THE BIOCHEMICAL BASIS OF CHANGES IN NORMAL AND MUTANT HUMAN SKIN FIBROBLASTS DURING AGEING IN CULTURE.

An investigation into the free radical theory of ageing

De biochemische basis van veranderingen in normale en gemuteerde menselijke huid fibroblasten gedurende veroudering in kweek.

Een onderzoek naar de vrije radicalen theorie van veroudering

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Everyone wants to reach an advanced age,
yet when it comes they all complain !

Cicero
Cato Major, De Senectute
44 BC

To: the late Professor Dr. Ch.M.A. Kuyper,
who introduced me to the thrills of cells
and their molecules

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Publication 1

Autofluorescence of human skin fibroblasts during growth inhibition and in vitro ageing
M. Poot, W.J. Visser, A. Verkerk and J.F. Jongkind
(1985) Gerontology 31, 158-165.

Publication 2

Reversible inhibition of DNA replication and protein synthesis by cumene hydroperoxide and 4-hydroxy-nonenal in human skin fibroblasts
M. Poot, A. Verkerk, J.F. Koster, H. Esterbauer and J.F. Jongkind
(1988) Mechanisms of Ageing and Development 43, 1-9.

Publication 3

Disturbance of cell proliferation by two model compounds of lipid peroxidation contradicts causative role in proliferative senscence
M. Poot, H. Esterbauer, P.S. Rabinovitch and H. Hoehn
(1988) Journal of Cellular Physiology 134, (in press).

Publication 4

Accumulation of a high molecular weight glycoprotein during in vitro ageing and contact inhibition of growth
M. Poot, A. Verkerk and J.F. Jongkind
(1986) Mechanisms of Ageing and Development 34, 219-232.

Publication 5

Glutathione content of cultured human fibroblasts during in vitro ageing
M. Poot, A. Verkerk and J.F. Jongkind
(1984) Mechanisms of Ageing and Development 27, 315-321.

Publication 6

De novo synthesis of glutathione during in vitro ageing and in some metabolic diseases as measured by a flow cytometric method
M. Poot, A. Verkerk, J.F. Koster and J.F. Jongkind
(1986) Biochim.Biophys.Acta 883, 580-584.

Publication 7

The influence of cumene hydroperoxide and 4-hydroxy-nonenal on the glutathione metabolism during in vitro ageing of human skin fibroblasts
M. Poot, A. Verkerk, J.F. Koster, H. Esterbauer and J.F. Jongkind
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Chapter 1

INTRODUCTION : BIOLOGICAL AGEING

Ageing, a however common and ubiquitous aspect of life it may be, is as yet poorly understood in biological terms. As a process, ageing is evident from the very moment of conception of new life, but it is visible only after maturation, as developmental changes are so dominant as to obscure changes pertaining to the ageing process. Necessarily, Hayflick (1987) argued that the time period of ageing should be divided in two: one comprising developmental ageing period and one post-developmental period.

In the human, ageing is experienced as wrinkling of the skin with localised pigment plaques; graying and thinning of hair; decrease in muscular strength and agility; shrinkage and sclerosis of tendons; flattening of intervertebral discs; loss of neurons and memory; decrease of sensory capabilities, especially of sight, hearing and heat sensing; a decreased control of blood pressure leading to essential hypertension and postural hypotension; lowered total and vital capacity of the lungs; reduced filtering by the kidneys. Some changes thought to accompany ageing, such as increased rates of incidence of atherosclerosis, cancer, emphysema, are not generally present in the population at large and seem rather to reflect adverse life conditions and habits (like unappropriate and deficient diets, polluted air and smoking) (Martin, 1985a).

Senescence, the final outcome of the ageing process has been defined as: "that change of the bodily faculties and sensibilities and energies which acompanies ageing, and which renders the individual progressively more likely to die from accidental causes of random incidence. Strictly speaking, the word "accidental" is redundant, for all deathts are in some degree accidental. No death is wholly "natural"; no one dies merely of the burden of the years" (Medawar, 1951). So, ageing mainly is an increase with time of the probabilty of death. Death itself is not intrinsically resulting from ageing, but is rather a manifestation of a bodily process discernible only after an external "stimulus" acted upon the body, that underwent this process.

In order to be applicable to research into events that might cause ageing this definition needs some elaboration. B.L. Strehler gave four basic criteria to identify such events causative to ageing (Strehler, 1977): (1) universality, (2) intrinsicality, (3) progressiveness and (4) deleteriousness. Via the principle of universality, diseases as cancer or atherosclerosis and genetically determined conditions are precluded from true ageing research. The principle of intrinsicality aims at excluding environmental factors like poisoning. The third criterion (progressiveness) aims at excluding suddenly occurring events as artery occlusion or cerebrovascular accidents. It also precludes all kinds of reversible changes. Finally, deleteriousness points towards an increasing probabilty of death. Medawar's definition, taken together with the considerations outlined

above will be used throughout this thesis, when it comes to decide whether or not an observed change is to be considered pertinent to the ageing process.

Cell culture systems, being systems of experimental hyperplasia, can be considered as models for decline and loss of function during ageing. A kind of functional decline particularly appropriate to study via cell culture systems is the loss of control over proliferative homeostasis and the increase of chromosomal instability (Martin, 1985). These two phenomena may contribute to the pathogenesis of neoplasia and hyperplasia of skin cells as evidenced by pigment and fat plaques. Furthermore, human skin fibroblasts tend to undergo some sort of an ageing process in vivo. This process seemingly resembles cellular ageing, as cells upon explantation from a person show a decreasing lifespan in culture, with increasing age of the donor (Martin et al., 1970).

Research into cellular ageing found its theoretical origin in 1881, with Weissmanns prediction that the somatic cells of higher organisms would have a limited proliferative potential (Kirkwood and Cremer, 1982). This idea is quite attractive in view of the limited lifespan of higher organisms. The experimental demonstration that cells in culture do have a limited replicative lifespan initiated serious consideration of Weissmanns prediction (Hayflick and Moorhead, 1961). The work of Hayflick and Moorhead with WI-38 cells is nowadays confirmed by other workers with a plethora of other cell strains of human (Jacobs et al., 1970; Nichols et al., 1977), avian (Hay and Strehler, 1967; Ponten, 1970; Lima et al., 1972) and hamster (Raes and Remacle, 1983) origin. During ageing of a human being skin fibroblasts tend to lose part of their proliferative capacity (Martin et al., 1970). However, factors such as site of biopsy, type of culture medium and batch of serum used, and genetic pathology lead to so large a variation as to preclude any conclusions to be drawn from an apparent maximal population doubling level (Martin et al., 1970). The progressive loss of proliferative capacity of a culture reflects a decrase in the number of cells capable of DNA replication (Cristofalo and Scharf, 1973; Rabinovitch, 1983) and a lengthening of the G1 compartment of the cell cycle (Rabinovitch, 1983). These two cell kinetic characteristics fully explain the ageing phenomenon (Angello and Porthero, 1987).

1.1 THEORIES OF AGEING

The field of ageing research abounds with theories, proposing a variety of causes to the ageing process. Theories about ageing, being defined as an universal, deleterious, intrinsic and progressive process taking place with time in the living species, should be based on assumptions sharing all these characteristics. These theories can be divided into two types based upon their starting point as either programmed or stochastic theories. Programmed theories of

ageing assume some sort of a programme being unleashed at a certain moment, which then engenders the sequence of events leading to senescence. Conversely, stochastic theories of ageing surmise a randomly arising event which should elicit ageing.

1.1.1 PROGRAMMED THEORIES OF AGEING

The two major theories of this kind either propose cells to reach a terminal stage (differentiation theory) or to drift away from their proper state (dysdifferentiation theory). Although both theories aim at the same observation, ie. cessation of growth in cell cultures during passage in vitro, they have a fundamentally different structure of reasoning. Differentiation implies attaining some sort of a "goal", while dysdifferentiation is based upon a kind of failure.

The differentiation theory of ageing was originally proposed by Martin et al. (1970) and assumes, that cells cease proliferation because they reach a terminal differentiation state. This theory received an overwhelming experimental support. Cell fusion experiments, in which heterodikaryons of proliferating and senescent cells were obtained, clearly showed that DNA synthesis is not reinitiated in the senescent nucleus. Also the rate of DNA synthesis decreases in the originally cycling nucleus (Norwood et al., 1974). This suggests dominance of the senescent phenotype (Norwood et al., 1974). This inference is buttressed by fusions of cells from permanent cell lines with senescent cells, which lead in the senescent nucleus to some extent of reinitiation of DNA synthesis, but never gave rise to a continuously proliferating cell clone (Stein and Yanishevsky, 1979; Stein et al., 1982; Pereira-Smith and Smith, 1982). Also, quiescent cells fused with cycling cells inhibited DNA synthesis in the cycling nucleus (Rabinovitch and Norwood, 1980; Stein and Yanishevsky, 1981). This suggests the existence of a diffusable factor able to inhibit DNA synthesis (Stein and Yanishevsky, 1979; Stein et al., 1982; Rabinovitch and Norwood, 1980).

With one possible exception (Lincoln et al., 1984), all attempts at identifying a protein that might serve as this putative inhibitory factor in senescent cells failed. In most cases a protein was spotted, which could also be detected in quiescent cells (Wang, 1985). This indicates certain similarities between senescence and quiescence, from which one might imply that the senescent and the quiescent state are one and the same. In support of this come data on cell cycle distribution demonstrating that senescent and quiescent cells exit from the G1 compartment of the cell cycle (Yanishevsky et al., 1974; Grove and Cristofalo, 1977; Grove and Cristofalo, 1986). However, quiescent G1 cells can be reactivated to DNA synthesis, whereas senescent G1 cells remain resistant to stimulation. In final analysis senescence

is distinct from quiesence and under strict genetic control (Smith and Lincoln, 1984). Reviewing several lines of evidence, Hayflick argued that the cellular lifespan determining entity is intranuclear (Hayflick, 1984). So, a change in gene expression has to be detected if senescence were to result from a differentiation-like process. The cultured fibroblast system does not allow analysis of differentiated functions, but cultured adrenocortical cells are quite amenable to this kind of investigation. Hornsby et al. showed that these cells express certain different cell functions throughout their lifespan in culture (Hornsby, 1984; Hornsby et al., 1984). The loss of response to ACTH and the loss of proliferative capacity appear to be independent stochastic events (Hornsby et al., 1986). This flaws the evidence in favour of the differentiation theory of cellular ageing, but some evidence on another level butresses the hypothesis. A high abundance mRNA has been isolated from senescent human diploid fibroblasts, which inhibited DNA synthesis upon microinjection into proliferating cells (Lumpkin et al, 1986). Unfortunately, this mRNA does also occur in quiescent, proliferation competent cells, albeit at a minor concentration (Lumpkin et al., 1986). By the same token, senescent cells have been shown to suppress tumour growth (O'Brien et al., 1986) and hybrids of SV-40 T antigen expressing human cells and tumour derived human cells having activated H-ras and N-ras oncogens still have a finite lifespan (Pereira-Smith and Smith, 1987). Finally , a membrane associated inhibitor of DNA synthesis has been found in senescent human diploid fibroblasts, which is distinct from the proliferation inhibitor of quiescent cells (Stein and Atkins, 1986). These data are in keeping with a positively acting, proliferation inhibiting factor.

A completely different avenue of programmed ageing was opened up by Cutler (Cutler, 1984a). He assumes cells to be gradually drifting away from their proper state of differentiation, which results from an accumulation of minute changes in the genetic apparatus of cells. These changes may be sufficient to alter highly differentiated features of cells but do not impair any vital functions (Cutler, 1984b). Ageing might primarily impair differentiated cell functions, leaving housekeeping functions unaffected. From this, one can imply that so-called "luxury" genes such as certain stabilising systems might be impaired. This hypothesis can be tested by looking for changes in functioning of putative stabilising systems during ageing or to analyse the activity and capacity of stabilising systems in animals of differing longevity. If the hypothesis is correct, longer living animals should contain more effective stabilising systems.

Cutler tested this hypothesis by comparing parameters of cell stabilising systems among animal species of differing longevity (Cutler, 1984a, 1984b). It should be pointed out that this kind of investigation suffers from a theoretical problem relating to the interpretation of correlations in general. Correlations are not proof of causal relationships,

but rather the inverse is true: the absence of a correlation proves the absence of a causal relationship. To shortly summarise the enormous data set generated by Cutler and co-workers (Cutler, 1985a): no correlation between maximal lifespan and blood concentration existed for ascorbate. So, vitamin C can safely be ruled out as a longevity determining factor. The concentrations of urate, carotenoids, alpha tocopherol, ceruloplasmin and the activity of superoxide dismutase (SOD) correlated positively with maximal lifespan, whereas the concentration of glutathione and the activities of catalase and glutathione peroxidase showed negative correlation. Although this does not necessarily indicate a causal relationship, it still indicates a route to be pursued in more detail. Also an inverse correlation between the oxidisability of kidney and brain tissue of 24 animal species and their maximal lifespan has been demonstrated (Cutler, 1985b). This means that an elevated amount of peroxidisable substrate correlated with a decreasing longevity. Here, Cutler encompasses the free radical theory of ageing. By the same token, the free radical theory of ageing becomes a special form of Cutler's dysdifferentiation theory.

