellular membrane of gram-negative bacteria that participate in the transportation of low-molecular-weight substances into the cell. Porines of many bacteria show strong anti-inflammatory effect via stimulation of synthesis of many anti-inflammatory mediators (cytokines, platelet-activating factor, and nitric oxide). However, their resorptional activity cannot be neutralized, neither by addition of the cyclooxygenase inhibitor indomethacin, nor by inhibition of the activity of the TNF. On the other hand, the effect of porines can be blocked by adding a natural inhibitor of IL-1-IL-1 receptor antagonist (M5).

Disturbances of the metabolism of bone tissue that usually exhibit not very specific clinical signs and symptoms are induced by the effects of both systemic and local agents. The role of hormones has already been described and defined many times, but news and new views keep appearing all the time. Without doubt, practically all systemic agents, hormones, but also drugs act on the bone tissue only through mediators. Their effect is provided by local, paracrine, and autocrine substances effective on the level of the cell, and by cellular components and on molecular level. Some of the hormones and local agents will be mentioned below.

2. Cytokines and Other Factors

Interleukin-6 (IL-6) is a principal cellular regulatory factor, and also plays a role in bone remodeling. IL-6 exercises its effect by binding to a receptor (IL-6R), which leads to signal transduction and activation of intracellular cascades. Information from studies performed both *in vitro* and *in vivo* show IL-6 as an autocrine/paracrine factor of the osteoclasts. Recent studies describe especially increased expression of IL-6R mRNA in the osteoclasts engaged in increased bone resorption. It can be judged that IL-6 itself is in a sophisticated way involved in bone resorption, and that especially the expression of its receptor (IL-6R) correlates with resorption activity of the osteoclasts (L1).

Estrogens play an important role in growth and maturation of bone, and thus in remodeling regulation. During bone growth, estrogens are essential for adequate closure of epiphyseal growth plates in maturing persons of both sexes. Decrease of estrogen levels in later periods of life leads to prompt increase in endocortical resorption. On the cellular level, estrogens inhibit differentiation of osteoclasts, reduces their number, and also reduces the number of active remodeling units. This effect is almost certainly mediated by cytokines; among them, the most serious candidates are the IL-1 and IL-6. Estrogens regulate the expression of IL-6 mRNA in bone marrow cells by a mechanism that is not quite known so far (V1). The effect of high doses of corticoids that interfere unfavorably with bone

modeling via reduction of bone formation and increase in bone resorption is also mediated by cytokines IL-6 and IL-1beta (S12). Taguchi *et al.* (T2) showed that cytokines that use glycoprotein 130 (gp 130) homodimers or gp 130/leukemia inhibitory factor (LIF) receptor of beta heterodimers for intracellular signal transfer are potent inductors of the development of osteoclasts, both *in vitro* and *in vivo*. A typical cytokine increasing osteoresorption is IL-6. Tests using the model of the culture of mice embryonic fibroblasts (EF) have proven that IL-6 and IL-11, combined with their soluble receptors (sIL-6R and sIL-11R) and depending on the dose used, increase the number of alkaline phosphatase-positive cells in cellular culture. Under certain conditions, mRNA for osteocalcin is also expressed. This "pro-differentiation effect" is specific for osteoblastic lines; any increase in the differentiation of chondrocytes, adipocytes, or muscle cells has not been proven. As opposed to IL-6/sIL-6R, other substances (LIF), oncostatin M (OSM), and cilliary neurotrophic factor (CNTF) have not shown pro-differentiative activity for osteoblasts in EF cells. Effect specificity of the IL-6 complex is attributed to a finding that the EF cells exprime gp 130 but neither ligand binding subunit of IL-6 receptor (gp 80) nor LIF receptor-beta. This observation, and the evidence of increased production of cytokines (and their soluble receptors) using gp 130 and also intracellular transcription factor NFκB (M1) for signaling under various pathological conditions, for example, in the deficit of sexual steroids, show that the above-mentioned cytokines are responsible not only for osteoclastogenesis, but also for osteoblastogenesis, and in that manner for general increase of bone remodeling degree (T2, T3). It is also interesting that both the very often discussed cytokines IL-6 and TNF-alpha probably do not play any role in increased bone resorption in patients with bone metastatic processes, such as those occurring in prostate cancer (A1). On the other hand, in patients with renal osteodystrophy, cytokines probably are of principal importance, not just the changes in secretion of PTH and production of calcitriol (M8).

Research has discovered that in patients with chronic idiopathic neutropenia the bone turnover is increased, and bone mineral density (BMD) drops. At the same time, increased concentration of cytokines IL-1 beta and TNF-alpha in serum were detected as a sign of a chronic inflammatory process. Their participation in the acceleration of bone remodeling and BMD reduction can be presumed (P6). Zheng *et al.* (Z1) followed the production of cytokines in the immune blood cells of postmenopausal osteoporotic women, and discovered increased production of IL-1 beta, IL-6, and TNF-alpha, while the production of IFN-gamma, GM-CSF, and LIF was unchanged. Other mediators from the cytokine group, frequently mentioned in connection with bone tissue remodeling, are leukemia inhibitory factor (LIF), and oncostatin M (OSM). It is known that LIF and OSM affect collagen synthesis in osteoblasts, but their exact role in collagenase expression and collagen degradation is not quite clear. Varghese and Canalis (V4) followed the influence of LIF and OSM on the expression of the metalloproteinases matrix (MMPs) and

tissue inhibitors of metalloproteinases (TIMPs) in osteoblasts isolated from fetal rat calvariae. LIF and OSM caused the increase of collagenase-3 mRNA and immunoreactive protein levels depending on time of delivery and dose. Based on these findings, it can be assumed that LIF and OSM are also engaged in the process of bone remodeling via collagenase-3 stimulation, and TIMP-1 expression in osteoblasts. Bohic *et al.* (B6) watched the effect of leukemia inhibitory factor (LIF) and oncostatin M (OSM) on physicochemical characteristics of the mineral phase formed in the rat bone marrow stromal cell culture model. Special detection methods (energy disperse X-ray microanalysis and Fourier transform infrared) have proven significant influence on physicochemical characteristics of the mineral phase, and at the same time, FT Raman spectroscopy has proven significant influence of LIF and OSM on vibration characteristics of the organic matrix. Jimenez *et al.* (J3) studied the above-mentioned collagenases and metalloproteinases. Collagenase-3 (MMP-3) was recently identified as a member of the metalloproteinase (MMP) gene family that are expressed on a large scale and at high levels in the cells of some types of human carcinomas, and in joint cartilage from arthritic patients. Besides these known expressions under pathological conditions, collagenase 3 has also been detected in osteoblasts and hypertrophic chondrocytes during fetal ossification. Another important transcription factor that probably plays an irreplaceable role in expression of osteoblastic specific genes is the core-binding factor 1 (Cbfa 1). The use of gel mobility shift assays has been proven that Cbfa 1 is the main stimulator of the expression of collagenase 3 in osteoblastic and chondrocytic cells. For its strong proteolytic activity on various collagenous and noncollagenous bone components, collagenase 3 is considered one of the key enzymes in the process of bone formation and remodelation (J3).

Osteopontin (OPN) also belongs to the group of cytokines, and its primary function is probably recovery facilitation after an insult (injury, infection) that generally causes an increase in its expression. However, OPN also stimulates intercellular signaling, and supports migration of a number of cells in bone tissue. OPN has the ability to extend cellular survival and to inhibit apoptosis, which can explain the increased metastatic ability in cells with increased expression of OPN. The role of OPN in bone remodeling is probably modulatory, but it has not been exactly defined so far (D8). Although the number of hormones and cytokines influences various aspects of osteoclast development, final effectors are the recently defined local factors of the osteoprotegerin type OPG (also called osteoclastogenesis inhibitory factor), and its ligand, osteoprotegerin ligand OPG-L/osteoclast differentiation factor (ODF). OPG-L/ODF is produced by osteoblast lines. It is a transmembrane protein. It performs its biological effect by binding to a receptor–osteoclast differentiation and activation receptor (ODAR)/receptor activator of NF-kB (RANK) in the osteoclast lines (receptors can be soluble, or membrane bound), when the RANK activation requires cell-to-cell contact. The binding to

these receptors leads to quick differentiation of osteoclast precursors to mature osteoclasts in the bone marrow, to the increase of their functional activity, and to reduction of the degree of apoptosis of mature osteoclasts. Binding of osteoprotegerin (OPG) neutralizes biological activity of OPG-L/ODF/osteoclastogenesis inhibitory factor (OCIF), included in the TNF-receptor superfamily that is also produced by osteoblast lines (H8). Recent studies have proven that osteoprotegerin is identical with a factor originally called osteoclastogenesis inhibitory factor (OCIF) (Y3). OPG-L was then identified as a factor of osteoclast differentiation. Mice missing the gene for OPG-L suffered from significant osteopetrosis, as well as defects in tooth eruption. In these mice, complete absence of osteoclasts was noticed as a sign of osteoblast inability to support osteoclastogenesis (K4). Similarly, Lomaga *et al.* (L4) describe how osteoclast differentiation and activation is mediated via the signals of the osteoprotegerin ligand (OPG-L), and its appropriate receptor RANK. Maturation and function of the osteoclasts is thus probably determined by the relative ratio of OPG-L/ODF to OPG/OCIF in the bone marrow microenvironment. Changes of this ratio may cause the loss of bone mass in a number of metabolic disorders, including deficit of estrogens or surplus of glucocorticoids (H8). The molecular mechanism of further intracellular signal transfer remains unclear for now. It is described that also mice with TNF receptor-associated factor 6 (TRAF6) deficit suffer from osteopetrosis. Other *in vitro* experiments have demonstrated that TRAF6 is an essential factor not only in IL-1 and CD-40 signaling, but is also, surprisingly, the essential factor for lipopolysaccharide (LPS) signaling. Many other factors associated with TNF receptor, especially TRAF2 and TRAF3 (and also TRAF6), play until now only a little explained role in cytokine signaling and in bone metabolism (H7, L4).

2.1. Heat Shock Proteins (HSP)

Molecular chaperones, also known as heat shock proteins (HSP), are intracellular proteins found in all cells that catalyze protein folding. Nair *et al.* (N1) discovered one class of bacterial molecular chaperone (the chaperonins) that exhibit strong inductory properties for bone resorption. The authors were trying to answer the question of whether this osteolytic activity is unique for the protein class discovered by them, or is a general property of molecular chaperones. To prove the osteolytic activity, they used murine calvarial bone resorption assay. It came out that molecular chaperones of *Escherichia coli* (marked as groEL, groES, and danK) exhibit osteolytic activity. This osteolytic activity was inhibited by indomethacin and a natural antagonist of IL-1 (interleukin-1 receptor antagonist), but was not influenced by neutralization of tumor necrosis factor or the inhibition of 5-lipooxygenase. The authors also tested mammalian molecular chaperones with a molecular weight of 27, 47, 70, and 90 kDa, and all of them—with the exception of the 47-kDa protein—exhibited activity on murine calvarial bone resorption assay.

Establishing the role of these proteins of the HSP class in the process of bone remodeling must be understood as a challenge for further studies (N1).

2.2. Growth Factors IGF, GH

Seck *et al.* (S6) observed the concentration of insulin-like growth factor-I (IGF-I) and IGF-II in human cortical bone, as well as histomorphometric parameters of the bone mass and the markers of the bone turnover. They examined 125 samples from the proximal part of the human femur and discovered that in females, the width of the cortical bone dropped in the period between the fourth and the ninth decade of life by 27%, and the porosity increased by 100%. Similar but less significant changes were found in the cortical bone in the males. The concentrations of both IGF species were correlated with the percentage of osteons undergoing bone remodeling. It was also found that oral and/or nasal application of 17-beta estradiol has various effects on serum concentration of insulin-like growth factor-I (IGF-I), which is considered a potent bone-forming growth factor. Garnero *et al.* (G5) discovered that the new route of administration of E2 with intranasal spray (S21400) influences bone turnover in a different manner, and leads to faster normalization of bone turnover in postmenopausal women to premenopausal levels. The authors explain this difference between intranasal and oral application by different dynamics of influencing circulating concentrations of IGF-I and IGF-binding protein-3 (IGFBP-3).

Hypertrophic chondrocytes in the epiphyseal growth plate exprime angiogenic protein vascular endothelial growth factor (VEGF). VEGF is an important coordinator of many functions: chondrocyte death, chondroclast function, extracellular matrix remodeling, angiogenesis, and bone formation in the growth plate (G6). Also, fibroblast growth factor-2 (FGF-2) has certain functions in bone formation and bone remodeling. In experiments on rabbits, FGF-2 application led to significant increase in bone mineral content (BMC) (O1). Parathormone (PTH) stimulates the expression of FGF-2 mRNA and FGF receptor (FGFR) in the osteoblasts. Probably, part of the effect on PTH on bone remodeling is mediated by the regulation of FGF-2 and FGFR expression in the osteoblasts (H9). In the literature, there is a lot of contradictory information about the modulating effect of transforming growth factor-beta (TGF-beta) in the process of bone remodeling. However, results of some recent studies indicate that the increased osteoclast activity has a permissive effect on TGF-beta-induced osteoblast differentiation in locations with intensified bone resorption. TGF-beta seems to be one of the physiological regulators of osteoblast differentiation, as well as one of the central components of coupling of formation to resorption during bone remodeling (E2). TGF-beta1 interferes with the expression of mRNA of some proteoglycans in osteoblasts (decorin and biglycan), and becomes the inhibitor of the expression of a gene for alkaline phosphatase during osteoblast differentiation (Y1).

2.3. Prostaglandins

Some histological studies indicate that endothelial cells in bone (BDECs) are involved in etiopathogenesis of some osteolytic bone diseases. The mechanism remains unclear. Kage *et al.* (K1) observed the reaction of murine BDECs that were isolated from femurs on the stimulation with basic fibroblast growth factor (bFGF, FGF-2). It came out that the application of bFGF induces the expression of mRNA for cyclooxygenase-2 (COX-2). In addition, bFGF stimulated the production of prostaglandin E2 (PGE2), which is a potent stimulator of bone resorption, and induces osteoclast formation. The authors of the study conclude that BDECs are engaged in bone remodeling by the modulation of expression of COX-2, and subsequently PGE2 production. Other studies prove (on cell cultures of rat osteoblastic cell line and fetal rat calvariae) that prostaglandins, especially PGF 2α, cause an increase of mRNA and protein of FGF-2 in the bone cells. The authors suppose that the regulation with FGF-2 prostaglandins is mediated by a PGF2α-selective receptor that affects protein kinase C (S2). Also, the PGE2 in the studies on cell cultures induces the expression by the IL-6 osteoblasts (synergically with LPS) (M7, R2).

2.4. Nitric Oxide

Nitric oxide (NO) is considered to be a factor that participates significantly in bone remodeling, especially as a mediator of cytokines, and their activities in bone tissue. In cell cultures, the presence of NO leads to an increase of activity of alkaline phosphatase, and to an increase of the number of calcified nodules in the primary line of bone osteoblasts (C3). Low NO concentration, on the other hand, led to increased osteoclast formation. Damoulis and Hauschka (D1) arrived at a similar conclusion: the proinflammatory cytokines induce production of NO in various types of cells, including osteoblasts and osteoclasts. It is obvious that each of these cytokines can interfere in the process of bone remodeling independently of the permissive effect of NO.

3. Regulation of the Apoptosis of Osteoclasts

Regulation of the degree of apoptosis, or programmed cellular death, is another possible mechanism of regulation of the activity of osteoclasts. The degree of osteoclastic apoptosis seems to be given by the depth of the resorbed regions that these cells form in the bone. This “apoptotic self-limitation” might be a result of insufficiency of the oxidative biosynthetic pathways in actively resorbing osteoclasts. The ability of osteoclasts to undergo apoptosis was first demonstrated in 1992, when it was proven that DNA of the osteoclasts and their precursors exhibits

fragmentation into characteristic 180-bp ladders. Apoptosis of the osteoclasts can be stimulated by a number of substances that block bone resorption, for example, estrogens, TGF-beta, bisphosphonates, and OPG/OCIF. On the other hand, the fact that calcitonin increases the viability of osteoclasts is rather surprising. Substances that stimulate bone resorption also suppress the apoptosis of the osteoclasts, and increase their viability. This group includes, for example, the already-mentioned osteoprotegerin-ligand (OPG-L), but also ascorbic acid, and some cytokines. Inhibition of apoptosis is mediated by the activation of NFκB, and by the inhibition of caspase activity (G11, G12).

