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AGEING—A BIOLOGICAL PERSPECTIVE

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Preface

Ageing has many facets. In the case of human beings, ageing is not only an important biological issue, but is also an important social and emotional issue that affects almost all aspects of our lives. A failure of maintenance underlies the biological process of ageing, during which random and progressive senescence of an organism's various constitutive parts eventually leads to its death. The property of homeostasis enables an organism to survive and remodel itself constantly in spite of perturbations from internal and external sources. Not all parts of the body become functionally exhausted with age. Rather, it is the interactions between individual components that sustain the body and determine its survival. The ultimate aim of biogerontological research is to understand why these changes occur, how they affect various other constituents of the network, and how these can be modulated in order to maintain the healthy span of life. The attainment of this goal will require the development of experimental approaches in which the interactions between mechanisms of maintenance at various levels is studied and the reasons for their failure are elucidated.

Chapter 1

A Question of Life and Death

All living systems have a unique property, called homeostasis, by virtue of which they maintain stability and functional integrity while adjusting to perturbations from internal and external sources. This property, at least theoretically, gives organisms the potential to survive for ever by maintaining a steady state. In reality, however, individual cells and organisms are mortal. Sooner or later, all individuals die out even though the apparent immortality of a population or of the germ line may overshadow the mortality of its individual members.

In nature, a vast variety in strategies for survival can be encountered and the spiral of life and death has innumerable variations. Rates of degenerative change fall into three main categories—rapid, negligible and gradual (Finch, 1990); these can explain most types of life history which culminate in the death of an individual. For example, rapid degeneration is common among species that die during or soon after reproduction. Such organisms generally undergo a single bout of reproduction during their life and are called semelparous in the case of animals, and annual or monocarp for plants. At the other end of the scale are organisms in which the rate of degeneration is imperceptible over very long periods and can be considered as negligible. Various vascular plants such as Bristlecone pine and other conifers; invertebrates, such as lobsters; and vertebrates, such as rockfish and tortoise, belong to this category.

The third category, found most commonly in animals, involves the growth and development of the organisms to adulthood and a period of reproduction followed by gradual ageing and senescence leading to death. Generally, species with repetitively reproducing (iteroparous) life histories experience ageing after completing a period of reproductive fitness. It is in this category of organisms, which includes human beings, that the phenomenon of progressive and intrinsic ageing is best manifested during the limited lifespan of the organism.

Is There a Species-Specific Maximum Lifespan?

It is generally believed that there is a species-specific maximum lifespan. This belief has frequently been challenged on the basis of both demographic–statistical analyses and experiments with very large cohorts of insects. For example, according to the Gompertz–Makeham equation which is the most widely used population model for mortality rates (the fraction of survivors that die in the next time interval) and lifespan, the mortality rate is considered to increase exponentially with age. It has been argued that if this model is correct, then at some stage the

probability of death (also called force of mortality) should become unity (Gavrilov and Gavrilova, 1991). However, extensive analyses of a large number of life tables generated for a wide range of species, including human beings, have shown that although the Gompertz–Makeham law holds true up to a certain age, at the most advanced ages there is no increase of mortality rates which may actually decline (Gavrilov and Gavrilova, 1991; Fukui *et al.*, 1993).

Experimental results of studies on large cohorts of insect populations also speak against the notion of a very rigid species-specific longevity. For example, studies with the medfly *Ceratitis capitata* have shown that a highly extended lifespan of 170 days could be achieved for the last one in a million surviving medflys, as compared with 20 days for 50% of the population, 64 days for 10% of the population and 103 days for 1% of the population (Carey *et al.*, 1992). These studies have shown that, contrary to the Gompertz law, life expectancy in older individuals increases rather than decreases with age. Similarly, analysis of ten genetically identical lines of *Drosophila melanogaster* also failed to show an age-specific increase in mortality rates at most advanced ages where, instead, the mortality rate levelled off (Curtsinger *et al.*, 1992). Further evidence that there is no continued acceleration in the rate of mortality at advanced ages comes from studies performed with highly inbred (for more than 70 generations) lines of *D. melanogaster* (Fukui *et al.*, 1993). All these observations suggest that a rigid species-specific limited lifespan paradigm is incorrect.

In the case of human beings too, the mortality rate stops increasing and either levels off or decreases after 85 years of age (Gavrilov and Gavrilova, 1991). As in the case of laboratory animals, the dependence of human mortality on age comprises three periods: (i) of high child mortality; (ii) of sexual maturity and a progressive increase in the force of mortality in accordance with the Gompertz–Mekham law; and (iii) a senile period of high mortality rate without any further acceleration in the force of mortality. However, a detailed analysis of available human life-tables shows that mortality stabilisation levels are different for different regions of the world and for different sexes (Gavrilov and Gavrilova, 1991).

Attempts at estimating the upper limits to human lifespan have failed to reach any definite conclusion. The so-called scientific estimates of human longevity give a value in the range 60–200 years (Gavrilov and Gavrilova, 1991). In contrast, guesses about human longevity found in different cultures are within a relatively narrow range from 100 (in the Indian Rigveda) to 120 years (in the Old Testament), which is closer to the observed and verified longevity of 120 years on 21 February 1995 for a French lady. It is now generally believed that an increasing number of individuals will be reaching ages beyond 100 years and that it will not be surprising if human longevity goes well beyond 120 years (Kannisto *et al.*, 1993; Olshansky *et al.*, 1993).

Therefore, concepts such as 'species-specific lifespan' or 'maximum lifespan potential' are of not much value when no reliable estimate of the maximum lifespan achievable by an individual can be made. Furthermore, since there are large differences in the lifespans of individuals within a single population, and lifespan distributions vary widely from population to population within the same species, the notion of a species-specific maximum lifespan has no reliable quantitative basis (Gavrilov and Gavrilova, 1991).

For practical purposes, however, the maximal lifespan for a population can be determined by selecting arbitrarily an age up to which only a fraction (for example, 0.1 or 0.001%) of the

initial number in the cohort survive. Although the value for the maximal lifespan obtained in this way does not define an absolute upper limit, a functional practical limit can be assigned within the context of the life history of a given species in a given condition. The studies by Carey *et al.* (1992), Curtsinger *et al.* (1992) and Fukui *et al.* (1993) discussed above show precisely that, whereas no absolute limit to longevity can be inferred from the data, there is still a practical limit to lifespan, and no fly could realistically attain longevity characteristics comparable to, say, those of mouse or a man. Furthermore, such a measure of the practical limit makes room for the possibility of alteration in maximum achievable lifespan with changing conditions of life, which, in the case of human beings, include social, psychological and cultural elements.

Evolution of Life and Death Mechanisms

In order to develop a complete understanding of ageing, it is important to discuss cellular and molecular determinants of longevity in the context of evolutionary theories. As discussed above, even in the absence of all accidental causes of death, there are intrinsic processes (such as the extent of damage accumulation) that in practice place a limit upon survival. This limit and its manifestation in death (either sudden and rapid, or following a period of progressive ageing and senescence) are linked with the life histories of various species.

In the context of the early evolution of ageing, it is incorrect to assume that ageing and the limited lifespan of an individual had some purpose or adaptive significance in terms of being advantageous for the species. In natural, wild populations the probability of death by accidental causes, including disease and predation, is so high that there is never a significant number of long-lived individuals left which might require special mechanisms to terminate life for the sake of newly born individuals. Furthermore, even if there were any life-terminating mechanisms that operated after a long period of survival, these would not be capable of resisting the spontaneous origin and evolution of non-ageing and immortal 'mutants', which in a given population would soon take over (also see Rose, 1991; Partridge and Barton, 1993; and Holliday, 1995).

In contrast to the adaptive theories of the evolution of ageing and lifespan, the non-adaptive theories state that ageing occurs either because natural selection is insufficient to prevent it, owing to its post-reproductive nature, or that senescence is a by-product of the expression of genes with early beneficial traits but deleterious and pleiotropic effects at later stages. Two major schools of thought (whose ideas are not mutually exclusive) in the non-adaptive theories of the evolution of ageing and lifespan are represented by *antagonistic pleiotropy* (Rose, 1991), and the *disposable soma theory* based on Weismann's distinction between the soma and the germ line (Kirkwood and Cremer, 1982).

According to these theories, evolutionary forces have optimised the conditions for efficient and successful reproduction either by (i) selecting for 'good' early genes that later have 'bad' effects (Rose, 1991); or (ii) selecting for efficient maintenance and repair of the germ cells at the cost of somatic maintenance (Kirkwood, 1992). However, the ideas of late-acting deleterious genes or of pleiotropic genes with early beneficial effects and late harmful effects both beg the question of what constitutes 'early' and 'late' in life. Any genes whose expression or effect is dependent upon certain signals of passing biological time can at best be a consequence of the ageing process rather than being its cause.

The disposable soma theory (Kirkwood and Holliday, 1979; Kirkwood, 1992) considers the evolved life history and the structural and functional design of the organism to be the basis of the evolution of ageing. According to this theory, evolutionary forces have selected genetic mechanisms for the survival of the soma only to the extent of fulfilling the Darwinian purpose of life, that is, assuring the reproduction and continuation of the germ line. One of the major predictions of the disposable soma theory is that potentially immortal germ-line cells should have more efficient and accurate processes of maintenance and repair than somatic cells. Secondly, there should be a direct relationship between longevity and the efficiency of maintenance mechanisms. Thirdly, there should be an inverse relationship between longevity and fecundity, because of the trade-off between the allocation of resources for the maintenance, survival and continuation of the germ line at the cost of reduced maintenance and survival of the soma.

Thus, evolutionary forces of natural selection have resulted in evolving mechanisms of maintenance that operate in concert with the complete structural (anatomical) and functional (physiological) design of the organism and assure the survival of the body until reproduction. After this point, evolutionary mechanisms will tend to prolong the period of reproductive competence, but not indefinitely, as the cost of this would be too high. There was no selection pressure to maintain the body for ever, nor was there a need to evolve special mechanisms for the termination of life. An inability to maintain the soma for ever or the failure of maintenance underlines the biological process of ageing during which random and progressive senescence and deterioration of various constitutive parts lead to death.

Mechanisms of Maintenance and Repair

An organism's property of homeostasis enables it to survive and to remodel itself constantly in the wake of perturbations from internal and external sources. There are several ways in which biological entities maintain homeostasis. These are also referred to as the longevity–assurance processes. Table 1 lists the major modes of maintenance at various levels of organisation. These mechanisms include the processes of cellular and sub-cellular repair, cell division, cell replacement, neuronal and hormonal responsiveness, immune response, detoxification, free-radical scavenging, stress-protein synthesis, macromolecular turnover and maintaining the fidelity of genetic information transfer. There may be other modes of maintenance which are more specific, such as thermoregulation, sleep and muscle fatigue developed during the evolution of different types of organism, each according to the particular conditions under which it lives.

Generally, these mechanisms of maintenance work efficiently during the major part of life, during which growth and development take place and reproductive maturity is attained. During the post-reproductive period, an ageing organism exhibits a broad spectrum of changes manifested mainly as an overall decline in most of its bodily functions. The process of ageing is the primary reason for the emergence of a wide variety of diseases with increasing age. For example, ageing of the brain can lead to cognitive impairments, memory loss and dementia; ageing of the heart can result in ischemia, angina and infarction; ageing of the lungs can give rise to emphysema and bronchitis; ageing of the eye tissue results in macular degeneration, glaucoma and cataract; ageing of the skeletal system is the cause of arthritis, osteoporosis and fractures, and so on for each and every organ and system.

This does not, however, imply that the intrinsic process of ageing is bound to result in various

Table 1. Major modes of maintenance and longevity assurance processes

Whole-body level
Neuronal and hormonal responsiveness
Immune defences
Thermoregulation
Repair, wound healing and regeneration
Tissue level
Neutralising and removing toxic chemicals
Tissue regeneration
Cell replacement and turnover
Cellular level
Stability of the differentiated state
Regulation of cell proliferation
Stability of the cellular milieu (viscosity, ion balance, pH)
Cell communication
Molecular level
Stability of the genome
Fidelity of genetic information transfer
Turnover and degradation of macromolecules
Stress-protein synthesis
Scavenging of free radicals

diseases in every elderly individual. The origin, occurrence and manifestation of a disease depends upon several interacting factors, such as the individual's genetic make up, environmental conditions and nutritional status. In the case of human beings, factors such as psychological state and life style, including socio-economic status, are also important modulators of health and disease (Taylor, 1992).

Understanding Ageing

The phenomenology of ageing is rich in empirical data about age-related changes in numerous systems. Innumerable papers have been published in the field of biogerontology describing age-related changes in cells, tissues and organisms. What these descriptive studies tell us about the nature of the process of ageing is that it is highly complex, and that it implicates both genetic and epigenetic stochastic causative factors. While the process and the end result of ageing are obvious, causes and mechanisms remain elusive, thus making the unravelling of the underlying mechanisms one of the most challenging tasks of modern biology.

This task is made even more difficult by the fact that there do not appear to be any deterministic, predictive or universal biomarkers of ageing. The diversity of biological systems used in ageing research, their widely varying rates of senescence and the fact that almost all characteristics undergo alteration with age make it very difficult to distinguish between 'specific' changes and 'universal' ones. Considered individually and in isolation from other parts, no part of the body becomes functionally exhausted, but it is the interactions between individual components that sustain the body and determine its survival. As a result, the organism becomes increasingly vulnerable to intrinsic and extrinsic risk factors, and the ability of the organism to survive diminishes.

The aim of this review is to contribute to a better understanding of the biological process of ageing in terms of its cellular and molecular determinants. For this purpose, a functional definition of ageing as 'the failure of maintenance' (Holliday, 1988) is used as a point of departure. Thus the fundamental characteristic of ageing is the failure of maintenance, and this is discussed at the levels of the whole body, of cell proliferation, of genomic stability and of the transfer of genetic information and cellular metabolism. It is not the aim of this article to give a comprehensive list of age-related changes in various organs, tissues and cells of the body. Our aim is to construct a biological perspective on ageing in the light of the available data and to achieve a better understanding of the phenomenon with a view to developing future strategies of intervention for attaining a healthy old age.

Chapter 2

Failure of Maintenance: Whole-body Level

Neuronal and hormonal responsiveness, immune defences, thermoregulation and repair, wound healing and regeneration are some of the major modes of maintenance at the whole-body level. Together, these form a homeostatic network that keeps the body in an ordered state of functional interdependence. Some of the main structural components of such a network include endocrine glands, cells and tissues of the immune system, the hypothalamus of the brain, and the replicative cells of the body. It is not intended here to give a detailed description of age-related changes in various structural components of the brain, the immune system and other tissues. Instead, alterations in the accuracy and efficiency of the above-mentioned homeostatic processes are discussed in the context of organismic ageing.

Altered Neuronal and Hormonal Responsiveness

A highly complex network of chemical mediators, particularly neurotransmitters and hormones from the endocrine glands, regulates many physiological and behavioural responses. It is primarily through this network that an organism is able to respond to internal and external stimuli in such a manner that normal processes of growth, maturation and development occur, leading to a successful period of reproduction. Alterations in the synthesis and the specificity of hormone action, including responsiveness to hormones and neurotransmitters by the target tissues, are major determinants of ageing. Some of the major hormones, classified on the basis of their chemical nature are listed in Table 2 (after Timiras, 1995).