1.1.2 STOCHASTIC THEORIES OF AGEING

Stochastic theories are entirely devoid of direction or meaning, thus absolutely non-teleologic and reductionistic. These theories assume an intrinsic failability, which inevitably provokes senescence. This intrinsic failure can either result from a continuous generation of errors by the system itself (error catastrophe) or from an uncomplete defense against errors induced by an external agent (eg. free radicals).

The error catastrophe theory as proposed by Orgel originates from the idea that no information transmission system is absolutely perfect (Orgel, 1963). So, if during transcription a single error is being made this will lead to an altered m-RNA, which, consequently, will be translated into an altered protein. If, however, a correct m-RNA is being transcribed, an error during translation might still lead to an altered protein. Provided this altered protein should saveguard the message contained in DNA or RNA, it is very well possible, that more error is being introduced. A catastrophe and senescence will ensue. This proposal has an enormous intuitive appeal in view of our knowledge of quite a number of the sophisticated "error control" or "error repair" systems in human cells. These systems then, would be highly important as "anti-ageing" checks and balances.

The error catastrophe theory has been analysed by looking into the failability of the information transmission systems. Several investigators attempted to show that during translation erroneous proteins were produced from "correct" m-RNAs (Harley et al., 1980; Mori et al., 1983). However, even the use of defined synthetic RNAs as a template could

not reveal an increase in error frequency in translation during in vitro ageing of fibroblasts (Mori et al., 1983). So, it seems unlikely that errors in translation will be responsible for the accumulation of "wrong" proteins during ageing of cells in culture. This does not rule out the possibility that other steps during manufacture of proteins and in the replication of genetic information might be defective and lead to an error catastrophe, although the very effects of such a catastrophe would be limited to enzymes involved in the regulation of substrate fluxes at branchpoints of metabolic pathways (Kurland, 1987).

The putative accumulation of altered proteins during ageing of cells in culture can also be explained by the free radical theory of ageing. This provident theory, is based upon some observations of effects from irradiation of animals (Harman, 1956). The hypothesis states that free radical reactions will lead to an intracellular accumulation of damages, which eventually will lead to the senescent phenotype.

The free radical theory of ageing is able to embrace several other damage theories of ageing. Among the explicit theories of this kind are: the membrane theory, the amino acid racemisation theory and the cross-linking theory. The membrane theory stipulates that ageing results from an increase in membrane rigidity (Zs.-Nagy, 1978). This increased rigidity will lead to an chnage in the intracellular ion-balance, which in turn might affect the functioning of enzymes and structural macromolecules. The amino acid racemisation hypothesis postulates, that through racemisation of amino acids the structural and functional properties of proteins will be lost gradually during ageing (DeLong and Poplin, 1977). Consequently, ageing will yield to a loss of three-dimensional structure and necesarily to an increase in entropy in cells. The cross-linking hypothesis, is supported by some data, but it remains unclear what significance the accumulation of clusters of covalently bound molecules of DNA, RNA and proteins might have (Bjorksten, 1974). Free radical reactions can oxidise PUFA in membranes, thus leading to an increased rigidity in membranes as proposed by Zs.-Nagy. Oxidised PUFA can decay to various aldehydes, which in turn can cross-link macromolecules containing amino residues, thus leading to the formation of crosslinks as proposed by Bjorksten. Conversely, free radicals can modify the amino acid residues of proteins and subsequently cause the racemisation, which was proposed (DeLong and Poplin, 1977). So, the free radical theory, if it were true, can easily accommodate the mechanisms of ageing stipulated by some other theories.

The three theories briefly outlined above all fall short at an important aspect. None of them do take into account cellular repair and turnover mechanisms, which might obviate the accumulation of rigidified membranes, "wrong" enantiomers containig proteins or cross-links of macrolecules.

1.1.2.1 THE FREE RADICAL THEORY

Free radical reactions result from cellular oxygen metabolism (Nohl and Hegner, 1978; Chance et al., 1979) and from the availability of ferritin, which acts as a physiological iron donor (Biemond et al., 1984; 1986). The reactive oxygen species thus formed can attack and damage lipids, nucleic acids and proteins. These damages might bear on cellular metabolism and structure. Among the effects ensuing are: lipid peroxidation (Pryor, 1976; Freeman and Crapo, 1982), the formation of aldehydic breakdown products (Benedetti et al., 1982; Poli et al., 1985), damaged DNA bases (Teoule, 1987; Kasai et al., 1986; Kuchino et al., 1987; Frenkel and Chrzan; 1987; Dizdoraglu et al., 1987), release of bases (Yamane et al., 1987; Lin et al., 1987), single strand breaks (Massie et al., 1972; Filho and Meneghini, 1984), impaired DNA replication (Clark and Beardsley, 1987), mutagenesis (Hsie et al., 1986) and finally enhanced turnover of proteins (Uchida and Kawakishi, 1986; Dean et al., 1986; Wolff and Dean, 1987; Davies and Goldberg, 1987a; 1987b; Vince and Dean, 1987; Dean, 1987; Wolff et al., 1986). In keeping with the hypothesis, senescent sequelae should ensue, provided protective and repair mechanism fail to prevent or to adequately dispose of the damage produced, and molecules damaged by any of these mechanisms should accumulate.

SCHEME 1

OXYGEN FREE RADICALS

↓

PROVOKE : LIPID PEROXIDATION
GENERATION OF ALDEHYDES
DAMAGED DNA BASES
RELEASE OF BASES FROM DNA
DNA SINGLE STRAND BREAKS
ENHANCED TURNOVER OF PROTEINS

↓

MAY ELICIT: ALTERATIONS IN MEMBRANE FUNCTION
CROSS-LINKING OF PROTEINS
RACEMISATION OF AMINO ACIDS

Possible unification of predictions from several theories of ageing by the free radical theory of ageing.

1.2 DATA ON FREE RADICALS AND AGEING

Evidence concerning the free radical theory of ageing has been reviewed recently (Balin, 1982; Harman, 1982; Melhorn and Cole, 1985, Pryor, 1987). It is of no use to repeat the points being made by these authors, but it should be noted, that most of the data in the reviews is of circumstancial nature, what precludes a final judgement at this stage. Direct evidence as to this theory is clearly needed (Takahashi and Zeydel, 1982).

Free radicals have been shown to inflict a plethora of damages to DNA; new types of DNA damage are being described continuously (see above). DNA damage, if it were to cause ageing, has to alter DNA coding properties. In other words, DNA damage will only be experienced during cellular ageing if it induces somatic mutations. The somatic mutation theory of ageing has been refuted a long time ago (Strehler, 1977). Recent experimental evidence (Hornsby and Harris, 1987) showed that the mutagenic effect of cumene hydroperoxide does not relate to ageing. Therefore, DNA damage induced by a direct effect of free radicals should be discarded as a possible mechanism by which ageing might originate.

A second mechanism for free radicals to provoke ageing is cross-linking of protein molecules. This phenomenon can either result from the formation of dityrosine links (Wolff, Garner and Dean, 1986) or secondarily from lipid peroxidation (Koster and Slee, 1980). In cellular ageing no evidence as to the first mechanism exists. The other mechanism requires a more detailed scrutiny.

Any oxidising free radical is likely to abstract a hydrogen atom from an aliphatic chain or from an aromatic ring. Fatty acids, especially those containing one or several unsaturated bonds are thus potential targets. The divinyl methane structure of a PUFA is particularly prone to this kind of attack (Stam and Koster, 1985). Once an allylic hydrogen is abstracted from the PUFA an alkyl radical is formed, which subsequently is rearranged in the diene configuration and then atracts a dioxygen molecule. The peroxy radical, thus formed, can abstract another hydrogen from a nucleophile function on DNA, lipid or protein and thus a lipid peroxide results. Although lipid peroxides are transiently stable, they can as well undergo a metal catalysed breakdown reaction which leads to the formation of alkoxy radicals and hydroxy lipids. Alkoxy radicals themselves can finally fragment to form reactive aldehydes (like hydroxynonenal) and lower hydrocarbons (Stam and Koster, 1985). The formation of the bifunctional aldehyde malondialdehyde, potentially able to cross-link proteins, has as well been implicated, but it remains to be seen what relative importance should be attributed to the various possible aldehydic decay products of lipid peroxidation. As research into these aldehydic breakdown products has been initiated recently, only a few reports are available. Those

point towards a plethora of deleterious effects upon the cell (Cadenas et al., 1983; Hauptlorenz et al., 1985). As aldehydes can react with amino functions to form imines (Schiffs bases), they might elicit cellular autofluorescence.

Stubel showed autofluorescent lipopigment to accumulate in brains of aged humans (Stubel, 1911). Deamer and Gonzales found a similar accumulation of lipopigment in acid phosphatase containing cytoplasmic granules during serial passage of WI-38 cells (Deamer and Gonzales, 1974). Isolated autofluorescent cells from a culture had elevated levels of lysosomal enzyme activity and a reduced growth capacity, thus confirming the previous suggestion, that this autofluoresnce accumulated as a result of ageing in culture (Jongkind et al., 1982). However, the change in lysosomal enzyme activity is not at all indicative of an ageing process, but might be a mere reflection of an impaired rate of proliferation (Heukels-Dully and Niermeyer, 1976). Cellular autofluorescnce has been quantitated and proposed as an index of in vitro ageing (Rattan et al., 1982). Moreover, autofluorescence was found to accumulate during ageing in albino rats (Katz et al., 1984) and in human brain (Goyal, 1982)). A nutritional deficiency in vitamin E potentiated this accumulation (Katz et al., 1984), while the addition of this lipophilic free radical scavenger to the culture medium could abolish the formation of autofluorescence (Thaw et al., 1984). Elevated oxygen tension and the addition of ascorbic acid plus ferri-chloride to the culture medium as well potentiated the accumulation of autofluorescence (Thaw et al., 1984). If this autofluorescence results from lipophilic free radical reactions, lipoperoxides or their breakdown products, like malondialdehyde, these should produce similarly fluorescent products if reacted with cellular costituents as proteins or amino acids. It was shown that reaction of malondialdehyde with amino acids indeed resulted in fluorescent products (Chio and Tappel, 1969; Esterbauer et al., 1986; Koster and Slee, 1980; Koster et al., 1982; Montfoort et al., 1987). However, the fluorescence spectra of these products differed strongly from the spectra of autofluorescent material found in cultured cells or biopsies. The latter autofluorescence contained at least two species (Aubin, 1979), which could be resolved into a whole variety of fluorescent compounds via thin layer chromatography (Eldred, 1982). Some authors suggested autofluorescence to be due to flavins (Benson et al., 1979) or to result from hydrolysis of FAD (Ragab et al., 1968). Hence, cellular autofluorescence should not necessarily result from lipid peroxidation.

1.3 CELLULAR DEFENCES AGAINST FREE RADICAL INDUCED DAMAGE

Cellular defense against free radical reactions can either be non-enzymatic or enzymatic. The first type of mechanism comprises metal chelators and anti-oxidants. The enzymatic defence against free radical reactions consists of superoxide dismutase, catalase and glutathione peroxidase. The first two

enzymes act upon the radicals directly, while glutathione peroxidase using glutathione as a low molecular reductant, deals with potentially deleterious sequelae of free radical reactions.

Metal storage proteins, like ferritin, and several proteins in extracellular fluids, such as ceruloplasmin (Halliwell, 1985), act via sequestration of transition metals thus reducing their availability for the generation of free radicals. Anti-oxidants react with existing free radicals, thus interfering with possible chain reactions. Among them are ascorbic acid, carotenes and tocopherol (known as vitamin E). Ascorbic acid has been shown to be an anti-oxidant at high concentrations, while at low concentrations it is a pro-oxidant (McCay, 1985). Carotenes and tocopherol, by virtue of their phenolic hydroxyl function, quench free radical chain reactions (McCay, 1985). As both compounds are highly lipophilic, they function as excellent scavengers for lipid peroxides and lipid radicals, as evidenced by their extremely high reaction constants towards these (McCay, 1985).

Superoxide dismutase (E.C. 1.15.1.1) has been studied extensively (Fridovich, 1975; 1983; Bannister et al. 1987). The enzyme limits intracellular superoxide concentration and thus prevent the formation of hydroxyl radical through the iron catalysed Haber-Weiss reaction. Increased activity of this enzyme reflects an elevated rate of generation of superoxide (Hassan and Fridovich, 1976). The activity of SOD was found to rise slightly during ageing of cultured cells (Somville and Remacle, 1980) and of erythrocytes (Glass and Gershon, 1981; Reiss and Gershon, 1976; Glass et al., 1983).

Catalase (hydrogenperoxide:hydrogenperoxide oxido-reductase; E.C. 1.11.1.6) is a tetrameric enzyme, which contains four tightly bound NADPH molecules (Kirkman and Gaetani, 1984). The NADPH is not converted during catalytic action, but maintains the enzyme in an active state. The enzyme is located in the mitochondrial matrix space (Nohl and Hegner, 1978), and in peroxisomes (Chance et al., 1979). Catalase has a relatively minor role in the catabolism of hydrogen peroxide in hepatocytes (Jones et al., 1981) and in antioxidant defence in cultured cells (Raes et al., 1987).

The glutathione system is the major route by which the cell can dispose of cytosolic electrophiles. Cells generally abound with GSH, although its concentration varies a lot among the different cell types analysed (Kosower and Kosower, 1978). GSH is reacted with peroxides by glutathione peroxi-dase (glutathione:H2O2 oxidoreductase: E.C. 1.11.1.9). The activity of glutathione peroxidase has been found to decrease during ageing in culture of human fibroblasts (Mbemba et al., 1985). Oxidised glutathione, resulting from the action of glutathione peroxidase, can be reduced by glutathione reductase (NADPH:GSSG oxidoreductase: E.C. 1.6.4.2). The activity of glutathione reductase decreases during in vitro ageing of cultured human fibroblasts (Mbemba et al., 1985).