4. Hormones

4.1. Dehydroepiandrosterone (DHEA)

During human life, changes in concentration and activity of many hormones occur. One of the very interesting hormones is the adrenal androgenic steroid dehydroepiandrosterone (DHEA). It is widely known that significant association exists between changes in circulating concentrations of DHEA and the incidence of malignant diseases, atherosclerosis, Alzheimer's disease, and other age-related diseases. Pharmacological activity of DHEA in rodents or rabbits includes many beneficial effects, for example, improvement of immune functions, prevention of atherosclerosis, cancer, diabetes, or obesity; even memory improvement has been described. Clinical studies with DHEA supplementation in elderly people have shown that DHEA application leads to increase of many hormones with a direct relationship to bone remodeling, for example, insulin-like growth factor-I (free and total), testosterone, dihydrotestosterone, estrone, and estradiol. However, the question remains unanswered to what degree these hormonal changes are beneficial for the organism (N4). Gordon *et al.* (G10) also proved subnormal concentration of DHEA in the female patients with anorexia nervosa and with subsequent loss of bone mineral density (BMD).

4.2. Phosphatonin

Renal elimination of phosphorus is regulated by PTH, calcitriol, growth hormone, insulin, and insulin-like growth factors. While the above-mentioned hormones show a number of other effects, the existence of a hormone affecting selectively the extracellular concentration of phosphorus is expected. The task of this hormone with phosphaturic effect has been speculated about from the beginning of the 1990s. It was named phosphatonin, and its increased concentration is found especially in patients with osteomalacia (tumor-induced osteomalacia, X-linked hypophosphatemic rickets, epidermal nevus). The phosphaturic effect of

phosphatonin is based on selective inhibition of sodium-dependent reabsorption of phosphates by the cells of proximal renal tubules without influencing other transport processes. The condition exhibits increased clearance, and increased fractional excretion of phosphate, hypophosphatemia, normal PTH, and PTHrP (parathormone-related peptide); there is osteomalacia as a clinical condition. Upon removing the source of phospatonin increase, both bone and laboratory findings return to normal. The condition may be also caused by different mechanisms. In the case of X-linked hypophosphatemia, we are dealing with a disturbance of the elimination of phosphatonin from the circulation in genetically based deficit of membrane endopeptidase. The particular gene is marked as the PHEX gene. In the case of tumor-induced hypophosphatemia, the condition is caused by excessive production of phosphatonin by the tumor. In patients with chronic renal failure and hyperphosphatemia, the concentration of phosphatonin is increased adequately as an expression of a homeostatic mechanism that can prevent the development of secondary hyperparathyroidism. Reduced concentration of phosphatonin is found in calcinosis of tumorous origin with hyperphosphatemia, increased level of calcitriol, and pathological deposits of calcium and phosphorus (D13, K6).

4.3. Prolactin

Prolactin is a polypeptide secreted by the pituitary gland and other tissues. Its activity is connected with binding on a specific membrane receptor (PRLR) belonging to the group of cytokine receptors (class I cytokine receptors). The receptor appears on cellular membranes of various tissues. Hyperprolactinemia, especially when untreated, is included among the causes of osteoporosis. The reason is especially hypoestrogenism induced by hyperprolactinemia, but neither is excluded direct effect of prolactin on the skeleton, probably by influencing calcium homeostasis. In conditions with prolactin increase (tumors of the pituitary gland, pregnancy, primary hypothyrosis, application of neuroleptic drugs, opioids, physical and mental strain, infection with herpes viruses, effect of vasoactive intestinal peptides), the concentration of the markers of both osteoresorption (NTx, deoxypyridinoline) and osteoformation (osteocalcin, bone alkaline phosphatase) increase, and the bone density of lumbar vertebrae and the hip drops. Normalization of prolactin in both men and women, induction of regular menstrual periods in women, and normalization of testosterone level in men can be achieved by surgical removal of the adenoma, or by the application of dopamine agonists. The latter include substances that stimulate dopaminergic D2 receptors (bromocryptine, cabergoline, pergolide, and quinagolide). When the prolactin level is reduced early, no long-term consequences to the skeleton will develop. It is being proven that after the application of dopamine agonists, bone density rises, and the markers of osteoresorption and osteoformation settle. However, these changes are not significant in all studies performed (D12, S8).

4.4. Gonadotropin-Releasing Hormone (LH-Releasing Hormone, Gonadorelin, GnRh)

GnRH is secreted by the hypothalamus, and acts through the specific receptor (GnRH receptor). GnRH increases the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH). Rarely, hypogonadism can present in a point gene mutation for the GnRH receptor, but more frequently, hypogonadism is induced by the application of synthetic superactive analogs of gonadoreline (busereline, gosereline, triptoreline, leuproreline) for various reasons (pubertas praecox, endometriosis, leiomyoma of the uterus, prostate cancer, breast cancer, sexual aberration). After a short stimulation phase, the GnRH agonists suppress the secretion of the gonadotrophic cells of the pituitary gland, and reduce both testicular and ovarian hormone production (chemical castration). Long-term application of the agonists of gonadoreline increases the risk of osteoporosis. Estrogen levels drop to postmenopausal values and the loss of bone mass accelerates, with uncoupling of the harmonization of osteoclastic resorption and osteoblastic formation (uncoupling effect); markers of bone resorption rise significantly, while markers of bone formation either stay unchanged or rise only slightly. At the same time, IGF-I and IGF-II rise after the application of GnRH agonists. The treatment of premenopausal women with gonadoreline agonists over a period of 16 weeks to 6 months led to a drop in bone density and a rise of pyridinoline, deoxypyridinoline, NTx, and CTx. The most substantial increase was marked by CTx; the increase continued for another 12–24 weeks after termination of the treatment. NTx has similar diagnostic ability in this condition, while pyridinoline and deoxypyridinoline are less reliable (N3, T1), and their increase is similar to the increase of bone ALP or osteocalcin (N3). The changes in the skeleton are reversible, but the restitution can last for up to several years. An initial value of T-score lower than -1.0 before the beginning of treatment is connected with the risk of fracture; this comes into consideration especially in postmenopausal women. Changes in the skeleton may not be obvious in all cases, or they can be prevented. When treatment with GnRH agonists was performed in young women for precocious puberty, changes in the skeleton were not proven, were reversible, or could be prevented by early supplementation with calcium. In men with androgen blockade for prostate cancer, the skeleton changes could be prevented with intermittent cyclic etidronate therapy (A2, D10, H5).

4.5. Growth Hormone (GH)

It is also obvious that growth hormone has an absolutely essential influence on BMD and bone turnover. GH treatment in GH-deficient adults led to an increase of BMD in 30–36 months, with a following plateau phase. In a similar period of time, bone turnover normalized. Adult patients with GH deficiency and osteopenia

or osteoporosis are recommended to be supplemented with GH for a period of 3–4 years (V2). Chapurlat and Delmas (C4), in their study, concluded that the growth hormone stimulates the osteoblasts to increase the synthesis of insulin-like growth factor-I, and so to increase bone formation (bone formation action of osteoblasts). The study of Toogood and Shalet (T4) led to the same conclusion. Although the GH/IGF-1 axis has now been relatively well explored, some authors assume that GH will not become a clinically utilizable substance for bone mineral density (BMD) improvement in patients with osteoporosis (M2).

Growth hormone influences especially the preosteoblasts, preosteoclasts, and mature chondrocytes. Chondrocytes under the influence of growth hormone produce IGF-I and TGF-beta. In epiphyseal growth cartilage, the uptake of sulfate is increased, as is mitogenesis, chondrocyte proliferation, and bone vascularization. Insulin-like growth factors (IGFs) and their receptors are under the influence of growth hormone. GH increases the concentration of IGF-I, IGF-II, IGFBP-3, and IGFBP-4, and reduces IGFBP-1 and IGFBP-2. Growth hormone supports, through IGF-I, the activity of osteoblasts, and stimulates the synthesis of collagen. Lower bone density of the lumbar vertebrae and the hip can be found more in patients with diabetes of type I than in those of type II. In type I diabetics, lower bone density is connected with higher concentrations of IGFBP-1 and IGFBP-4, and lower concentrations of IGF-I and IGFBP-5.

GH participates in the stimulation of calcitriol production. Application of GH has a positive effect on bone markers and bone density with treatment duration for at least 6, but better 12–36 months. The anabolic effect of GH is more apparent in young individuals and in males; good effect can be proven in menstruating females with sufficient estrogen level. After the application, bone markers of osteoformation and osteoresorption rise concomitantly; however, with continuing treatment the concentrations may return to the initial values. During the whole treatment, IGF-I remains increased. In postmenopausal women, the effect of the application of GH is questionable. Markers of osteoformation (osteocalcin and propeptide of procollagen type I) rise, IGF-I also rises, but the bone mass does not increase, and in some locations it can even decrease (S1, S5).

4.6. Parathyrine, Parathormone, Hormone of the Parathyroid, Intact Molecule of PTH

PTH, besides its well-known activity, increases the number of osteoblasts, as well as the number and activity of osteoclasts, but it reduces the activity of osteoblasts. PTH causes the activation of nonlysosomal protease calpain. Calpain attacks the osteoblasts, and provides direct access to the surface of mineralized bone for osteoclasts, with resulting increase in bone resorption. PTH activates renal D-1-α-hydroxylase, and so it increases the conversion of 25-hydroxyvitamin D3 to 1,25-dihydroxyvitamin D3. Increased concentration of 1,25-dihydroxyvitamin D3

leads to increased intestinal calcium absorption. For the above-mentioned reason, and also because of the increased reabsorption of calcium in renal tubules caused by PTH, as well as osteoresorption, the activity of Ca^{2+} in plasma rises, and as a result of a feedback, the secretion of PTH drops. The following drop of Ca^{2+} causes release of PTH, and the concentration of Ca^{2+} normalizes again in the above-mentioned way. PTH also exhibits a direct phosphaturic effect, with the development of hypophosphatemia (stimulation of adenylate cyclase with the increase of intracellular cAMP in renal tubules).

PTH is synthesized in the parathyroid gland as a chain of 115 amino acids (Pre–Pro–PTH), broken on the chain of 90 amino acids (Pro–PTH), and eventually on intact PTH (84 amino acids, MW 9425) which is stored in secretory granules; from there, it is released as a bolus when the Ca^{2+} level in the ECF drops. Biological activity is connected with the first 34 amino acids of the N-terminal part of the molecule. Degradation of the intact PTH takes place in the parathyroid after the break-off of the C-terminal fragment, which is biologically inactive. The circulating pool of PTH consists of 5–25% intact PTH (concentration about 1–2 pmol/liter), and 75–95% C-terminal fragments (concentration about 10–20 pmol/liter). The pool of intact molecules is completed only from secretory granules; the pool of C-terminal fragments comes from the parathyroid, liver, and kidneys (in the liver and kidneys, the C-terminal fragment also breaks off the intact molecule). C-terminal fragments are eliminated by glomerular filtration. The pool of N-terminal fragments (biologically active) is very small; it is the biologically active part of the molecule which is preferentially caught by the bone. Intact PTH has a half-life of 5 min. The C-terminal fragment has a half-life of 30–40 min. Half-life is extended in renal failure to 1–2 h. The drop in calcemia leads to the release of PTH from secretory granules. Hypercalcemia blocks the release of PTH. The gene for PTH receptor type-1 (PTHR-1) is one of the candidate genes for primary osteoporosis because a relationship exists between PTHR-1 and bone density (D14). A sensitive indicator of the activation of the receptors for PTH, either by biologically active PTH or by PTHrP, is the urinary cyclic adenosine monophosphate (U-cAMP). Renal tubules are activated by the N-terminal fragment of PTH (or PTHRP), and in direct connection with this stimulation, cAMP is secreted in the tubules (99%). U-cAMP is excreted in direct dependence on the stimulation by PTH.

Dispersion of physiological concentrations of parathyrine in blood serum influence diurnal rhythm, hourly pulsation, oral load with calcium, exercise, pregnancy, age, and menstrual cycle. There are no sex differences. Medication, resection of a tumor causing hypercalcemia, hypertension, and urolithiasis are the causes of pathophysiological mechanisms that also influence the blood level of parathyrine. Establishing the intact PTH provides sufficient reliability and comparability of the results provided the preanalytic phase is maintained.

Hypoparathyroidism manifestates as osteomalacia with a low number and activity of both osteoclasts and osteoblasts. Osteoid formation is reduced, and its

mineralization is even lower, and that is why the result is a surplus of unmineralized osteoid. From the histological point of view, both mineralization and osteoid formation are disturbed in the sense of the formation of unmineralized osteoid. Laboratory signs include reduction of 1,25-dihydroxyvitamin D3; in the PTH deficit, its stimulatory effect on the osteoclasts and osteoblasts is absent, and the number of osteoblasts and osteoclasts is reduced. A drop in concentration of PICP, ICTP, bone ALP, and osteocalcin is found. The spectrum of musculoskeletal abnormalities is interesting, including the calcification and ossification of the soft tissues, osteosclerosis, intracranial calcification, and ankylosing spondylitis. These signs are less common in iatrogenic-induced hypoparathyroidism.

Hyperparathyroidism can be primary (primary adenoma of the parathyroid), secondary (increased synthesis of PTH, for example, in chronic hypocalcemia, or in the defect of the receptors for calcitriol), or tertiary (hyperplasia of permanently stimulated parathyroid). In usual slight or moderate forms of primary hyperparathyrosis, bone density must not be significantly reduced; the changes are predominantly slight, and expressed on the appendicular skeleton. Typical X-ray finding can be found in approximately 20% of individuals; scintigraphic changes are more common (in about 50% of patients). First X-ray findings are on phalanges, where subperiostal resorption and increased cortical porosity can be found. Cortical stripes or cystic changes of the type of Recklinghausen's bone disease are found in more advanced forms, and osteosclerosis is sometimes found. The number of remodeling units is increased, and histologically, an increased number of the osteoclasts and osteoblasts is found, but the activity of the individual osteoblasts is reduced.

4.7. Parathormone-Related Peptide (PTHrP)

Humoral hypercalcemia in malignant diseases (HHM) can be caused by the presence of a peptide structurally similar to parathormone (PTHrP). In malignant disorders, the direct destruction of the bone by the tumor can also be responsible for hypercalcemia, although the increase of the level of PTH is not excluded as its cause (PTH is usually suppressed). The concentration of PTHrP can be increased even without hypercalcemia, but usually the changes in its level follow the changes of calcemia, including relapse of the tumor, or the drop of calcemia after successful resection. PTHrP is a protein (MW 16 to 17,000, 1–139, 1–141, 1–173 amino acids) synthesized in tumors, but also in normal tissues, for example, endocrine tissue (pancreatic islets, keratinocytes, fibroblasts, bone marrow cells, parathyroid, pituitary gland and hypothalamus, cortex and medulla of the adrenals, brain, gastric mucosa, lactating mammary gland, and placenta). The N-terminal part of the molecule (at least 8 of the first 13 amino acids) is identical with parathormone and the effect is identical with PTH, because PTHrP is able to bind on the receptors for PTH with their subsequent activation. The effect of PTHrP is identical with the

bone and renal effects of PTH, that is, increased bone resorption and increased renal excretion of phosphates and cAMP with usual laboratory findings (hypercalcemia, hypercalciuria, hypophosphatemia, hyperphosphaturia, increased urinary cAMP). With increased concentration of PTHrP, the receptors for PTH are activated, and the normal relationship between the bone resorption and formation is disconnected in disorders of bone collagen formation. The serum level of osteocalcin in tumors with hypercalcemia is usually low, and the levels of urinary hydroxyproline are high. Levels of PTH (when only the intact molecules of PTH are included) as a result of eutopic or ectopic secretion rise in tumors very rarely; on the contrary, in hypercalcemia they are reduced or undetectable (D5, P2).