The neurotransmission network

Neurons, glial cells and the connective tissue intercommunicate and thereby function as a single unit, the neurotransmission network. Alterations in any of these components will result in the disturbance of balance, the interruption of intercellular relationships and finally the disruption of function. Age-related changes in the structural and functional aspects of neurons and glial cells, including a loss of specific neurons, have been well documented (Duara *et al.*, 1985; Goudsmit *et al.*, 1990). As regards the neurotransmission network, age-related changes in dopaminergic, noradrenergic, serotoninergic, cholinergic, neuropeptidic, GABAergic, and amino-excitory systems have been reported (Allain and Bentué-Ferrer, 1995). Significant decreases in the densities of major subtypes of striatal dopaminergic receptors D_1 and D_2 have been observed, which are associated with impairments in motor skills requiring balance, strength and

Table 2. A list of major hormones grouped by chemical structure

Steroids
Aldosterone, cortisol, oestradiol, progesterone, testosterone, vitamin D
Amines
Epinephrine, norepinephrine, melatonin, thyroxine (T4), triiodothyronine (T3)
Glycoproteins
Human chorionic gonadotropin (hCG), follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyroid-stimulating hormone (TSH)
Polypeptides
Adrenocorticotropic hormone (ACTH), calcitonin, erythropoietin, gonadotropin-stimulating hormone, growth hormone, insulin, nerve growth factor, oxytocin, parathyroid hormone, prolactin, somatostatin, TSH-stimulating hormone (TRH), vasopressin

co-ordination (Roth, 1995). Similarly, a decline in the synthesis and activity of cholinergic system components, for example, acetylcholine (ACh) and variations in receptor response to ACh have been reported, and these correlate with cognitive disorders of ageing (Allain and Bentué-Ferrer, 1995).

In the case of α_1-adrenergic and muscarinic–cholinergic systems, a number of G-protein-linked receptor systems show altered affinity states and coupling–uncoupling problems during ageing (Roth, 1995). A decrease in the α_1-adrenergic responsiveness is associated with functional alterations in the coupling of G-proteins with α_1-adrenergic receptors. A similar age-related reduction is reported for muscarinic cholinergic stimulated dopamine release, which is not due to the loss of muscarinic receptors but is due to impaired coupling between G proteins and their receptors during ageing.

Other neurotransmitters also undergo changes to varying extents during ageing. For example, the level of γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter, declines only slightly during ageing, while the extent of the GABA receptor binding does not change (Amenta *et al.*, 1992). Opiate peptides such as β-endorphin, met-enkaphalin and leu-enkephalin, substance P (a peptide chain of 11 amino acids), somatostatin, vasopressin and oxytocin do not undergo major age-related alterations.

Hormonal responsiveness

The synthesis and secretion of hormones and their action on target cells, along with a number of feedback mechanisms, make up one of the most complex homeostatic processes in the body. Hormone secretion (i) by endocrine glands such as the pituitary, thyroid, parathyroid, pancreas, gonads and adrenals; (ii) by diffuse endocrines such as gastrointestinal hormones gastrin, gastric inhibitory polypeptide, secretin and somatostatin; and (iii) by atypical endocrine glands (the pineal and the thymus) are the major components of the hormonal responsiveness machinery.

Ageing brings about changes in the endocrine glands both directly and indirectly. Glandular

atrophy, vascular changes and fibrosis are some of the direct effects of ageing. Indirectly, alterations in other endocrines, the nervous system, the immune system, body metabolism, body composition and cellular and molecular processes may affect the hormonal responsiveness of the cells. Much information is available regarding age-related changes in various glands and cells producing hormones . Many studies have been made which describe changes in the amounts, activities, specificity, and receptor binding and feedback aspects of hormone action. Altered hormonal responsiveness is also the basis of some of the major age-related impairments and diseases, including diabetes, osteoporosis, hyperthyroidism, masked hypothyroidism, breast cancer, and various disorders associated with cognition and motor skills. Similarly, ageing of the hypothalamus leads to a reduced capacity for thermoregulation and then to increased sensitivity to sudden changes in environmental temperatures (Timiras *et al.*, 1995).

Recently, a resurgence of interest in the pineal gland as an important site of ageing regulation has drawn attention to this component of the hormonal responsiveness network. This is primarily because of significant new advances in the field of chronobiology, which have shown that circadian rhythms are a fundamental aspect of homeostasis. The pineal gland is a neuroendocrine transducer whose main function is to synchronise endogeneous circadian rhythms through the secretion of the melatonin hormone. A decline in the amount of melatonin and a disturbance in its light–dark rhythm of synthesis are associated with disturbed bodily rhythms and increased susceptibility to age-related diseases (Reiter, 1995). Therefore, it appears that the pineal gland as a component of the hormonal responsiveness network has important role in the regulation of ageing, which demands further investigation.

Immune Defences

The immune system is a key component of the homeostatic machinery, and its progressive failure is considered integral in the ageing process. Alterations in the functions of the immune system are generally seen either as defects and immunodeficiencies or as excesses such as allergy and autoimmunity. A decreased response to vaccines and an increased proneness to infections, higher levels of autoantibodies and increased incidence of diseases in the elderly point toward a failure of the immune system during ageing. However, the data obtained regarding age-related changes in the number and function of B- and T-lymphocytes and their subsets are controversial, particularly when conclusions drawn from studies performed on rats and mice are compared with data from human beings.

In the case of ageing mice, a population of T-cells can be viewed as a changing mosaic of cells in which higher proportions of native or virgin cells in young animals change to a higher proportion of memory T-cells in the old (Miller, 1994). Such a shift is related to the decreased responsiveness of older animals to certain classes of stimuli, including Con A and staphylococcal enterotoxin B. However, in human beings, although an accumulation of T-cells with memory markers has been observed, comparable shifts in the subsets $CD4^+$ and $CD8^+$ and in the absolute number of virgin $CD45RA^+$ and memory $CD45R0^+$ have not been seen (Miller, 1994; Franceschi *et al.*, 1995). Similarly, although some changes in calcium signal production affect T-cells in ageing mice, it is not clear whether this is an important factor in human immunosenescence (Miller, 1994).

Studies with cultured T-cells have shown that they have a limited capacity to divide (Pawelec, 1995). A similar proliferative limitation *in vivo* is seen by the increase in the number of CD28-negative cells in the peripheral blood of elderly donors (Effros *et al.*, 1994). Furthermore, there

is a reduced expression of interleukin 2 (IL 2), which is indicative of failing immune response with age (Effros *et al.*, 1994). A decreasing pool of naive T-cells may also be due to thymic involution during ageing, which also results in a decline of serum thymulin levels along with a decrease in the production of other hormones such as thymopoietin and thymosin (Pawelec, 1995).

Other cells of the immune defense system, such as cytolytic T lymphocytes (CTL), natural killer (NK) cells and lymphokine-activated killer (LAK) cells, also undergo a decrease in number and loss of function (Bloom, 1994). Although the exact reasons for such a change during ageing are not known at present, it is suggested that altered Ca^{2+} flux and defects in phosphatidylinositol metabolism are its important contributors. In the case of antibody-producing B lymphocytes, only a slight decrease in number during ageing has been reported, accompanied by a decline in their Fc-receptor-mediated response (Homo-Delarche *et al.*, 1995). Furthermore, the distribution of Ig classes changes during ageing; for example, whereas IgG and IgA levels increase, IgM levels generally decrease. There is also an increase in the production of auto-anti-idiotypic antibodies (Homo-Delarche *et al.*, 1995).

A failing immune system makes the body vulnerable to all kinds of opportunistic infections and allows cancerous cells to develop into cancers, as examplified in an extreme way by the development of AIDS following HIV infection. With regard to ageing, it is difficult to make the generalisation that the changes in the immune system are totally deteriorative. Instead, it has been suggested that immunosenescence should be viewed as a process of continuous remodelling and complex reshaping (Franceschi *et al.*, 1995). Yet, as a part of the total homeostatic network of maintenance and repair, a failing immune system is an integral part of the process of ageing.

Detoxification, Wound Healing and Regeneration

An organism is constantly exposed to various kinds of damaging agent such as nutritional and environmental toxins along with other causes of wear and tear. A complex system of detoxifying enzymes consisting of a series of mono-oxygenases and the P450 cytochromes carry out a wide range of chemical reactions, mainly in the liver cells, in order to neutralise toxic materials (Jakoby and Ziegler, 1990). Much information is available regarding altered drug response in the elderly. Increased adverse drug reaction in old age is a sign of failing mechanisms of drug uptake, absorption, clearance and detoxification. Extensive studies on the pharmacokinetics (drug delivery) and pharmacodynamics (drug action) during ageing have shown age-related alterations and impairments, including changes in the α- and β-adrenoreceptors (Meyer and Reidenberg, 1992).

Wound-healing and regeneration of damaged tissue parts are further aspects of homeostatic processes of maintenance and repair which becomes less efficient with age (Bruce and Deamond, 1991; Reenstra *et al.*, 1993). Since both these processes depend upon efficient cell proliferation, age-related decline in the capacity of cells to divide is an important determinant of the ageing process. Much research has been done on the biochemical and molecular mechanisms of cellular ageing *in vitro*, which is discussed in detail in the next chapter.

Chapter 3

Loss of Cell Proliferation

Many cell types in the body retain the capacity to divide during most of the adult lifespan, and are required to divide repeatedly in carrying out various functions of the body, such as immune response, blood formation, bone formation, and repair and regeneration of various tissues. For example, epithelial cells, epidermal basal cells, fibroblasts, bone marrow cells, lymphocytes, osteoblasts, myoblasts and glial cells constitute some of the most important dividing cell compartments of an organism. It is not only their differentiated and specialised functions that are critical for the organism; their capacity to divide is an integral part of their role in organismic growth, development, maintenance and survival. A loss of proliferative capacity of any one of these cell types has a deteriorative impact on the functioning and survival of the entire organism. A loss or slowing-down of proliferation of osteoblasts, glial cells, myoblasts, epithelial cells, lymphocytes and fibroblasts can lead to the onset of many age-related diseases and impairments including osteoporosis, arthritis, immune deficiency, altered drug clearance, delayed wound healing and altered functioning of the brain.

The study of age-related changes in the physiology, biochemistry and molecular biology of isolated cell populations has greatly expanded our understanding of some of the fundamental aspects of ageing. In modern biogerontology, the term 'cellular ageing' most commonly implies the study of normal diploid cells in culture which during serial subcultivation undergo a multitude of changes culminating in the cessation of cell division. This process of cellular ageing *in vitro* (Hayflick, 1965) is generally known as the Hayflick phenomenon, and the limited division potential of normal cells is called the Hayflick limit (Rattan and Stacey, 1994).

Figure 1 is a graphical representation of the Hayflick phenomenon comprising Phase I, the period of establishment of the primary culture from normal tissue; Phase II, a long period of growth and cell proliferation at a constant rate; and Phase III, a period in which the growth slows down, resulting in the cessation of cell division. To date, there has been no example of the unlimited proliferative lifespan of normal diploid cells of any type from any species without genetic recombination and other alterations. Only spontaneous or induced cell transformation can lead to the path of immortalisation and indefinite proliferative potential. Although most of the studies performed on the Hayflick system of diploid cell cultures consider the end of replicative lifespan as the terminal stage of cellular ageing, there have been some attempts to analyse post-replicative events to find out the role of terminal differentiation and apoptosis in such systems (Bayreuther *et al.*, 1991).

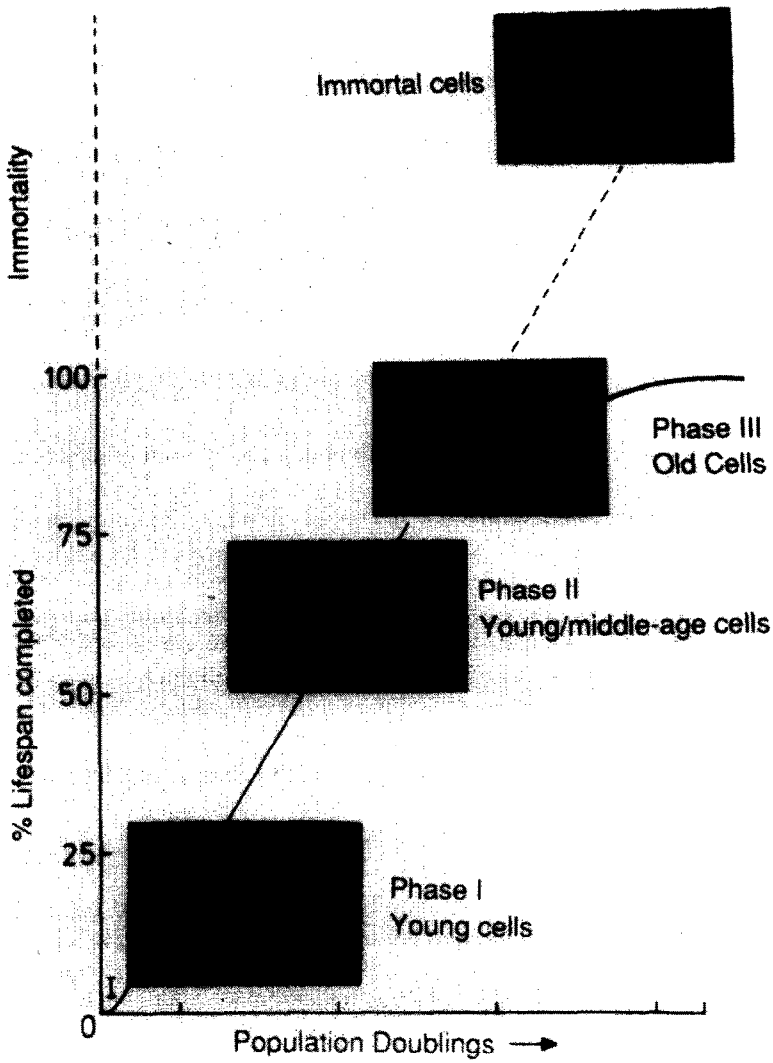

Fig. 1. Graphical representation of the ageing of normal diploid cells in culture undergoing the classical Hayflick phenomenon of progressive loss of proliferative potential. Only immortalisation lets the cells escape ageing.

Since 1961, studies performed on a wide variety of cell types have established unequivocally that normal diploid cells have a limited proliferative potential. These cells include fibroblasts, epithelial cells, endothelial cells, chondrocytes, keratinocytes, melanocytes, articular cartilage cells, periosteal osteoblasts, colonic epithelial cells, glial cells, bone marrow cells, and lymphocytes from human and other animals. Volumes of data have been collected on age-related changes in various characteristics of cells, and these range from cellular morphology, cell-cycle kinetics and cytoskeletal organization to changes in enzyme activities, gene expression, and macromolecular synthesis and turnover. There are more than 200 such structural, physiological, biochemical and molecular characteristics which have been studied during cellular ageing

(Rattan, 1991). Table 3 gives a list of selected major characteristics that appear progressively in cell cultures and distinguish young and senescent cells generally *before* the end of proliferative lifespan and their irreversible arrest in the G_1 phase of the cell cycle.