But it remains to be seen whether the activites of glutathione reductase or glutathione peroxidase limit the rate of removal of peroxides or whether a possible shortage in the supply with NADPH is of greater importance, as has been suggested in studies with isolated rat hepatocytes (Ekloew et al., 1984).

1.4 TWO GENETIC SYNDROMES

With respect to free radical metabolism or to ageing two human mutants leading to overt disease are known: Werner's syndrome, or segmental premature ageing and the Spielmeyer-Vogt syndrome, or neuronal ceroid lipofuscinosis. Both syndromes are rare autosomal recessive diseases, and do not share any clinical characterics.

The classical signs of Werner's syndrome include: skin atrophy, early graying and loss of hair, telangiectasia, muscular atrophy and most prominently cataract (Werner, 1904). Although many patients experience hypogonadism, their reproductivity is rather good. This can partially be accounted for by the relatively late onset of signs (generally in the third or fourth decade). The patients show never any kind of mental retardation or other neuronal disturbancy, which is definetely at variance with ageing as experienced within the general population. This led Martin to consider Werner's as a segmental progeroid syndrome, rather than as a form of accelerated ageing (Martin, 1985b). The features of Werner's seem to be a caricature rather than a true picture of ageing. Normal ageing is considered as a condition with apparent multifactorial autosomal dominant inheritance (Sacher, 1978; Schneider, 1987). In this view, the single, recessive mutation causing Werner's syndrome can explain only a few aspects of senescence and is, therefore, to be excluded from normal ageing.

Due to the rather late onset of clinical signs, which implies a transmitance of the genotype to offspring before the disease can be recognised, an interest in prenatal diagnosis of Werner's syndrome is obvious. Prenatal diagnosis relies on the detection of some aberration in amniotic fluid fibroblast like cells. So, it is necessary to define Werner's syndrome in a cultured fibroblast system. Skin derived fibroblast like cells show a clearly protracted growth rate (Salk et al., 1981a; Salk et al., 1985, Nakao et al., 1978; Norwood et al., 1979). By fusion with cells from healthy donors, this characteristic of cells from Werner's syndrome can be reversed (Pendergrass et al., 1985); a finding which differentiates normal senescent cells from cells of Werner's. The decreased growth can be explained by a prolongation of the S phase (Hanaoka et al., 1983) and a decrease in average replicon size (Takeuchi et al., 1982). Along with a protracted growth rate cultured cells from Werner's show aberrant karyotypes (Salk, 1982)). These are stable within a clone, but on the level of a mass culture the karyotype is seemingly

instable. Therefore, Werner's cannot be classified among the instable karyotype syndromes, such as ataxia telangiectasia, Fanconi's anemia and Bloom's syndrome (Salk, 1982). Attempts to find the cause of the protracted growth rate and the chromosomal instability of Werner's syndrome within the field of free radical metabolism failed. Cells contained normal levels of CuZn and Mn superoxide dismutase, catalase and glutathione peroxidase (Marklund et al., 1981). Addition of SOD or catalase to the culture medium or lowering the oxygen concentration could not abolish chromosomal instability or the protracted growth rate (Salk et al., 1981b).

Spielmeyer-Vogt syndrome, or Batten-Mayou's disease, or Spielmeyer-Sjoegrens, or Santavuori syndrome involves an amaurotic idiocy, characterised by atrophy of the N. opticus, a progressive degeneration of the retina and an impairment of verbal intelligence. The disease, showing autosomal recessive inheritance, is generally classified as amaurotic idiocy of unknown aetiology, such to differentiate it from Tay-Sachs disease. The age of onset of the disease varies from two months to 18 years. This characteristic has led to considerable confusion and the variety in labelling of this syndrome. In the USA the disease is named Neuronal Ceroid Lipofuscinosis (NCL) after the presence of some autofluorescent material (ceroid or lipofuscin) in post-mortem specimens from the brain of these patients. From this pecularity one can imply an affected lipid metabolism to play a role in this disease. However, its mode of inheritance and its clinical signs indicate no genotypic overlap between Spielmeyer-Vogt syndrome and normal ageing. Cultured fibroblasts from patients with this syndrome have as yet not been studied, but some controversy exists as to a putatively impaired defence against oxygen free radicals (Gutteridge et al., 1982; Garg et al., 1982).

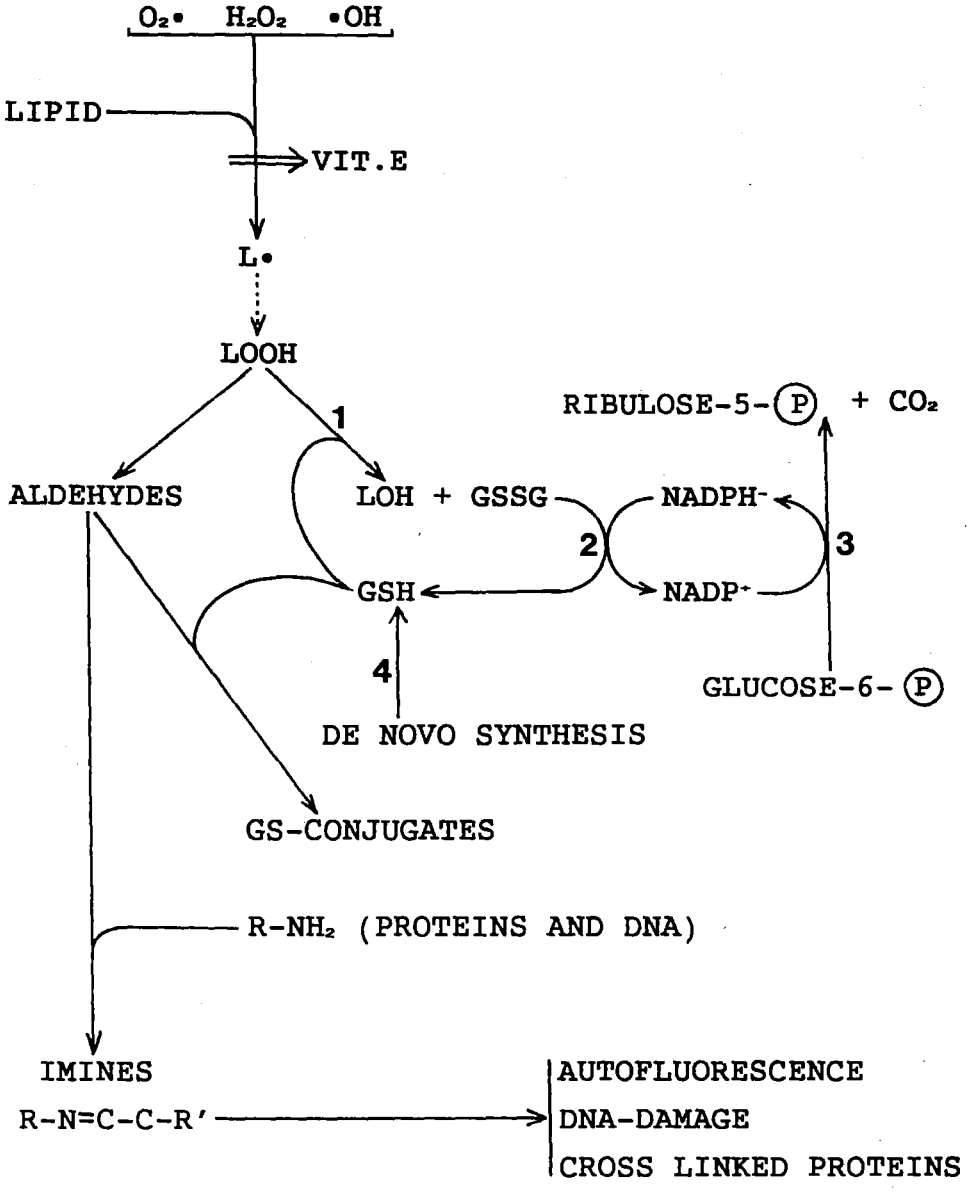
1.5 SCOPE OF THIS THESIS

From the pattern of inheritance of normal ageing, this process inevitably results from either abnormal aggregation of subunits of enzymes, abnormal feedback inhibition of enzymes, receptor mutations, membrane defects, or deposition of abnormal fibrillar proteins (Vogel and Motulsky, 1986). The process of ageing should, due to the principle of universality, occur at a site common to all cells : general metabolism, chromatin structure and function (eg. DNA replication and gene expression) or the physiology of the membrane. The cell type to be studied should be fully functional as to these three sites, and the theories potentially able to explain the ageing process should all encompass changes at any of these aspects of cellular function. The system to be used throughout this thesis is the cultured human skin fibroblast, which still expresses genes, replicates DNA, has a general metabolism and has functional membranes. This cell type alos undergoes a loss of proliferative potential both in vivo and in culture (Martin et al.,

1970). Furthermore, any kind of a phenotype expressed in fibroblast-like cells can theoretically be used for prenatal diagnosis of human genotypes (eg. Werner's syndrome and Spielmeyer-Vogt syndrome).

The following postulates can be derived now. First, free radicals should cause the accumulation of lipid peroxides or decay products, which should further yield to the accumulation of autofluorescence. Second, free radicals, or the reactive products resulting from them should impair cellular proliferation to the extent as to cause an irreversible growth arrest of initially dividing cells. Third, cross-linking of proteins by reactive breakdown products of lipid peroxides should occur. Fourth, the capacity of the systems implied in the detoxification of free radicals, or the reactive products thereof, should decrease in capacity during ageing of cultured cells.

SCHEME 2



Legend

1 = glutathione peroxidase
2 = glutathione reductase
3 = pentose phosphate shunt
4 = glutathione synthetase

Chapter 2

DISCUSSION OF THE APPENDIX PAPERS

In the appendix papers an answer to the following five questions was sought.

1. Do free radical reactions at all take place during ageing of cells in culture ?

2. Do free radical reactions lead to irreversible inhibition of cellular proliferation ?

3. Does the protein pattern of cells change during ageing in a way that can be accounted for by free radical reactions ?

4. Does ageing in culture affect the capacity of the glutathione redox system in detoxifying free radicals and the reactive products thereof ?

5. Do cells from the syndromes of Werner and of Spielmeyer-Vogt show impaired defences against oxygen free radicals ?

2.1 THE RELATIONSHIP BETWEEN AUTOFLUORESCENCE AND AGEING

From the introduction should be retained that the accumulation of autofluorescence might be the proof, that free radical reactions do at all take place during cellular ageing. If this turns out to be right, one is prompted to ask whether this autofluorescence is the cause or the effect of the loss of cellular proliferation. In appendix paper 1 this questions is investigated. An inverse relationship between the number of rapidly dividing cells and the autofluorescence of the whole culture was found. From this, one cannot decide as to cause and effect. However, growth inhibition, by maintaining cells at confluency, caused a rise in autofluorescence similar to that of ageing cultures. Hence, the accumulation of autofluorescence is an effect of growth inhibiton. The origin of this autofluorescence is indeed the peroxidation of lipids, as shown in experiments with vitamin E. This lipophilic free radical scavenger reacts with organic peroxides at diffusion controlled rates. If a certain process can be inhibited by culturing cells with this lipophilic free radical scavenger, this process necessarily involves the formation of a lipid radical or equivalent species. In the experiment in appendix paper 1, vitamin E indeed inhibits the formation of cellular autofluorescence; hence a lipid radical or related species is to be involved in this process. So, the final conclusion is, that autofluorescence results from lipid peroxidation occurring during culture of cells and that this autofluorescence accumulates as an epiphenomenon of a reduced rate of cellular proliferation.

2.2 EFFECTS OF FREE RADICALS UPON DNA REPLICATIVE CAPACITY

Now it has been shown, that free radical reactions, yielding to lipid peroxidation do at all take place in cultured cells, the precise role of these reactions during

cellular ageing is to be analysed. The characteristic to define the process of ageing in culture of fibroblast-like cells is their loss of proliferative capacity (Hayflick and Moorhead, 1961; Jacobs et al., 1970; Nichols et al., 1977; Hay and Streheler, 1967; Ponten, 1970; Lima et al., 1972; Raes and Remacle, 1983). Explanations for this phenomenon have been sought either assuming a programme to be the cause or assuming a randomly ocurring damage to be at the origin.

The proponents of the "ageing programme" (Martin et al., 1974) tried to substantiate their proposal with cell fusion experiments (Martin et al., 1975). This kind of experiments can reveal whether cells express different genetically determined programmes and which of these programmes is dominant over other programmes. An excellent review of the data is available (Smith and Lincoln, 1984). Their conclusion is that the finite lifespan of cultured cells is under strict genetic control, which can only partially be overriden by fusion of non-proliferating cells with "immortalised" cells. However, after a period of proliferation roughly equivalent to the total in vitro lifespan of normal cells these hybrids also cease proliferation. Hence, cessation of proliferation is dominant over continuous proliferation. A cytoplasmically accumulating inhibitor has been proposed (Smith and Lincoln, 1984) and further research has been directed towards the characterisation of this compound. The synthesis and the accumulation of this compound is thought be controlled by the nucleus, thus the senescent phenotype is an expression of a senescent genotype (Hayflick, 1984).