4.8. 1,25-Dihydroxyvitamin D3 (1,25-Calcitriol), 25-Hydroxyvitamin D3 (25-OH-D)

Vitamin D is a hormone, not a real vitamin. Two principal forms with the same biological properties and metabolism exist, vitamin D2 and vitamin D3. They differ only by the structure of the side chains. Chemically, they are fat-soluble secosteroids. Humans obtain vitamin D from food, and also produce it under the influence of ultraviolet rays (with a wavelength of 230–313 nm) in the skin. Everyday exposure to the sun for a period of 15 min will do to reach sufficient plasmatic levels. Active metabolite 1,25-dihydroxyvitamin D3 increases the absorption of calcium in the small intestine and increases the resorption of calcium in the bone. Vitamin D formed in the skin or taken in with food reaches the circulation, where it is bound to protein (DBP, vitamin D-binding protein). It is transported to the liver, where it is hydroxylated in position 25 to 25-hydroxyvitamin D. This 25-hydroxyvitamin D is the main form of vitamin D in circulation, and it is a precursor for dihydroxylated metabolites. In the circulation, 25-hydroxyvitamin D is bound again to a binding protein, and it is transported to the kidneys where it is hydroxylated in position 1 to a biologically active metabolite, 1,25-dihydroxyvitamin D. It can also be produced in other tissues in which the active D-1-α-hydroxylase is present (placenta, activated macrophages and monocytes, in tuberculosis and non-Hodgkin lymphomas). Besides 1,25-dihydroxyvitamin D, in the circulation can be found other metabolites, being formed also in the kidney. Production of 1,25-dihydroxyvitamin D is stimulated by PTH, and probably by the growth hormone, prolactine, and estrogens; it is inhibited by its own product, phosphates (for example, in renal failure with hyperphosphatemia), and probably by calcium ions.

4.9. Glucocorticoids

The principal result of the effect of glucocorticoids on the bone and its metabolism *in vivo* is in the reduction of number of bone cells, drop in quantity of remodeling units, reduction of the absorption of calcium in the bowel, and development

of renal hypercalciuria caused by decreased reabsorption of calcium in the kidney. The changes in calcium homeostasis lead to hyperparathyroidism, which can be prevented with the application of calcitriol, or calcium supplementation. Therapy with corticoids does not interfere significantly with metabolism of vitamin D; however, changes in synthesis of the hormones of the adrenal cortex may appear, as well as in the production of testosterone. In chondrocytes, synthesis of mucopolysaccharides decreases. The effect on bone cells demonstrates as divergence of formation and resorption (uncoupling effect) when the activity of osteoblasts with reduced osteoformation decreases while the resorption either remains unchanged or may rise in some cases. Negative effect on osteoformation may appear after small even doses of corticoids, equivalent to 5–10 mg of prednisone per day. Most often it is the trabecular structure in the axial skeleton that is affected, however, with the application of the corticoids; also, the increased risk of hip fracture exists. Feedback drop in the secretion of ACTH during treatment with glucocorticoids leads to secondary drop of the secretion of dehydroepiandrosterone (DHEA) in the adrenal cortex. The anabolic effect of DHEA is thus reduced, and, on the other hand, the catabolic effect of glucocorticoids rises. The situation is worse in females, where DHEA is necessary for androgen-dependent anabolic processes. In males, predominant production of androgens (testosterone) is maintained in the testicles, and that is why the consequences of the decrease in DHEA production are not so important. With the application of glucocorticoids, decrease of the markers of osteoformation is described (osteocalcin, bone ALP, PICP). The changes in osteoresorption markers are not so unambiguous. All the increases, decreases, and unimportant changes have been described (ICTP, hydroxyproline, pyridinoline, deoxypyridinoline). If glucocorticoid therapy cannot be interrupted, or substitution with preparations with little negative effect on the skeleton (deflazacort) is impossible, supplementation with vitamin D and calcium is necessary, as well as the application of calcitonin, DHEA, and estrogen in females, and testosterone in males, respectively, therapy with bisphosphonates (L2, S5).

4.10. Hormones of the Thyroid Gland

Hormones of the thyroid stimulate bone remodeling; both trabecular and cortical bone are damaged. Bone damage caused by thyroidal hormones originates from disorder of the homeostasis of calcium, and its effect on the bone cells. Calcium homeostasis changes with regard to reduced intestinal calcium absorption. Because of the bone calcium resorption, there is a shift toward hypercalcemia, and this is why the production of PTH decreases. It is also why the activity of D-1-α-hydroxylase in the kidneys declines, as well as the concentration of 1,25-dihydroxyvitamin D3. Its shortage contributes to a decrease in the intestinal resorption of calcium. Osteoclasts and osteoblasts are activated, and cytokines play the principal role in this activation (IL-1, IL-6). Together with osteoresorption,

osteoformation is also activated. That is why the level of the markers of osteoformation and osteoresorption can be higher. The activation of the osteoblastic T3 receptor supports the production of signal peptides and the differentiation of the osteoclasts. Their increased activity is compensated to only a certain degree by the increase in osteoblastic activity. When the T3 receptors are stimulated permanently, osteoresorption outweighs osteoformation. The surface of the bone, where the resorption is in progress, grows larger. Hyperthyroidism (early manifested with the suppression of TSH), and the developed thyrotoxicosis are connected with lower bone density and intensified bone remodeling. After treatment of thyrotoxicosis is initiated, osteoresorption decreases very quickly (in the course of a few months), as can be evaluated with a help of changes in the levels of particular bone markers (deoxypyridinoline, telopeptide, ICTP). However, accelerated osteoformation is adjusted more slowly, and that is why the divergence of formation and resorption can be observed, as in this case, with positive consequences for the skeleton in the situation of persisting increased osteoformation. The situation is complicated after total strumectomy, when the changes in the skeleton can be a result of the decrease in calcitonin, previous hyperthyroidism, or can be caused by iatrogenic subclinical hyperthyroidism after thyroidectomy. Reduced bone density in suppressive therapy with L-thyroxin is not the rule, though. In hypothyroidism, growth retardation is observed, the number of osteoblasts and osteoclasts is in decline, and the bone is inactive. As opposed to hyperthyroidism, in hypothyroidism there is a tendency toward an increase in PTH.

4.11. Anabolic Steroids

With regard to the development of osteopenia or osteoporosis with hypogonadism in males, lack of androgens is considered as a risk factor from the point of view of the quality of the bone. Anabolic steroids act on the bone probably through the androgen receptors on the osteoblasts, but an inhibitory effect on the osteoclasts is also possible. The condition affects mainly the cortical phase of the bone. When anabolic steroids are applied in females, they are usually combined with estrogens. Their application acts preventively against the loss of bone mass, stimulates osteoformation, and reduces the risk of hip fracture.

5. Proinsulin, Insulin, Insulin-like Growth Factors (IGF), IGF-Binding Proteins, and Changes in Bone Metabolism in Diabetes Mellitus

Osteopenia and osteoporosis are parts of the symptomatology of diabetes mellitus. Although the relationship between the occurrence of fractures, the degree

of reduction of bone density, the markers of the formation and resorption, and the compensation of diabetes has not been confirmed, many pathophysiological processes exist that contribute to osteopenia and osteoporosis in diabetics. The basis for osteopenia is probably different in diabetes type I and type II. In diabetes type I, genetic factors play a role, for example, the polymorphism of the receptor for vitamin D. In type II diabetics, the development of osteopenia can decelerate with regard to hyperinsulinism and obesity. In addition, in women with diabetes type II, higher androgenic activity plays a role. Generally, the bone of a diabetic is inactive, has a small number of remodeling units, histologically, the tissue is poor in cells, and little osteoid is formed. Hypertrophy of the intestinal mucosa leads to increased absorption of calcium in the bowel. Resulting reduction of PTH and calcitriol contributes to inactivity of the osteoblasts, and the secretion of collagen and mucopolysaccharides is reduced. Lower concentrations of the markers of osteoblastic activity (PICP, bone ALP, and osteocalcin) may be a sign of reduced osteoformation. Reduced osteoformation probably appears in utero in fetuses of diabetic mothers, where lowered concentrations of the markers of osteoblastic activity in the amniotic fluid were established. Markers of increased osteoresorption report only a part of studies in diabetics. Setting the genotype COL1A1 could contribute to evaluation of the risk of osteopenia in diabetics. The data about bone density are not constant, and some of the studies do not prove a reduction in density, neither in diabetes type I nor in type II. Proinsulin concentration in type 2 diabetics correlates positively with bone density. When albuminuria is used as an indicator of the compensation of diabetes, the more significant albuminuria is associated wih lower bone density, lower IGFBP-5, and higher IGFBP-4 (J1). Diabetics with low concentrations of IGF-I, IGFBP-3, and IGFBP-5 can be considered high-risk patients; the risk is also higher in diabetics with a higher concentration of IGFBP-4. The role of PTH is important for the production of IGF (H4, J1).

6. Biochemical Markers of the Metabolism of Bone Tissue

Monitoring bone turnover and bone remodeling using biochemical methods is possible with the help of follow-up of two types of parameters.

1. Activity of the cells participating in bone remodeling. Usually it is the activity of the specific enzymes characteristic for the osteoblasts (especially alkaline phosphatase and its bone isoenzyme) or the osteoclasts, respectively (acid phosphatase or its bone isoenzyme)
2. Measuring the concentration of the components of the bone matrix that are partially released during the buildup of new bone (osteocalcin, some types of

procollagen), or during its resorption (telopeptides, pyridinoline, and deoxypyridinoline).

These parameters are the most often used indicators of the laboratory monitoring of bone remodeling; they can be used both for considering the speed of bone turnover (which is a "bad omen" for the quality of the bone), and for monitoring therapeutic effect. Methods used to establish their activity and concentration are not homogenous so far and not well standardized. In addition, all laboratory parameters of bone metabolism possess significant intra- and interindividual variability, diurnal rhythm, and are influenced by many factors. Those important of them will be mentioned in the following text.

Systemic markers of bone remodeling can be divided into (C2, D9, E1)

Markers of bone formation produced by osteoblasts
Markers of bone resorption produced by osteoclasts
Products of collagen type I degradation

The fact that the mentioned markers of bone remodeling change their concentration and activity in various diseases of the bone tissue leads to the following table.

Changes of Markers of Bone Remodeling in Some Common Types of Bone Diseases (P1, R1)

Diagnosis	Bone formation	Bone resorption
Osteoporosis	↑↑	↑
Hyperparathyroidism	↑↑	↑↑
Hyperthyroidism	↑↑	↑↑
Paget's disease	↑↑	↑↑
Inflammatory processes	↑↑	↓

6.1. General Potential of Bone Markers in the Diagnosis and Treatment of Bone Diseases

Results of the measuring of bone markers can provide us with more or less straight answers for the following questions (B4, W2):

Has the patient an increased level of bone remodeling?
Can the patient be diagnosed with osteoporosis?
Can we evaluate the extent and the speed of the bone mass loss, and the connected risk of a fracture with it?
Can the treatment be optimized with the help of the results?
Can we perform quick evaluation of the patient's response to treatment?

6.1.1. *Tartrate-Resistant Acid Phosphatase, Marker of Bone Resorption (TRACP, EC.3.1.3.2 5-TRACP)*

5-TRACP is the bone isoform of the enzyme EC.3.1.3.2, synthesized on the membrane of the osteoclast. It is one of the five known isoforms of this enzyme. We can measure its catalytic concentration (activity) or mass concentration by immunoanalytic methods. Measuring catalytic concentrations after the inhibition of other isoforms with the addition of sodium-potassium tartrate exhibits low analytical selectivity and sensitivity, and suffers from low stability of the activity in biological material. It can be unfavorably influenced by hemolysis, and the differences between the serum and the plasma (ACP form the blood platelets). That is why the use of immunoanalytical methods, measuring the mass concentration of the isoenzyme 5-TRACP, is necessary. Competitive methods type EIA (C5) were used; currently, noncompetitive methods with two antibodies have been developed in the form of diagnostic kits. Cheung *et al.* (C5) used a competitive method with one catch antibody marked with peroxidase, anchored on microtitration platelets with a colored detection reaction. Analytic signal was recounted with the help of TRACP calibrators, obtained by purification and isolation from the umbilical cord blood in μg/liter of protein TRACP. Nakasato *et al*. (N2) described both a competitive method with one antibody (EIA) and a noncompetitive method with two antibodies. They used the competitive method to measure the catalytic activity of TRACP using the 4-nitrophenylphosphate substrate, and the noncompetitive method to measure the TRACP protein. Measuring the TRACP protein with the use of two antibodies was also described by Halleen *et al*. (H1). In addition, they performed testing of several alternative antibodies and the choice of the most selective of them with the lowest share of the cross-reaction with other isoforms of ACP or ALP, respectively. Currently, the following statements seem to be conclusive:

In children and postmenopausal women, TRACP is significantly higher than in men and premenopausal women.
With estrogen therapy, a decrease of TRACP by up to 70% was observed.

The usefulness of TRACP in clinical practice is not evident so far, but the development of sufficiently selective and reliable methods, being the first precondition of this usefulness, has currently made significant steps ahead.

6.1.2. *Deoxypyridinoline, Pyridinoline (DPD, PYD)*

There are pyridine cross-links between the parallel chains of collagen of bone mass, namely, between the lysine amino acid residues (PYD), and between the lysine and hydroxylysine residues (DPD). They are found in nonhelical terminal (N- and C-terminal) regions of collagen macromolecules—telopeptides. They are the final products of the biodegradation of collagen excreted to urine in the kidneys.

While DPD is generally considered a degradation product of the bone collagen type I, the presence of PYD in urine can be also attributed to the degradation of collagen of other connective tissues (cartilage, skin, etc.). DPD and PYD are generally considered useful indicators of increased bone remodeling, with DPD being considered more selective for the above-mentioned reasons (R4, S7, W4). In urine, both analytes are found either in the free form (f-DPD, PYD), or bound on peptides, with the free fraction being reported at about 40% of the total level. Most often, and in practice most easily, the free fractions are set with a method of competitive EIA. To measure the total level, acid hydrolysis must be performed to release the DPD and PYD from the peptide binding. In this case, especially HPLC with fluorescent detection is used; this method uses the natural fluorescence of the measured analytes.

6.1.3. *Hydroxyproline*

Collagen contains large amount of hydroxyproline, a substance created by post-translational hydroxylation of proline. That is why hydroxyproline has long been used as an indicator of bone resorption. It is, however, released not only from the bone tissue but also from other connective tissues, from the components of the complement, and in addition, there is a share coming from nutrition, and also from special gelatine diet. Only about 50% of hydroxyproline in urine originates from the degradation of bone collagen type I (C8, D2). For this reason, measurement of hydroxyproline in urine seems to be obsolete, because other products of collagen degradation are not so influenced.

6.1.4. *N-terminal Telopeptides*

N-terminal telopeptides are released to urine during bone mass resorption. Their origin is in telopeptide areas of collagen type I. Telopeptides contain the above-mentioned pyridine cross-links. They are marked as NTx. NTx is part of the N-terminal telopeptide limited by the immunoreaction with a monoclonal antibody oriented against the α-2 chain, isolated from the urine or bone by incubation with bacterial collagenase and used as an immunogen. They are measured in urine with competitive EIA or in serum with the same method, or using chemiluminiscent detection. The methods for measurement in urine are well known; measurement of NTx in serum belongs to the new means of follow-up of bone remodeling, and there is not too much data about it yet (G8, H3, S4). NTx measurement results are given in units called bone collagen equivalents (BCE). This is the molar concentration of collagen type I that provides the same immunoreactivity as the equivalent NTx.

6.1.5. *C-terminal Telopeptides*

C-terminal telopeptides are analogs of the NTx from the C-terminal area of telopeptides. They enter the circulation, and are excreted into urine under the same conditions. The appropriate analyte is marked as CTx (with the original company

name CrossLaps). It is limited by a monoclonal antibody against the octapeptide of the α-1 chain of the C-terminal telopeptide with a sequence of amino acids given by the epitope EKAHDGGR (Glu-Lys-Ala-His-Asp-Gly-Gly-Arg). CTx is not a homogeneous analyte, and, under various conditions it contains various concentration of a β-isomer, when the isomeration appears at the sequence β Asp-Gly. Nonisomerized CTx is sometimes called α-CTx. Methods of measurement in urine are usually based on the principle of competitive EIA; measuring methods in serum use the format of noncompetitive EIA (ELISA), and usually determine β-CTx. As in the case of NTx, neither by determination of CTx is the benefit of the newly introduced measurement in serum against the well-established measurement in urine not confirmed (B7, B8, C6, F1). It is known that the ratio β-CTx/CTx increases with age and lower quality of bone mass (G4).