Altered Cellular Responsiveness

Altered cellular responsiveness is one of the critical aspects of cellular ageing. The mitogenic and growth-stimulating effects of growth factors, hormones and other agents are reduced significantly during cellular ageing. The stimulation of protein synthesis and induction of several cell-cycle-related genes and DNA synthesis in response to extracellular serum, insulin, hydrocortisones, epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and other hormones is reduced significantly in ageing cells (Rattan and Derventzi, 1991; Cristofalo *et al.*, 1992). In contrast, the sensitivity of ageing cells to toxic agents including antibiotics, phorbol esters, free-radical-inducers, irradiation and heat shock increases (Rattan and Derventzi, 1991).

The causes of age-related changes in the responsiveness of cells is a matter of continuing debate. The decreasing response of old cells to hormones and other growth factors was in earlier studies generally attributed to a loss of cell surface receptors. However, later studies show that the diminished response of cells to externally supplied factors may be due not to any decrease in the number of receptors, but instead to defective pathways of signal transduction during ageing. For example, no age-related changes in the binding and other characteristics of various growth-factor receptor complexes during the ageing of human fibroblasts and rat liver cells have been observed (De Tata *et al.*, 1993; Ishigami *et al.*, 1993; Sell *et al.*, 1993).

Recent studies have shown the significance of post-receptor events during ageing. It has been

Table 3. Major characteristics of senescent normal diploid cells undergoing cellular ageing *in vitro*

Morphology
Large cells, loss of fingerprint-like arrangement in parallel arrays, irregular shape, increased granularity, increased number of vacuoles, increased number of dense lysosomal granules with high autofluorescence, multinucleation and heteroploidy, cytoskeletal actin filaments highly polymerised, and disorganised microtubules.

Cell cycle characteristics
Reduced [^{3}H]-thymidine labelling, cessation of DNA synthesis, cells arrested in G_1 unable to pass the restriction point, accumulation of inactive complexes of cyclin E/Cdk2, high levels of cyclin-dependent kinase inhibitor SDI1 or p21.

Physiology
Reduced response to growth factors and other mitogens, increased sensitivity to toxins and drugs, altered calcium flux and pH, altered viscosity, altered membrane characteristics.

Biochemical and molecular characteristics
Reduced activity, specificity and fidelity of various enzymes, accumulation of altered and abnormal proteins, reduced rates of DNA and protein synthesis, increased DNA damage, altered pattern of post-translational modifications, reduced rate of protein degradation, altered expression of several genes, loss of cytosine methylation in DNA, reduced length of telomeres.

demonstrated that the ability of steroid receptor complexes to bind to nuclear acceptor sites is impaired and that changes in calcium mobilization occur during ageing. It has been suggested that high calcium concentrations inside old cells may impede normal fluxes of free calcium, thus resulting in impaired responsiveness to hormones and neurotransmitters. However, it has been shown that the intracellular calcium concentration either stays the same (Takahashi *et al.*, 1992; Brooks-Frederich *et al.*, 1993) or decreases during ageing, and the defect may lie at the level of the cell's capacity to mobilize calcium as a result of extracellular stimuli.

The role of protein kinases as modulators of signal transduction is also well known. In the case of calcium-dependent protein kinase C (PKC), no age-related changes have been observed in mouse organs and human cells (De Tata *et al.*, 1993). Furthermore, there is evidence that major pathways of signal transduction remain intact in old cells that can respond to extracellular stimuli. For example, several growth-regulated genes can be induced fully in senescent cells by treatment with serum and other growth factors (Stein and Dulic, 1995). Similarly, phorbol esters can stimulate the synthesis of DNA, RNA, proteins and protein elongation factors and induce c-*fos* expression and the translocation of PKC from the cytosol to the particulate compartment (Rattan, 1995a). These studies underline a very important point in ageing research by demonstrating that during cellular ageing, the integrity of the DNA-, RNA- and protein-synthesizing machineries remains intact along with the structural and functional stability of the receptor system.

Since several studies have shown a disorganised cytoskeleton in continuously dividing transformed cells, the age-related loss of proliferative capacity shown by normal cells is thought to be related to the integrity of the cytoskeleton. It has also been reported that the photo-ageing of human skin cells induced by environmental wavelengths of UV light is related to disruption of the cytoskeletal microtubule complex. Furthermore, observations that the aggregates of paired helical filaments (PHF) in the neurones of patients with Alzheimer's disease contain microtubule-associated proteins MAP2 and tau suggest a possible role of aberrant or modified cytoskeletal proteins in the age-related pathology of the brain (Crawford and Goate, 1992). Thus it is becoming increasingly clear that the components of the cytoskeleton, their organization, stability and integrity, along with their ability to maintain an equilibrium between their different structural and chemical forms have far-reaching implications for the regulation of cell shape, cell motility, cell division, cell survival and cell transformation.

Senescence-Specific Markers

A large number of studies have been undertaken with the aim of identifying the genes and gene products responsible for limiting and regulating cell proliferation. Based on studies performed by cell fusion and micro-injection methods, there is evidence that senescence is dominant over unlimited proliferation, and that negative growth effectors are involved in the pathway to cellular senescence (Shay *et al.*, 1992; Smith, 1992). Several markers of mortality have been identified by comparative studies on mortal, immortal, and hybrid cells. For example, tumour suppressor genes, human chromosomes 1, 4, 6, 7 and X, and a novel member of mouse hsp70 protein termed mortalin have been associated with the mortal phenotype of normal and hybrid cells (Wadhwa *et al.*, 1994, 1995). On the basis of experiments using human diploid fibroblasts, a two-stage theory of cellular senescence has been proposed, whereby in the first stage (termed M1) the cells are arrested at the G_1/S interface and rarely escape senescence unless treated with SV40 T antigen or human papillomavirus 16 E6/E7. The second stage (M2) is

considered to involve failure of replication during the period of crisis, and these mechanisms together make escape of senescence a very rare event (Wright and Shay, 1992).

A large number of genes have been studied in relation to their expression during cellular ageing. As in the case of measuring enzyme activities in 1970s and 1980s, in recent years, comparing the levels of mRNAs in young and senescent cells has resulted in a long list of increasing/decreasing/unchanging levels of various mRNAs during cellular ageing. However, a comparison of cDNA libraries made from young and senescent diploid cells have resulted in the isolation of differentially regulated gene products whose levels may be increased or decreased in old cells. Many of these mRNAs and proteins could be identified as well-known cellular products such as fibronectin, pro-collagen, ferritin heavy chain, insulin-like growth factor binding protein-3, plasminogen-activator inhibitor type 1, thrombospondin, crystallin, cathapsin B, and the mitochondrial genes for NADH dehydrogenase subunit 4 and for cytochrome *b* (Rattan, 1995a). In other cases, in which no similarities between the cDNA or the protein and any known gene products were found, new names were given to various gene products capable of inhibiting DNA synthesis, for example, statin, terminin, prohibitin, SUSM-1 factor, SDI1 or p21, which is an inhibitor of cyclin-dependent kinase, a growth-inhibitory glycopeptide and a so-called senescence-associated gene, SAG (Stein and Dulic, 1995). There are several other overexpressed genes in normally senescent and prematurely aged Werner's syndrome fibroblasts, whose identities have not yet been confirmed (Linskens *et al.*, 1995).

All such studies have shown a quantitative change in the amounts of certain gene products during cellular ageing without identifying any products unique to old cells. Furthermore, since the inhibitors of DNA synthesis are generally produced in cells that have already completed more than 90% of their lifespan *in vitro*, it is not clear whether these inhibitors are a cause of cellular ageing or are a result of other age-related changes in gene expression, regulation and metabolism during serial passaging of normal diploid cells.

Cell-Division-Counting Mechanisms

Since the proliferative capacity of normal diploid cells is related to the maximum lifespan of the species, the existence of some kind of counting mechanism has often been presumed. For example, a progressive loss of 5-methylcytosine (5mC) during serial passaging of cells has been considered as a mechanism for counting the number of completed cell divisions (Holliday, 1986). An inverse relationship has been observed between the rate of loss of 5mC and the maximum lifespan of a species, along with the life-shortening effects of a demethylating agent, 5-azacytidine, on cultured cells (Wilson *et al.*, 1987; Holliday, 1995).

Recently, telomeres, which are the terminal repetitive sequences at the ends of the eukaryotic chromosomes, have been suggested to be a kind of determining mechanism for the number of divisions a cell can undergo (Harley, 1991). Several lines of evidence indicate that the shortening of telomeres may play a crucial role in cellular ageing. A loss of terminal restriction fragment (TRF) telomeric DNA during ageing has been reported for human fibroblasts, peripheral blood leukocytes, colon mucosa epithelia, skin, and lymphocytes (Harley, 1991; Allsopp and Harley, 1995; Wright and Shay, 1995). A strong relationship has been established between telomere length, donor age, and the proliferative capacity of fibroblasts from normal donors and from patients with Hutchinson–Gilford premature ageing syndrome. These studies have shown that in the case of fibroblasts, an average loss of 50±20 TRF base pairs (bp) per cell

division *in vitro* and a decrease of 15±6 TRF bp per year of life *in vivo* is a more accurate predictor of replicative lifespan than any other marker, such as donor age. Similarly, the rate of telomere loss in human lymphocytes was higher in subjects with Down's syndrome (133±15 bp) as compared with normal subjects (41±7 bp) per year of life (Vaziri *et al.*, 1993).

The hypotheses that telomere length is a predictor of the replicative lifespan of normal somatic cells, and that the rate of loss of telomeres can be used as a molecular marker of ageing are attractive suggestions. Furthermore, the observations that (i) sperm telomeres are longer than somatic telomeres; and (ii) that immortal cells with unlimited proliferative capacity have telomeres that are shorter but whose length is maintained during proliferation by virtue of telomere polymerase (telomerase) activity, have raised the possibility of testing whether telomere loss and telomerase expression are coincidental or causal in cellular mortality and immortality (Wright and Shay, 1995).

Concluding Remarks

The correlation between cellular ageing *in vitro* and *in vivo* has already been reviewed above on the basis of ample evidence gathered from studies on the effects of donor age, species lifespan and premature ageing syndromes upon cellular proliferative capacity in culture. These studies indicate that the genetic and intrinsic Hayflick limit of diploid cell strains in culture is a true reflection of what is going on during the ageing of an organism. In some ways, the ageing of the organism can be considered as the clonal ageing of the zygote having a limited proliferative capacity. Considering a Hayflick limit of between 70 and 80 population doublings for the newly formed diploid zygote, the total number of cells that can be produced (10^{21}), is several orders of magnitude more than the estimated 10^{17} cells required for growth, development and survival of a human being for more than 100 years!

It is thus clear that studies performed on diploid cell cultures undergoing ageing *in vitro* have had a major influence on the development of ideas and theories about ageing. It must also be pointed out that no one has ever claimed that organisms become old and die because their fibroblasts or some other cell type stops dividing. The capacity of some cells to divide *in vitro* and *in vivo* is an integral component of the overall survival strategy evolved in many organisms. Those tissues which undergo regular turnover in the adult are dependent on the proliferative capacity of their cells to maintain homeostasis. The loss of a cellular function as important as division, along with a large number of other physiological, biochemical and molecular changes which precede and/or accompany this loss, is one of the fundamental determinants of ageing.

Chapter 4

Instability of the Genome

Instability of the genome is an important aspect of the failure of homeostasis. This is because the survival and continued existence of any lifeform depends upon the stability of its genome, at least until its genetic information has been transmitted faithfully to the next generation. Therefore, the inability of organisms to live for ever has often been linked to the possibility of progressive instability of their genome. Until recently, most attention was paid to the stability and instability of the nuclear genome as the primary cause of ageing. However, it is now becoming increasingly clear that the mitochondria and the mitochondrial genome have a crucial role to play in the regulation of cellular metabolism and survival.

Various physical, chemical and biological factors are continuously challenging the DNA in all cells of the body. At the same time, several DNA repair systems operate in the cell which counteract the effects of various DNA-damaging agents. Major DNA-damaging agents include solar UV radiation, background ionizing radiation, a wide range of chemicals in food and in the environment, and several endogenous agents such as aldehydes, active oxygen species and other free radicals which are the result of metabolic pathways. In addition to that, damage to DNA in a cell can also result from several spontaneous chemical changes in the DNA, such as those due to hydrolysis, deamination, methylation, demethylation and glycation, along with the errors that can occur during DNA duplication and repair, as a result of innate limits on the accuracy of any biochemical processes (Lindahl, 1993).

The biological consequences of damage to DNA are generally very similar, irrespective of the nature of the damaging agent. For example, several DNA-damaging agents can be the cause of mutations, depurination, depyrimidation, dimers, strand breaks, crosslinks and epimutations. At present, there are no reliable methods available that can establish a cause-and-effect relationship between the origin of DNA damage and its consequences in a cell. Therefore, most studies measuring age-related changes in the levels of DNA damage have been unable to pinpoint the source of that damage—a factor that might be crucial in explaining differences in the type and rate of damage accumulation in different cell types, organs and species.

DNA Damage During Ageing

DNA can be considered as damaged if there is any change or modification in its base sequence or in its structure which changes or modifies its information content or its normal pattern of

replication and transcription. According to this rather all-encompassing definition, three major categories of DNA damage can be identified: (1) mispairing of bases due to the innate physicochemical properties of the nucleotides; (2) spontaneous chemical changes caused by the intrinsic thermodynamic instability of DNA; and (3) damage caused by exogenous and endogenous chemical agents. Various types of DNA damage, their origins and, in the absence of repair, their consequences at the level of DNA integrity are listed in Table 4, modified from Rattan (1989).

A large number of studies have shown that some kinds of DNA damage do accumulate during ageing. For example, single-strand breaks of DNA have been observed to increase in cultured cells of rodent and human origin in the cells isolated from various rodent organs, including brain, heart, kidney, liver, spleen and thymus, and in human lymphocytes and muscle tissue during ageing (Rattan, 1989; Gaubatz, 1995). An age-related increase in the number of alkali-labile sites in DNA occurred only in post-mitotic rat liver parenchymal cells and not in actively dividing non-parenchymal cells in the same tissue. Various other investigators have reported age-related increases in DNA crosslinks, dicentric chromosomes, aneuploidy, polyploidy, loss of centromeric tandem repeats, and shorter linker regions between nucleosomes (Rattan, 1989; Gaubatz, 1995). DNA adduct-like covalent modifications called indigenous (I)-compounds have been shown to increase during ageing (Randerath *et al.*, 1992).

An increase in the free radical-induced oxidative damage, particularly the formation of 8-hydroxy-2'-deoxyguanosine (8OHdG) (Barciszewski *et al.*, 1995) in the nuclear and mitochondrial (mt) DNAs has been reported to occur in various organs of ageing rats and in human hearts (Shigenaga *et al.*, 1994; Ozawa, 1995). Levels of 8OHdG also increase with age in the nuclear and

Table 4. Types, origin, and molecular consequences of DNA damage

Type	Origin	Consequences
Base mispairing	Tautomerization, base rotation, base analogs, ionization	Transition, transversion, mutations, insertions, deletions, mispairs
Spontaneous chemical changes	Deamination, demethylation methylation, glycation, hydrolysis	Depurination, crosslinks, depyrimidation, strand breaks, epimutations
Induced chemical changes	Exogenous agents (solar UV radiation, background ionizing radiation, natural mutagens in food and environment)	Pyrimidine dimers, single- and double-strand breaks, mutations, crosslinks
	Endogenous agents (alkylating agents, aldehydes, reactive oxygen species and other free radicals)	DNA adducts, crosslinks, thymine glycols, 7-methylguanine, 8-hydroxydeoxyguanosine, N^6-furfuryladenine, hydroxymethyluracil

mtDNAs of houseflies and in human serum (Agarwal and Sohal, 1994; Rattan *et al.*, 1995). Another oxidative damage product, 7-methylguanine adduct also increases in nuclear and mtDNAs during ageing (Shigenaga *et al.*, 1994). A five-fold increase in the frequency of deletions and additions was found in mtDNA isolated from livers of old mice as compared with those from young mice. An age-related increase in a somatic deletion of $mtDNA^{4977}$ in ageing human nervous and muscle tissues has been reported (Arnheim and Cortopassi, 1992; Cortopassi *et al.*, 1992). Deletions of mtDNA have also been reported for fungi undergoing ageing (Osiewacz *et al.*, 1989).