The free radical theory of ageing presumes a randomly occurring damage to cell function to be the origin of the ageing process. Early experiments (Cristofalo and Scharf, 1973) suggest that a fraction of cells ceases to divide. Results obtained with the BrUdR-Hoechst flow cytometric assay for cellular proliferation (Rabinovitch, 1983), clearly demonstrate that a reduction in the number of cells responding to serum stimulation is at least one of the cell kinetic alterations accounting for the senescent phenotype.

The cell strains used throughout this thesis all exhibit a reduction in 24 hours labelling indices during extended culture, which suggests that
the fraction of rapidly dividing cells decreases during in vitro ageing (appendix paper 1). To test the hypothesis, that this phenomenon is caused by free radical reactions, cells were incubated with either the lipophilic hydroperoxide, cumene hydroperoxide (Chp), or with the breakdown product of lipid peroxidation, 4-hydroxy-2,3-trans-nonenal (HNE). Both compounds inhibited DNA replication in a dose dependent way (appendix paper 2). The action of Chp could not be prevented by culturing cells with the lipophilic free radical scavenger vitamin E. Under the incubation circumstances used vitamin E was an effective antioxidant (Packer and Smith, 1977) and prevented fully the formation of cellular autofluorescence (appendix paper 1). Hence, Chp exerted its action upon

cellular DNA replication via a route, which is not dependent upon the secondary formation of lipid radicals. Besides, the effect of Chp and HNE could be reversed by the cells within one day of culture without Chp or HNE (appendix paper 2). This reversal was complete if cells were growth inhibited during exposure with Chp or HNE, but only partial if cells were continuously proliferating during the experiment. Furthermore, literature data (Packer and Smith, 1974; Balin et al., 1977) on vitamin E speak as well against a lipophilic free radical mechanism as the cause of the cessation of proliferation of cells in culture, whereas the effects of lowered oxygen tension upon the proliferative lifespan of cells in culture are subject to some dispute (Packer and Fuehr, 1977; Balin et al., 1977). An early paper (Packer and Smith, 1974) implying a beneficiary effect of vitamin E upon in vitro lifespan resulted from a mis-interpretation of results and has been retracted (Packer and Smith, 1977). So, neither lipophilic hydroperoxides nor a diffusable breakdown product of lipid peroxidation may cause irreversible inhibition of the 24-hours labelling index. However, BrdU-Hoechst flow cytometry revealed that both Chp and HNE disturb cell proliferation by inducing permanent arrests in certain compartments of the cell cycle (appendix paper 3). The persistence of this inhibitory effect is in keeping with the irreversible reduction in clone forming ability imparted upon lung fibroblasts exposed to HNE during one hour (Krokan et al., 1985). Upon ageing in culture fibroblasts leave continuous cycling by an arrest in the G1 compartment of the cell cycle (Yanishevsky et al., 1974; Grove and Cristofalo, 1977; Gorman and Cristofalo, 1986), a pattern reminiscent of cell differentiation. Both Chp and HNE, however, provoke an arrest in the G2 compartment of the cell cycle, a result commensurate with literature data on the effects of oxygen upon cell proliferation (Balin et al., 1977). This pattern of disturbed proliferation clearly contradicts the hypothesis that lipid peroxidation might cause the senescent phenotype.

As to the mechanism of the inhibition of DNA replication by Chp and HNE various possibilities exist. Firstly, as DNA replication is intimately linked with concomitant protein synthesis (Kim et al., 1968), an inhibition of protein synthesis by these compounds might cause the cells to run out of the necessary protein. Secondly, Chp and HNE might deplete cells of ATP, thus preventing cells to form the nucleotides to be incorporated into the DNA. Thirdly, both compounds might overwhelme the cellular defence mechanisms against compounds of this kind and thus might be able to destroy certain factors required for DNA replication. DNA polymerase alpha, one of the enzymes involved in DNA replication, is particularly sensitive towards thiol oxidising agents (Sheinin and Humbert, 1978). These three possible mechanisms have been analysed in appendix paper 2. Both compounds do indeed inhibit protein synthesis, albeit to a lesser extent than DNA replication. A full inhibition of protein synthesis gives only a partial inhibition of DNA replication (Sheinin and Humbert, 1978). Hence, the moderately reduced rate of

protein synthesis cannot possibly limit the level of DNA replication. Incubated with each of the compounds cells did not fall short of ATP, nor were their energy charges altered. This speaks against a shortage in nucleotides as a possible mechanism for the effect of Chp or HNE upon DNA replication. The third possibility seems as well unlikely, as GSH levels are maintained during culture with Chp or HNE. But, one cannot exclude that both compounds might be able to circumvent the GSH system and still damage the DNA replication system. Thus, the GSH system might not at all protect the enzymes and the chromatin needed for DNA repliction against these reactive compounds. This means that the glutathione system is intrinsically fallible if it comes to protect the DNA replication system of cells against free radical induced damages.

2.3 DO FREE RADICAL REACTIONS AFFECT THE CELLULAR PROTEIN PATTERN DURING IN VITRO AGEING ?

An alteration in cellular protein patterns is a prediction of any theory of programmed ageing like the differentiation theory of cellular ageing (Martin et al., 1974; 1975). This prediction lead to a considerable effort to pinpoint a presumed "senescence protein". Most of these endeavours with the system of cultured fibroblasts have disclosed the existence of a protein, which after some time has been challenged as a protein specific to the process of cellular ageing. The necessary test to be applied to any claim of an "senescence specific protein" is that this protein should not accumulate in cells if they are growth inhibited by any outer cause, such as confluency or serum starvation. Hitherto, only one report on a possible "senescence related protein" has remained unchallenged, which menas that this particular protein is not a quiescence related protein (Lincoln et al., 1984).

Another theory predicting the existence of at least one protein resulting from cellular ageing is the error catastrophe theory (Orgel, 1963). In this scheme the protein results not from differential gene expression but from the progressive accumulation of errors during ageing of cells in culture. The comparison of two dimensional protein patterns (showing distributions of isoelectric points and molecular weights of proteins) has been critisized as being insufficiently sensitive to detect possible error induced alterations in cellular protein patterns (VanKeuren et al., 1983). Another approach, analysing the fidelity of information transfer during translation of mRNA to protein, has lead to results disproving the error catastrophe theory (Harley et al., 1980; Mori et al., 1983)

Finally, the free radical theory of ageing predicts as well alterations in cellular protein patterns to occur during ageing of cells in culture. In this case these altered proteins are a consequence of free radical reactions (Wolff

et al., 1986). Free radical reactions can either produce a scission in the peptide backbone or a cross-linking of amino acid residues of proteins (Wolff et al., 1986). Scission of peptide backbones leads to the degradation of proteins and the resultant accumulation of protein fragments. However, changes in activity of proteases in cultured cells might act to the same effect. On the other hand an attack by free radicals can render a protein more to susceptible to degradation by proteases (Dean et al., 1986; Davies and Goldberg, 1987a; 1987b). This makes it hard to asses a free radical dependent degradation of proteins during in vitro ageing. Cross-linking of proteins through free radical attack is more open to analysis. The outcome of cross-linking of proteins is the formation of high molecular weight "garbage". Hitherto, free radical reactions, like linkage of two tyrosine residues or cross-linking of amino groups by aldehydes resulting from lipid peroxidation, are the only way to explain the existence of this "garbage".

Appendix paper 4 reports the results of a search for cross-linkage of proteins during cellular ageing. Use was made of discontinuous polyacrylamide gel electrophoresis (PAGE) system containing a 3 % polyacrylamide (PAA) stacking gel and a 5 to 10 % PAA gradient separation gel. This system can separate rather large protein molecules (from 60,000 to 400,000 molecular weight) and can show the accumulation of cross-linked proteins larger than 400,000 molecular weight on the border between stacking and separation gel. Neither sorted autofluorescent cells, nor cells from cultures with senescent cells, or cells from cultures kept in a confluent state showed an elevated level of material at this border region. However, all these three conditions induced the advent of a protein, which was absent from continuously proliferating cells. This protein showed up as a discrete band with an apparent molecular weight of 240,000 and a heterogenous isoelectric point (IEP) of about 5.3. This IEP resulted partially from neuraminic acid residues on the surface of the protein, which could be removed by neuraminidase treatment, leading to a homogenous IEP of 5.85. This protein, apparently being a glycoprotein, did not contain any terminal mannose residues, as it did not bind to Concanavalin A Sepharose. The protein could be recovered in the microsomal pellet, which indicates that it accumulated inside the endoplasic reticulum or in the Golgi system. Its appearance as a sharp band makes it unlikely that the protein results from protein cross-linking. As culturing cells with the lipophilic free radical scavenger vitamin E did not affect the accumulation of this protein, nor did culture during a week with either Chp or HNE (results not shown), it can be ruled out that the accumulation of this protein results from lipid peroxidation and subsequent protein cross-linking. Another hypothesis, that this protein results from differential gene expression, is disproven as the protein is synthesized in cells from cultures with a high PDL (population doubling level) and in cells with a low PDL kept in a growth inhibited state. These data suggests that the accumulation of this

protein is the result of a decreased rate of processing or breakdown.

2.4 THE PROTECTIVE CAPACITY AGAINST FREE RADICAL REACTIONS OF THE GLUTATHIONE SYSTEM

From cell microinjection studies it is clear that the glutathione system is the major cytosolic system to detoxify oxygen derived radicals and organic peroxides (Raes et al., 1987). Letting alone the importance of this system, it also has the advantage that its activity can be analysed by pursuing the fate of the reduced and oxidised forms of glutathione. Appendix paper 5 through 7 report the results of this endeavour.

The first aspect to be analysed is the evolution of reduced and oxidised glutathione during ageing in culture. The data in appendix paper 5 clearly indicate that the glutathione contents of the seven cell strains analysed increase slightly during cellular ageing of the cells. This result is supported by the similar findings in WI-38 cells (Mbemba et al., 1985). However, a decrease with cellular age of the maximum activity of both glutathione reductase and glutathione peroxidase (Mbemba et al., 1985), the two enzymes which make up the glutathione redox cycle, might imply that the turnover of glutathione through the redox cycle is altered. In addition to this, an alteration in the rate of de novo synthesis of glutathione during cellular ageing can be envisaged. So, the dynamics of both systems are to be investigated.

Appendix paper 6 reports the results obtained with an assay for the capacity of de novo synthesis of GSH. The assay consists of a full depletion of reduced glutathione with diamide from the cells and a subsequent recovery. During the recovery period samples are taken and assayed for their content of reduced glutathione by flow cytometric analysis of monobromobimane stained cells. Doing a similar study with erythrocytes Sass found a decrease in the rate of GSH recovery (Sass, 1966). However, the fibroblats strains used in this study had the same rate of GSH recovery after in vitro ageing as before. As the oxidised glutathione formed during the depletion period is fully extruded from the cells the assay measures specifically the rate of de novo synthesis of GSH. This conclusion is further butressed by the findings with a cell strain with a diminished rate of de novo synthesis of glutathione. These cell show a strongly protracted recovery of reduced glutathione upon depletion with diamide. Hence, during cellular ageing of cultured human skin fibroblasts the rate of de novo synthesis of GSH is not altered.

The third aspect of the glutathione metabolism to be analysed is the turnover of glutathione through the glutathione redox cycle when cells are exposed to lipophilic,

organic hydroperoxides or to a breakdown product of lipid peroxidation. This kind of analysis gives information as to the capacity of the glutathione system to respond to elevated levels of hydroperoxides generated during metabolism and as to the levels of the products thereof. The two model compounds to probe the glutathione system are cumene hydroperoxide (Chp) and 4-hydroxy-2,3-trans-nonenal (HNE). The reaction of CHP with GSH yields to oxidised glutathione, which can either be reduced to GSH or can be extruded from the cell. HNE reacts with GSH to form a stable adduct. So, Chp is a probe for the capacity of the glutathione redox cycle, and HNE gives information as to the cellular capacity to replenish GSH by de novo synthesis.

As is evident from appendix paper 7, both compounds react avidly with GSH. But, after a week of culture in the presence of of Chp or HNE at concentrations up to 40 micromolar cells had compensated the loss of GSH caused by the compounds. Cells cultured with Chp responded with an increase in the generation of NADPH via the pentose phosphate shunt. By the substrate flux through the pentose phosphate shunt cells will obviate the Chp applied in approximately 48.3 hours. So, Chp was added every 72 hours, and 24 hours before assay, and finally, to the assay medium as well. Thus, cells are almost permanently exposed to Chp. The rise in flux through the pentose phosphate shunt is much more pronounced in senescent relative to young cells. It stands to reason that a decrease in activity of both glutathione peroxidase and glutathione reductase (Mbemba et al., 1985) during in vitro ageing cannot yield to an increase in turnover of GSH. It is also improbable that a putatively higher level of lipid peroxidation in senescent relative to young cells will be metabolised at a higher rate by enzymes with a lowered activity. So, the amount of GSSG formed in senescent cells should be equal to or less than in young cells. It should be noted, that the turnover of glutathione through the redox cycle as deduced from the data on the pentose phosphate shunt is still below the maximum capacity of this system as derived from the data by Mbemba et al. (Mbemba et al., 1985)(glutathione reductase and glutathione peroxidase operating at 43 and 74 % of their maximal capacity respectively). Hence, the difference in rise in the flux through the pentose phosphate shunt can only be explained as the result of an increase in the requirement for NADPH to reduce the GSSG formed.