6.1.6. *Bone Alkaline Phoshatase (BALP EC3.1.3.1.)*

Alkaline phosphatase is an enzyme of the cellular membranes. Its isoforms can be found in liver, digestive tract, placenta, and some tumor tissues. Bone isoform, BALP, is a membrane enzyme of the osteoblasts. Bone, liver, and intestinal isoforms are the posttranslational modifications of the same isoenzyme exprimed by the same gene, and the difference among them lies in various ways of reaction with a saccharide component, sialic acid (M9).

Establishing the total ALP as an indicator of bone remodeling can be useful only in some limited cases when the activity of ALP is significantly increased, and the shares of other isoforms stay unchanged (as in Paget's disease). In other cases, the differentiation of BALP from other isoforms is necessary. Thermoinhibition uses the lower stability of the bone fraction at increased temperature, but for the purpose of monitoring of bone remodeling it is only slightly reproducible, and practically useless for quantification. To quantify BALP, methods were developed using lectin precipitation, electrophoretic separation, and especially immunoanalysis.

The method of lectin precipitation is based on selective precipitation of BALP with wheat germ lectin. The difference between the catalytic concentration before the precipitation and after can relatively successfully quantified in the form of the catalytic concentration of BALP (R7).

Electrophoretic separation is preceded by adjustment of the sample, usually by enzymatic cleaving with neuraminidase. Separation can be further combined with use of lectin, or with thermoinactivation. Excellent analytical selectivity of the method can be achieved by electrophoretic separation, but for routine use it provides only barely reproducible results in connection with its analytical robustness. An example of a successful application of the electrophoretic separation of BALP as a bone marker are the studies of VanHoof (V3). Currently, the need for an immunoanalytical measuring as a method of choice clearly dominates.

Immunochemical methods can be considered exact, fast, sensitive, and robust. However, they still show decreased analytical specificity because of the

cross-reaction with the hepatic isoform of ALP. The intensity of the cross-reaction depends on the catalytic concentration of the hepatic form in the sample, and also on the type of analytical system used (B10, G1, G9). Another certain disadvantage of the immunoanalytical methods is the nonuniform expression of the results of measurement. While the AlkPhase (Metra) diagnostic kits use activity units (U/liter), defined for the temperature of 25°C, the Tandem-R-Ostase and Tandem-MP-Ostase (Beckman) diagnostic kits produce the measurement results as a mass concentration of proteins (μg/liter).

6.1.7. *Osteocalcin*

Osteocalcin is an essential noncollagen protein of bone mass, synthesized exclusively in the osteoblasts. The intensity of the biosynthesis depends on the concentration of 1,25-dihydroxyvitamin D. The molecule consists of 49 residues of amino acids, and has a relative molecular weight of 58,000. Post translation, three gamma-carboxyglutamyl residues are added. In this reaction, vitamin K plays an important role as a cofactor of the enzyme carboxylase. Posttranslational binding of gamma-carboxyglutamyl takes place at positions 17, 21, and 24. Osteocalcin forms about 10% of the protein matrix of bone mass. With regard to the location of its production, an effect on the production of bone mass, and osteoformation, is expected; practically, however, under the influence of many circumstances, a clear causal relationship between the concentration of OC and the intensity cannot be proven. Synthesized and posttranslationally changed osteocalcin is incorporated into the bone matrix, where it is firmly bound with hydroxyapatite and contributes to the bone mineralization. Some amount is also released to the circulation. The shares of free and bound component are not constant, changing in the interval from 60% to 90%. The part released to the circulation depends not only on the amount of synthesized osteocalcin but also on the amount of osteocalcin incorporated into the bone, on the share of the undercarboxylated osteocalcin that is worse to be built into the bone matrix because of the reduced affinity to hydroxyapatite, and also on renal function (D4, D7, P10).

An important source of disproportion between osteocalcin synthesized in the osteoblasts and that in the circulation is its instability, caused by quick proteolytic breakdown. Only the circulating osteocalcine can in causal relationship with the bone formation. However, its level is difficult to set. The difficulty of the situation is increased by a very short biological half-life. Osteocalcin is quickly eliminated from the circulation by the kidneys. In the molecule of osteocalcin there are two arginine residues that mark the regions accessible to proteolytic-tryptic hydrolysis—amino acid residues 19 and 43. The result is formation of peptide fragments 1-19, 20-43, 1-43, 44-49, and 20-49. The easiest to break off is the C-terminal fragment OC 44-49. The most stable region of the molecule is the peptide OC 20-43 (mid-fragment). Various peptide fragments exhibit different immunoreactivities (G2), see Table 1.

TABLE 1
IMMUNOREACTIVITY OF VARIOUS FRAGMENTS OF OSTEOCALCIN

Fragment	Peptide	Relative immunoreactivity (%)
Intact	1–49	36
N-terminal-mid	1–43	30
N-terminal	1–19	14
Mid	20–43	15
Mid-C-terminal	20–49	5
C-terminal	44–49	5

Source: Garnero *et al.* (G2).

Fragmentation of osteocalcin has an important effect on its stability in samples of biological material (Table 1). Using antibodies against the C-terminal fragment, the initial concentration in 24 h dropped at the temperature of 4°C to approximately 50%; using antibodies oriented toward the N-terminal peptides, the concentration dropped only to about 96–98%.

When the antibodies—or one of them—are oriented against the epitopes of the C-terminal end, their low immunoreactivity shows up. When the antibodies are oriented in such a way that they cover the whole intact molecule, a significant part of the molecule escapes the immunoreaction as a result of the fast split on C-terminal low-reactive fragments. When the antibodies are oriented against the N-terminal epitopes, intact osteocalcin will split but the resulting sum of the intact and N-terminal mid-fragment will change only slightly for a significantly long period of time. The result of the problems with osteocalcin fragmentation was an absolute incomparability of the results of measurements performed with the diagnostic kits of various producers (D11, M4), and their totally different clinical validity. Important progress in the immunoanalysis of osteocalcin was the use of antibodies oriented toward the stable areas of the molecule. Rosenquist *et al.* (R9) used monoclonal antibodies against the sequence of amino acids 20–43 and another antibody against the sequence 7–19 for the pickup of calcitonin from the sample. This way, they set the total of the intact and the stable osteocalcine. Such a method is clearly preferred today in the monitoring of bone remodeling.

Undercarboxylated osteocalcine is probably a result of the lack of vitamin K, and it can be its indicator (S10). On the contrary, decrease in affinity of the undercarboxylated osteocalcine to apatite can explain the ability to predict bone fractures (V5).

6.1.8. *Propeptides of Collagen Type I (PICP, PINP)*

During the biosynthesis of collagen, these propeptides break off of procollagen, both from its C-terminal end (PICP) and from the N-terminal end (PINP).

In collagen biosynthesis, they are released to the circulation as a result of the effect of proteases, and can be the indicator of bone formation. PICP is a globulin of relative molecular weight 117 kDa, while PINP has a relatively low molecular weight of 27 kDa. The concentration of both markers rises not only in increased bone formation but also with the accelerated remodeling of non-bone (skin, muscular) collagen. The result is low clinical specificity. Of importance may be the fact that both markers are typically increased in osteogenesis imperfecta.

Competitive RIA and, lately, ELISA are the methods used to measure the serum concentration of these markers. Systematic differences between the RIA and ELISA in establishing PINP are explained by the existence of two molecular forms, and different antigenic reaction of the antibodies of both methods with them (J2, P7).

6.2. General Variability of the Results of Measurements of Bone Markers

6.2.1. *Collagen Degradation Products (Pyridinoline, PYD, and Deoxypyridinoline)*

Probably the most important source of variability in the measurement of bone markers is biological variability. The consequences of the combination of analytical and biological variability are the values of critical differences of two consequent measurements. Only the changes in the concentration of the analyte higher than the value of the critical difference can be with certainty assigned to the condition of the disease, or the effectiveness or ineffectiveness of treatment. The values of critical differences can be calculated by the equation:

$$\mathrm{CD}\% = 2.77 \times \left(\mathrm{CV}_\mathrm{a}^2 + \mathrm{CV}_\mathrm{i}^2\right)^{1/2}$$

Markers of bone remodeling are subject to strong diurnal rhythms expressed as the average of percent amplitudes of daily minimums and maximums. For example, diurnal rhythm for U-DPD/creatinin is 37% when using the immunoanalytical measuring Metra (J4). DPD and PYD are stable at the temperature of -20°C for several years (G7). Limiting condition is preventing the urine samples from light exposure (W1). Correlation between HPLC and immunoanalytical measuring is reported differently by different authors, and probably depends more on composition of the patient groups than on the method itself. In the literature, correlations of $r > 0.95$ (R6) as well as $r < 0.38$ can be found (C1).

Similarly can be explained different data about a reportedly stable ratio of free and total deoxypyridinoline. In literature commonly reported, 40% of free DPD of the total is probably unstable, and can vary in some groups of patients or in

TABLE 2
VALUES OF CRITICAL DIFFERENCES OF REPEATED DPD/PYD MEASUREMENTS

Analyte[a]	Method	Critical difference (%)	Reference
t-DPD/Cr	HPLC	60	(K2)
t-PYD/Cr	HPLC	45	(K2)
f-DPD/Cr	EIA	47	(K2)
f-DPD/Cr	EIA	38	(B3)
f-DPD/Cr	EIA	28	(P8)
f-PYD/Cr	EIA	54[b]	(P5)
		102[c]	
		53[d]	
		57[e]	

[a] t = Total concentration, f = concentration of free fraction, Cr = urinary creatinine.
[b] First morning urine.
[c] Second morning urine without normalization on creatinine.
[d] Second morning urine.
[e] 24-h collection sample.

individual patients in general. Garnero *et al*. have found different ratios of free and total DPD depending on age, condition of bone remodeling, and therapy (G3).

Reproducibility of the immunoanalytical measurements of DPD can be characterized with a median of CV percent values found in the literature at 9.0%. For HPLC, the similarly found value of the median of CV percent was little higher; 10.5%. Chemiluminiscent measurement performed on a Chiron analytical system showed better accuracy, ranging—depending on concentration—from 3% to 9% (R8).

The results of DPD/ PYD measurements are usually normalized for the concentration of creatinine in urine, and presented as μmol DPD(PYD)/mol of creatinine. This normalization will reduce the variability of results by elimination of the influence of diuresis, but the creatinine excretion itself exhibits individual biological variability of approximately 11% (R3).

DPD and PYD are the only markers of bone remodeling for which the exactly characterized primary referential material is available. This material is a preparation isolated from bovine bone submitted to intensive purification, and characterized with molar absorption coefficients, elementary organic analysis, mass spectrometry, and NMR spectra (R5). The values of critical differences of DPD/ PYD measurements are summarized in Table 2.

From the data in Table 2 we can deduce the following:

Critical differences in measurements are very high, and cause very difficult interpretations of the changes in concentration.

TABLE 3.1
REPRODUCIBILITY OF TELOPEPTIDES MEASUREMENTS IN URINE

Method	Median CV(%)
U-CTx/Cr	8.0
U-NTx/Cr	9.0

Sources: B7, C7, H3, J4, S9, W5.

Normalization of measurements on urinary creatinine concentration is necessary.

Also necessary is to optimize the type of material sample used for the analysis.

6.2.2. *Telopeptides NTx-CTx*

The results of measurements of urinary NTx and CTx are normalized on the concentration of creatinine in urine (U-NTx/Cr, U-CTx/Cr). The fact that the diagnostic kits for CTx measuring are produced almost only by Osteometer Biotech (Denmark), while the diagnostic sets for NTx measurement are the domain of Osteomark, Ostex Int. (USA), may help the comparability of the results of the measurements.

Ju *et al*. (J4) established diurnal rhythms for U-NTx at 37%, and for U-CTx at 57%. Originally it was supposed that the diurnal rhythms of serum concentrations of NTx and CTx would be lower, but results published to date do not support this expectation. Gertz *et al*. (G8) established the diurnal rhythm for S-NTx at about 50%, Wichers *et al*. (W3) for S-β-CTx at 60–66%.

It must be repeated that the variability of the results is huge, and in some data quite inconsistent. Problems with clinical interpretation of the results of measurements are their logical consequence. The expected introduction of methods for measuring serum concentration of NTx and CTx was connected with certain hope, and with the expectation of significantly lower variability. Current results do not indicate unambiguous meeting of these expectations, as shown in Tables 3.1, 3.2, and 3.3.

TABLE 3.2
REPRODUCIBILITY OF TELOPEPTIDE MEASUREMENTS IN SERUM

Method	Median CV(%)
S-β-CTx	8.0
S-NTx	7.0

Sources: B8, C6, C7, G8, S4, W5.

TABLE 3.3
CRITICAL DIFFERENCES OF THE TWO SUBSEQUENT MEASUREMENTS OF TELOPEPTIDES

Marker	Manufacturer	CD (%)	Reference
U-NTx /Cr	Osteomark	36	(K2)
U-CTx /Cr	Osteometer	51	(K2)
U-NTx /Cr	Osteometer	41	(K7)
S-β-CTx	Osteometer	54	(K7)
U-NTx /Cr	Osteomark	20	(D3)
U-NTx /Cr	Osteomark	43	(P8)
U-NTx /Cr	Osteomark	70	(H2)
U-CTx /Cr	Cis-Bio Int	133	(H2)

6.3. SERUM MARKERS PRODUCED BY OSTEOBLASTS AND OSTEOCLASTS

6.3.1. *Alkaline Phosphatase and Its Bone Isoenzyme*

BALP (B10, K5, P3) is stable for at least 100 days at −20°C when using the diagnostic kits Alkphase-B-Metra and Beckman Tandem-MP-Ostase. With the use of the Beckman Tandem-R-Ostase kit, the stability at −20°C is supposed to be unchanged even for 48 months. Cross-reactivity of different isoforms of BALP is shown in Table 4.

6.3.2. *Osteocalcin*

Stability of osteocalcin depends strongly on the method used. As has already been mentioned, from this point of view, it is best to measure the sum of the intact osteocalcin and the N-terminal mid-region (peptide 1–43). When the serum is obtained right after the blood is taken, and stored at −20°C, regardless of the method used there will be no changes in concentration for at least 1 month. However, that requires very demanding ways of sample taking and transportation (on ice) in the methods using the antibodies that recognize the unstable C-terminal part of the molecule (B5).

Stability of osteocalcin *in vitro* can be provided with the addition of an appropriate amount of suitable antiproteolytic agent to the sample tube. Banfi and Daverio

TABLE 4
CROSS-REACTION OF BALP WITH HEPATIC ISOFORM

Method	Percentage of cross-reaction
Alkphase-B-Metra	5.0–8.7
Beckman Tandem-R-Ostase	12.7–16.0
Beckman Tandem-MP-Ostase	8.0–16.0

Sources: B10, G1, G9, P9.

TABLE 5
REPRODUCIBILITY OF MEASUREMENTS

Method	Median CV (%)
BALP—immunoanalysis	7.0
Osteocalcin	6.0
TRACP	9.0
PICP	7.0

(B1) have successfully used the addition of aprotinine, and proved 300-min stability at room temperature, compared with the same samples without aprotinine, with a loss of more then 50% of measured concentration during the same period of time.

6.4. VARIABILITY OF THE RESULTS OF MEASUREMENTS OF THE MARKERS OF BONE TURNOVER

6.4.1. *Reproducibility of Measurements*

Critical differences of two subsequent measurements for serum analytes produced by the osteoblasts and osteoclasts are recorded in Table 6. Variability of the serum markers produced by the osteoblasts and osteoclasts is significantly—about two times—lower than the variability of urinary, and probably also serum markers, of the degradation of collagen type I. See Table 5.