One of the most common results of DNA damage is the causation of mutations. An exponential increase in the number of rare variants with enhanced levels of glucose-6-phosphate dehydrogenase (G6PD) were reported in serially passaged cultures of human fibroblasts. Similarly, several-fold increases in the frequency of diphtheria toxin- and thioguanine-resistant mutants during cellular ageing of human fibroblasts have been observed. In human T lymphocytes isolated from blood obtained from donors of different ages, an exponential increase in the frequency of mutated cells at the HPRT locus was reported. Using allele-specific PCR, the occurrence of a point mutation by base substitution (from A to G) at nucleotide position 3243 on mtDNA has been demonstrated in several human tissues from old subjects as compared with infant tissues . Other kinds of DNA damage which have been studied in relation to ageing are the so-called epimutations (Holliday, 1987). These include the presence of 5-methyl cytosine and 5-methyldeoxycytidine (5mC and 5mdC). There is evidence that levels of 5mC and 5mdC decline during the ageing of cells in culture and in tissues and organs isolated from old rats, mice and cattle (Catania and Fairweather, 1991).

DNA Damaging Agents and Cellular Ageing

The proposition that DNA damage may be the primary cause of cellular ageing has been frequently put to the test by assuming that experimental induction of DNA damage may cause premature cellular ageing and a reduction of lifespan. As with other studies on DNA damage and repair, conflicting results have come out of these studies too. Various DNA-damaging agents which include ionizing and non-ionizing radiation, chemical mutagens, and alkylating and oxidative agents have been tested for their ability to induce premature ageing in cells and organisms.

Some earlier studies on diploid fibroblast cultures of avian, rodent and human origin have claimed life-shortening and ageing-accelerating effects of UV, X- and γ-rays, the extent of the effects varying according to the nature and origin of the cells. Similarly, a reduction in the growth potential of human skin fibroblasts in culture established from the skin biopsies from UV-exposed regions as compared with those from unexposed regions of the body has been reported. Later studies, however, have reported either no effects or, paradoxically, life-prolonging effects of low-dose chronic ionizing radiation on human fibroblasts (Rattan, 1989; Holliday, 1995).

Studies with other DNA-damaging agents have also provided conflicting data. For example, the treatment of ageing human fibroblasts with mutagenic doses of either EMS or MNNG did not reduce their lifespan in culture, in spite of the fact that many mutations were introduced and could be detected in the cells by these treatments. In contrast to this, bovine adrenocortical cells exposed to cumene hydroperoxide which induced DNA strand breaks and crosslinks, had

severely reduced cloning efficiency, an indirect indication of the reduced proliferative potential. Similarly, the inhibition of DNA and protein synthesis in human fibroblasts treated with cumene hydroperoxide, and their recovery on the removal of the damaging agent, had no subsequent effects on the proliferative capacity of cells (Rattan, 1989). Furthermore, the induction of epimutations by the treatment of cells with 5-azacytidine induces premature ageing of human cells in culture (Holliday, 1986; Catania and Fairweather, 1991).

DNA Repair During Ageing

With respect to the repair of DNA damage during ageing, only a few repair pathways have been studied to date. These include the reversal of damage through photo-reactivation or through the removal of modifications from a base, excision repair of damaged bases and nucleotides, repair of single- and double-strand breaks, and repair of free-radical- and oxidation-induced damage. One of the most widely studied DNA repair pathways during ageing is repair by nucleotide excision. In this kind of DNA repair, the recognition of the damaged site and the creation of a nick by an endonuclease enzyme is followed by its excision by an exonuclease. In most cases, DNA repair during ageing has been measured by the capacity of cells to repair DNA damage induced by a short, intense treatment with UV, X-rays, γ-irradiation, mutagens or free radicals. Although some investigators have reported some decline in the DNA repair capacity of very old cells in culture, or of cells isolated from old animals, the general consensus is that there is no age-related decline in the overall capacity of cells to repair DNA damage during ageing (Rattan, 1989; Niedermüller, 1995). As regards the mitochondrial genome, although there is no repair of UV-induced pyrimidine dimers in mtDNA, a number of repair enzymes such as AP endonuclease and uracil glycosylase have been detected and gene-specific repair in mtDNA has been reported (Bohr and Anson, 1995). It will be important to find out if mtDNA repair is altered during ageing.

The notion that declining repair capacity may be the primary defect of ageing is further undermined by the absence of a strong relationship between the extent of DNA repair and the proliferative lifespan of cells. For example, cells from xeroderma pigmentosum patients do have severe DNA repair deficiency, but do not show either a reduced proliferative capacity or any other symptoms of accelerated ageing (Rattan, 1989; Niedermüller, 1995). Conversely, cells from patients with premature ageing syndromes do not always have reduced DNA repair capacity and in some cases, such as lymphocytes from Down's syndrome patients, the rate of DNA repair of γ-radiation-induced damage was much higher than in cells from normal individuals (Chiricolo *et al.*, 1993).

Thus, there is no significant reduction in overall DNA repair capacity in old cells and tissues which could be considered to be the cause of ageing. It must, however, be pointed out that the DNA repair capacity that has been studied until now gives only a gross overview of what might be happening in the genome as a whole, irrespective of the fact that only a small proportion of the genomic DNA is actually active and expressed in any cell type. Recent studies have shown that there is a strong intragenomic heterogeneity between the repair of active and inactive regions of the genome. This phenomenon of preferential gene repair is extended to strand-specific repair, in which the transcribed strand is repaired much more efficiently than the nontranscribed DNA strand (Link *et al.*, 1992; Bohr and Anson, 1995). Therefore, it is important to study the repair, maintenance and functioning of individual genes, such as those involved in DNA repair, or the genes involved in genetic information transfer pathways, for example the genes for DNA/RNA polymerases, protein synthetic factors and free radical scavengers.

Species Lifespan Versus DNA Repair Capacity

A crucial test of any theory of ageing is whether it is able to provide a satisfactory explanation of the evolutionary fact that the maximum achievable lifespan of individuals is species-specific. DNA repair theories of ageing, therefore, propose that species-specific longevity is a function of a species-specific capacity to repair DNA damage and to maintain its genomic integrity efficiently. Several studies have been performed, using a wide range of methods for measuring DNA repair, in order to test this hypothesis and to establish the relationship between a species' lifespan and its capacity to repair DNA.

There is evidence that establishes a positive correlation between a species' lifespan and its capacity to repair certain kinds of DNA damage. Various lines of evidence in this regard include the repair of UV-induced damage in fibroblasts and lymphocytes; the capacity of cells to metabolise 7,12-dimethylbenz(a)anthracene; the activity of methyltransferase in removing the methyl group from damaged O^6-methylguanine in liver and in chondrocytes; the rate of loss of 5mC and 5mdC during ageing; and the extent of oxidative damage to DNA in various species. Similarly, the extent of DNA repair measured by determining the rate of removal of benzo(a)pyrene-induced DNA adducts in the liver of three different congenic mice strains correlated well with their differences in longevity (Rattan, 1989; Niedermüller, 1995). The activity of poly(ADP-ribose) polymerase involved in DNA repair is positively related with species lifespan (Grube and Bürkle, 1992). Furthermore, observations that the frequency of spontaneous or induced transformation of normal cultured cells into cancerous immortal cells is inversely proportional to the maximum lifespan of the species (Holliday, 1995), suggests that those species best equipped to resist genomic change and to maintain their genomic integrity are generally those which also have longer lifespans.

Altered Gene Expression

Each cell type in an organism comes to acquire a unique pattern of gene expression through differentiation during development. It is obvious that this pattern of gene expression must be maintained for the normal functioning of cells and for the survival of the organism. Since ageing is considered to be a result of the failure of maintenance at all levels, attempts have been made to look for an age-related drifting-away of cells from their proper state of differentiation, i.e. dysdifferentiation, in terms of changes in the pattern of gene expression during ageing.

Several studies have been undertaken in order to determine the extent of expression of different genes during ageing. In most cases, the mRNA levels of different genes have been estimated by RNA–DNA hybridisation, using cDNA or genomic probes for specific genes. The results obtained show that during ageing, the expression of some genes increases, of some it decreases, and of others it remains constant (Van Remmen *et al.*, 1995). In all such studies on measuring the levels of mRNA in young and old cells and tissues, it is assumed that this estimate is a direct measure of gene activity. This is a simplistic notion, because it is well known that post-transcriptional changes, such as the processing, transport and turnover of RNA, significantly change the levels of mRNA.

A better way to find out if the stability of the genome decreases and if the pattern of gene expression changes during ageing is to study the expression of a cell-type-specific gene. For

example, the globin gene is normally repressed in fibroblasts, and no globin-like RNA could be detected in either young or old human fibroblasts, showing thereby that the stability of gene expression remains unchanged during ageing (Kator *et al.*, 1985). Similar results have been reported for five tissue-specific genes (myelin basic protein in the brain, atrial natriuretic factor in the heart, albumin in the liver, kappa immunoglobulin in the spleen and a skin-specific keratin), the fidelity of whose expression was maintained during the ageing of Wistar rats (Sato *et al.*, 1990). In contrast to this, some relaxation of the expression of endogenous murine leukemia virus-related RNA and globin RNA was found in mouse brain and liver (Ono *et al.*, 1989). However, no reactivation of repressed α-fetoprotein genes was observed in adult rat livers during ageing (Richardson *et al.*, 1985). More studies of a similar nature and with a wide range of cell-type-specific genes will be required in order to resolve this important issue of the stability of gene expression during ageing.

Reorganization of the Chromatin

The eukaryotic genome consists largely of the chromatin, which is a complex of DNA, histones and non-histone proteins that are arranged in a series of repeating units, termed nucleosomes. The stability of the structure and organization of the chromatin is crucial both for the maintenance of the state of differentiation of a cell and for its function throughout the lifespan of the cell. In a large number of studies, the composition, structure and function of the chromatin in young and old organisms, tissues and cells have been compared. These include studies on age-related changes in DNA content; DNA synthesis; levels of histones and non-histone proteins; nucleosome spacing and linker regions; protein–DNA interactions; and physical properties of the chromatin, such as thermal denaturation and template activity. It is now well established that the chromatin becomes more condensed during ageing, and this can have wide implications for DNA repair, DNA replication and gene expression (Thakur, 1984; Macieira-Coelho, 1995). It has been suggested that the reorganization of chromatin structure is characterized by a succession of subtle changes through the lifespan and a final short stage with abrupt events. These changes include an increased fragility of the chromatin structure and an increase in sensitivity to strong detergents (Macieira-Coelho, 1995).

At the gross level of chromosomal changes during ageing, the failure of cells to maintain their diploid status has been observed in ageing peripheral lymphocytes, bone marrow cells, fibroblasts and mouse kidney cells and hepatocytes. Other major chromosomal aberrations, such as deletions, rearrangements, endoreduplication and sister chromatid exchange have not been observed in all ageing systems without treatment with high doses of various DNA-damaging agents. Another line of research that indicates the possibility of the decreased stability of chromatin is the reactivation of inactive X-chromosomes during ageing. For example, in the livers of old mice, a spontaneous 50-fold reactivation and expression of the autosomal ornithine carbamoyl transferase (OTC) gene, which had been translocated to the inactive X-chromosome and had therefore become silent, was taken as a proof of instability in the genome during ageing, possibly as a result of demethylation (Wareham *et al.*, 1987). However, no such spontaneous reactivation of the human X-linked HPRT gene was observed in skin fibroblasts isolated from women with severe deficiency of this enzyme (Migeon *et al.*, 1988). It is possible that the stability and maintenance of individual genes is a function of their cell-type and species specificity, as reflected in the great heterogeneity of the extent of DNA repair observed for various genes in the same genome.

At the level of genes, studies on age-related changes in the structural organization of genes are

just beginning. For example, no difference in the restriction patterns of histone H3 and H4 genes in ageing human fibroblasts has been observed (Green *et al.*, 1986). Similarly, no sequence rearrangements of nine DNA regions, including genes for actin, dihydrofolate reductase, immunoglobulin μ constant region, and c-*abl* and c-*ras* proto-oncogenes were detected in various tissues of young and old mice (Ono *et al.*, 1985). However, a decreased intensity of hybridisation due to a possible loss of sequences of actin and globin genes, but not of interferon gene, has been reported for senescent cultures of human fibroblasts (Icard-Liepkalns *et al.*, 1986). Altered splicing of fibronectin mRNA during cellular ageing (Burke and Danner, 1991) may be related to alterations in trans-acting factors that bind to cAMP-responsive elements (CRE) at the 5' end of the fibronectin gene and regulate its expression.

Thus, although at a gross level, there are no major alterations in the genomic organisation, it does not rule out the involvement of subtle alterations at the level of a single gene or other specific regions of the genome, such as telomeres (discussed in Chapter 3) in ageing. At present, indirect evidence shows that the failure of maintenance at the level of nuclear and mitochondrial genomes is important for ageing. For example, some of the maintenance pathways which become improved or are better preserved by life-prolonging calorie restriction in rodents include DNA repair pathways (Haley-Zitlin and Richardson, 1993; Masoro, 1995). Future studies on the repair and maintenance of individual genes will clarify the issue of genomic instability during ageing.

Chapter 5

Misregulation of Genetic Information Transfer

Although the genomic instructions of life are written in the language of nucleic acids, the life is actually 'lived' in the language of proteins. The genetic information encoded in DNA becomes functionally meaningful only when it is accurately transcribed and translated into RNA and proteins, respectively. Whereas two types of RNA, transfer (t)RNA and ribosomal (r)RNA, are themselves functional molecules, the genetic information transcribed into the third RNA, messenger (m)RNA, has to be generally translated from a language of nucleic acids into a language of amino acids in order to produce proteins which are the functional gene products. It has been estimated that in a human cell, there are about 80,000 genes per haploid genome, of which about 22,000 are housekeeping genes and the rest are tissue-specific (Antequera and Bird, 1993). Furthermore, in order to become a functional protein, a newly synthesised polypeptide chain has to undergo a wide variety of post-translational modifications that determine its activity, stability, specificity and transportability. Misregulation of genetic information transfer at any of these steps can be critical for the failure of homeostasis.