Apparently, the GSH redox cycle functions in a less economic way after cellular ageing. So, although the glutathione redox cycle remains fully capable to maintain cellular GSH levels during exposure to lipophilic, organic hydroperoxides, this occurs only at the expense of more NADPH when cells age in culture. These findings are the first example of a partial uncoupling of metabolic pathways during cellular ageing. In this way, metabolic control mechanisms will be jeopardized. During in vitro ageing the turnover of NADPH decreases (appendix paper 7), whereas culture with Chp induces an increase in this turnover. Hence, incubation of

cells with organic hydroperoxides does not lead to metabolic alteration reflecting cellular ageing. Therefore, the change in turnover of NADPH during cellular ageing cannot result from an increased turnover of organic hydroperoxides, nor might it merely result from damages induced by oxygen free radicals.

As HNE forms a stable adduct with glutathione, this compound can be used to test the capacity of cells to synthesize glutathione de novo. Fibroblasts of all cellular ages from healthy persons cultured with HNE are quite able to maintain their GSH level (appendix paper 6). HNE did not cause a rise in the flux of substrate through the pentose phosphate shunt, which is consistent with a role of this metabolic system to generate NADPH for the reduction of oxidised glutathione. As reduced glutathione levels rise during culture with HNE (80 to 170 % above initial levels), de novo synthesis of glutathione is the most likely route of response towards HNE. Cells with severely diminished activity of glutathione synthetase (residual activity 4.4 % of control cells) were not able to survive in the presence of moderate concentrations of HNE. These cells, however, did survive at all concentrations of Chp used. Hence, only cells with a severely diminished activity of glutathione synthetase might be unable to maintain GSH levels in the presence of HNE. So, in cultured fibroblasts de novo synthesis of GSH is of relatively little importance to the maintenance of cellular GSH levels during detoxification of oxygen free radicals and organic peroxides. On may conclude that during cellular ageing the capacity of de novo synthesis remains unaffected and that de novo syntheis of GSH is only of importance in the defence against oxygen free radicals once the GSH content of cells in fully depleted.

2.5 CELLS FROM TWO GENETIC SYNDROMES

Cells from Werner's syndrome and from the Spielmeyer-Vogt syndrome have been routinely tested in all assays to which other cells have been subjected. Neither of the two human mutant cell types showed a different rate of accumulation of autofluorescence, but they both showed a particularly different appearance of their residual bodies. It remains to be seen whether this appearance is an artifact of the cell culture system or it might constitute something typical for these syndromes (appendix paper 1).

Cells from Werner's syndrome failed to accumulate a high molecular weight glycoprotein typical for cells with impaired proliferation. It remains to be seen whether the absence of this protein relates to the protracted growth of these cells. Cells from Spielmeyer-Vogt syndrome exhibit a protein pattern similar to that of control fibroblasts (appendix paper 4).

Neither contents of reduced or oxidised glutathione nor the capacity of de novo synthesis of reduced glutathione is

altered in cells from these syndromes in comparison to cells from healthy donors (appendix papers 5 and 6).

In conclusion, in fibroblasts from both syndromes no altered glutathione matabolism in relation to oxygen free radicals has been found. Hence, it is unlikely, that cells from these two syndromes might suffer more oxygen free radical induced damages than do diploid human skin fibroblasts, and by consequence that these damages are the primary cause of the signs exhibited by these patients. The findings concerning the protein related to growth inhibition might merit further study.

2.6 CONCLUSIONS

The work reported in the appendix papers and analysed in this chapter allows the following conclusions to be drawn:

1. Autofluorescence results from lipid peroxidation and accumulates as a result of impaired proliferation. It is not related to ageing in culture as such and cannnot serve as an index of cellular ageing.

2. Neither an organic, lipophilic hydroperoxide nor a breakdown product of lipid peroxidation cause an irreversible decrease in 24-hours labelling indices. The pattern of disturbed proliferation induced by the two model compounds lead to an arrest of cells in the G2 phase of the cell cycle, whereas during in culture cells uniquely exit from cycling via the G1 phase. So, lipid peroxidation cannot be the cause of the irreversible loss of proliferative capacity typical for ageing of cells in culture.

3. During in vitro ageing no accumulation of cross-linked proteins is detectable. The protein found to accumulate in growth inhibited cells is presumably a result of growth inhibition rather than the cause of this phenomenon.

4. The glutathione system remains fully capable to cope with challenges resulting from a lipophilic peroxide or a breakdown product of lipid peroxidation, although this occurs at the expense of more NADPH after cellular ageing of fibroblasts. The glutathione system fails to protect the DNA replication system against both intermediates of lipid peroxidation.

5. Fibroblasts from Werner's syndrome are similar to control fibroblasts with respect to their autofluorescence and their glutathione system. They fail to accumulate a protein specific to growth inhibition and they exhibit an altered appearance of their residual bodies. Cells from the Spielmeyer-Vogt syndrome are similar to those from Werner's syndrome, except that they do accumulate the protein related to growth inhibition.

Chapter 3

SUMMARY/SAMENVATTING

3 SUMMARY

Ageing is a common and inevitable physical destiny of all multicellular entities. From old age on, many ideas were proposed to explain the origin and the course of this hideous process. A century ago, Weismann, surrounded by discoveries in the new science of cellular biology, purported that ageing might be due to the loss of cellular proliferation. It took until 1961 before this idea was finally substantiated by experimental evidence. The advent of the findings of Hayflick and Moorhead, that cells in culture had a finite lifespan, initiated the science of cellular gerontology. This thesis is an investigation into the possible causes of the "Hayflick" phenomenon.

In chapter one a definiton of ageing is analysed. Thus, ageing is thought to be a process based on intrinsic, general, progressive and deleterious changes in the physique. In view of this definition the major theories trying to explain ageing are discussed. The differentiation theory of ageing, proposed by Martin, has met with an overwhelming experimental support, whereas the error catastrophe hypothesis of Orgel can be ruled out in view of present data. The dysdifferentiation hypothesis of Cutler suffers from much methodological difficulty and therefore progresses only slowly. The free radical theory of Harman prompted a lot of controversy. This theory is analysed by deriving some postulates from it, which are scrutinized in the second chapter of this thesis.

This examination went basically along three lines. The relationship between lipid peroxidation and cellular ageing was attacked firstly. Secondly, the effects upon DNA replication of two products of free radical reactions (lipophilic, organic hydroperoxides and diffusable aldehydes resulting from lipid peroxidation) was investigated. The advent of a protein related to both ageing and contact inhibition of growth is discussed. This protein was not detected in cells from a patient with Werner's syndrome. The fourth type of analysis was into the capacity of detoxifying free radical induced damage by the glutathione system.

The major conclusions are that the detoxifying capacity of the glutathione system is maintained during cellular ageing, albeit at the expense of more reductive equivalent. Secondly, the irreversible reduction in proliferative capacity of cells during in vitro ageing does not appear to be caused by lipophilic, organic hydroperoxides nor by difusable breakdown products of lipid peroxidation. The glutathione system intrinsically failed to protect the DNA replication system against sequelae resulting from free radical reactions, but the direct effects of free radicals upon DNA and chromatin should be subjected to further study. At present, the free radical theory of ageing, not having stood a single test to which it was subjected, and being contradicted by the vast majority of literature data, should be discarded entirely.

Veroudering is het algemene en onvermijdelijke, physieke lot van alle veelcellige levensvormen. Door de eeuwen heen werden verschillende ideeen geopperd om de oorsprong en het verloop van dit sluipend proces te verklaren. Een eeuw geleden, opperde Weissmann, omgeven door de ontdekkingen van de nieuwe wetenschap van de cellulaire biologie, dat veroudering zou kunnen voortkomen uit een verlies van cellulair groeivermogen. Het duurde tot 1961 vooraleer deze gedachte werd ondersteund door experimentele uitkomsten. Het verschijnen van de uitkomsten van Hayflick en Moorhead, dat cellen in kweek een beperkte levensduur hebben, deed de wetenschap van de cellulaire verouderingsleer ontstaan.

In hoofdstuk 1 van dit proefschrift wordt een omschrijving van veroudering besproken. Volgens deze is veroudering gedacht een proces te zijn dat berust op innerlijke, algemene, toenemende en schadelijke veranderingen in het lichaam. In het licht van deze omschrijving worden de belangrijkste theorieen van veroudering besproken. De differentiatie-theorie van veroudering, voorgesteld door Martin, heeft een enorme experimentele ondersteuning ontmoet, terwijl de fouten-catastrophe hypothese van Orgel uitgesloten moet worden in het licht van de beschikbare uitkomsten. De dysdifferentiatie hypothese van Cutler lijdt onder ernstige methodologishe moeilijkheden en boekt daarom slechts langzame vooruitgang. Rondom de vrije radicaal theorie van Harman ontstond veel tegenstelling. Deze theorie is ontrafeld door er enige postulaten van af te geleiden, dewelke daarna in het tweede hoofdtsuk van dit proefschrift nader onderzocht worden.

Dit onderzoek volgde in het algemeen drie lijnen. De verhouding van vet-peroxidering tot cellulaire veroudering was het eerste punt dat aangevat werd. Ten tweede werd de invloed op de DNA verdubbeling van twee producten van vrije radicaal reacties (vetminnnende, organische hydroperoxiden en diffunderende aldehyden voortkomend uit vet-peroxidering) onderzocht. Het optreden van een eiwit voortkomend uit zowel veroudering als groeiremming door omderling celcontact werd besproken. Dit eiwit werd niet waargenomen in cellen van een lijdende aan de ziekte van Werner. Het vierde soort van onderzoek was naar het vermogen tot ontgifting van door vrije radicaal reacties opgeroepen schade door het glutathion stelsel.

De voornaamste besluiten zijn, dat het ontgiftend vermogen van het glutathion stelsel behouden blijft gedurende veroudering van cellen in kweek, hoewel tegen een verhoogd verbruik aan reductief vermogen. Ten tweede blijkt de afname van groeivermogen van cellen gedurende veroudering in kweek niet te zijn veroorzaakt door vet-minnende, organische peroxiden noch diffundeerbare afbraakproducten van vet-peroxdering. Het glutathion stelsel was innerlijk niet in staat het DNA verdubbelingstelsel te beschermen tegen de gevolgen van vrije radicaal reacties. Onbemiddelde invloed van vrije radicaal reacties op DNA en het chromatine zou nog

verder onderzocht moeten worden. De vrije radicaal theorie van veroudering, die geen enkele van de proeven, waaraan zij was onderworpen, heeft doorstaan en die tegengesproken wordt door de overgrote meerderheid van de uitkomsten gemeld in de literatuur dient geheel te worden verworpen.

Chapter 4

REFERENCES

J.E. Aubin (1979) Autofluorescence of Viable Cultured Mammalian Cells, J.Histochem.Cytochem. 27, 36-43.

A.K. Balin, D.G. Goodman, H. Rasmussen and V.J.Cristofalo (1977) The effect of oxygen tension and vitamin E on the lifespan of human diploid WI-38 cells, J.Cell.Biol. 74, 58-67.

A.K. Balin (1982) Testing the free radical theory of aging, In: Testing the theories of aging, Eds. R.C. Adelman and G.S. Roth, pp. 137-182, CRC Press, Boca Raton.

J.V. Bannister, W.H. Bannister and G. Rotilio (1987) Aspects of the structure, function, and applications of superoxide dismutase, CRC Crit.Rev.Biochem. 22, 111-180.

A. Benedetti, H. Esterbauer, M. Ferrali, R. Fulceri and M. Comporti (1982) Evidence for aldehydes bound to liver microsomal protein following CCl4 or BrCCl3 poisoning, Biochim.Biophys.Acta 710, 345-356.

R.C. Benson, R.A. Meyer, M.E. Zaruba and G.M. McKhann (1979) J.Histochem.Cytochem. 27, 44-48.

J. Bjorksten (1974) Crosslinkage and the Aging Process, in: Theoretical Aspects of Aging, pp.43-59, Eds. M. Rockstein, M.L.Sussman and J. Cheskey, Academic Press, New York.

E. Cadenas, A. Mueller, R. Brigelius, H. Esterbauer, and H. Sies (1983) Effects of 4-Hydroxynonenal on Isolated Hepatocytes. Studies on Chemiluminescence Response, Alkane Production and Glutathione Status, Biochem.J. 214, 479-487.

E. Cadenas, A. Mueller, R. Brigelius, H. Esterbauer and H. Sies (1983) Effects of 4-hydroxynonenal on Isolated Hepatocytes, Studies on Chemiluminescence Response, Alkane Evolution and Glutathione Status, Biochem.J. 214, 479-487.

B. Chance, H. Sies and A. Boveris (1979) Hydroperoxide Metabolism in Mammalian Organs, Physiol.Rev. 59, 527-605.

K.S. Chio and A.L. Tappel (1969) Synthesis and Characterisation of the Fluorescent Products Derived from Malonaldehyde and Amino Acids, Biochem. 8, 2821-2832.

J.H. Clark and G.P. Beardsley (1987) Functional effects of cis-thymine glycol lesions on DNA synthesis in vitro, Biochemistry 26, 5398-5403.

V.J. Cristofalo and B.B. Scharf (1973) Cellular Senescence and DNA synthesis, Thymidine Incorporation as a Measure of Population Age in Human Diploid Cells, Exp.Cell Res. 76, 419-427.

R.G. Cutler (1984a) Evolutionary Biology of Aging and Longevity in Mammalian Species, in: Aging and Cell Function, Ed. J.E. Johnson Jr., Plenum Press, New York and London.