6.5. CLINICAL USEFULNESS OF THE MARKERS OF BONE REMODELING

Markers of bone remodeling were very useful when used in clinical studies that were intended to learn and understand the pathogenesis of osteoporosis, and the

TABLE 6
CRITICAL DIFFERENCES OF THE TWO SUBSEQUENT MEASUREMENTS FOR SERUM ANALYTES

Marker	Method /manufacturer	CD (%)	Reference
BALP	ELFO	20	(P4)
BALP	Tandem-R-Ostase	26	(H2)
BALP	Tandem-R-Ostase	25	(K5)
BALP	Tandem-R-Ostase	30	
BALP	Alkphase-B-Metra	29	
Osteocalcine	Intact+N-Mid	29	(P4)
Osteocalcine	Intact+A-Mid	21	(H2)
TRACP	Catalytic concentration	35	(P4)
TRACP	Catalytic concentration	17	(H2)
PICP	RIA Orion	24	(H2)
PINP	RIA Orion	21	(H2)

mechanisms of therapy (W2). Noninvasivity of their measuring, the possibility of frequent repetition, quick changes of concentration during the therapy, and relatively low cost are their main advantages. The principal disadvantage remains rather too high variability of their values, both analytical and biological, which makes interpretation of their results very difficult and ambiguous (B4). With the application of the measurements in clinical practice we must answer the question, what testimonial value has the result for the individual patient; answering this question is extremely difficult, and it requires a lot of care.

6.5.1. *Are Bone Markers Useful in the Diagnosis of Osteopenia and Osteoporosis?*

The results of a number of studies show very low correlation between bone markers and total body density of bone mineral (BMD), with the exception of the population of elderly postmenopausal women, where lower bone density and increased bone remodeling can already be expected with high probability a priori (M6). From these studies can be drawn a conclusion that parallel measuring of BMD and the bone markers can provide better information. However, it cannot be claimed unambiguously that measuring the bone markers has contributed to diagnosing osteoporosis. According to Woitge *et al*. (W5), serum concentration of NTx and CTx are capable of substantially better discrimination between the healthy population and the individuals with osteoporosis, but the increase in clinical sensitivity was barely noticeable: from 0% in U-CTx/Cr to 17% in S-CTx, and from 16% in U-NTx/Cr to 31% in S-CTx. The values of specificity and sensitivity of S-CTx and U-CTx/Cr, according to a study of Christgau *et al*. (C6), were even statistically insignificant. So, though some authors put their expectation into the development of methods for measuring the collagen degradation products in serum instead of urine, their expectations have not been met so far (B4).

6.5.2. *Can Bone Markers Predict the Speed of Loss of Bone Tissue in the Future? Can They Distinguish Fast Losers and Slow Losers of Bone Tissue?*

Several publications exist that consider bone markers capable of this differentiation. An example is the report of Moss *et al*. (M9) concerning the serum markers BALP and osteocalcine, and urinary markers DPD and PYD. Major prospective studies—PEPI (Postmenopausal Estrogen-Progestin Intervention) (M3), Fracture Intervention Trial (B2), and the alendronate study (H6)—have not found a causal relationship between the bone markers and change in BMD, or found a relatively weak one.

6.5.3. *Follow-up of the Patient's Response to Treatment*

Follow-up represents the most common and the most important application for measuring bone markers in routine clinical practice. Because the results are loaded with high variability, and the patient's response to treatment is very individual, in agreement with the formulations of Blumsohn and Eastell (B4) it

remains to evaluate the changes during therapy against baseline values. Evaluation of the changes themselves must respect critical differences in measurements. Bone markers react to therapeutic intervention much more quickly than BMD, and by following the above-mentioned rules, they can provide valid information about its successfulness.

Hannon *et al*. (H2) evaluated the response to estrogen substitution therapy after 6 months of treatment by comparing the measurement results with the values before treatment, with respect to the critical differences. The effectiveness of therapy was best followed by the values of osteocalcine and PINP (87% of the results with the drop below the values CD percent). Collagen degradation products U-NTx/Cr and U-CTx/Cr showed only 27% and 18% responses to the treatment, respectively; BMD change was significant only for the lumbar spine area in 36% of cases. No answer was reached by measuring the total and femoral value of BMD.

In a similar experiment (D3) with a follow-up of the response to alendronate therapy (10 mg a day for a period of 25 weeks), the best-answering marker was U-NTx/Cr (100% of responders); DPD, BALP, PICP, and osteocalcine reacted very similarly (81–88% of responders). In this case, the response to the treatment also provided the measurement of BMD, although not as effectively as the bone markers (maximum response was provided by the values of lumber BMD, in 44% of responders. Kyd *et al*. (K7) achieved, in practically identically organized follow-up of the effectiveness of alendronate therapy in months, 66% of responders with the help of U-NTx, but only 23% of responders for U-DPD. As well, they used establishing the CTx in serum, with a 66% response to treatment. Kress *et al*. (K5) watched the course of therapeutic intervention with application of 10 mg of alendronate per day for a period of 6 months with the help of BALP, and obtained 85% of responders. Hannon *et al*. had in the above-mentioned study (H2) only 45% of responders to the same therapy when BALP was used for evaluation.

From the above-mentioned data, we can conclude that even the procedure evaluating the changes against the baseline before the therapeutic intervention, with respect to biological variability, may not lead to the same conclusion.

Table 7 attempts to explain hypothetically the possible causes of different conclusions. Unequal CD values and even various methods can be the source of different clinical conclusions.

TABLE 7
POSSIBLE CAUSES OF DIFFERENT CONCLUSIONS

Marker	Author	Method	Critical difference used (%)
BALP	(K5)	Tandem-R-Ostase	25
BALP	(H2)	AlkPhase-B-Metra	26
NTx	(H2)	Osteomark	70
NTx	(K7)	Osteomark	41

Follow-up of the response to treatment is the principal current application of the measurement of the markers of bone remodeling. The condition for success is careful establishing of the baseline values before the treatment, and respecting the critical differences of measuring. The data about them do not seem to be generally acceptable. For other potential applications for establishing bone markers (diagnosis of osteoporosis, prediction of loss of bone mass, and the risk of fractures), there is not enough scientific evidence, especially answers to the questions:

1. What markers to choose
2. In what time interval to measure them
3. What cutoff values to use

Only the answer to these and other questions may bring the decisive facts about the possibilities that the establishing of bone markers in serum and in urine brings for diagnostics and follow-up of the therapeutic response in patients with osteoporosis.

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LABORATORY MARKERS OF OVARIAN FUNCTION

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1. Introduction

After the age of 35 in women, fecundity decreases sharply. Delaying childbearing until the age of 35 or beyond is therefore one of the major reasons why in the last decade attention has been drawn to the decline in fertility in older women (G1, S8, S26, V2). The number of couples seeking help for infertility problems after their 30s and even 40s is increasing (P6). People of advancing age are destined to end up in a sort of age trap of declining fecundity and therefore the need for more time to conceive on the one hand and, on the other hand, less time available to conceive because of declining fecundity. The older the people are who need infertility treatment, the more they will feel the pressure to conceive within a short period of time (P7); the demand for assisted reproduction techniques, such as intrauterine insemination (IUI) after ovarian hyperstimulation or *in-vitro* fertilization (IVF) and embryo transfer, will therefore increase.

With the use of assisted reproductive techniques, the need to assess the chance of success of the treatment has increased (S18). Determining the chance of success serves two goals: first, it can help the couple realize that assisted reproduction can in no way guarantee offspring and that a realistic approach toward infertility therapy means accepting the chance of failure. Second, it can lighten the burden of waiting 2 weeks between oocyte retrieval and embryo transfer in an IVF treatment.

The chance of a spontaneous conception as well as the prognosis of infertility therapy can only be determined when there is some knowledge about the remaining capacity for reproduction (S8). Measuring ovarian reserve is the cornerstone of most tests designed to predict the success of infertility therapy. In Fig. 1, a representation of the hypothalamic–pituitary–ovarian axis is shown. However, there are more factors determining the chance of success in assisted reproduction. The

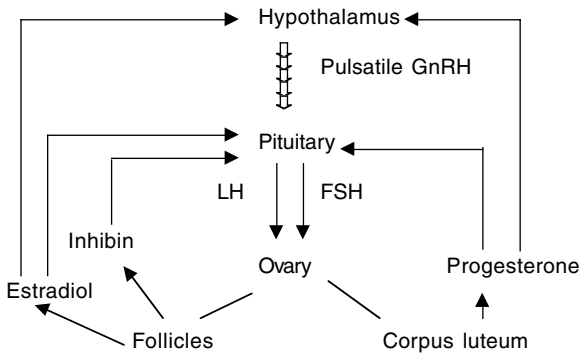

FIG. 1. The major hypothalamic–pituitary–ovarian interactions mentioned in different parts of this review. Ovarian steroids exert effects on the secretion of GnRH and gonadotropins.

best biological evidence for adequate ovarian function is the live birth of a baby. Unfortunately, not every couple is able to conceive without medical aid. These are the patients who present themselves in fertility clinics. These couples are also the richest sources of information in clinical studies, because they are in contact with physicians and motivated to participate in studies, whereas couples who conceive without medical aid are often not available or willing to serve as volunteers. Because of this handicap, almost all information on reproductive endocrinology is drawn from infertility patients. This review will discuss the tests most commonly used to assess ovarian function in infertility patients, as well as some experimental tests.

In this review, "success" of an assisted reproduction treatment is defined as pregnancy rate, ongoing pregnancy rate, and, the ultimate goal of assisted reproduction, live birth of a baby. Assisted reproduction is defined as treatment protocols using IUI after treatment with gonadotropins and IVF treatments. Insemination protocols (either with insemination of the husband's sperm or with donor sperm) without treatment with gonadotropins are not the subject of this review, although these might be mentioned now and then for illustration. As studies have shown, success in IVF treatment can also be measured by the number of ampoules of FSH needed to achieve a situation suitable for oocyte retrieval, the number of follicles on the day of oocyte retrieval, the number of oocytes harvested, and number of embryos (C12, R7). These parameters were also taken into account in this review, as some studies use them to measure the effect of the treatment.

2. Age

The first functional test used on all couples attending a fertility clinic is age of the woman. As age increases, fecundity drops. In subfertile couples, the woman's age is significantly inversely correlated with the chance of a spontaneous conception (C9, E5). We have found no reports about the age of the male partners in relation to conception rates. This is at the least interesting, as almost all factors one could think of, including female income (C9), were studied in relation to conception rates. In assisted reproduction, the woman's age is related to success of treatment. This generally acknowledged statement is supported by data on women—whose husbands were totally sterile—receiving donor insemination in natural as well as stimulated cycles (F4, S26, V2, V6). After the age of 30, fecundity drops significantly, and after the age of 35, even more sharply. The lower fecundity rate in older women can be compensated by more insemination cycles, resulting in the same pregnancy rates in older women compared to younger women (V2).

In several other studies, pregnancy rates drop as age increases in insemination programs, whether ovulation induction has been used or not, and whether donor semen or husband's semen has been used (H13, Y1).

Also, in a program of timed intercourse, intrauterine insemination, and cup insemination of donor semen following ovulation induction, a significant lower number of women beyond the age of 40 became pregnant or had live births (P6). Beyond the age of 44, no woman became pregnant.

It could be expected that IVF fecundity will also prove to drop as age increases. In IVF studies, pregnancy rates are sometimes calculated as a percentage of egg retrievals, thereby biasing in favor of older patients, as they more frequently might have cycle cancellations than younger patients. Or, in some programs, older patients have more embryos transferred than younger patients, again biasing the outcome in favor of older patients (R5).

Numerous studies have found a clear picture of age-related decline in the results of IVF: more ampoules of follicle-stimulating hormone (FSH) needed, fewer oocytes, fewer embryos transferred, fewer embryos available for cryopreservation, and lower ongoing pregnancy rates (A4, C1, F8, H1, H15, H16, L6, L8, P4, P7, S19, T5, T6, W3). Even after adjusting the numbers for number of embryos transferred, husband's age, IVF attempt rank, and infertility diagnosis, the decline in fecundity with advancing age is significant (P7). There is still discussion whether higher cancellation rates are related to advancing age. Some studies cannot find a higher cancellation rate in older patients (S19, T5), while other studies do (G8).

Interestingly, Templeton *et al*. have shown that the highest livebirth rates in IVF come from age group 25–30 years (T2). Women younger than 25 years have lower success rates in their report. This could not be confirmed by other studies (H15, W3).

The age calculated as a cutoff point after which pregnancy rates drop more differs between studies: some calculate the age of 34 as a cutoff point (L8), others 36 (G8), 37 (F8, P7), or 40 (C1, F8). Toner *et al*. (T5), who studied 1478 IVF cycles, found no change in the slope of the decline in pregnancy rates, but did find a significant decline in total and ongoing pregnancy rates as age increases.

The decline in fecundity is reported not to be due to a higher abortion rate, as one study found no difference in miscarriage rates between different age groups (T5). Others, however, did find significant more miscarriages in older women (H15, R5, V6).

3. Basal Hormone Levels

3.1. FSH

Measuring serum FSH on cycle day 3 is probably the most widely used invasive test to measure ovarian reserve (S8, T4). When natural cycles are monitored, follicular growth rate is slower in young women with high FSH levels compared to controls with normal FSH levels (A1). In natural IVF cycles, raised FSH is associated with an increase in cancellation rates for poor follicular development, although

after embryo transfer, clinical pregnancy rates are not affected (S23). Despite a poor response to gonadotropin stimulation, women with raised FSH levels are capable of achieving pregnancy in treatment as well as spontaneous cycles (B8, R6). So the main problem in women with raised FSH levels is poor response to stimulation, resulting in low pregnancy rates in assisted reproduction. In women of advanced age undergoing ovulation induction therapy with IUI, basal FSH is shown to be a useful predictor of live birth when estradiol is measured simultaneously (B11).

In IVF programs using ovulation induction, both overall (L6, S10, T5) and ongoing (M2, S10, T5) pregnancy rates are higher in women with normal FSH levels compared to women with elevated FSH levels. One study, however, found that the significant association between FSH and pregnancy rate disappeared when controlled for age (S19). Another study found no significant difference between patients with normal FSH and high FSH levels when conception rates and term pregnancy rates are calculated per embryo transfer (H16). High basal FSH levels again seem to have more impact on responsiveness of the ovary to stimulation than to the ability of the uterus to provide implantation once the embryo has been transferred. Although high FSH predicts low pregnancy rates, FSH is much better at predicting ovarian response than pregnancy (E1, S19, T5).

Cancellation rates are higher in women with high FSH levels (G8, S10, S19, T5). This is possibly due to a poorer response, since peak estradiol concentrations are lower in women with elevated FSH levels (C1, E1, G8, H16, M11, R2, S10, T5). Some studies find no difference in cycle failures between patients with elevated and those with normal FSH levels (E1). This can be explained by the fact that different IVF programs have different reasons for cycle cancellation.

The number of follicles aspirated (C1, E1, H16, S10, T5), number of oocytes retrieved (C1, E1, H16, R2, S10, S19, T5), the number of embryos (S10, T5), and the number of embryos available for cryopreservation (T6) are also higher in women with normal FSH levels.

Some studies find a higher total number of ampoules of FSH administered before oocyte retrieval in patients with elevated FSH levels (S19), but others do not (C1, E1, H16, S10). When the influence of age and FSH is compared, FSH is a better predictor for response in assisted reproduction than age (C1, G8, T5), although this is not the case for predicting pregnancy in all studies (H1, S19).

Usually, basal FSH is measured on cycle day 3. Hansen *et al.,* however, found no statistical difference in FSH levels on cycle days 2, 3, 4, and 5 (H3). It seems that within this time range, any moment is appropriate to determine basal FSH. However, as estradiol levels raise significantly during this early follicular stage, caution must be taken in interpreting FSH levels as normal, because raised estradiol levels may suppress FSH concentrations (S22).

Different studies use different cutoff levels for normal FSH. This is only logical, as different assays are used, and populations and laboratories differ, too. When measured in different laboratories using different assays, FSH in the same

serum is reported to have significant different values (H6). It has been shown that an immunometric assay using a double monoclonal antibody is superior than a radioimmunoassay using a polyclonal antibody in predicting ovarian response (mature oocytes and peak estradiol) (R2).

The upper limit of normal FSH is reported to be 9 IU/liter (C1, H15), 9.5 IU/liter (R2), 10.8 IU/liter (S19), 11.5 IU/liter (E1), 11.7 IU/liter (H16), and 12 IU/liter (T1). There are two different ways to determine the upper limit of basal FSH: a statistical one, calculating the 95th percentile, and a clinical one, calculating the FSH value above which a decline in ovarian response is noted. Using the latter method, a decline in fertility appears when FSH is above 10 (A1) to 15 (M2) IU/liter.