Transcription and Processing

Studies on the synthesis of RNA and on its processing during ageing have been few. There are three types of RNA in a cell, of which about 70–80% is rRNA, 10–15% is tRNA and 5–7% is mRNA. Although the level of total transcription is generally reduced during ageing, the proportion of different RNAs does not change significantly. Furthermore, the endogenous nucleotide pool and the activities of the enzymes involved in RNA synthesis are also reduced during ageing (Müller *et al.*, 1995). For each type of RNA, some age-related changes have been observed in various ageing systems. For example, a significant decline in the content and synthesis of rRNAs has been reported in ageing beagles, rodent organs and cultured cells (Medvedev, 1986). This decline in rRNAs was previously thought to be associated with the loss of ribosomal genes. However, no age-related decline in the gene copy number of rRNA has been observed in human fibroblasts or in mouse myocytes. Furthermore, the number of rRNA genes in a cell is already in great excess (between 200 and 1000 copies), and a small loss with age may not have any serious consequences for cell function and survival. However, whether there is a differential loss of various rRNA species during ageing, and what effects such a loss might have, is not known (Rattan, 1995e).

Another step in the transfer of genetic information that can be rate-limiting is the availability

of mRNAs for translation. Post-transcriptional processing of eukaryotic mRNA is highly complex and is still not very well understood. However, a few reports are available on changes in various aspects of mRNA processing during ageing. For example, a decrease in the total poly(A^+) mRNA has been reported in ageing rat brain and liver tissues, in rabbit liver, in the liver, heart and oviduct of quails and in the post-mortem brain tissues obtained from patients with Alzheimer's disease (Rattan, 1995e). This is thought to be due to both a decrease in the length of the poly(A) tail and the rate of polyadenylation of mRNA during ageing. However, an application of more sensitive methods for measuring the age-related changes in the length of the poly(A) tail have shown no significant differences in rat hepatocytes, and in the liver, kidney and brain samples obtained from calorie-restricted and freely-fed rats of different ages (Kristal *et al.*, 1993). Similarly, no age-related differences in mRNA cap structure and its translatability *in vitro* have been observed. Thus although it appears that at a gross level there are no major alterations in mRNA characteristics, it is possible that individual mRNA species do undergo changes, including splicing, transport from nucleus to cytoplasm, binding to ribosomes, stability and turnover during ageing (Müller *et al.*, 1995; Van Remmen *et al.*, 1995).

Translation

Proteins are the most versatile macromolecules necessary for the organization of internal cellular structures, for the formation of the energy-creating and metabolic utilising systems in the cell, for the transport of ions and larger molecules over the cell membranes and for maintaining intracellular and intercellular communication pathways. Proteins interact with all other macromolecules including DNA, RNA, carbohydrates and lipids, and are required for maintenance and repair at all levels of biological organization. Protein synthesis is thus crucial for the survival of a living system, and any disturbance at this level can cause large imbalances and deficiencies.

A decline in the rate of total protein synthesis is one of the most common age-associated biochemical changes that has been observed in a wide variety of cells, tissues, organs and organisms, including human beings. The implications and consequences of slower rates of protein synthesis are manifold in the context of ageing and age-related pathology (Table 5). Although there is a considerable variability among different tissues and cell types in the extent of decline (varying from 20 to 80%), the fact remains that bulk protein synthesis slows down during ageing (Ward and Richardson, 1991; Van Remmen *et al.*, 1995). Furthermore, it has been shown that the conditions, such as calorie-restriction, which increase the lifespan and retard the ageing process in many organisms, also slow down the age-related decline in protein synthesis (Van Remmen *et al.*, 1995). These observations reinforce the view that the slowing down of protein synthesis is an integral part of the ageing process.

Table 5. Consequences of slowing-down of protein synthesis

- Decrease in the availability of enzymes.
- Inefficiency in the removal of intracellular damage.
- Accumulation of abnormal and defective molecules.
- Inefficient intra- and inter-cellular communication.
- Decrease in the production of hormones and growth factors.
- Decrease in the production of antibodies.
- Decrease in the production of extracellular matrix.

However, it should be pointed out that the age-related slowing down of bulk protein synthesis does not mean that the synthesis of each and every protein becomes slower uniformly during ageing. Although no senescence-specific unique proteins have been detected during ageing, a significant increase in the heterogeneity of protein synthesis during ageing has been observed (Van Remmen *et al.*, 1995). Furthermore, even though bulk protein synthesis slows down with age, the total protein content of a cell generally increases because of an accumulation of abnormal proteins during ageing. Age-related changes in protein synthesis are regulated both at the transcriptional and pre-translational levels in terms of the availability of individual mRNA species for translation, and at the translational and post-translational levels in terms of alterations in the components of the protein-synthesising machinery and the pattern of post-synthetic modifications which determine the activity, specificity and stability of a protein.

The efficiency and accuracy of protein synthesis

Eukaryotic protein synthesis is a highly complex process which requires about 200 small and large components to function effectively and accurately in order to translate one mRNA molecule while using large quantities of cellular energy. There are three major components of the translational apparatus: (1) the translational particle, the ribosome; (2) the amino acid transfer system or charging system; and (3) the translational factors. The protein-synthesising apparatus is highly organised and its macromolecular components are not freely diffusible within cells. Table 6 gives an overview of the components and subcomponents involved in eukaryotic protein synthesis, along with their major functional characteristics.

The rate and accuracy of protein synthesis (as well as those of DNA and RNA synthesis) have been presumably obtained through natural selection and evolved to optimal levels according to the overall life history of an organism. Since the error frequency of amino acid misincorporation is generally considered to be quite high (10^{-3}–10^{-4}) as compared with nucleotide misincorporation, the role of protein error feedback in ageing has been a widely discussed issue. At

Table 6. Major components of the translational machinery

Component	Subcomponents	Function
(A) *Translational particle*		
Ribosome	40S and 60S subunits, 4 rRNAs and approximately 80 ribosomal proteins	recognizing and translating the genetic codons in mRNA
(B) *Charging system*		
Amino acids	at least 20	building blocks for proteins
tRNAs	approximately 60	matching codons with respective amino acids
Aminoacyl-tRNA-synthetases	at least 20	adding correct amino acids to specific tRNAs
(C) *Translational factors*		
Initiation factors	approximately 24 proteins	making 80S initiation complex
Elongation factors	4 proteins	addition of amino acids to growing peptide chain
Release factor	1 protein	terminating protein synthesis

present, no direct estimates of protein error levels in any ageing system have been made, primarily due to the lack of appropriate methods to determine spontaneous levels of errors in a normal situation.

Several indirect attempts have, however, been made to determine the accuracy of translation in cell-free extracts, using synthetic templates or natural mRNAs. The studies on the accuracy of protein synthesis during ageing which have been performed on animal tissues, such as chick brain, mouse liver, and rat brain, liver and kidney, did not reveal any major age-related differences in the capacity and accuracy of ribosomes to translate poly(U) in cell-free extracts (Rattan, 1995d). However, these attempts to estimate the error frequencies during translation *in vitro* of poly(U) template were inconclusive because the error frequencies encountered in the assays were several times greater than the estimates of natural error frequencies . Another indirect method that has been used to detect the misincorporation of amino acids during ageing is the method of two-dimensional (2D) gel electrophoresis of proteins, by which at least one kind of error, that is the misincorporation of a charged amino acid for an uncharged one (or vice versa) can be demonstrated because of 'stuttering' of the protein spot on 2D gels. Using this method, no age-related increase in amino acid misincorporation affecting the net charge on proteins was observed in histidine-starved human fibroblasts and in nematodes (Rattan, 1995d).

In contrast to this, using mRNA of CcTMV coat protein for translation by cell extracts prepared from young and old human fibroblasts, a seven-fold increase in cysteine misincorporation during cellular ageing has been observed (Luce and Bunn, 1987, 1989). These studies also showed that an aminoglycoside antibiotic paromomycin (Pm), which is known to reduce ribosomal accuracy during translation *in vivo* and *in vitro* induces more errors in the translation of CcTMV coat protein mRNA by cell extracts prepared from senescent human fibroblasts than those from young cells. Further indirect evidence that indicates the role of protein errors in cellular ageing can be drawn from studies on the increase in the sensitivity of human fibroblasts to the life-shortening and ageing-inducing effects of Pm and another aminoglycoside antibiotic G418 (Holliday and Rattan, 1984; Buchanan *et al.*, 1987). Similarly, the increased longevity of high-fidelity mutants in *Podospora anserina* indicates the role of protein errors in lifespan (Silar and Picard, 1994).

Although a global 'error catastrophe' as a cause of ageing due to errors in each and every macromolecule is considered unlikely, it is not ruled out that some kinds of error in various components of the protein synthesising machinery, including tRNA charging, may have long-term effects on cellular stability and survival (Kowald and Kirkwood, 1993). Better methods are still required for measuring the basal levels of translational errors in young and old cells, tissues and organisms.

Initiation of protein synthesis

The translational process can be envisaged as proceeding in three steps—initiation, elongation and termination, followed by post-translational modifications, including folding, which give the protein a functional tertiary structure. The translation of an mRNA begins with the formation of a so-called initiation complex between the ribosome and the initiator codon. It is an intricate process, which consumes energy and involves at least seven initiation factors (eIFs; e stands for eukaryotic) consisting of 24 different subunits, two subunits of ribosomes, and an initiating tRNA called methionyl (Met)-$tRNA_i$. The whole process of the formation of the 80S

initiation complex takes about 2–3 sec in cell-free assays and is thought to occur much faster *in vivo* (Merrick, 1992). The initiation step is considered to be the main target for the regulation of protein synthesis during the cell cycle: growth, development, hormonal response, and under stress conditions including heat shock, irradiation and starvation.

With respect to ageing, however, the rate of initiation appears to remain unaltered. For example, using *in vitro* assays, the conversion of isolated 40S and 60S ribosomal subunits into the 80S initiation complex has been reported to decrease by less than 15% in old *Drosophila*, rat liver and kidney, and mouse liver and kidney. On the other hand, since polysomal fraction of the ribosomes decreases during ageing, it implies that the activity of an anti-ribosomal-association factor eIF-3 may increase during ageing. The activity of eIF-2, which is required for the formation of the ternary complex of Met-tRNA$_i$, GTP and eIF-2, has been reported to decrease in rat tissues during development and ageing (Rattan, 1995d). Similar studies on other eIFs and in other ageing systems are yet to be performed and it is necessary that detailed studies on eIFs are also undertaken in the context of ageing and the question of the regulation of protein synthesis at the level of initiation is reinvestigated.

Several studies have been performed on the age-related changes in the number of ribosomes, their thermal stability, binding to aminoacyl-tRNA, the level of ribosomal proteins and rRNAs, their sensitivity to aminoglycoside antibiotics, and the fidelity of ribosomes (Rattan, 1995e). Although there is a slight decrease in the number of ribosomes in old animals, this does not appear to be a rate-limiting factor for protein synthesis due to a ribosomal abundance in the cell. Instead, several studies indicate that the biochemical and biophysical changes in ribosomal characteristics may be more important for translational regulation during ageing. For example, the ability of aged ribosomes to translate synthetic poly(U) or natural globin mRNA decreases significantly. A decrease in the translational capacity of ribosomes has also been observed in rodent tissues such as muscle, brain, liver, lens, testis and parotid gland and in various organs of *Drosophila* (Ward and Richardson, 1991; Van Remmen *et al.*, 1995).

The reasons for the functional changes observed in ageing ribosomes are not known at present. Some attempts have been made to study the effect of ageing on rRNAs and ribosomal proteins. Although a three-fold increase in the content of rRNA has been reported in late-passage senescent human fibroblasts (Adam *et al.*, 1987), it is not clear if the quantity and quality of individual rRNA species undergo alterations during ageing, and what effect such changes might have on the functioning of ribosomes. Similarly, although an increase in the levels of mRNA for ribosomal protein L7 has been reported in aged human fibroblasts and rat preadipocytes, there are no differences in the electrophoretic patterns of the ribosomal proteins in young and old *Drosophila* and mouse liver (Rattan, 1995e).

Elongation of protein synthesis

The formation of the 80S initiation complex is followed by the repetitive cyclic event of peptide chain elongation, which is a series of reactions catalysed by elongation factors (EFs; also abbreviated as eEFs). Various estimates of the elongation rates in eukaryotic cells give a value in the range 3–6 amino acids incorporated per ribosome per sec, which is several times slower than the prokaryotic elongation rate of 15–18 amino acids incorporated per second (Merrick, 1992).

With regard to ageing, a slowing-down of the elongation phase of protein synthesis has been

suggested to be crucial in bringing about the age-related decline in total protein synthesis. This is because a decline of up to 80% in the rate of protein elongation has been reported by estimating the rate of phenylalanyl-tRNA binding to ribosomes in poly(U)-translating cell-free extracts from old *Drosophila*, nematodes and rodent organs (Richardson and Semsei, 1987). *In vivo*, a two-fold decrease in the rate of polypeptide chain elongation in old WAG albino rat liver and brain cortex has been reported. Similarly, a decline of 31% in the rate of protein elongation in the livers of male Sprague–Dawley rats has been reported by measuring the rate of polypeptide chain assembly, which was 5.7 amino acids per sec in young animals, and 4.5 amino acids per sec in two-year-old animals (Merry and Holehan, 1991). However, these estimates of protein elongation rates have been made for 'average' sized proteins. It will be important to see if there is differential regulation of protein elongation rates for different proteins during ageing.

The elongation of a polypeptide chain is mediated by two elongation factors: EF-1 and EF-2 in eukaryotes (a third factor, EF-3, is reported only in yeast), which are highly conserved during evolution (Riis *et al.*, 1990a). Depending on the relative amounts of EF-1α and EF-1βγ, EF-1 is found in differently aggregated heavy or light forms that might reflect the overall protein synthetic activity of the tissue. There is a high abundance of EF-1α (between 3 and 10% of the soluble protein), and there are multiple copies or isoforms of the gene that undergo cell-type- and/or developmental-stage-specific expression reported in yeast, fungi, brine-shrimp, *Drosophila*, toad and mammalian cells and tissues (Knudsen *et al.*, 1993). EF-1α has several other functions in addition to its requirement in protein synthesis. For example, EF-1α has been reported to bind to cytoskeletal elements; it is associated with endoplasmic reticulum; it is part of the valyl-tRNA-synthetase complex; it is associated with mitotic apparatus; it is involved in maintaining the accuracy of protein synthesis and protein degradation; it binds calmodulin in protozoan parasites; it induces rapid fragmentation of cytoplasmic microtubule arrays in fibroblasts; and its overexpression increases the susceptibility of mammalian cells to transformation (Gonen *et al.*, 1994; Shiina *et al.*, 1994; Rattan, 1995c).

The activity of EF-1 declines with age in rat livers and *Drosophila*, and the drop parallels the decrease in protein synthesis (Rattan, 1995e; Van Remmen *et al.*, 1995). This decline in the activity of EF-1 has been correlated only to EF-1α as no changes were observed in the EF-1βγ-mediated activity. Using more specific cell-free stoichiometric and catalytic assays, a 35–45% decrease in the activity and amounts of active EF-1α has been reported for serially passaged senescent human fibroblasts, old mouse and rat livers and brains . The germ line insertion of an extra copy of the EF-1α gene under the regulation of a heat-shock promoter resulted in a better survival of transgenic *Drosophila* at high temperature (Shepherd *et al.*, 1989). However, this relative increase in the lifespan of transgenic insects at high temperature was not accompanied by any increase in the levels of mRNA or the amount and activity of EF-1α (Shikama *et al.*, 1994). Similarly, no increased expression of EF-1α genes was observed in *Drosophila* with extended longevity phenotype in a long-lived strain (Dudas and Arking, 1994). However, the increased longevity of EF-1α high-fidelity mutants of a fungus *Podospora anserina* suggest that the life prolonging effects of EF-1α may be due to its role in maintaining the fidelity of protein synthesis (Silar and Picard, 1994). Future studies on other ageing systems, particularly human cells and rodents will clarify the role of EF-1α in the regulation of both protein synthesis and longevity.