R.G. Cutler (1984b) Dysdifferentiation hypothesis of aging: A review, In: Aging vol. 29, Molecular Biology of Aging, gene stability and gene expression, pp.307-340, Eds. R.M. Sohal, L.S. Birnbaum and R.G. Cutler, Raven Press, New York.

R.G. Cutler (1985a) Antioxidants and longevity of mammalian species, In: Molecular biology of aging, Eds. A.D. Woodhead, A.D. Blackett and A. Hollander, pp. 15-73, Raven Press, New York.

R.G. Cutler (1985b) Peroxide-producing potential of tissues: Inverse correlation with longevity of mammalian species, Proc.Natl.Acad.Sci.USA 82, 4798-4802.

K.J.A. Davies and A.L. Goldberg (1987a) Oxygen radicals stimulate intracellelular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes, J.Biol.Chem. 262, 8220-8226.

K.J.A. Davies and A.L. Goldberg (1987b) Proteins damaged by oxygen radicals are rapidly degraded in extracts of red blood cells, J.Biol.Chem. 262, 8227-8234.

D.W. Deamer and J. Gonzales (1974) Autofluorescent Structures in Cultured WI-38 Cells, Arch.Biochem.Biophys. 165, 421-426.

R.T Dean (1987) A mechanism for accelerated degradation of intracellular proteins after limited damage by free radicals, FEBS Letters 220, 278-282.

R.T. Dean, S.M. Thomas and A. Garner (1986) Free-radical-mediated fragmentation of monoamine oxidase in the mitochondrial membrane, Biochem.J. 240, 489-494.

R. DeLong and L.Poplin (1977) On the etiology of aging, J.Theor.Biol. 67, 111-120.

L. Ekloew, P. Moldeus and S. Orrenius (1984) Oxidation of glutathione during hydroperoxide metabolism, A study using isolated hepatocytes and the glutathione reductase inhibitor 1,3-bis-(2-chloroethyl)-1-nitrosourea, Eur.J.Biochem. 138, 459-463.

G.E. Eldred, G.V. Miller, W.S. Stark and L. Feeny-Burns (1982) Lipofuscin: Resolution of discrepant fluorescence data, Science 216, 757-759.

H. Esterbauer, E. Koller, R.G. Slee and J.F. Koster (1986) Possible involvement of the lipid-peroxidation product 4-hydroxynonenal in the formation of fluorescent chromolipids, Biochem.J. 239, 405-409.
A.C.M. Filho and R. Meneghini (1984) In vivo formation of

single-strand breaks in DNA by hydrogen peroxide is mediated by the Haber-Weiss reaction, Biochim.Biophys.Acta 781, 56-63.

B.A Freeman and J.D. Crapo (1982) Biology of disease. Free radicals and tissue injury, Lab.Invest. 47, 412-426.

K. Frenkel and K. Chzran (1987) Hydrogen peroxide formation and base modification by tumor promoter-activated polymorphnuclear leukocytes, Carcinogenesis 8, 455-460.

I. Fridovich (1975) Superoxide Dismutases, Annu.Rev.Biochem. 44, 147-159.

I. Fridovich (1983) Superoxide Radical: An Endogenous Toxicant, Annu.Rev.Pharmacol.Toxicol. 23, 239-257.

H.S. Garg, Y.C. Awasthi, W.A. Neff, N.H. Ansari and S.K. Srivastava (1982) Studies in neuronal ceroid lipofuscinosis: Enzymes of liver and brain tissues involved in the defence against oxidative damage, J.Neurosc.Res. 7, 305-311.

G.A. Glass and D. Gershon (1981) Enzymatic changes in rat erythrocytes with increasing cell donor age: loss of superoxide dismutase activity associated with increases in catalytically defective forms, Biochem.Biophys.Res.Comm. 103, 1245.

G.A. Glass, L. Lavie, A. Dovrat, S. Shpund and D. Gershon (1983) Further studies on SOD function and properties in different tissues of animals of different ages, In: Oxy-radicals and their scavengers sytems, cellular and medical aspects, Vol. 2, Eds. R.A. Greenwald and G. Cohen, Elsevier, New York, 1.

S.D. Gorman and V.J. Cristofalo (1986) Analysis of the G1 arrest position of senescent WI-38 cells by quinacrine dihydrochloride nuclear fluorescence. Evidence for a late G1 arrest, Exp.Cell Res. 167, 87-94.

V.K. Goyal (1982) Lipofuscin Pigment Accumulation in Human Brain during Aging, Exp.Gerontol. 17, 481-487.

G.L. Grove and V.J. Cristofalo (1977) Characterization of the cell cycle of cultured human diploid cells: effects of aging and hydrocortisone, J.Cell.Physiol. 90, 415-422.

J.M.C. Gutteridge, D.A. Rowley, B. Halliwell and T. Westermarck (1982) Increased non-protein-bound iron and decreased protection against superoxide-radical damage in cerebrospinal fluid from patients with neuronal ceroid lipofuscinoses, Lancet II, 459-460.

F. Hanaoka, F. Takeuchi, T. Matsumura, M. Goto, T. Miyamoto and Yamada (1983) Decrease in Average Size of Replicons in a Werner Syndrome Cell Line by Simian Virus 40 Infection, Exp.Cell Res. 144, 463-467.

C.B. Harley, J.W. Pollard, J.W. Chamberlain, C.P. Stanners and S. Goldstein (1980) Protein Syntetic Errors do not Increase during Aging of Cultured Human Fibroblasts, Proc.Natl.Acad.Sci.USA 77, 1885-1889.

D.Harman (1956) Aging: A Theory Based on Free Radical Radiation Chemistry, J.Geront. 11, 298-300.

D. Harman (1982) The Free Radical Theory of Aging, in: Free Radicals in Biology, volume V, pp. 255-275, Ed. W.A. Pryor, Academic Press, New York.

H.M. Hassan and I. Fridovich (1977) Regulation of the synthesis of superoxide dismutase in Escherichia coli. Induction by methyl viologen, J.Biol.Chem. 252, 7667-7672.

S. Hauptflorenz, H. Esterbauer, W. Moll, R. Puempel, E. Schauenstein and B. Puschendorf (1985) Effects of the Lipid-peroxidation Product 4-Hydroxynonenal and Related Aldehydes on Proliferation and Viability of Cultured Ehrlich Ascites Tumor Cells, Biochem.Pharm. 34, 3803-3809.

R.J. Hay and B.L. Strehler (1967) The Limited Growth Span of Cell Strains Isolated from Chick Embryo, Exp.Gerontol. 2, 123-135.

L. Hayflick and P.S. Moorhead (1961) The Serial Cultivation of Human Diploid Cell Strains, Exp.Cell Res. 25, 585-621.

L. Hayflick (1984) Intracellular Determinants of Cell Ageing, Mech.Ageing Dev. 28, 177-185.

M.J. Heukels-Dully and M.F. Niermeyer (1976) Variation in Lysosomal Enzyme Activity during Growth in Culture of Human Fibroblasts and Amniotic Fluid Cells, Exp.Cell Res. 97, 304-312.

R. Holliday and G.M. Tarrant (1972) Altered Enzymes in Ageing Human Fibroblasts, Nature 238, 26-30.

P.J. Hornsby (1984) Regulation of Adrenocortical Cell Proliferation in Culture, Endocine Res. 10, 259-281.

P.J. Hornsby, K.A. Aldern and S.E. Harris (1984) Adrenocortical Cultures as Model Systems for Investigating Cellular Aging, In: Free Radicals in Molecular Biology, Aging and Disease, pp.203-222, Ed. D. Armstrong et al. Raven Press, New York.

P.J. Hornsby, K.A Aldern and S.E. Harris (1986) Clonal variation in response to adrenocorticotropin in cultured bovine adrenocortocal cells: relationship to senescence, J.Cell.Physiol. 129, 395-402.

P.J. Hornsby and S.E. Harris (1987) Oxidative damage to DNA and replicative lifespan in cultured adrenocortical cells, Exp.Cell Res. 168, 203-217.

A.W. Hsie, L. Recio, D.S.Katz, C.Q. Lee, M. Wagner and R.L Schenley (1986) Evidence for reactive oxygen species iducing mutations in mammalian cells, Proc.Natl.Acad.Sci.USA 83, 6916-9620.

J.P. Jacobs, C.M. Jones and J.P. Baille (1970) Characterisation of a Human Diploid Cell Designated MRC-5, Nature 227, 168-170.

D.P. Jones, L. Eklow, H. Thor and S. Orrenius (1981) Metabolism of Hydrogen Peroxide in Isolated Hepatocytes: Relative Contributions of Catalase and Glutathione Peroxidase in Decomposition of Endogenously Generated H2O2, Arch.Biochem.Biophys. 210, 505-516.

J.F. Jongkind, A. Verkerk, W.J. Visser and J.M. van Dongen (1982) Isolation of Autofluorescent "Aged" Human Fibroblasts by Flow Sorting. Morphology, Enzyme Activity and Proliferative Capacity, Exp.Cell Res. 138, 409-417.

H. Kasai, P.F. Crain, Y. Kuchino, S. Nishimura, A. Ootsuyama and H. Tanooka (1986) Formation of 8-hydroxy-uridine moiety in cellular DNA by agents producing oxygen radicals and evidence for its repair, Carcinogenesis 7, 1849-1851.

M.L Katz, W.G. Robison Jr., R.K. Hermann, A.B. Groome and J.G. Bieri (1984) Lipofuscin Accumulation Resulting from Senescence and Vitamin E Deficiency: Spectral Properties and Tissue Distribution, Mech.Ageing Dev. 25, 149-159.

J.H. Kim, A.S. Gelbard and A.G. Perez (1968) Inhibition of DNA Synthesis by Actinomycin D and Cycloheximide in Synchronized HeLa Cells, Exp.Cell Res. 53, 478-487.

H.N. Kirkman and G.F. Gaetani (1984) Catalase: A Tetrameric Enzyme with Four Tightly Bound Molecules of NADPH, Proc.Natl.Acad.Sci.USA 81, 4343-4347.

T.B.L. Kirkwood and T. Cremer (1982) Cytogerontology Since 1881: A Reappraisal of August Weissmann and a Review of Modern Progress, Hum.Genet. 60, 101-121.

N.S. Kosower and E.M. Kosower (1978) The glutathione status of cells, Int.Rev.Cytol. 54, 109-160.

J.F. Koster and R.G. Slee (1980) Biochim.Biophys.Acta 620, 489-499.

J.F. Koster, R.G. Slee and T.J.C. Van Berkel (1982) Biochim.Biophys.Acta 710, 230-235.

Y. Kuchino, F. Mori, H. Kasai, H. Inoue, S. Iwai, K.Miura, E. Ohtsuka and S. Nishimura (1987) Misreading of DNA templates containing 8-hydroxyguanosine at the modified and at adjacent residues, Nature 327, 77-79.

C.G. Kurland (1987) The error catstrophe: a molecular fata morgana, BioEssays 6, 33-35.

L. Lima, E. Malaise and A. Macieira-Coelho (1972) Aging in Vitro, Effect of Low Dose Rate Irradiation on the Division Potential of Chick Embryonic Fibroblasts, Exp.Cell Res. 73, 345-350.

W.S. Lin, F. Wong and R. Anderson (1987) Role of superoxide in radiation-killing of Escherichia coli and in thyminne release from thymidine, Biochim.Biophys.Res.Comm. 147, 778-786.

D.W Lincoln II, K.I Braunschweiger, W.R.Braunschweiger and J.R. Smith (1984) Two Dimensional Polypeptide Profile of Terminally non-Dividing Human Diploid Cells, Exp.Cell Res. 154, 134-146.

C.K. Lumpkin, Jr., J.K. McClung, O.M. Pereira-Smith and J.R. Smith (1986) Existence of high abundance antiproliferative mRNA's in senescent human diploid fibroblasts, Science232, 393-395.

S. Marklund, I. Nordensson and O. Baeck (1981) Normal CuZn Superoxide Dismutase, Mn Superoxide Dismutase, Catalase and Glutathione Peroxidase in Werner's Syndrome, J.Gerontol. 36, 405-409.

H.R. Massie, H.V. Samis and M.B. Baird (1972) The kinetics of degradation of DNA and RNA by hydrogen peroxide, Biochim.Biophys.Acta 272, 539-548.

F. Mbemba, A. Houbion, M. Raes and J. Remacle (1985) Subcellular Localization and Modification with Ageing of Glutathione, Glutathione Peroxidase and Glutathione Reductase Activities in Human Fibroblasts, Biochim.Biophys.Acta 838, 211-220.

G.M. Martin, C.A. Sprague, T.H. Norwood and W.R. Pendergrass (1974) Clonal Selection, Attenuation and Differentiation in an in vitro model of hyperplasia, Am.J.Pathol. 74, 137-150.

G.M. Martin, C.A. Sprague, T.H. Norwood, W.R. Pendergrass, P. Bornstein, H.Hoehn and W.P. Arend (1975) Do hyperplastoid Cell Lines "Differentiate to Death"? Adv.Exp.Med.Biol. 53, 67-90.

G.M. Martin (1985a) Overview of the Pathology of Aging, In: Interrelationship among Aging, Cancer and Differentiation, pp. 23-34, Eds. B. Pullman, P.O.P. Ts'o and E.L. Schneider, D. Reidel Publishing Company, Dordrecht.