Normal FSH is normal FSH. Patients with FSH levels low in the normal range have no higher pregnancy rates than patients who have FSH levels higher up in the normal range (H1, S10), although there appears to be a trend toward a poorer response with increasing FSH level, even within the normal range (C1).

Intercycle variation of FSH is also predictive of IVF outcome. Patients with high intercycle variability of FSH have lower mean FSH levels, lower peak estradiol concentrations, require more ampoules of FSH, and have fewer follicles aspirated and fewer oocytes recovered than patients with low intercycle variability (S11). There is no difference in response to stimulation between cycles with high or low FSH levels in the same patient, once cycles with raised FSH have started to appear (S11). In low-FSH cycles, only the number of follicles aspirated is higher, but peak estradiol, number of ampoules of FSH, and number of oocytes is the same in low- and high-FSH cycles (S11). Van Os and Jansen, however, have shown that conception rates are higher in cycles with low FSH levels compared to high-FSH cycles (V3).

It can be concluded that raised basal FSH identifies those women who will have a poor ovarian response to gonadotropin stimulation, but once embryos have been achieved, pregnancy rates are not much lower than in women with normal FSH levels. Once FSH is elevated, ovarian response will be poor, even though some cycles might have normal FSH levels. If FSH levels are normal, there is no difference between women with low-normal FSH levels and high-normal FSH levels in response to stimulation.

3.1.1. *FSH Measured in Urine*

In order to investigate the possibility of a noninvasive way to determine basal FSH, we have measured FSH in urine (O2). The variation in serum FSH levels is substantial between different menstrual cycles in the same person (V3). Although this may be caused by a true variation in FSH levels between cycles, the variation in FSH secretion during the day may also be the cause (F7). The gold standard for FSH level in serum is serial sampling, which of course is expensive and cumbersome. Also, measuring serum FSH is invasive, and will therefore decrease patient compliance and volunteer availability in the case of endocrinological studies. Sampling of urine, on the other hand, is noninvasive and simple.

Others have also tried to measure FSH in urine, but this was done with double-antibody radioimmunoassays or Delfia IFMA assays (C5, D1, E6, H4, K6, K9, L1, M1, M3, S1, S3). These assays are complicated and the urine needs to be extracted, causing the procedure to be less suitable for routine use.

We have measured FSH in unextracted urine on an AxSYM random-access immunoassay analyzer (Abbott laboratories, Abbott Park, IL) with a MEIA (microparticle enzyme immuno assay) reagent kit. In order to correct for dilution, creatinine was measured, and the urinary FSH was normalized for creatinine concentration. Urine and serum samples were obtained from 40 women between 32 and 55 years of age. All women were healthy, except for a benign gynecological illness for which they were admitted to our hospital. All women had normal renal function. On the day of operation, we took six serum samples from each patient, each at least an hour apart, in order to calculate the mean serum FSH concentration. During the same day, we collected an early-morning urine sample, 24-h urine sample, and a random void urine sample.

Serum was either used for measuring FSH immediately or frozen until hormone determination. FSH and creatinine in urine were determined immediately. Additional urine samples were obtained to determine assay characteristics. In order to determine storage conditions, urine samples were stored at room temperature, at 4°C, at −20°C without any addition, and at −20°C with addition of glycerol. After 1 and 4 weeks, FSH and creatinine were determined again.

Serial dilutions of urine samples showed linear dilution curves, and the correlation between expected and measured FSH levels was 99.95%. The analytical recovery also showed a linear curve, and the correlation between expected and measured FSH levels was 99.94% for the standard calibrators and 99.03% for the 3rd International Standard for FSH and LH (urinary). Concordance between FSH concentrations in extracted and unextracted urine samples was high, the correlation being 98.9%.

Storage conditions are shown in Fig. 2. Storage at 4°C caused no decline in FSH immunoreactivity, even after 4 weeks: still 98.9% ± 7.7% of the immunoreactivity remained detectable.

The correlation between urinary FSH and serum FSH was high, Pearson's correlation coefficient being 0.915 for random void urine ($p < 0.0001$), 0.904 for early-morning urine ($p < 0.001$), and 0.857 for 24-h urine ($p < 0.001$) (Fig. 3).

The high correlation we found between urine FSH and serial sampling serum FSH shows that the assay used is suitable for measuring FSH reliably. The fact that the best correlation is found with random void urine adds to patient comfort. Also, the excellent storage conditions make the assay practical for use in large studies. As urine samples provide a practical method for monitoring the endocrine function of patients and volunteers, we believe the assay we presented will be able to improve volunteer availability and patient compliance in studies regarding ovarian function.

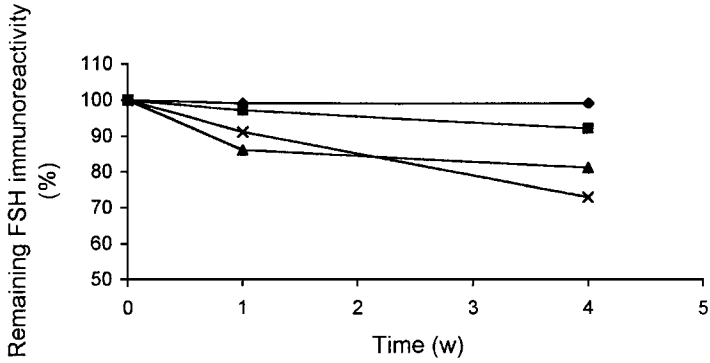

FIG. 2. Storage conditions of urinary FSH. The decline of FSH immunoreactivity in urine samples stored at −20°C without additive (x), at −20°C with addition of 0.25 ml glycerol in 3 ml urine (▲), at 4°C (♦), and at room temperature (■) after 1 and 4 weeks. From Oosterhuis *et al.* (O2, Fig. 4, p. 546), used with permission.

3.2. BASAL LH

High basal luteinizing hormone (LH) levels are associated with high pregnancy loss and low conception rates in women attempting to achieve pregnancy in non-therapeutic cycles (R4). The possible explanation of this phenomenon is that high LH levels during the follicular phase cause a prolonged interval between the completion of the first meiotic division of the oocyte (which is caused by the LH surge) and fertilization. Such an extended interval causes a decreased quality of the oocyte, resulting in poor fertilization rates and high pregnancy loss.

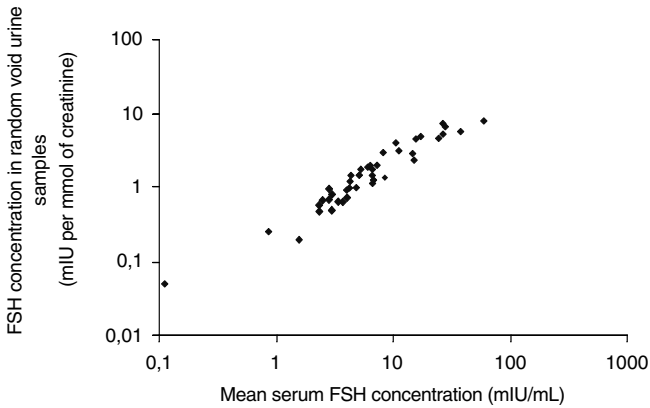

FIG. 3. The correlation between the mean of 6 serum FSH measurements and FSH measured in a random void urine sample on the same day in 40 perimenopausal women. $R = 0.915$, $p < 0.0001$. From Oosterhuis *et al.* (O2, Fig. 5, p. 547), used with permission.

In assisted reproduction, one would not expect that ovarian response is reduced given the previous explanation. Only one study finds a relation between ovarian response and basal LH level: a significant correlation between basal LH and the total number of ampoules of FSH administered (M11). Oocyte yield and pregnancy rates were not correlated to basal LH in this study. One other study finds significantly lower pregnancy rates caused by lower fertilization rates and lower cleavage rates in women with elevated basal LH levels (S24), which might very well be in line with an extended time span between the completion of the first meoitic division of the oocyte and fertilization. In this study, no pituitary desensitization is carried out and patients receive either clomiphene citrate alone or clomiphene citrate with human menopausal gonadotropin. Comparison with modern protocols is therefore hard, and one could argue that these results cannot be extrapolated to modern treatment protocols.

In no other study is basal LH related to treatment outcome in assisted reproduction, neither to ovarian response nor to pregnancy rates (C1, E1, H16, S10). It is claimed that this could be caused by the pulsatile excretion of LH compared with its short half-life, giving a misleading estimation of basal LH level when measured only once in serum (S10). Also, in modern treatment protocols using pituitary desensitization, elevated LH levels during the treatment cycle will not occur often. Thereby, the relation between LH and poor treatment oucome will be lost.

3.3. Estradiol

Estradiol can be measured in the luteal phase of a menstrual cycle preceding a treatment cycle. When expressed as area under the curve (time $\times$ level), estradiol in the luteal phase can be seen as a sign of impaired ovarian response. Women who do not become pregnant during an IVF treatment have lower estradiol levels in the luteal phase of a preceding cycle compared to women who do become pregnant (C12, C13). The predictive value of luteal-phase estradiol is even better than that of basal FSH or the clomiphene citrate challenge test (C13, C14). The problem with using luteal-phase estradiol as a predictor is the numerous blood samples which need to be taken, making it a costly and unfriendly way of predicting treatment outcome.

Women who respond poorly to ovulation induction strategies in assisted reproduction often have menstrual cycles resembling women approaching menopause (A1). As women approach menopause, menstrual cycles become shorter and follicular development consequently starts earlier in the cycle, resulting in a rise in estradiol concentration early in the cycle (S20), even in women with normal menstrual cycles (H3). When basal estradiol concentrations (measured on cycle day 3) in young women are elevated, it might be a sign of ovarian failure and a prediction of a poor response to gonadotropin stimulation.

There are conflicting results in different studies addressing this subject. No correlation between basal estradiol levels (determined before pituitary desensitization

was started) and response in terms of pregnancy rates in assisted reproduction programs has been found in several studies (E1, H1, P6, S10). A (weak) statistically significant inverse relation was found between basal estradiol levels and number of ampoules of FSH administered (H16). One study even reports a statistically significant positive correlation between basal estradiol levels and oocyte yield (H16), a finding for which no physiological explanation can be found. These results are in contrast with more recent studies, which found significant lower pregnancy rates (S22), higher cancellation rates (S22), and a lower number of oocytes retrieved (L7) in women with elevated basal estradiol levels despite lower FSH levels in IVF. Finally, one study only found a significant lower pregnancy rate in women with high estradiol levels when the patients were older than 35 years (V4). In the total patient groups, estradiol levels had no effect on implantation rates, pregnancy rates, or cancellation rates (V4).

The reason for this difference in results between different studies is not clear. The additional value in measuring basal estradiol in order to predict ovarian response to stimulation seems to be to identify those women who seem to have normal FSH levels, but due to negative feedback from raised estradiol levels (S22), FSH concentration in these women is suppressed.

3.4. Progesterone

Progesterone, as well as estradiol, can be measured in a nontreatment cycle in the luteal phase, and be used as a way to identify impaired ovarian function. Women with ovarian failure despite regular menstrual cycles might have not only high basal FSH levels, but also lower estradiol levels and lower progesterone levels in their natural cycles compared to women without ovarian failure. When progesterone is measured on several days in the luteal phase of a cycle preceding an IVF treatment cycle, and expressed as area under the curve (time $\times$ level), it is significantly correlated with IVF outcome (C12, C13), and even a better predictor for treatment outcome than basal FSH or the clomiphene citrate challenge test (C14). These studies were all performed by the same research group. The authors hypothesize that impaired ovarian function is shown not only by elevated basal FSH levels, but also by lower progesterone levels. The problem with this assay is the numerous blood samples which need to be taken, making it an impractical method to be carried out routinely. Also, the number of studies on the relation between basal progesterone levels and ovarian response is too limited to draw hard conclusions.

3.5. Inhibin

Serum inhibin B levels in normal-cycling women tend to rise during the luteal–follicular transition and peak in the mid-follicular phase, and serum inhibin

A levels rise during the late follicular phase (L10). After pituitary desensitization, inhibin levels drop significantly (L10). It is believed that follicular-phase inhibin levels reflect the quality of the cohort of follicles growing and might therefore be a marker for success in assisted reproduction, as granulosa cells from women with high basal FSH levels secrete significantly less inhibin compared to granulosa cells from women with low basal FSH levels (S15). Basal inhibin B concentration (measured in a cycle before pituitary desensitization) might be predictive of ART outcome. In one study, inhibin B, measured in a cycle preceding an IVF treatment cycle, was significantly higher in pregnant vs nonpregnant women, but only in multivariate models controlling for several factors such as age could inhibin B predict the outcome of treatment (H1). One other study also found significant lower inhibin B concentrations in nonpregnant women compared to pregnant women (S17). Basal inhibin (B) is also significantly correlated with the number of ampoules of FSH administered, peak estradiol concentration, number of oocytes and of embryos, and cancellation rate (B1, B2, S17). In these studies, basal inhibin is measured in a cycle preceding the treatment cycle and can therefore be used to predict treatment outcome before treatment has started. One study has measured total inhibin concentration on the third day of a IVF/GIFT treatment cycle during which no pituitary desensitization was carried out (T7). The number of follicles grown and the number of oocytes obtained correlate significantly with serum inhibin levels in this study.

In nonanalog IVF cycles, inhibin B levels can predict premature LH surges (F1). Nonanalog IVF, however, is not practiced in many treatment centers. Basal inhibin B levels can probably predict ovarian response in IVF, and might be suitable to predict pregnancy, although not all studies agree on this issue. The question can be raised how inhibin B can predict success in assisted reproduction treatment a few menstrual cycles away, as it is produced by the growing cohort of follicles. It might be that women with high basal inhibin levels in a cycle preceding the IVF treatment cycle have a better ovarian reserve than women with low inhibin levels, and will therefore perform better in assisted reproduction cycles. Inhibin levels measured during a treatment cycle will be discussed later.

The role of basal inhibin as a marker for success in treatment looks promising, but needs to be investigated further.

3.6. Insulin-like Growth Factor

The growth hormone/insulin–like growth factor (IGF) axis plays a role in ovarian responsiveness. The IGF system comprises of IGF-I and -II and their binding-proteins, IGF-binding proteins (BP)-1 to -6 (G3).

IGF-I measured in serum in the early follicular phase of the cycle preceding an IVF treatment is not correlated with the outcome of the IVF treatment (S2).

IGFBP-3 measured in serum in the early follicular phase of the cycle preceding an IVF treatment is significantly correlated with number of follicles on the day of hCG, and with peak estradiol concentrations (S2). Although it is hard to believe that IGFBP-3 itself is responsible for mediating ovarian function, IGFBP-3 might very well serve as a potentiator of the effect of IGF-I or growth hormone in the ovary. The IGF system may play a significant role in folliculogenesis, but there is no evidence that it can serve as a marker for pregnancy in assisted reproduction.

3.7. Placenta Protein 14

Placenta protein 14 is a product of the secretory endometrium, and might serve as a biochemical marker for endometrial function. Women who conceive during assisted reproduction treatment (IUI cycles or IVF) have significant lower levels of placenta protein 14 in their natural cycles—cycles preceding IVF or IUI—than women who do not conceive (W1). This report is about only a small number of patients, so more work is needed to provide evidence that placenta protein 14 is an adequate marker for pregnancy in assisted reproduction.

4. Function of the Pituitary–Ovarian Axis

The most useful markers for a successful outcome of assisted reproduction are those that can be assessed before the treatment begins. The following tests are therefore less useful from a clinical point of view. However, sometimes a stimulation or challenge test can give more detailed information about the functioning of the endocrine system of a subject, or can predict pregnancy more precisely than baseline tests.

4.1. Clomiphene Citrate Challenge Test

In order to establish a functional test for fertility potential, Navot *et al.* (N3) have described the clomiphene citrate challenge test (CCCT). In short, FSH is measured on cycle day 3 and on cycle day 10, while on cycle days 5–9, 100 mg of clomiphene citrate is administered daily. When response FSH is high, ovarian reserve is considered diminished. The reason for the overshoot of FSH in women with diminished ovarian reserve is claimed to be a reduced capacity to secrete inhibin in these women (H9, N3).