In the case of EF-2, which catalyses the translocation of peptidyl-tRNA on the ribosome during

the elongation cycle, conflicting data are available regarding the changes during ageing. For example, a lack of difference in the rate of translocation has been observed during the translation of poly(U) by cell-free extracts prepared from young and old *Drosophila* and from rodent organs (Webster, 1985, 1986). Similarly, although the proportion of heat-labile EF-2 increases during ageing, the specific activity of EF-2 purified from old rat and mouse liver remains unchanged (Takahashi *et al.*, 1985a). In contrast, a decline of more than 60% in the amount of active EF-2 has been reported during the ageing of human fibroblasts in culture, measured by determining the content of diphtheria toxin-mediated ADP-ribosylatable EF-2 in cell lysates (Riis *et al.*, 1990b). However, using the same assay, no age-related change in the amount of ADP-ribosylatable EF-2 was detected in rat livers (Rattan *et al.*, 1991). Further studies are required to determine if there are any qualitative and quantitative changes in EF-2 at the levels of transcription, translation and post-translational modifications, and how such changes are related to the regulation of protein synthesis during ageing.

Other major components of the protein synthesis machinery are tRNAs and aminoacyl-tRNA synthetases (aaRS). There is at least one tRNA for each codon which is translatable into an amino acid, but there is no tRNA for the stop codons. For several amino acids, for example glycine, alanine, valine, leucine, serine and arginine, there are 4–6 isoacceptor tRNA species. The function of a tRNA in transferring the amino acid to the ribosome–mRNA complex is dependent upon the enzymes aaRS. Levels of tRNAs and aaRS have been considered to be rate limiting for protein synthesis.

According to one of the molecular theories of ageing, called the codon restriction theory (Strehler *et al.*, 1971), a random loss of various isoaccepting tRNAs will progressively restrict the readability of codons resulting in the inefficiency and inaccuracy of protein synthesis. There is some evidence that a shift in the pattern of isoaccepting tRNAs occurs during development and ageing in some plants, nematodes, insects and rat liver and skeletal muscle (Rattan, 1995e; Van Remmen *et al.*, 1995). Similarly, a 30- to 60-fold increase in the amount of UAG suppressor tRNA has been reported in the brain, spleen and liver of old mice, and has been related to increased expression of Moloney murine leukemia virus (MO-MuLV) in fibroblasts (Schröder *et al.*, 1992). Other characteristics of tRNAs which have been studied during ageing include the rate of synthesis, total levels, aminoacylation capacity and nucleoside composition (Table 7). There is no generalised pattern that emerges from these studies, and the reported changes vary significantly among different species.

The aminoacylation capacity of different tRNAs varies to different extents during ageing, and the reasons for such variability are not known. However, the fidelity of aminoacylation did not differ significantly in cell-free extracts prepared from young and old rat livers (Takahashi and Goto, 1988). Therefore, more studies are required to establish the changes in the structural and functional aspects of individual tRNAs, including their stability, accuracy and turnover.

In the case of aaRS, an increase or decrease in the specific activities of almost all of them has been reported in various organs of ageing mice without any apparent correlation with tissue/cell type and its protein synthesis activity. A significant decline in the specific activities of 17 aminoacyl-tRNA synthetases has been reported in the liver, lung, heart, spleen, kidney, small intestine and skeletal muscle of ageing female mice, and during development and ageing of nematodes (Gabius *et al.*, 1983). An increase in the proportions of the heat-labile fraction of several of these enzymes has been reported in the liver, kidney and brain of old rats (Takahashi

Table 7. Age-related changes in the characteristics of tRNA

Characteristic	Change	Aging systems*
Total levels	decrease	mouse liver, kidney, heart, muscle
	increase	mouse brain
Rate of synthesis	decrease	mouse liver, kidney, heart, muscle
Capacity to accept amino acids	variable	rat liver
Pattern of isoacceptors	unstable	soybean cotyledon; nematodes; *Drosophila*; rat liver
	stable	mouse liver, brain; *Drosophila*
Methylation	decrease	nematodes; rat and mouse liver, kidney; human fibroblasts
Nucleoside composition	no change	mosquitoes; mouse liver
Modified nucleoside queuine (Q) level	decrease	mosquitoes; mouse liver
	increase	rat liver
6-isopentenyl adenosine level	increase	rat liver

*for complete references to each system, see Rattan, 1995e.

et al., 1985b). However, no universal pattern can be seen for the changes in the activities of various synthetases in different organs and in different animals. Although an age-related decrease in the efficiency of aaRS can be crucial in determining the rate and accuracy of protein synthesis, direct evidence in this respect is lacking at present.

The cycle of peptide chain elongation continues until one of the three stop codons (UAA, UAG, UGA) is reached. There is no aa-tRNA complementary to these codons, and instead a termination factor or a release factor (RF) binds to the ribosome and induces the hydrolysis of both the aminoacyl linkage and the GTP, releasing the completed polypeptide chain from the ribosome. Studies on ageing *Drosophila* and old rat livers and kidneys have shown that the release of ribosome-bound N-formylmethionine, a measure of the rate of termination, was not affected with age (Webster, 1986). Direct estimates of the activity of the termination factor during ageing have not been yet made.

Chapter 6

Post-translational Modifications and Protein Turnover

Age-related changes in the functioning of proteins can be due to inefficient protein synthesis, altered patterns of post-translational modification, and altered rates of protein turnover. For example, although total protein synthesis slows down during ageing, the translational processes are never shut down completely. An accurate translation of mRNA followed by appropriate modifications of the polypeptide chain are essential for its normal folding, targeting and specificity. A misregulation in any of these steps can have far reaching biological consequences, including effects on cell growth, division and survival. A large number of post-translational modifications of proteins have been described which determine the activity, stability, specificity, transportability and lifespan of a protein. A brief discussion of some of the major protein modifications follows.

Phosphorylation

Phosphorylation of serine, threonine and tyrosine residues is one of the best studied modifications of proteins. The coordinated activities of protein kinases which catalyse phosphorylation, and protein phosphatases which catalyse dephosphorylation, regulate several biological processes, including protein synthesis, cell division, signal transduction, cell growth and development. Altered patterns of protein phosphorylation may be one of the reasons for age-related alterations in protein function and activity, and can be a major cause of the failure of homeostasis and ageing.

The inhibition of DNA synthesis and the loss of proliferative capacity is the ultimate characteristic of normal diploid cells undergoing ageing *in vitro*. Although several putative inhibitors of DNA synthesis have been identified in senescent cells, little is known about the mechanisms of action and the regulation of activity of these inhibitors. It is possible that the activity of several of these inhibitors is regulated by phosphorylation. For example, several studies have shown age-related alterations in cell-cycle-regulated gene expression of various genes such as c-*fos*, c-*jun*, *JunB*, c-*myc*, c-Ha-*ras*, *p*53, *cdc*2, *cycA*, *cycB*, *cycD* and retinoblastoma gene *RB1*. Although phosphorylation is involved in regulating the activities of the gene products of almost all these genes, a decrease in phosphorylated cyclin E and Cdk2, and failure to phosphorylate *RB1* gene product $p110^{Rb}$, and *cdc*2 product $p34^{cdc2}$ during cellular ageing have been reported to date (Stein and Dulic, 1995). It will be important to find out if there are age-related alterations in the phosphorylation state of other cell-cycle-related gene products, and proteins involved in DNA and RNA synthesis, including various transcription factors.

Various components of the protein synthesis apparatus undergo phosphorylation and dephosphorylation and thus regulate the rates of protein synthesis (Merrick, 1992). For example, phosphorylation of eIF-2 correlates with inhibition of initiation reactions and consequently the inhibition of protein synthesis. Conditions like starvation, heat shock and viral infection, which inhibit the initiation of protein synthesis, induce the phosphorylation of eIF-2 in various cells. Stimuli such as insulin and phorbol esters modulate the phosphorylation of eIF-3, eIF-4B and eIF-4F by activating various protein kinases (Merrick, 1992). Since the activity of eIF-2 has been reported to decrease during ageing, it is possible that the phosphorylation status of eIF-2 also changes during ageing. However, no studies are available on age-related changes in the phosphorylation pattern of initiation factors.

At the level of protein elongation, the phosphorylation of elongation factors EF-1α and EF-2 appears to be involved in regulating their activities (Ryazanov *et al.*, 1991). It will be interesting to see whether the age-related decline in the activities of elongation factors is accompanied by a parallel change in the extent of phosphorylation of these enzymes. Incidentally, it has been reported that there is an increase in the levels of phosphorylated EF-1 and EF-2 during mitosis when minimal protein synthesis occurs. Furthermore, there is indirect evidence that alterations in the phosphorylation and dephosphorylation of EF-2 due to changes in the activities of EF-2-specific protein kinase III (Riis *et al.*, 1993), and PP2A phosphatase (Riis *et al.*, 1995) may affect the rates of protein synthesis during ageing in rat livers.

Phosphorylation also occurs in other proteins that participate in the translational process. For example, the regulatory role of phosphorylation of aa-tRNA synthetase in protein synthesis has been suggested (Meinnel *et al.*, 1995). However, to what extent the decline in the activity and the accumulation of heat-labile aa-tRNA synthetases reported in studies performed on various organs of ageing mice and rats is related to their phosphorylation is not known. Furthermore, since the phosphorylation of the S6 ribosomal protein correlates with the activation of protein synthesis, failure to phosphorylate S6 protein in senescent human fibroblasts in response to serum (Kihara *et al.*, 1986) can be one of the reasons for the decline in the rate of protein synthesis observed during ageing.

Pathways of intracellular signal transduction depend on sequential phosphorylation and dephosphorylation of a wide variety of proteins. All phosphorylation reactions result from the action of single or multiple kinases, and the ratio between two interconvertible, active and inactive, forms of kinase acts as a control mechanism for many cellular functions. Studies performed on ageing cells have not shown any deficiency in the amount, activity or ability of PKC to elicit a signalling pathway (Blumenthal *et al.*, 1993). There is also evidence that senescent human fibroblasts retain their ability to phosphorylate proteins in the PKC signal transduction pathway (Shigeoka and Yang, 1990). It appears that the PKCs are largely unaltered in fibroblasts, although the body of information about phosphorylation mechanisms is very limited still.

Growth factor receptors for EGF, FGF, PDGF, insulin, glucocorticoids and several other hormones also possess protein kinase activity. Therefore, deficiencies in the phosphorylation process of receptors would be a logical explanation for the age-related decline of responsiveness to hormonal action and growth stimulation. However, there is no age-related decline in the autophosphorylation activity of various growth factor receptors (De Tata *et al.*, 1993; Farber *et al.*, 1993). Similarly, most of the PKC-mediated pathways of intracellular signal transduction in response to various mitogens including phorbol esters appear to remain unaltered in senescent fibroblasts (De Tata *et al.*, 1993; Derventzi *et al.*, 1993; Farber *et al.*, 1993). However, a

decline in both serine/threonine- and tyrosine-specific protein kinase signals after activation has been observed in the case of T lymphocytes in ageing mice (Miller, 1994). Similarly, alterations in PKC phosphorylation have been observed in pathological ageing, including Alzheimer's disease and neurodegenerative processes (Battaini *et al.*, 1995). Thus, phosphorylation of a wide variety of proteins has significant influence in biological processes and it will be extremely useful to undertake detailed studies on this post-translational modification of various proteins in relation to the process of ageing.

Oxidation

It is often observed that inactive and abnormal protein accumulates in old cells and tissues. This increased amount of debris in the cytoplasm can be inhibitory for cell growth and normal metabolism, and thus can contribute towards the failure of homeostasis. One of the reasons for the inactivation of enzymes can be their oxidative modification by oxygen free radicals and by mixed-function oxidation (MFO) systems or metal catalysed oxidation (MCO) systems (Stadtman, 1992). Since some amino acid residues, particularly proline, arginine and lysine, are oxidized to carbonyl derivatives, the carbonyl content of proteins has been used as an estimate of protein oxidation during ageing.

An increase in the levels of oxidatively modified proteins has been reported in old human erythrocytes of higher density, and in cultured human fibroblasts from normal old donors and from individuals suffering from progeria and Werner's syndrome (Stadtman, 1992). Similarly, there was a two-fold increase in the protein carbonyl content of the brain proteins of retired breeder Mongolian gerbils, which was reversed by treatment with a spin-trapping compound N-*tert*-butyl-α-phenylnitrone (PBN) (Carney *et al.*, 1991). An age-related increase in the carbonyl content has also been reported for houseflies, mouse organs, and *Drosophila* (Sohal *et al.*, 1993a,b).

The loss of activity of 6-phosphogluconate dehydrogenase and liver malic enzyme during ageing is related to the loss of lysine and histidine residues by oxidation (Gordillo *et al.*, 1989). The oxidation of a cysteine residue in glyceraldehyde-3-phosphate dehydrogenase may be responsible for its inactivation during ageing in rat muscles (Gafni, 1990). It has also been reported that the concentration of the oxidation products of human lens proteins and skin collagen increases along with the accumulation of oxidative forms of α crystallin in patients with age-related cataracts (Stadtman, 1992). However, the content of *ortho*-tyrosine and dityrosine, formed by the oxidation of phenylalanine and tyrosine, respectively, did not increase in the ageing human lens (Wells-Knecht *et al.*, 1993).

Structural alterations introduced into proteins by oxidation can lead to the aggregation, fragmentation, denaturation, distortion of secondary and tertiary structure, increasing thereby the proteolytic susceptibility of oxidized proteins. Furthermore, toxic products of carbonyl modifications can react with other macromolecules and affect various metabolic processes (Yin and Brunk, 1995).

Glycation

Glycation is one of the most prevalent covalent modifications in which the free amino groups of proteins react with glucose forming a ketoamine called Amadori product. This is followed

by a sequence of further reactions and rearrangements producing the so-called advanced glycosylation end products (AGEs). Most commonly, it is the long-lived structural proteins such as lens crystallins, collagen and basement membrane proteins which are more susceptible to glycation. The glycated proteins are then more prone to form crosslinks with other proteins, leading to structural and functional alterations.

An increase in the levels of glycated proteins during ageing has been observed in a wide variety of systems. For example, there is an increase in the level of glycated lysine residues of rat sciatic nerve, aorta and skin collagen during ageing (Oimomi *et al.*, 1988). There is an increase in the glycation of human collagen and osteocalcin during ageing (Miksík and Deyl, 1991). The formation and accumulation of AGEs is implicated in the physiology and pathology of senescence. It has been observed that pentosidine (crosslinked glycated lysine and arginine), carboxylmethyllysine (glycated and oxidated proteins), and pyrroline increase with age in normal and diabetic humans (Lee and Cerami, 1992). By using AGE-specific antibodies, an AGE-modified form of human hemoglobin has been identified whose levels increase during ageing and in patients with diabetes-induced hyperglycemia (Makita *et al.*, 1992). More studies are required in order to understand differences in the rates of formation and removal of glycated proteins in different species with different lifespans and rates of ageing.