G.M. Martin (1985b) Genetics and Aging: Werner's Syndrome as a Segmental Progeroid Syndrome in: Werner's Syndrome and Human Aging, Eds. D. Salk, Y. Fujiwara and G.M. Martin, Plenum Press, New York and London.

G.M. Martin, C.A. Sprague and C.J. Epstein (1970) Replicative Lifespan of Cultivated Human Cells. Effects of Donor's Age, Tissue and Genotype, Lab.Invest. 23, 86-92.

P.B. McCay (1985) Vitamin E: Interactions With Free Radicals and Ascorbate, Annu.Rev.Nutr. 5, 323-340.

P.B. Medawar (1951) The Uniqueness of the Individual, Methuen and Co., London.

R.J. Melhorn and G. Cole (1985) The free radical theory of aging: A critical review, Adv.Free Radical Biol.Med. 1, 165-223.

A. Montfoort, K. Bezstarosti, M.M.J. Groh and J.F. Koster (1987) The influence of the chain length of aldehydes on the fluorescence of chromolipids, FEBS Letters 226, 101-104.

N. Mori, K. Hiruta, Y. Funatsu and S. Goto, (1983) Codon Recognition Fidelity of Ribosomes at the First and Second Positions does not Decrease during Ageing, Mech.Ageing Dev. 22, 1-10.

Y. Nakao, M. Kishihara, H. Yoshimi, Y. Inoue, K. Tanaka, N. Sakamoto, S. Matsukura, H. Imura, M. Ichihashi and Y. Fujiwara (1978) Werner's Syndrome, In Vivo and In Vitro Characteristics as a Model of Aging, Am.J.Med. 65, 919-932.

W.W. Nichols, D.G. Murphy, V.J. Cristofalo, L.H. Toji, A.E. Greene and S.A. Dwight (1977) Characterisation of a new Human Diploid Cell Strain, IMR-90, Science 196, 60-63.

H. Nohl and D. Hegner (1978) Do Mitochondria Produce Oxygen Radicals in vivo ? Eur.J.Biochem. 82, 563-567.

H. Nohl and D. Hegner (1978) Evidence for the Existence of Catalase in the Matrix Space of Rat-Heart Mitochondria, FEBS Letters 89, 126-130.

H. Nohl, D. Hegner and K.-H. Summer (1979) Responses of Mitochondrial Superoxide Dismutase, Catalase and Glutathione Peroxidase Activities to Aging, Mech.Ageing Dev. 11, 145-151.

T.H. Norwood, W.R. Pendergrass, C.A. Sprague and G.M. Martin (1974) Dominance of the senescent phenotype in heterokaryons between replicative and post-replicative human fibroblast-like cells, Proc.Natl.Acad.Sci.USA 71, 2231-2235.

T. Norwood, H. Hoehn, D. Salk and G.M. Martin (1979) Cellular Aging in Werner's Syndrome: A Unique Phenotype ? J.Invest.Dermatol. 73, 92-96.

W. O'Brien, G. Stenman and R. Sager (1986) Suppression of tumor growth by senescence in virally transformed human fibroblasts, Proc.Natl.Acad.Sci.USA 83, 8659-8663.

L.E. Orgel (1963) The Maintenance of the Accuracy of Protein Synhtesis and its Relevance to Ageing, Proc.Natl.Acad.Sci.USA 49, 517-521.

L. Packer and J.R. Smith (1974) Extension of the Lifespan of Cultured Normal Diploid Cells by Vitamin E, Proc.Natl.Acad.Sci.USA 71, 4763-4767.

L. Packer and J.R. Smith (1977) Extension of the Lifespan of Cultured Normal Diploid Cells by Vitamin E: A Reevaluation, Proc.Natl.Acad.Sci.USA 74, 1640-1641.

L. Packer and K. Fuehr (1977) Low Oxygen Concentration Extends the Lifespan of Cultured Human Diploid Cells, Nature 267, 423-424.

W. Pendergrass, D. Salk and T. Norwood (1985) Cell Fusion Studies and Biochemical Analysis of DNA Synthesis in Werner and Non-Werner Cultured Cells, in: Werner's Syndrome and Human Aging, Eds. D. Salk, Y. Fujiwara and G.M. Martin, Plenum Press, New York and London.

O.M. Pereira-Smith and J.R. Smith (1982) The phenotype of low proliferative potential is dominant in hybrids of normal human fibroblasts, Somat.Cell Genet. 6, 731-742.

O.M. Pereira-Smith and J.R. Smith (1987) Functional simian virus T antigen is expressed in hybrid cells having finite proliferative potential, Mol.Cell.Biol. 7, 1541-1544.

J. Ponten (1970) The Growth Capacity of Normal and Rous-Virus-Transformed Chicken Fibroblasts in vitro, Int.J.Cancer Res. 6, 323-332.

W.A. Pryor (1976) The Role of Free Radical Reactions in Biological Systems, In: Free Radicals in Biology, volume 1, pp.1-49, Editor W.A. Pryor, Academic Press, New York.

W.A. Pryor (1987) The free radical theory of aging revisited:

A critique and a suggested disease-specific theory, In: Modern biological theories of aging, Eds. H.R. Warner, R.N. Butler, R.L. Sprott and E.L. Schneider, pp.89-112, Raven Press, New York.

P.S. Rabinovitch (1983) Regulation of Human Fibroblast Growth Rate by both Noncycling Cell Fraction and Transition Probability is Shown by Growth in 5-Bromodeoxyuridine Followed by Hoechst 33258 Flow Cytometry, Proc.Natl.Acad.Sci.USA 80, 2951-2955.

P.S. Rabinovitch and T.H. Norwood (1980) Comparative heterokaryon study of cellular senescence and the serum deprived state, Exp.Cell Res. 130, 101-109.

M. Raes and J. Remacle (1983) Ageing of Hamster Embryo Fibroblasts as the Result of Both Differentiation and Stochastic Mechanisms, Exp.Gerontol. 18, 223-240.

M. Raes, C. Michiels and J. Remacle (1987) Comparative study of the enzymatic defence systems against oxygen-derived free radicals: the key role of glutathione peroxidase, Free Radical Biol.Med. 3, 3-7.

M.H. Ragab, R. Brightwell and A.L. Tappel (1968) Hydrolysis of Flavin-Adenine Dinucleotide by Rat Liver Lysosomes, Arch.Biochem.Biophys. 123, 179-185.

S.I.S. Rattan, K.D. Keeler. J.H. Buchanan and R. Holliday (1982) Autofluorescence as an Index of Ageing of Human Fibroblasts in Culture, Biosc.Rep. 2, 561-567.

U. Reiss and D. Gershon (1976) Rat-liver superoxide dismutase. Purification and age-related modification, Eur.J.Biochem. 63, 617.

G.A. Sacher (1978) Evolution of longevity and survival characteristics in mammals, In: The genetics of Aging, Ed. E.L. Schneider, pp. 151-167, Plenum Press, New York.

D. Salk, E. Bryant, K. Au, H. Hoehn and G.M. Martin (1981a) Systematic Growth Studies, Cocultivation, and Cell Hybridization Studies, of Werner Syndrome Cultured Skin Fibroblasts, Hum.Genet. 58, 310-316.

D. Salk, K. Au, H. Hoehn and G.M. Martin (1981b) Effects of Free Radical-Scavenging Enzymes and Reduced Oxygen Exposure on Growth and Chromsome Abnormalities of Werner's Syndrome Cultured Skin Fibroblasts, Hum.Genet. 57, 269-275.

D. Salk (1982) Werner's Syndrome: A Review of Recent Research with an Analysis of Connective Metabolism, Growth Control of Cultured Cells, and Chromosomal Aberrations, Hum.Genet. 62, 1-15.

D. Salk, E. Bryant, H. Hoehn, P. Johnston and G.M. Martin (1985) Growth Characteristics of Werner Syndrome Cells in

Vitro in: Werner's Syndrome and Human Aging, Eds. D. Salk, Y. Fujiwara and G.M. Martin, Plenum Press, New York and London.

M.D. Sass (1966) Clin.Chim.Acta 15, 1-6.

R. Sheinin and J.Humbert (1978) Some Aspects of Eukaryotic DNA Replication, Annu.Rev.Biochem. 47, 277-316.

E.L. Schneider (1987) Theories of aging: a perspective, In: Modern biological theories of aging, Eds. H.R. Warner, R.N. Butler, R.L. Sprott and E.L. Schneider, pp. 1-4, Raven Press, New York.

J.R. Smith and D.W. Lincoln II (1984) Aging of Cells in Culture, Int.Rev.Cytol. 89, 151-177.

M. Somville and J. Remacle (1980) Comparative Study of Mitochondrial and Cytoplasmic Superoxide Dismutase during Ageing of Human Fibroblasts in Culture, In: Biological and Clinical Aspects of Superoxide and Superoxide Dismutase, Eds. W.H. Bannister and J.V. Bannister, Elsevier/North-Holland, New York.

S.P. Spielberg, L.I. Kramer, S.I. Goodman, J. Butler, F. Tietze, P. Quinn and J. Schulman (1977) 5-Oxoprolinuria: Biochemical Observations and Case Report, J.Ped. 91, 237-241.

H. Stam and J.F. Koster (1985) Fatty Acid Peroxidation in Ischemia, In: Prostaglandins and Other Eicosanoids in the Cardiovascular System, pp. 131-148, Editor Schroer, Karger, Basel.

G.H. Stein and R. M. Yanishevsky (1979) Entry into S phase is inhibited in two immortal cell lines fused to senescent human diploid cells, Exp.Cell Res. 120, 155-165.

G.H. Stein and R.M. Yanishevsky (1981) Quiescent human diploid cells can inhibit entry into S-phase in replicative nuclei of heterodikaryons, Proc.Natl.Acad.Sci.USA 78, 3025-3029.

G.H. Stein, R.M. Yanishevsky, L.Gordon and M. Beeson (1982) Carcinogen-transformed human cells are inhibited from entry into S-phase by fusion to senescent cells but cells transformed by DNA viruses overcome the inhibition, Proc.Natl.Acad.Sci.USA 79, 5287-5291.

G.H. Stein and L. Atkins (1986) Membrane-associated inhibitor of DNA synthesis in senescent human diploid fibroblasts: Characterization and comparison to quiescent cell inhibitor, Proc.Natl.Acad.Sci.USA 83, 9030-9034.

B.L.Strehler (1977) Time, Cells and Aging, 2nd Edition, Academic Press, New York.

H. Stubel (1911) Archiv Ges.Physiol. 142, 1.

S. Takahashi and M. Zeydel (1982) Gamma-glutamyl transpeptidase and glutathione in aging IMR-90 fibroblasts and in differentiating 3T3L1 preadipocytes, Arch.Biochem.Biophys. 214, 260-267.

F. Takeuchi, F. Hanaoka, M. Goto, M. Yamada and T. Miyamoto (1982) Prolongation of S Phase and Whole Cell Cycle in Werner's Syndrome Fibroblasts, Mech.Ageing Dev. 17, 473-480.

R. Teoule (1987) Radiation induced DNA damage and its repair, Int.J.Radiat.Biol. 51, 573-589.

H.H. Thaw, V.P. Collins and U.T. Brunk (1984) Influence of oxygen tension, pro-oxidants and antioxidants on the formation of lipid peroxidation products (lipofuscin) in individual cultivated human glial cells, Mech.Ageing Dev. 24, 211-223.

K. Uchida and S. Kawakishi (1986) Selective oxidation of imidazole ring in histidine residues by the ascorbic acid-copper ion system, Biochem.Biophys.Res.Comm. 138, 659-665.

M.L. VanKeuren, C.R. Merill and D. Goldman (1983) Protein Variations Associated with In Vitro Ageing, Limitations to the Error Catastrophe Hypothesis, J.Gerontol. 38, 645-652.

G.S. Vince and R.T. Dean (1987) Is enhanced free radical flux associated with increased intracellular proteolysis ? FEBS Letters 216, 253-256.

E. Wang (1985) A 57,000 mol-wt protein uniquely present in nonproliferating cells and senescent human fibroblasts, J.Cell Biol. 100, 545-551.

Otto Werner (1904) On Cataract in Conjunction with Scleroderma, Translated by Holger Hoehn (1985) in: Werner's Syndrome and Human Aging, Eds. D. Salk, Y. Fujiwara and G.M. Martin, Plenum Press, New York and London.

S.P. Wolff, A. Garner and R.T Dean (1986) Free Radicals, Lipids and Protein Degradation, T.I.B.S. 11, 27-31.

S.P. Wolff and R.T. Dean (1987) Glucose autoxidation and protein modification. The potential role of "autoxidative glycosylation" in diabetes, Biochem.J. 245, 243-250.

H. Yamane, N. Yada, E. Katori, T. Mashino, T. Nagano and M. Hirobe (1987) Base liberation from nucleotides by superoxide and intramolecular enhancement effect of phosphate group, Biochem.Biophys.Res.Comm. 142, 1104-1110.

R.M. Yanishevsky, M.L. Mendelsohn, B.H. Mayal and V.J.

Cristofalo (1974) Proliferative capacity and DNA content of aging human diploid cells in culture: A cytophotometric and autoradiographic analysis, J.Cell.Physiol. 84, 165-170.