The CCCT can be interpreted in several ways: as originally described, the test is abnormal if the second FSH level is high, but others consider the test also abnormal if either the first or the second FSH level is high, if the second FSH level is higher than the first one, or if the sum of basal and stimulated FSH is above a certain level. Finally, estradiol concentration can be taken into account in the interpretation of

the test: estradiol level should rise significantly after 5 days of clomiphene citrate. If not, the test is considered abnormal, even if both FSH levels are normal.

In a group of 51 patients, of whom 18 had an abnormal CCCT, significantly more women in the group with adequate ovarian reserve conceived during subsequent fertility treatment (not IVF) (N3). FSH on cycle day 3 as well as on cycle day 10 after CCCT is correlated with pregnancy in subsequent IVF treatment cycles (C13). More cycles are cancelled, fewer oocytes are retrieved, peak estradiol levels are lower, but pregnancy rates are not different in subsequent IVF when the CCCT is abnormal in another study, where the test is considered abnormal if either basal or stimulated FSH level is $\geq$10 U/liter (K2). However, if the same definition for abnormal CCCT is used in the general infertility population, an abnormal CCCT correlates significantly with pregnancy (H11, S9), although type of treatment is not described in these studies (H10, H12, M7). If the stimulated FSH level is elevated, more cycles are cancelled, peak estradiol levels are lower, and no pregnancies are obtained, compared to 10% pregnancies when the stimulated FSH level is normal, in one study (T1). Basal FSH + stimulated FSH correlates significantly with number of ampoules of FSH administered, number of follicles aspirated, number of oocytes, and number of embryos obtained, as well as with pregnancy (L12).

Stimulated estradiol levels after clomiphene citrate stimulation are lower in older patients with regular cycles compared to younger women (G2), but they are not correlated with any outcome parameter in IVF (S12). Therefore, although the response of estradiol in a CCCT might give some insight into the function of the ovaries, it is of no prognostic value in IVF.

The CCCT is able to detect those patients with normal basal FSH levels who have a high risk of a poor response in assisted reproduction.

4.2. GnRH Stimulation Test

To evaluate the response of the pituitary and the ovary to a gonadotropin releasing hormone (GnRH) agonist, a GnRH-agonist stimulation test is used. From cycle day 2 on, a GnRH agonist is administered daily in an IVF program ("flare-up"). Estradiol, FSH, and LH are determined in serum during cycle days 2–5. From cycle day 5 on, exogenous FSH is administered daily.

The response of both FSH and LH to a GnRH agonist is not related to pregnancy rates (P1).

Some studies report a significantly higher pregnancy rate when there is a significant (early) response of estradiol to a GnRH agonist (P1), but others do not (C8, W2).

Conclusively, there are not enough studies on the GnRH-agonist stimulation test to decide whether the test is predictive enough of treatment outcome. Even if the test is proven to be as reliable as basal FSH, it is not simple enough to compete with basal FSH to be used routinely.

4.3. EFORT

In order to improve the predictive value of basal FSH, Fanchin *et al*. (F2, O1) have introduced the exogenous follicle-stimulating hormone ovarian reserve test (EFORT). On cycle day 3, two menstrual cycles before IVF treatment, basal FSH as well as estradiol concentrations are determined in serum, and 300 IU of FSH are administered i.m. After 24 h, estradiol levels are determined again. The predictive value of basal FSH <12 U/liter and a rise in estradiol >30 pg/ml is more predictive of adequate ovarian response than basal FSH alone.

The flaws in this study are that only a small group of patients has been studied, and that pregnancy has not been taken into account as a study endpoint.

4.4. Delayed Pituitary Desensitization

The purpose of pituitary desensitization is to prevent untimely LH surges from occurring. Using the so-called long protocol, GnRH agonist is used to suppress the pituitary–ovarian axis completely. Usually, this is achieved after 10–18 days of GnRH-agonist treatment.

If pituitary or ovarian desensitization is not complete, does this affect treatment outcome? Goswami *et al*. (G7) have found that if LH levels on day 12 of GnRH-agonist administration are elevated, this does not affect pregnancy rates in IVF, whether GnRH-agonist administration is prolonged until complete hypogonadotropic state is reached or not. Others have also found that pregnancy rates are not affected by LH level on the day FSH administration commences (L13). However, if estradiol levels are raised on day 12, pregnancy rates are lower, whether GnRH-agonist administration is prolonged until complete hypogonadal state is reached or not. It can therefore be concluded that raised estradiol levels on day 12 of GnRH-a treatment are predictive of poor response to IVF. A lack of sensitivity of the ovaries is worse than a lack of sensitivity of the pituitary gland, so to say.

Develioglu *et al*. (D3) have shown that after incomplete pituitary suppression, peak estradiol concentrations are higher and number of ampoules of FSH needed is lower, but number of oocytes retrieved does not differ compared to patient with complete suppression.

Incomplete suppression of pituitary function might have the beneficial effect of some endogenous FSH activity facilitating follicular growth, but also the possibility of raised progesterone levels on the day of hCG, resulting in postmaturity of the oocytes. Both effects seem to compensate each other more or less, resulting in conflicting study results.

4.5. Peak Estradiol Levels

Estradiol level on the day of hCG administration reflects the aromatase activity of the cohort of follicles growing. It might therefore be that pregnancy rates are

correlated with peak estradiol levels. Many authors even define poor response by a failure to achieve a particular serum estradiol level following gonadotropin stimulation (K4). Although estradiol levels are closely correlated to number of follicles, oocytes, and embryos, there is no significant correlation between peak estradiol level and pregnancy rates in most studies (B4, F9, F10, F11, R1, S2, S21, W3). Even in a study in which no limit is set to the number of embryos transferred, peak estradiol concentration is not correlated with pregnancy (H1). Some studies, however, show significant higher peak estradiol levels in patients conceiving (D5, L2). These are older studies with lower ovarian stimulation protocols, and stimulation was done without pituitary desensitization.

If estradiol levels are divided by the number of oocytes, pregnancy rates are significantly lower when the estradiol–oocyte ratio is 0–250 pmol/liter, compared to >250 pmol/liter (L13). The same study shows that pregnancy rates are significantly higher when the estradiol–oocyte ratio is 250–500 pmol/liter, compared to estradiol–oocyte ratios of <250 and >500 pmol/liter (L13).

The pattern of estradiol concentration during the days before hCG reflects the degree of maturity of the follicles (R7). When estradiol levels on the days before hCG administration plateau, significantly more oocytes are atretic and significantly fewer oocytes fertilize compared to those cycles where estradiol levels still increase (B7, L2). The percentage of pregnancies is not correlated to trend in estradiol concentration (B7).

Because of conflicting results in different studies, there is not enough evidence that peak estradiol levels are an adequate prognosticator for assisted reproduction outcome.

4.6. Peak Inhibin Levels

As inhibin concentration in serum is believed to be a marker for oocyte quality, it is interesting to learn whether inhibin A or B level during a treatment cycle is related to treatment outcome.

Total inhibin, measured on day 8 of human menopausal gonadotropin (hMG) in down-regulated cycles, is significantly correlated with estradiol levels, and is correlated with pregnancy (M5).

Peak inhibin A and B levels (measured on the day of hCG administration) are significantly higher in pregnant than in nonpregnant women (H1). Both inhibin A and B and total inhibin are significantly correlated with number of follicles (L10, M5, M6) and oocytes (H1, H2, M5, M6) also. Inhibin A is also significantly correlated with number of embryos (H1).

After controlling for age and number of oocytes, however, inhibin A and B concentrations on the day of hCG administration give no additional information in predicting pregnancy (H1).

Inhibin seems to be a promising predictor of ovarian response during IVF treatment, and therefore also of treatment outcome.

4.7. Progesterone

Untimely LH surges in IVF are associated with adverse treatment outcome (E4, L5). The effect of untimely LH surges can be premature luteinization of follicles. It is also argued that premature luteinization has not so much an adverse effect on the oocyte, but on endometrial receptivity, causing low pregnancy rates in patients (L4).

In order to prevent LH surges, desensitization with GnRH agonists is routinely performed. However, even after desensitization with a GnRH agonist, premature luteinization of follicles might occur and cause postmaturity of oocytes and subsequently poor results.

Elevated LH levels might cause elevated progesterone levels on the day of hCG administration. Elevated progesterone levels on the day of hCG administration could thus be correlated with adverse outcome of treatment.

It might be that elevated serum progesterone levels are caused by an exaggerated ovarian response to exogenous gonadotropins, as even recombinant FSH preparations have some LH bioactivity. A FSH-induced increased LH receptivity in granulosa cells might play a role in elevated progesterone levels (U1). Incomplete pituitary desensitization is not the reason for raised progesterone, as is suggested (F6, H8), as raised progesterone levels are even observed in patients treated with a GnRH antagonist (U2). On the other hand, in cycles with raised LH levels on the day of hCG administration, just as many have raised progesterone levels compared to cycles with normal LH levels on the day of hCG (L9). So the raise in progesterone does not seem to come from LH surges. The mechanisms responsible for subtle progesterone rise are unclear.

The effect of elevated progesterone levels in the late follicular phase of the treatment cycle on the outcome of IVF is unclear in clinical studies. Some studies report higher peak estradiol levels in patients with elevated progesterone levels (C10, G4, R3), while others report no difference (H8, U3) or lower peak estradiol levels (E3). Other studies found significantly lower pregnancy rates in cycles with elevated progesterone levels (D4, K1, R3, S6, S21), or no correlation between progesterone levels and pregnancy rates (B4, H10, R1, U3).

Elevated progesterone levels have an effect on both endometrium and ovary. The effect on the ovary seems to be more oocytes, as one study reports significantly higher pregnancy rates and more oocytes in oocyte donation cycles with elevated serum progesterone levels in the late follicular phase (L4). On the other hand, raised progesterone levels are also correlated with low fertilization rates, so there is also some negative effect on the oocyte (K1).

The effect on the endometrium is transformation of proliferative endometrium into secretory endometrium (C4). There is also a correlation between raised progesterone levels and pregnancy failure (D4), again drawing attention to the negative effect of raised progesterone levels on the endometrium. In controlled ovarian

hyperstimulation cycles, progesterone levels on the day of hCG administration as well as early luteal-phase progesterone levels are significantly higher compared to natural cycles, and endometrial histological features are also advanced compared to nonstimulated cycles (K8).

Besides the fact that progesterone has an effect on both the endometrium and the ovary, the different findings in different studies on the effect of progesterone on treatment outcome might also be the difference in progesterone assays, as some assays do not perform well in the lower ranges.

Most but not all studies point toward the general conclusion that elevated serum progesterone levels on the day of hCG administration reflect a good ovarian response (higher estradiol levels, more oocytes) but has a bad effect on the endometrial receptivity (lower ongoing pregnancy rate) (H7). Serum progesterone level in the late follicular phase is not fit to be used as an indicator for treatment outcome in assisted reproduction.

Paired serum progesterone measurements, however, one 12 h before and the other 12 h after hCG administration, can predict the chance of success of the cycle. A threefold or more increase in progesterone levels after hCG administration correlates significantly with implantation and ongoing pregnancy rates (P9).

4.8. Human Chorionic Gonadotropin

One rare phenomenon occurring in patients undergoing IVF is empty follicle syndrome, the failure to aspirate oocytes from follicles. This can be predicted by measuring beta human chorionic gonadotropin levels 36 h after hCG administration (i.e., at the time of oocyte retrieval) (N4). Women with empty follicle syndrome have significantly lower levels of beta-hCG (range 0–9 mU/mliter) than controls without the empty follicle syndrome (range 106–290 IU/liter) (N4). The fact that this syndrome occurs only very rarely, and that at the time the beta-hCG level is known, the laboratory also has concluded that no oocytes are present in the follicular fluid, makes this test not very practical for everyday use.

4.9. Cortisol Binding Protein

As cortisol is known to have a deleterious effect on oocyte quality (F3), cortisol binding protein is measured in serum in an attempt to identify cycles that have the best chances of resulting in pregnancy. Serum cortisol binding protein is significantly higher in women who become pregnant during IVF treatment compared to those who do not (A2). The lack of supporting studies and the relatively high p value (<0.05) makes cortisol binding protein not a good candidate for a marker of treatment outcome.

4.10. CA-125

CA-125 might be a marker for endometrial receptivity, and therefore serve as a marker for implantation and pregnancy in IVF treatment. The outcomes of different studies concerning CA-125 are conflicting. Serum CA-125 levels before and on the day of hCG, ovum pickup, and embryo transfer are not correlated with IVF outcome in some studies (B10, M4), while other studies report significant association between serum CA-125 levels on the day of hCG and conception (C6) and pregnancy (M10). The existence of a significant correlation, however, does not mean that CA-125 is a useful predictor of IVF outcome. Noci *et al.* (N5) have performed a receiver operating characteristic curve analysis, and showed that there is no useful cutoff value for CA-125. CA-125 is therefore not a useful tool in predicting the outcome of IVF.

4.11. Placenta Protein 14

Placenta protein 14 concentrations after gonadotropin stimulation for IVF might serve as a prognosticator for treatment outcome. On the day of oocyte retrieval, placenta protein 14 levels are significantly higher in patients conceiving compared to patients not conceiving (C6). Further, on the day of embryo transfer, concentrations are significantly increased compared to the day of ovum pickup.

Further studies have to be carried out on placenta protein 14 levels before it can be concluded that it is a useful marker for IVF outcome.

4.12. Number and Volume of Follicles

Ovarian response to stimulation can be measured by counting the number of antral follicles. Several studies have been done to assess whether a larger number of antral follicles is associated with success in treatment. The number of follicles >14 mm in diameter on the day of hCG is significantly correlated with peak estradiol levels and oocyte and embryo numbers in one study (F10). Surprisingly, however, pregnancy rates were significantly higher when only two follicles were >14 mm than when nine or more follicles were >14 mm (F10). This is in contrast with the results from other studies, showing that a large number of follicles (and a high mean follicular volume) is positively correlated with pregnancy (A4, D5).

Not all studies confirm these results, however. Several studies show that number of follicles and follicle size are not correlated with IVF outcome (F9, F11, S4).

4.13. Oocyte Number and Quality

In IVF, the number of oocytes retrieved is significantly correlated with pregnancy in some studies (C12, F9, H1, W3). Not all studies agree on this, however

(F11). Besides oocyte quantity, oocyte quality is also an important factor. Although immature oocytes can be fertilized *in vitro,* the incidence of pregnancy per transfer of immature eggs is half that of mature eggs (P8, V5). Oocytes with cytoplasmic abnormalities can be inseminated using intracytoplasmic sperm injection (ICSI), but almost never lead to pregnancy (K3).

5. Follicular Fluid

Biochemical markers in follicular fluid could be reliable criteria to select those oocytes with optimal quality for transfer.

There is evidence that oocytes which originate from follicles with high levels of FSH and LH have higher rates of fertilization (P8), but not all studies agree on this (H5). In pooled follicular fluid, neither FSH nor LH is correlated with pregnancy (F11). Some studies report that high progesterone levels in follicular fluid and a high progesterone/estradiol ratio in follicular fluid is associated with fertilization or pregnancy in IVF (B4, E7, H5, W4). The reason for this finding is not clear; as progesterone in follicular fluid is correlated with oocyte maturity (B9), high progesterone levels in follicular fluid might also be a sign of postmaturity. This correlation between progesterone level in follicular fluid and treatment parameters is lost when GnRH agonist is used for pituitary down-regulation (A3, B9, F11, H5, M12).

Estradiol levels in follicular fluid correlate significantly with cleavage rate and with pregnancy in some studies (A3, B9, C2, W4), but not all (H5). In pooled follicular fluid, estradiol level is not associated with pregnancy (F11, M12). It seems that the most estrogenic follicles are the major source of fertilizable oocytes.

IGF-I is found in follicular fluid, and dominant follicles have three times higher IGF-I concentrations in follicular fluid compared to cohort follicles in normal-cycling women (E2).