Deamidation, Racemization and Isomerization

Age-related changes in the catalytic activity, heat stability, affinity for substrate and other physical characteristics, such as the conformation of proteins, may also be due to the charge change introduced by conversion of a neutral amide group to an acidic group by deamidation. Spontaneous deamidation of asparaginyl and glutaminyl residues of several proteins has been related with the observed accumulation of their inactive and heat-labile isoforms during ageing (Gafni, 1990). The sequential deamidation of two asparagine residues of triphosphate isomerase is responsible for the differences of the isoenzymes present in ageing cells and tissues, such as bovine eye lens, and human skin fibroblasts from old donors and patients with progeria and Werner's syndrome (Gracy *et al.*, 1985). Deamidation of glucose-6-phosphate isomerase produces the variant of the enzyme that accumulates in ageing bovine lenses (Cini and Gracy, 1986).

The interconversion of optical isoforms of amino acids, called racemization, has been reported to increase during ageing. For example, the concentration of D-aspartate in protein hydrolysates from human teeth, erythrocytes and eye lens increases with age (Brunauer and Clarke, 1986). Racemization of tyrosine has been reported to occur in the ageing brunescent human cataract lenses (Luthra *et al.*, 1994). The spontaneous prolyl *cis–trans* isomerization in proteins that may cause some of the so-called spontaneous conformational changes has been implicated in the age-related decline in the activity of certain enzymes. However, no definitive examples of enzymes undergoing this kind of post-translational modification during ageing are available. It is also not known to what extent the conformational changes associated with old rat muscle phosphoglycerate kinase, enolase and other enzymes are associated with racemization and isomerization.

ADP-Ribosylation

The structure and function of many proteins such as nuclear proteins topoisomerase I, DNA ligase II, endonuclease, histones H1, H2B and H4, DNA polymerases α and β, and cytoplasmic proteins adenyl cyclase and elongation factor EF-2 is modulated by ADP-ribosylation.

ADP-ribosylation of proteins is involved in various cellular processes such as maintenance of chromatin structure, DNA repair, protein synthesis, cell differentiation and cell transformation (Shall, 1995).

Indirect evidence suggests that poly-ADP-ribosylation of proteins may decrease during ageing because the activity of poly(ADP-ribose)polymerase (PARP) decreases in ageing human fibroblasts both as a function of donor age and during serial passaging *in vitro* (Dell'Orco and Anderson, 1991). Similarly, the direct relationship observed between the maximum lifespan of a species and the activity of PARP in mononuclear leukocytes of 13 mammalian species indicates its important role in ageing and longevity (Grube and Bürkle, 1992).

One cytoplasmic protein that can be specifically ribosylated by diphtheria toxin and exotoxin A is the protein elongation factor EF-2. ADP-ribosylation of the diphthamide (modified histidine 715) residue of EF-2 results in the complete abolition of its catalytic activity (Riis *et al.*, 1990a). There is evidence that increased ADP-ribosylation of EF-2 is correlated with cellular ageing. For example, the amount of EF-2 that can be ADP-ribosylated in the presence of diphtheria toxin in cell-free extracts decreases significantly during the ageing of human fibroblasts in culture (Riis *et al.*, 1990b). Many more studies are required to establish the role of ADP-ribosylation on the activity of various proteins during ageing.

Methylation

The methylation of nitrogens of arginine, lysine and histidine, and carboxyls of glutamate and aspartate residues is a widely observed post-translational modification that is involved in many cellular functions. Although most of our present understanding regarding the significance of protein methylation has come from studies on bacterial chemotaxis, muscle contraction, electron transport, the processing of pituitary hormones and gene expression, its role in ageing is beginning to emerge.

Proteins whose activities are increased by methylation include alcohol dehydrogenase, histones, ribosomal proteins, cytochrome C, elongation factor EF-1α, myosin, myelin and rhodopsin. Of these, decreased methylation of histones in the livers and brains of ageing rats has been reported. On the other hand, there is no difference in the extent of methylation of newly synthesised histones during cellular ageing of human fibroblasts in culture. Studies on the levels of methylated histidine, arginine and lysine of myosin isolated from the leg muscles of ageing rats, mice and hamsters showed unchanged levels of histidine, decreased levels of arginine and trimethyllysine, and increased levels of monomethyllysine .

During the ageing of erythrocytes, there is an increase in the number of methyl groups per molecule of band 2.1 (ankyrin) and band 3 protein, which correlates with increased membrane rigidity of erythrocytes during ageing (Mays-Hoopes, 1985). Similarly, there is a several-fold increase in the number of methyl acceptor proteins in the eye lenses from aged humans and people suffering from cataracts (McFadden and Clarke, 1986). The number of carboxylmethylatable sites of cerebral membrane-bound proteins also increases in rat brain during ageing (Sellinger *et al.*, 1988). At present, age-related changes in the methylation of other proteins such as ribosomal proteins, calmodulin, cytochrome C and myosin have not been studied. It is clear that protein methylation is involved in diverse functions including protein synthesis and turnover, and that it should be studied thoroughly in relation to the process of ageing.

Proteolytic Processing

Many newly synthesised proteins undergo post-translational proteolytic processing by which a certain conformational restraint on the inactive precursor is released and a biologically-active protein is generated. Several inactive precursors of enzymes called zymogens, precursors of growth factors, peptide and protein hormones such as insulin, precursors of extracellular matrix and many other secretory proteins including various proteases such as collagenase undergo proteolytic processing.

There have been no systematic studies performed on age-related changes in post-translational proteolytic processing of any proteins. However, there is some evidence that alteration in proteolytic processing may be one of the reasons for the appearance or disappearance of certain proteins during ageing. For example, the appearance of the 'senescent cell antigen' on the surface of a wide variety of ageing cells is considered to be derived from the proteolysis of band 3 protein (Kay, 1990). The exposure of senescent cell-specific epitopes on fibronectin (Porter *et al.*, 1992) may also be due to altered proteolytic processing. Progressive proteolysis of a 90 kDa protein, Tp-90 terminin, into Tp-60 and Tp-30 terminin in senescent cells and in cells committed to apoptosis has been reported (Hébert *et al.*, 1994). Proteolytic cleavage of the β-amyloid precursor protein is well known to play an important role in the pathogenesis of Alzheimer's disease (Selkoe, 1992). Increased proteolysis of a conformationally more labile single-chain form of the lysosomal protease cathepsin B has been suggested as a reason for the age-related decline in its activity during ageing of human fibroblasts (DiPaolo *et al.*, 1992). Similarly, alterations in the activity of collagenase during ageing of human fibroblasts has been suggested to be due to structural and catalytic changes (Sottile *et al.*, 1989).

Other Modifications

In addition to the types of post-translational modification mentioned above, there are some other modifications that determine the structure and function of various proteins and may have a role to play during ageing. For example, the incorporation of ethanolamine into protein elongation factor EF-1α may be involved in determining its stability and interaction with intracellular membranes (Riis *et al.*, 1990a). Whether this modification has any role in the regulation of the activity of EF-1α is not known at present. Similarly, the protein initiation factor eIF-5A contains an unusual amino acid, hypusine, which is synthesised post-translationally as a result of a series of enzymatically catalysed alterations of a lysine residue (Park *et al.*, 1993). Since the absence of hypusine in eIF-5A blocks the initiation of protein synthesis, it will be interesting to investigate changes in this modification during ageing when total protein synthesis slows down.

Detyrosination of microtubules, affecting the cytoskeletal organization and many other cellular functions, may also be important during ageing. Furthermore, the roles of chaperones in protein folding and conformational organization are yet to be studied in relation to the ageing process. According to the crosslinking theory of ageing, the progressive linking together of large vital molecules, especially proteins, results in the loss of cellular functions (Bjorksten and Tenhu, 1990). There is some evidence that both the pentose-mediated protein crosslinking and transglutaminase-mediated crosslinking of proteins is involved in ageing. For example, there is a high correlation between pentosidine protein crosslinks and pigmentation in senescent and cataract-affected human lens (Nagaraj *et al.*, 1991). Similarly, an increase in transglutaminase

activity during cellular apoptosis, differentiation and ageing of human keratinocytes (Norsgaard *et al.*, 1995) indicates an important role of this modification in the process of ageing.

Protein tyrosine sulfation is another post-translational modification that may have significance in protein alteration during ageing because it is involved in determining the biological activity of neuropeptides and the intracellular transportation of a secretory protein (Huttner, 1987). Similarly, prenylation, the covalent attachment of isoprenoid lipids on cysteine-rich proteins, is involved in the regulation of the activity of some proto-oncogenic *ras* proteins and the nuclear lamins A and B (Marshall, 1993). These studies have indicated a critical role for prenylation in the regulation of oncogenesis, nuclear structure, signal transduction and cell cycle progression, functions very much related with the causative aspects of ageing. Recent evidence shows an age-dependent decrease in the activity of prenyltransferases in the rat liver, which may account for the changes in the synthesis and turnover of mevalonate pathway lipids including cholesterol, ubiquinone and dolichol (Thelin *et al.*, 1994).

In conclusion, it is clear that an efficient and accurate translational machinery and the post-translational modification of proteins constitute the fundamental biochemical processes for cellular functioning and survival. Alterations at the level of protein synthesis and their post-synthetic modifications can have global detrimental effects on the maintenance and survival of cells, tissues, organs and organisms leading to ageing and death.

Protein Turnover

Efficient macromolecular turnover is integral to the normal functioning and survival of a biological system. Protein degradation during ageing is a relatively little researched topic as compared with RNA and protein synthesis. Although there are several-hundred-fold variations in the rates of degradation of individual proteins, it is generally believed that protein turnover slows down during ageing . The physiological consequences of decreased protein turnover include the accumulation of altered and abnormal proteins in the cell, an altered pattern of post-translational modifications due to increased dwell time, and a disruption of the organisation of the cytoskeleton and extracellular matrix.

Age-related decline in protein turnover is generally due to a decrease in the proteolytic activity of various lysosomal and cytoplasmic proteases (Dice, 1989). It is only recently that the molecular details of various pathways of protein degradation, such as the proteasome-mediated, ubiquitin-mediated and lysosome-mediated pathways, have begun to be studied in relation to ageing. For example, ubiquitin marking of proteins for degradation and ubiquitin-mediated proteolysis did not decline in ageing human fibroblasts and no change in the levels of ubiquitin mRNA and ubiquitin pools was detected (Pan *et al.*, 1993).

Other reasons for age-related changes in the activities of various proteases leading to a decrease in the rate of protein turnover include slower transcription, reduced rates of synthesis and altered patterns of post-synthetic modification, as discussed above. Furthermore, there is evidence that certain inhibitors of various proteases, such as the tissue inhibitor of metalloproteinases (TIMP) (Wick *et al.*, 1994), and trypsin inhibitor (Hearn *et al.*, 1994) had increased levels of expression and activities during ageing of human fibroblasts. This will also lead to a decrease in the activities of proteases leading to decreased protein degradation during ageing.

Finally, the synthesis, modification and turnover of proteins are interdependent processes which,

for practical purposes, set a limit on the efficiency of genetic information transfer from coded molecules to functional molecules. Ageing as the failure of maintenance can be fully understood by studying various enzymatic and other protein mechanisms and networks of repair and maintenance, which progressively become inefficient during ageing. The analysis of the synthesis of proteins, their modification which determines their activity, stability and specificity, and the turnover of inactive and altered proteins is central in this regard.

Chapter 7

Gerontogenes and Modulation of Ageing

The critical discussion of the major biological characteristics of ageing presented in the previous six chapters has, it is hoped, established that the failure of maintenance and repair mechanisms underlies the ageing process. Almost all theories of ageing imply directly or indirectly that the progressive failure of homeostatic mechanisms is crucial for the process of ageing. For example, the build-up of oxidative damage in macromolecules, the accumulation of abnormal, erroneous and defective proteins, defects in the signal transduction pathways, deficiency of the immune system and several other similar hypotheses point towards the failure of maintenance at all levels of organization as a crucial determinant of ageing and lifespan (Rattan and Clark, 1988; Medvedev, 1990; Holliday, 1995).

The diversity of the forms and variations in which age-related alterations are manifested, at all levels of organisation, indicate that ageing is stochastic in nature. Yet the apparent practical limit to maximum lifespan within a species, along with the evidence from studies on twins (McGue *et al.*, 1993; Yashin and Iachine, 1995), and from linkage studies performed on *Drosophila* (Buck *et al.*, 1993), that longevity is at least partly inheritable, suggest that there is genetic regulation of ageing and longevity. Furthermore, recent studies on human centenarians have shown a correlation between their exceptional longevity and certain genotypes, e.g. for the apolipoprotein (APO-E) gene and the angiotensin converting enzyme (ACE) gene (Schächter *et al.*, 1994). The genetic aspects of ageing and longevity on the one hand and the stochastic nature of age-related changes on the other are easily brought together if ageing is seen as the ultimate failure of maintenance and loss of homeostasis due to interactions between genetic mechanisms of defence (longevity assurance processes) and stochastic causes of damage and perturbations. In this context, the term gerontogenes (Rattan, 1985) refers to any such genetic elements which are involved in ageing.

The idea of gerontogenes does not contradict the non-adaptive nature of ageing discussed in the section on the evolution of life and death mechanisms in Chapter 1. Rather, it reasserts the importance of the genetic mechanisms of somatic maintenance in assuring germ-line continuity, as envisaged by both the antagonistic pleiotropy theory and the disposable-soma theory of the evolution of ageing and lifespan. Since the existence of genes for programmed self-destruction is on the whole discounted, on the basis of evolutionary arguments against the notion of the adaptive nature of ageing (Rose, 1991; Kirkwood, 1992), the concept of gerontogenes is closely linked with the genes involved in homeostasis and longevity assurance, instead of certain special genes for ageing.

The Nature of Gerontogenes

The term *gerontogenes* does not refer to a tangible physical reality of real genes for ageing, but refers to an emergent functional property of a number of genes which influence ageing. For this purpose, the term 'virtual' gerontogenes has been suggested (Rattan, 1995b). In science, the term 'virtual' is used for entities which it is helpful to regard as being present although they have no physical existence. The paradigm of such an entity is the virtual image of optics. Another example is the currently popular 'virtual reality', which fulfils the same definition.

The concept of virtual genes therefore refers to the emergent property of several genes whose functions are tightly coupled and whose combined action and interaction resemble the effect of one gene. Treating such a group as a virtual gene is a useful conceptual tool while the search continues for the genetic elements of regulation of complex biological processes, such as ageing. Although differentiation and development provide good examples of highly complex systems involving a large number of genes, it may be inappropriate to apply the concept of virtual genes to them, because these processes are under direct genetic control and have evolved as a result of natural selection. This situation is unlike ageing, where no natural selection for any specific genes is envisaged. Therefore, the concept of virtual genes is appropriate only for phenomena such as ageing, in which a genetic involvement is expected without direct genetic control open to natural selection.

The idea that gerontogenes are virtual implies that every time a gene is discovered which appears to have a role in the process of ageing, it will, on sequencing and identification, turn out to be a familiar normal gene with a defined function. Its role as a gerontogene can only be realised in the context of its emergent property in relation to several other genes which influence its activity and interactivity. Such genes cannot be hidden or cryptic, because their identities can in principle become well known. Individually, the functions of such genes can in principle be clearly established. Yet, as a result of concerted action and interaction, the combined effect of these genes resembles that of a 'gene for ageing', although these were not specially designed or naturally selected for causing ageing. This idea of virtual gerontogenes is in line with the evolutionary explanation of the ageing process as being an emergent phenomenon caused by the lack of eternal maintenance and repair instead of being an active process caused by evolutionary adaptation.

Candidate Gerontogenes

Obviously, not every gene is potentially a virtual gerontogene. However, potentially every gene can affect the survival of an organism. Therefore, a distinction must be made between immediate survival or death on the one hand and the process of ageing on the other. The inactivation of any essential gene will result in the death of an organism without having anything to do with the process of ageing.

The set of possible virtual gerontogenes can be narrowed down to sets of genes involved in the maintenance and repair of the cellular and sub-cellular components. Evidence for the hypothesis that candidate virtual gerontogenes operate through one or more of the mechanisms of somatic maintenance and repair comes from experiments performed to retard ageing and to increase the lifespan of organisms. For example, the anti-ageing and life-prolonging effects of calorie restriction are seen to be accompanied by the stimulation of various maintenance mechanisms. These include increased efficiency of DNA repair (Weraarchakul *et al.*, 1989), increased

fidelity of genetic information transfer (Srivastava *et al.*, 1992), more efficient protein synthesis (Merry *et al.*, 1992), more efficient protein degradation (Ishigami and Goto, 1990), more effective cell replacement and regeneration, improved cellular responsiveness (Chatterjee *et al.*, 1989), fortification of the immune system, and enhanced protection from free-radical- and oxidation-induced damage (Yu, 1990; Youngman *et al.*, 1992; Youngman, 1993).

The genetic selection of *Drosophila* for longer lifespan also appears to work mainly through an increase in the efficiency of maintenance mechanisms, such as antioxidation potential (Rose and Graves, 1990; Luckinbill, 1993). An increase in the lifespan of transgenic *Drosophila* containing extra copies of Cu–Zn superoxide dismutase (SOD) and catalase genes is due primarily to enhanced defences against oxidative damage (Orr and Sohal, 1994). Similarly, the anti-ageing effects of a dipeptide, carnosine (McFarland and Holliday, 1994) and a cytokinin, kinetin (Rattan and Clark, 1994) on human diploid fibroblasts also appear to be due to the effect of these chemicals on maintaining the efficiency of defence mechanisms, including efficient protein synthesis and turnover and the removal of oxidative damage.

Attempts at identifying the determinants of longevity by finding a correlation between the maximum lifespan of a species and other biological characteristics have also shown that it is the efficiency of the various defence mechanisms that correlates best with longevity. Some of the well-known maintenance mechanisms whose activity levels and efficiencies are directly correlated with species lifespan include DNA repair (Grube and Bürkle, 1992; Whitehead and Grigliatti, 1993; Bohr and Anson, 1995), cell proliferative capacity (Rattan and Stacey, 1994) and antioxidative potential (Sohal *et al.*, 1993a).

Further experimental evidence in favour of the concept of virtual gerontogenes comes from several studies whose purpose was to identify senescence-specific genes in old cells and tissues. Almost all such studies have resulted in the identification of genes, such as those of the components of the extracellular matrix, which are known to have other functions in cell metabolism and physiology (see Chapter 3). Studies on the extension of lifespan and the slowing-down of various age-related biochemical and functional alterations of *D. melanogaster* by simultaneous over-expression of Cu–Zn–SOD and catalase (Orr and Sohal, 1994) indicate that these free-radical-scavenging and antioxidant genes are part of the gerontogene family, by virtue of their role in influencing ageing and lifespan.

The identification of long-lived mutants of the nematode *Caenorhabditis elegans*, involving the *age*-1 (Johnson, 1990), dauer-constitutive *daf*-2 (Kenyon *et al.*, 1993) and spermatogenesis-defective *spe*-26 (Van Voorhies, 1992) genes may provide other examples of virtual gerontogenes. Although the exact nature of the final protein products of these genes is at present unknown, the *age*-1 mutant strain of *C. elegans* shows increased resistance to hydrogen peroxide-induced oxidative damage, an increase in the activities of SOD and catalase enzymes, and an increase in thermotolerance (Larsen, 1993; Lithgow *et al.*, 1995). Other possibilities may be found in the complex regulatory mechanisms known to exist in connection with the end-replication problem of telomeres and the post-replicative processing of DNA, such as methylation (see Chapters 3 and 4). Molecular studies using a comparative approach, including the use of transgenic organisms, will be useful in identifying the genes that are most important in this respect, and can be expected to form the basis of appropriate strategies for future gerontological research.

Modulating Ageing and Lifespan

Whatever academic and other noble reasons there may be for the study of ageing, significant driving forces have always been the search for ways to modulate the process of human ageing, to delay its onset and to reverse its course. From the philosophical query about old age that put Prince Siddharatha of India on to the path of becoming the Buddha, and the failed attempt by Gilgamesh of Mesopotamia to attain immortality to the continuing search for the fountain of youth in its modern scientific disguise, the modulation of ageing and lifespan has occupied the human mind since its inception.

Throughout human history, including the present times, search for a means to prevent or retard ageing has followed three main lines: (i) cleansing from impurities and wastes; (ii) nutritional supplements, including the use of medicinal plants; and (iii) replacement therapy. The immense popularity of various spas and water therapies, even today, is an example of the first type of anti-ageing approach. Also, the claims made for various herbal and other medicinal plant products, such as ginseng, ginkgo biloba and garlic, as nutritional supplements and anti-ageing drugs have received some support, however preliminary, from laboratory and/or clinical tests (Bardhan *et al.*, 1985; Lu and Dice, 1985; Petkov *et al.*, 1993; Xiao *et al.*, 1993; Svendsen *et al.*, 1994).

Replacement therapy

Replacement therapy, especially hormonal replacement therapy, as an anti-ageing treatment has been used and misused for quite some time. The logic behind this approach has been that since the levels of various hormones decreases during ageing, supplying these hormones externally may compensate for this loss and rejuvenate the system (Everitt and Meites, 1989). Various therapeutic procedures have been followed to replace the lost hormones, such as gland transplantation, secretory-cell injections and hormone injections. Whereas many of these approaches, such as monkey testicle transplants in the 1950s, have been frauds (Hamilton, 1986), others have been refined to some extent and are still in use. For example, the subcutaneous injection of recombinant biosynthetic human growth hormone for six months in elderly men resulted in a 9% increase in lean body mass, a 14% decrease in adipose-tissue mass, and a 1.6% increase in vertebral bone density (Rudman *et al.*, 1990). Similarly, there have been some claims regarding the anti-ageing effects of dehydroepiandrosterone (DHEA), which is the primary precursor of sex steroids (Regelson and Kalimi, 1994). However, several questions remain unanswered regarding the wider applicability of such an approach because of our present lack of understanding of the regulation of synthesis of various hormones, their modes of action, metabolism and interrelations with other hormones.

Recently, one of the hormonal glands, the pineal gland, has received much attention for its role as an anti-ageing endocrine (Reiter, 1995). In a small-scale study, the pineal gland's main hormonal product, melatonin, is shown to delay ageing and prolong the lifespan of C57BL mice when they are fed orally with this hormone from early age (Pierpaoli and Regelson, 1994). In the same study it was also shown that grafting of pineals from young mice into the thymus of syngenic old mice increased their survival and inhibited age-related structural changes in the thymus (Pierpaoli and Regelson, 1994). Extensive studies need to be done on the effects of melatonin and the pineal gland on the survival and ageing of a wide range of species before the usefulness of melatonin for human beings can be guaranteed.

Calorie restriction

Experimentally, the most effective anti-ageing and life-prolonging strategy has proved to be calorie restriction. Although most of the studies on dietary restriction and ageing have been performed on rats and mice, results have started to emerge from studies on long-term dietary restriction of non-human primates, rhesus monkeys (Bodkin *et al.*, 1995). A large number of studies have established that calorie restriction delays ageing and prolongs the lifespan of various animals (Yu, 1990; Masoro, 1992; Masoro, 1995). Most important, the anti-ageing effects of dietary restriction operate mainly by improving the efficiency of various mechanisms of maintenance and repair such as DNA repair, fidelity of genetic information transfer, rate of protein synthesis and degradation, cell replacement and regeneration, cellular responsiveness, the immune system, and protection from free-radical- and oxidation-induced damage (for cross references, see the earlier section on candidate gerontogenes). However, at this stage, it is very difficult to say what kind of voluntary dietary-restriction regimes will have similar anti-ageing and life-prolonging effects in human beings, considering that there are significant differences in the biology and, perhaps more importantly, the sociology of human beings as compared with other animals.

Antioxidants

Some other attempts to modulate ageing and lifespan include antioxidant supplementation in insects and rodents, and supplementation of cell-culture-media with growth factors, hormones and other compounds. Studies performed to see the effects of various antioxidants on the lifespan and ageing of organisms have given inconclusive results (Massie *et al.*, 1991, 1993; Hayflick, 1994). In cell cultures, various growth factors including fibroblast growth factor, dexamethasone and epidermal growth factor (Hasegawa and Yamamoto, 1992), hyaluronidase (Moczar and Robert, 1993), a dipeptide carnosine (β-alanyl-L-histidine) (McFarland and Holliday, 1994), and a plant hormone kinetin (Rattan and Clark, 1994), have been reported to have anti-ageing effects. However, it is not clear how any of these compounds brings about various anti-ageing effects and whether these compounds will have any application at the whole-body level.

Gene therapy

Attempts to develop gene therapeutic approaches to delay the signs of ageing and prolong lifespan have had only limited success until now. Most of these studies have been performed on fruitflies only. One of the first such studies to report lifespan extension by gene manipulation employed protein elongation factor EF-1α the gene for making transgenic *Drosophila* which lived longer at higher temperatures (Shepherd *et al.*, 1989). However, this effect was seen only under specific conditions of heat-shock promoter regulation of EF-1α gene and not under normal conditions of fruitfly survival (Shikama *et al.*, 1994). The other successful attempt at delaying ageing and prolonging the lifespan of *Drosophila* is described by Orr and Sohal (1994), who made long-lived transgenic lines which overexpressed SOD and catalase genes. Although genetic selection of natural and induced mutants in nematodes (Johnson, 1990) and selective breeding of late-reproducing *Drosophila* (Luckinbill, 1993) have given rise to long-lived strains, no other studies have so far been published reporting any effects of gene manipulation on the prevention of ageing and the prolongation of lifespan.

As discussed throughout this article, individually, no tissue, organ or system becomes functionally exhausted, even in very old organisms, yet it is their combined interaction and interdependence that determines the survival of the whole. The same logic needs to be applied to studies

at the molecular and genetic levels, particularly with respect to developing gene therapy to delay ageing. The search for an all-encompassing ageing gene(s) is unlikely to be successful. Estimates of the number of genes which could influence the ageing and lifespan of mammals run up to a few hundred out of about one hundred thousand genes, and their allelic variants (Martin, 1992). Therefore, manipulating any single gene or a few genes that show some effects on ageing and lifespan will help to identify genes that might qualify as being a part of the virtual gerontogene family. Of course, such studies will be valuable in finding ways to 'fine-tune' the network and to prevent the onset of various age-related diseases and impairments by maintaining the efficiency of homeostatic processes. In the short term, such studies will also result in the development of a variety of so-called anti-ageing products, by concentrating on individual members of the gerontogene family. In contrast, direct gene therapy directed at of the overall ageing process seems to hold little promise. The concept of virtual gerontogenes can be useful in the design of new experiments and help to search for the genetic 'hand of cards' that provides the best possible combination to prevent succumbing to perturbations from internal and external sources.

Some Final Comments

In order to unravel the molecular basis of ageing and modulate the process, it is proposed that the most promising research strategies will incorporate an analysis of the formation and functioning of maintenance and repair networks (Fig. 2). Some of the mechanisms expected to be crucial in this regard are those involved in maintaining: (i) the structural and functional integrity of the nuclear and mitochondrial genome; (ii) the accuracy and speed of transfer of genetic information from genes to gene products; (iii) the turnover of defective and abnormal macromolecules; and (iv) the efficiency of intracellular and extracellular communication and responsiveness.

Much is known about each of the above-mentioned categories of maintenance mechanism. The application of modern, more sensitive methods can further establish in detail what happens to

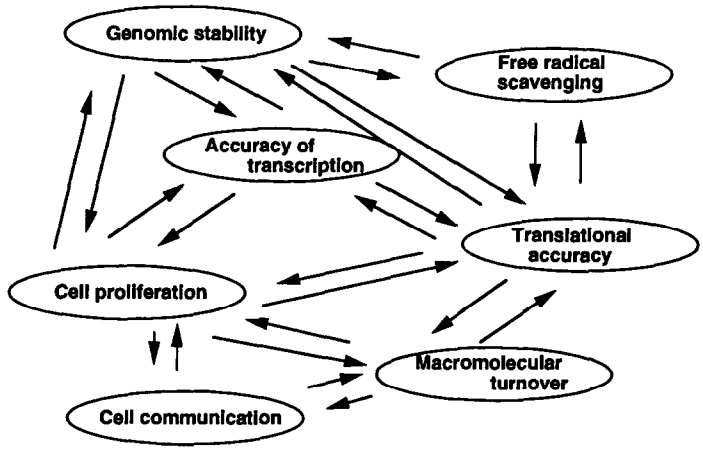

Fig. 2. Major molecular and cellular components of the maintenance and repair network that are essential for homeostasis.

these processes during ageing. However, the ultimate aim of biogerontological research is to understand why these changes occur, how they affect various other constituents of the network, and how these can be modulated in order to maintain the healthy span of life. The attainment of this goal will require the development of experimental approaches in which the interaction between mechanisms of maintenance at various levels is studied and the reasons for their failure are elucidated.

The following lines of research can form the basis of a promising strategy to understand ageing:

(1) the study of the extent of maintenance and repair of the important genes involved in maintaining the stability of the nuclear and mitochondrial genome;

(2) the study of the efficiency of transcription of these genes and post-transcriptional processing of their transcripts during ageing;

(3) the study of the accuracy and efficiency of translation of these genes and the analysis of the specificity and stability of their gene products, including post-translational modifications;

(4) the experimental modulation of various types of maintenance mechanism (for example, responsiveness to stress) and the study of its effects on other levels such as gene stability and gene product synthesis and turnover;

(5) the search for natural or induced mutants (including in transgenic and knockout organisms) with altered levels of maintenance and repair of the crucial genes; and

(6) the search for age-specific and age-related disease-specific biomarkers for diagnostic purposes and for monitoring the effects of potential therapeutic agents.

The above approaches can narrow down the number of genes and the critical processes which are central to forming an optimum network for survival. Elucidating the nature and components of such a network will open up new possibilities of interfering with the gerontogene network and fine tuning the system for both successful ageing and prolonged survival.

In a Nutshell

Ageing.
Descending notes of
the jeewan-raga.*
Rhythmic restrains transcended.

Slower to respond,
slower to adapt.
A failure of maintenance,
of homeostasis.

Not a cycle of life and death.
Not even a spiral of time.
The reality of birth, growth, continuity,
a disappearing mime.
In virtual gerontogenes,
which are there,
but they don't exist.

Ageing, a journey
from real to virtual,
from heard to unheard.

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*jeewan-raga = the life-raga (*raga is the fundamental structural unit of Indian music*)
(reproduced from Rattan, S. I. S. (1994) in: *The National Library of Poetry – Echoes of Yesterday*, pp. 398, Library of Congress, USA.)

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