We have also measured IGF-I in follicular fluid and correlated the outcome with responsiveness to gonadotropin treatment in IVF(O4). We obtained follicular fluid from 70 women during an IVF treatment. All were under 40 years of age, and received a gonadotropin-releasing hormone analog (Decapeptyl, Ferring B.V., Hoofddorp, The Netherlands). For stimulation of follicular growth, patients received either human menopausal gonadotropin (Humegon, Organon, Oss, The Netherlands) or highly purified urinary FSH (Metrodin HP, Serono Benelux, The Hague, The Netherlands). Follicle size was determined by ultrasound until at least three follicles were $\geq$16 mm in diameter. HCG (10 000 IU i.m., Profasi, Serono) was given 35 h before oocyte retrieval.

After isolation of the oocytes, the follicular fluid from each patient was pooled, and after isolating the contaminating cells from the follicular fluid, the fluid was stored at -70°C until IGF-I determination. IGF-I was measured by radioimmunoassay (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA).

TABLE 1
DIFFERENCE IN IGF-I LEVELS BETWEEN PATIENTS WHEN LOOKING AT NUMBER OF AMPOULES OF FSH ADMINISTERED, NUMBER OF FOLLICLES ASPIRATED, AND DURATION OF STIMULATION

	IGF-I level (ng/ml)
≤36 ampoules FSH	151 ± 70.3[a]
>36 ampoules FSH	97.6 ± 47.8
≤12 follicles aspirated	116 ± 57.3[b]
>12 follicles aspirated	155 ± 78.1
≤12 days stimulated	140 ± 70.6[c]
>12 days stimulated	110 ± 56.6

Numbers are means ± standard deviations.
[a] $p = 0.001$, IGF-I levels ≤36 ampoules vs >36 ampoules.
[b] $p = 0.05$, IGF-I levels ≤12 follicles vs >12 follicles.
[c] $p = 0.06$, IGF-I levels ≤12 days vs >12 days.

The mean concentration of IGF-I in follicular fluid was 129 ± 67 ng/ml (mean ± SD). Linear regression analysis showed that IGF-I concentrations were significantly and inversely correlated with both the number of ampoules of FSH administered (Pearson's correlation coefficient $= -0.405$, $p = 0.001$) and with the number of days of FSH administration (Pearson's correaltion coefficient $= -0.249$, $p = 0.039$).

IGF-I levels in follicular fluid correlated significantly with the number of follicles aspirated ($R = 0.317$, $p = 0.008$). Patients who needed >36 ampoules of FSH had significantly lower concentration IGF-I levels in their follicular fluid compared to patients needing ≤36 ampoules of FSH (97.6 ± 47.8 vs 151 ± 70.3 ng/ml, $p = 0.0001$). Patients with ≤12 follicles had significantly lower IGF-I concentrations than patients with >12 follicles (116 ± 57.3 vs 155 ± 78.1 ng.ml, $p = 0.05$). There was an apparent but not significant trend toward lower IGF-I concentrations in follicular fluid from patients stimulated for >12 days compared with patients stimulated ≤12 days (110 ± 56.6 vs 140 ± 70.6 ng/ml, $p = 0.06$)(Table 1). This study is in line with evidence that IGF-I concentrations in follicular fluid are higher in patients who respond better to treatment. Other have found that IGF-I concentrations in follicular fluid correlate with oocyte maturity, but not with treatment outcome (R8). There are also studies, however, that find no supportive evidence for IGF-I playing a role in follicular maturation or oocyte quality (S16, V1). These conflicting results stress the uncertainty about the exact role IGF-I plays in folliculogenesis.

Glycosaminoglycanes are present in follicular fluid, and the glycosaminoglycanes chondroitin sulfate and heparan sulfate have been shown to be related to

follicular maturation in animals. In humans, a weak but statistically significant relation has been found between chondroitin sulfate concentrations in follicular fluid and fertilization rates (B6). No other relation could be found between glycosaminoglycane concentrations and development of embryos in humans. Glycosaminoglycane concentration is therefore no predictor for success in IVF treatment.

Placenta protein 14 was measured in follicular fluid and found to be significantly higher in conception compared to nonconception cycles (C6).

In follicular fluid, renin–angiotensin is produced by the theca cell (D6, P5). Its levels are independent of sodium intake (B5). The renin–angiotensin system in the follicle is possibly involved in the neovascularization of the follicle (F5). Although the variation in renin concentration in follicular fluid is large, total renin concentration in follicular fluid is significantly correlated with delivery after IVF in one study (C11). However, this finding is not confirmed by other studies (B5, I1, P5). It can be concluded that although (pro)renin probably plays a role in follicle maturation (D2, G5, I1, I2, P3), follicular fluid concentration of renin is no suitable prognosticator for IVF outcome.

An increase in alfa1-antitrypsin in follicular fluid is associated with pregnancy (P8), but the correlation is not strong enough for alfa1-antitrypsine to be an adequate marker for IVF treatment outcome.

The adrenal axis seems to play a role in reproductive function in women as well as in rats. Therefore, several components of the corticotropic axis have been measured in plasma and follicular fluid, and correlated with the outcome of treatment in IVF patients. There is no significant difference in change of components of the corticotropic axis between women who become pregnant and those who do not (K5). Cortisol-binding protein and sex-hormone binding globulin are significantly higher in follicular fluid from women who become pregnant after IVF compared to those who do not (A2). The reason might be that both proteins bind cortisol and other factors which have negative effects on oocyte quality. It is therefore conflicting that high cortisol levels in pooled follicular fluid are associated with pregnancy (M12). Transforming growth factor beta1 is a member of a family of peptide growth factors that plays a role in regulating growth and differentiation of cells. Transforming growth factor beta1 has been measured in pooled follicular fluid from IVF patients, and is significantly higher in patients who become pregnant (F10). For transforming growth factor beta1 in follicular fluid to be a marker for success in IVF, there need to be more studies done showing the same outcome.

6. Granulosa Cells

Follicular fluid and granulosa cells derived at ovum pickup in an IVF treatment, can reflect the qualities of the follicle, and therefore the oocyte.

In vitro, granulosa cells can be cultured and secrete hormones. Estradiol and progesterone secreted *in vitro* by granulosa cells are not correlated with treatment outcome, but relaxin secretion is correlated with conception and with term pregnancy (S25). Relaxin has a stimulating effect on endometrium, and this might be the reason for the reported correlation.

As cortisol has a deleterious effect on embryo quality (A2), 11beta-hydroxysteroid dehydrogenase—which inactivates cortisol by conversion to cortisone—might play a positive role in oocyte development. When 11beta-hydroxysteroid dehydrogenase production by granulosa cells is determined, none of the patients with 11beta-hydroxysteroid dehydrogenase-positive granulosa cells achieve clinical pregnancies, while more than 60% of patients with 11beta-hydroxysteroid dehydrogenase-negative granulosa cells achieve clinical pregnancies (M8, M9). This finding could not be confirmed by another study (T3). When 11beta-hydoxysteroid dehydrogenase activity is measured (not by measuring 11beta-hydroxysteroid dehydrogenase directly, but by measuring enzyme activity), no correlation is found between enzyme activity and pregnancy rates, not even on a per-follicle basis (K7). Besides as a source of hormone production, granulosa cells themselves can be measured. Seifer *et al*. (S13) have measured the proliferative index of granulosa cells and compared the outcome with basal FSH. Granulosa cells from women with basal FSH levels $\geq$18 IU/liter have a significantly smaller proliferative index compared to granulosa cells from women with basal FSH $\leq$6 IU/liter. The clinical pregnancy rates were not different between the two groups.

Apoptosis is the main destination of follicles (H14). In IVF, some follicles are saved from this fate by exogenous administration of supernatural amounts of FSH, the main survival factor from follicle apoptosis (C7). Apoptosis in granulosa cells might reflect the vitality of the oocytes harvested. In women with high basal serum FSH levels, a significant larger percentage of granulosa cells are apoptotic compared to women with low basal serum FSH levels (S14). Also, the incidence of apoptotic bodies from mural granulosa cells is significantly correlated with oocyte quality, fertilization, and pregnancy (N1, N2). In these studies, apoptotic bodies are counted by examination of the nuclei of recovered granulosa cells by fluorescence microscopy. Using this method limits the number of cells determined compared to other methods such as flow cytometry.

We have determined apoptotsis in granulosa cells by flow cytometry (O3). Granulosa cells were obtained from patients undergoing ovarian hyperstimulation in an IVF program. Study endpoints were age, basal serum FSH, number of ampoules of FSH needed for adequate ovarian hyperstimulation, treatment duration, number of follicles and oocytes, and percentage apoptotic granulosa cells. The granulosa cells were collected from follicular fluid obtained via ultrasound-guided transvaginal oocyte retrieval. After the oocytes were isolated from the follicular fluid, the follicular fluid from each patient was pooled, in order to be able to relate the response of each patient and not the individual follicle to the number of apoptotic granulosa

TABLE 2
OUTCOME IN THE PREGNANT VS THE NONPREGNANT GROUP

Variable	Pregnant ($n = 6$)	Not pregnant ($n = 9$)	p value
Age (y)	32.8 ± 2.3	32.2 ± 3.6	NS
Basal serum FSH (mlU/ml)	6.8 ± 1.8	5.8 ± 1.4	NS
Duration of treatment (d)	10.7 ± 1.2	11.8 ± 2.2	NS
No. of ampoules of FSH	27.8 ± 5.2	36.6 ± 20.5	NS
No. of follicles	13.0 ± 4.2	12.6 ± 4.3	NS
No. of oocytes	10.3 ± 3.4	9.9 ± 2.9	NS
No. of oocytes fertilized	5.8 ± 3.4	4.0 ± 2.4	NS
Apoptotic granulosa-lutein cells (%)	7.1 ± 5.1	20.7 ± 13.7	0.021

All values are means $\pm$ standard deviation. NS = not significant.

cells. The granulosa cells were isolated from the red blood cells by a 60% Percoll solution (Pharmacia Biotech, Uppsala, Sweden) and from the leukocytes with the use of magnetic cell sorting.

Apoptosis was measured by TdT-mediated dUTP nick end labeling (TUNEL) by flow cytometry as described by Gorczyca *et al*. (G6). Every measurement was done in duplo, and a negative and postive control were carried out with each experiment. All patients had normal basal FSH levels, and their partners had normal semen parameters.

We found a statistical significant difference in apoptosis when we compared the patients who became pregnant with those who did not ($p = 0.021$) (Table 2). We also found a significant inverse correlation between the number of embryos obtained and the percentage of apoptotic granulosa cells detected ($p < 0.05$). This is the first study to find a significant relation between number of apoptotic granulosa cells measured by flow cytometry and the outcome of IVF in patients with normal basal FSH levels. Patients who became pregnant did not differ in any parameter measured from patients who did not become pregnant, except for apoptosis in granulosa cells. The cutoff level we found is 13% apoptotic granulosa cells. All women who became pregnant had <13% apoptotic granulosa cells, whereas all women who had a higher percentage of apoptotic granulosa cells did not become pregnant.

It can be concluded that in IVF, women who have more apoptosis in their follicles, reflected by percentage apoptotic granulosa cells, also have less chance of pregnancy.

7. Quality of Embryos

Choosing the right embryo(s) for embryo transfer is essential in optimizing pregnancy rates in IVF. If transfer of only one embryo in order to prevent multiple

pregnancies will be the practice in the near future, assessing embryo quality will become even more important than today.

7.1. Embryo Quality Scoring

Embryo quality is routinely used in IVF laboratories to assess which embryo(s) have the best chance of implanting and therefore need to be transferred, and embryo quality is correlated with pregnancy (S7, S25).

Zona pellucida thickness is probably influenced by the preovulatory hormonal environment (L11), and its variation is significantly correlated with pregnancy (P2). If zona pellucida thickness variation $\{[(Z_{max} - Z_{mean})/Z_{mean}] \times 100\}$ is below 15%, pregnancy rate is only 4.5%, even if three embryos are transferred. If zona pellucida thickness variation of any of the replaced embryos is 15–20%, pregnancy rate rises to 24.1%, and if zona pellucida thickness variation is more than 20%, the pregnancy rate is 76.5% (P2). The reason for thickness variation being important is probably the ability to hatch, which is presumed to be larger in embryos with more variation in zona pellucida thickness.

The number of visible mononucleated blastomeres is also a significant predictor of pregnancy. If at least one blastomere of a transferred embryo has a single visible nucleus, pregnancy rate is 59.2%, while it is 13.9% in the group with no single visible nucleus (P2). Visible mononucleated blastomeres represent blastomere developmental potential, and are therefore correlated with pregnancy. In contrast, when embryos have multinucleated blastomeres, pregnancy rates are reduced, even though multinucleated embryos are correlated with a better ovarian response to gonadotropin therapy (J1).

Regularity in blastomere shape and identical blastomere size are not significant predictors of pregnancy (P2).

The rate of cleavage during the first 2 days after fertilization is not a reliable prognosticator for embryo quality (P8).

Blastocyst formation is correlated with age and pregnancy (W5). This is further evidence that the negative effect that age has on treatment outcome has to do with oocyte and embryo quality.

7.2. Embryo Culture Media

The quality of embryo culture media is essential for optimizing treatment outcome in IVF. Comparing different culture media is outside the scope of this review. However, several factors found in culture media of embryos, released by the embryo, might reflect qualities of the embryo associated with implantation.

Interleukin-1beta is present in culture media of embryos, and the level correlates significantly with pregnancy (B3).

8. Hormone Levels after Embryo Transplant

8.1. Human Chorionic Gonadotropin

In order to test the viability of a pregnancy resulting after IVF or gamete intrafallopian tube transfer, a serum beta-hCG concentration can be determined 2 weeks after embryo transplant (S5). There is a statistically significant higher beta-hCG concentration in women with pregnancies continuing after the first trimester compared to women with nonviable pregnancies. When a second measurement is done 1 week later, the diagnostic accuracy for prediction of pregnancy outcome is significantly higher (C3). This test might reduce anxiety in the patient, as the authors claim. It is, however, only applicable 2 weeks after embryo transplant.

8.2. Vascular Endothelial Growth Factor

Vascular endothelial growth factor is a growth factor playing a role in corpus luteum development. Measured in serum on the day of ovum pickup, vascular endothelial growth factor is the same in women who become pregnant after IVF and those who do not (L3). However, when measured on days 11–14 after embryo transfer, women who become pregnant have significantly higher levels than women who do not become pregnant (L3).

9. Discussion and Conclusion

An ideal prognosticator for ovarian function is cheap, noninvasive, easy to perform, and reliable. As far as reliability goes, almost all of the above-mentioned tests provide only a probability of a poor response in assisted reproduction. Most patients and most doctors would not make any final decision about treatment based on the outcome of one of these tests alone. However, carrying out one or two tests might help in counselling before treatment.

The earlier in the diagnostic or therapeutic process a prognosticator can be applied, the more valuable it becomes. If, on the basis of the outcome of a prognostic test, a specific treatment can be chosen or patients can be counselled to refrain from any treatment, this test is more valuable than a test which can only be done during or after a treatment.

The studies can be placed in one of two categories. Some studies are carried out from the scientist's point of view, and are designed to gain more information about the processes playing a role in physiological and pathological fertility. These studies provide a lot of information about fertilization and implantation, but generally lack patient- and laboratory-worker friendliness. An example of these studies is determining hormone levels in follicular fluid per on a per-follicle basis: this

gives a lot of information about what qualities determine whether an oocyte leads to a pregnancy, but it is not very practical for routine use. The second category of studies is those that are performed from a clinician's point of view, and are designed to help the clinician in determining the prognosis of the treatment. An example is basal FSH.

The general impression of most studies is that as reliability improves, noninvasiveness and ease of performance decrease. The predictive value of basal FSH can be improved by the clomiphene citrate challenge test, but this also adds to invasiveness and patient discomfort.

In this review, many possible prognosticators have been discussed. Age and basal FSH combined with basal estradiol are well-established prognosticators. FSH measured in urine seems to add to patient comfort without decreasing reliability, but more work has to be done to examine the value of urinary FSH as a determinator of ovarian function.

Besides these, the most promising factor is basal inhibin in the female. In order to assess the quality of the oocyte or embryo in IVF, apoptosis-related factors might become important. We have shown that apoptosis in the follicle is a major contributor to a lack of success in assisted reproduction. Also, 11beta-hydroxysteroid dehydrogenase measured in embryo culture media seems to be a promising factor in selecting the best embryo for transplant.

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