I. Zs.-Nagy (1978) A Membrane Hypothesis of Ageing, J.Theor.Biol. 75, 189-195.

Chapter 5

APPENDIX

APPENDIX PAPER 1

AUTOFLUORESCENCE OF HUMAN SKIN FIBROBLASTS DURING GROWTH INHIBITION AND IN VITRO AGEING

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Autofluorescence of Human Skin Fibroblasts during Growth Inhibition and in vitro Ageing

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Key Words. Human skin fibroblasts · Autofluorescence · Ultrastructure · Werner's syndrome · Spielmeyer-Vogt syndrome · Vitamin E

Abstract. The increase in autofluorescence (AF) of human skin fibroblasts during their in vitro ageing and growth inhibition was investigated by means of flow cytophotometry. The cellular AF of in vitro ageing cultures increased while the relative number of (^{3}H)-thymidine incorporating cells decreased. Therefore, the rate of accumulation of cellular AF during in vitro ageing of the cultures is inversely related to the proliferation rate of the culture. The rates of increase of AF varied widely among the cell strains, being the highest in cells from patients with Werner's syndrome. Upon growth inhibition in a confluent culture the net rates of increase of cellular AF were found to vary widely among the cell strains. The respective net rates of increase of AF of the cells from patients with Werner's syndrome and the Spielmeyer-Vogt syndrome were within the range covered by the normal cell strains. The ultrastructure of the bright AF cells from patients with Werner's syndrome and the Spielmeyer-Vogt syndrome differed from the ultrastructure of AF cells from control persons with regard to the morphology of their residual bodies, those from the patients contained more multilamellar and multivesicular structures.

In sorted non-AF cells vitamin E was found to completely inhibit the accumulation of AF without affecting the formation of 'residual bodies'. We infer that cellular AF is caused by lipid peroxidative reactions and that the accumulation of AF is due to a decrease in cellular proliferation rate.

Introduction

Among the many changes cell populations undergo during in vitro ageing there is an increase in the relative number of autofluorescent (AF) cells. This AF is localized in acid phosphatase-containing cytoplasmic granules [3]. When bright AF cells were sorted from a cell population with an intermediate population doubling level (PDL), these cells were shown to exhibit properties of in vitro aged cells, such as a decreased growth rate and a

decreased level of (^{3}H)-thymidine incorporation [4]. The AF of MRC5 fibroblasts has been shown to increase exponentially in a culture during in vitro ageing [8]. So, it can be implied that among other factors a decreased cellular proliferation rate is responsible for the occurrence of AF in cell cultures.

In the present study we measured the AF of skin fibroblast strains during their in vitro ageing, and we determined the (^{3}H)-thymidine incorporation of the same cultures. In order to investigate the effect of growth inhibition on cellular AF we analyzed the AF of nonfluorescent (NF) cells during 3 weeks in a confluent culture. In this way we compared the accumulation of AF in cells from patients with Werner's syndrome and the Spielmeyer-Vogt syndrome with fibroblasts from 3 normal subjects. A possible role of lipid peroxidation in the causation of cellular AF was investigated using vitamin E (*D,L*-α-tocopherol), a lipophilic radical scavenger.

Materials and Methods

Cells and Culture

Skin fibroblasts of unspecified origin were derived from 4 healthy subjects (C5RO: female, 30 years; 79RD200: male, 5 years; 80RD143: male, 30 years; 80RD174: male, 33 years). The patient material consisted of fibroblasts from 2 patients with the Spielmeyer-Vogt syndrome (494LAD: male, 5 years; 75RD97: female, 25 years) and from 2 patients suffering from Werner's syndrome (525LAD: male, 25 years; 531LAD: male, 30 years). All cell strains were cultured with Ham's F10 medium supplemented with 8% fetal calf serum (FCS) and antibiotics (100 µg streptomycin and 100 U penicillin/ml of culture medium). For the normal strains serum from Boehringer (lot No. 66.3902.02) and for the patient strains serum from Sera Lab (lot No. 101107) was used. Cultures were subcultivated by 1:3 splits. Culture media were replenished twice a week.

Flow Cytometry and Cell Sorting

Cells were harvested by trypsinization just before cell sorting. They were suspended in culture medium containing 8% FCS. Flow cytometry and sorting were carried out using a FACS II cell sorter (Becton & Dickinson) equipped with an argon ion laser (Spectra-physics 164–05) at 457 nm using a 70 µm nozzle. Fluorescence was measured above 520 nm using a combination of K515, K510 (Schott) and LP520 (Ditric Optics) filters. The instrument alignment was optimized and calibrated using bright fluorescent microspheres (Polysciences: 1.51 µm; No. 9719, lot No. 3-0826). A computer (Minc II, Digital) was linked to the FACS to store and to process the fluorescence profiles obtained from the cultures. In each measurement 20,000 cells were used. The percentage of bright AF cells (% AF) was defined as the percentage of cells having a fluorescence of more than that of channel 145 of the FACS. The mean AF ($\overline{A}$) of a culture was calculated as follows:

$$\overline{A} = \sum_{1}^{256} \frac{\text{number of cells in a channel} \times \text{channel number}}{\text{total number of cells}}$$

NF cells were defined as cells having a fluorescence below channel 30 of the FACS.

Labeling Index

The 24-hour (^{3}H)-thymidine labeling index of our cultures was determined according to *Christofalo and Scharf* [2]. For each determination at least 2,000 cells were counted.

Growth Inhibition

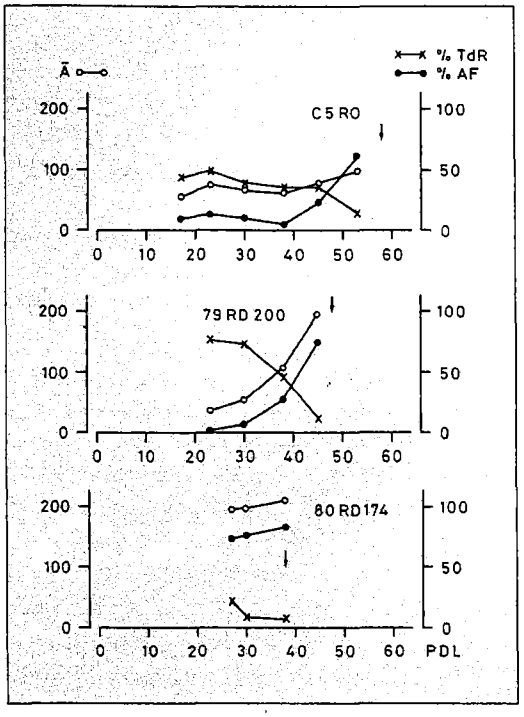
Sorted NF cells were seeded at a density of 200,000 cells/hole on glass coverslips (diameter 24 mm) in holes of plastic culture trays (Costar 3524). The experiments were done in culture media containing the Sera Lab serum. They were replenished twice a week.

Ultrastructure

Electron microscopy of sorted and cultured AF and NF cells was carried out as described previously [4].

Vitamin E

Incubations were done with sorted NF cells from a normal cell strain (C5RO). These were cultured in a confluent monolayer (see above) in culture medium containing 10 µg *D,L*-α-tocopherol/ml (Sigma). Vitamin E-containing culture media, which were prepared immediately before use, were replenished twice a week.



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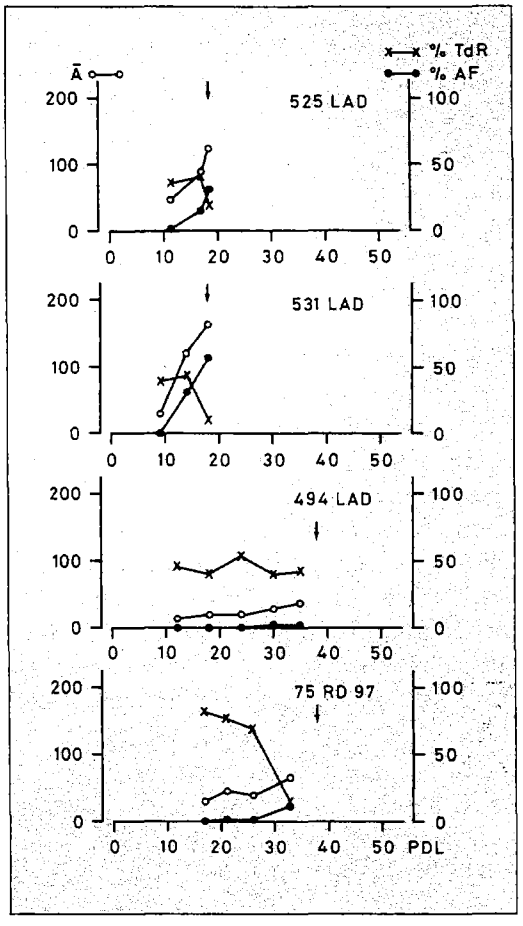

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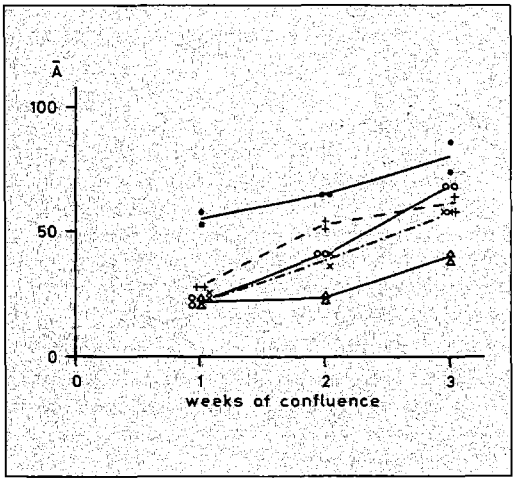
Fig. 1. AF and (^{3}H-thymidine labeling indices of three normal cell strains. Arrows indicate the maximum population doubling level of each strain. × = Percentage of (^{3}H)-thymidine incorporating nuclei (right-hand axis); ● = percentage of bright AF cells (% AF) (right-hand axis); ○ = mean AF ($\bar{A}$) (left-hand axis).

Fig. 2. AF and (^{3}H)-thymidine labeling indices of patient cell strains. 525LAD and 531LAD are from Werner's syndrome; 494LAD and 75RD97 are from the Spielmeyer-Vogt syndrome. Arrows indicate the maximum population doubling level of each strain. × = percentage of (^{3}H)-thymidine incorporating nuclei (right-hand axis); ● = percentage of bright AF cells % AF (right-hand axis); ○ = mean AF ($\bar{A}$) (left-hand axis).

Fig. 3. AF during confluence of sorted NF cells from 5 different cell strains. Normal cell strains are: C5RO (○), 79RD200 (△) and 80RD143 (●); Werner's syndrome: 525LAD (+), and Spielmeyer-Vogt syndrome: 494LAD (×). The serum used is from Boehringer (lot No. 66.3902.02).



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Results

AF and Labeling Indices

AF was determined by flow cytometry of human skin fibroblasts, derived from healthy subjects and patients, during their in vitro ageing (fig. 1, 2). In cultures of fibroblasts from healthy subjects the percentage of AF cells increases when a high PDL was attained. The cultures of 80RD174 (fig. 1) form an exception as the percentage of AF cells was already high in the first PDL studied. The pattern, as described for the percentage of AF cells, is reflected in the mean AF of the cultures.

In the cultures of fibroblasts from patients with Werner's syndrome a rapid increase in percentage of AF cells or mean AF is found. At PDL 18, cessation of in vitro growth occurs in the cells of these patients. The mean AF of both these cell strains at PDL 18 is then as high as that of cell cultures from healthy subjects at PDL 30 or over. Both cell strains from the patients with the Spielmeyer-Vogt syndrome showed only a slight increase in mean AF or percentage of AF cells (fig. 2).

The rates of increase of mean AF varies widely among the cultures, being the highest in the cultures of fibroblasts from patients with Werner's syndrome (table I). The labeling indices of the cell cultures, a measure of fast dividing cells, from healthy subjects show a clear decrease when the mean AF of these cultures increases. This pattern is particularly clear in the 79RD200 strain (fig. 1). The cultures of patient-derived fibroblasts show a similar pattern with respect to these parameters. Summarizing, we find an indication of an inverse relationship between mean AF and proliferation rate of the cultures.

Growth Inhibition

As there is an indication of an inverse relationship between mean AF and proliferation rate of a culture (see above) we investigated the increase in mean AF in confluent cultures of fibroblasts from healthy subjects and patients in order to compare the net accumulation rates of cellular AF. The mean AF of the cultures of sorted NF cells (mean AF <30) increased in all cases during the period of confluence (3 weeks). The mean fluorescence values attained in this way are comparable to the values obtained in cultures with a high PDL. The individual values for each cell strain were highly reproducible but the values for the different cell strains varied widely. The values of the patient-derived cell strains fell within the range covered by the cell strains from the healthy controls (fig. 3).

Table I. Rate of increase of cellular AF during in vitro ageing

Cell strain		Rate l/PDL[1]
Normal	C5RO	0.031
	79RD200	0.078
	80RD174	0.007
Werner's syndrome	525LAD	0.110
	531LAD	0.194
Spielmeyer-Vogt syndrome	494LAD	0.063
	75RD97	0.043

[1] The rate of increase in AF was calculated using the mean AF values of the last three PDLs displayed in figures 1 and 2. These values were fitted into the exponential curve $F_t = F_o \times e^{kt}$ and k was calculated using least squares linear regression analysis.

Ultrastructure

We compared the ultrastructure of NF and AF cells from a normal strain (C5RO), a strain from Werner's syndrome (525LAD) and a strain from the Spielmeyer-Vogt syndrome (494LAD). There is no qualitative difference among the NF cells of the three cell strains. In all cell strains the AF cells differed from the NF cells with respect to their content of residual bodies. There are always

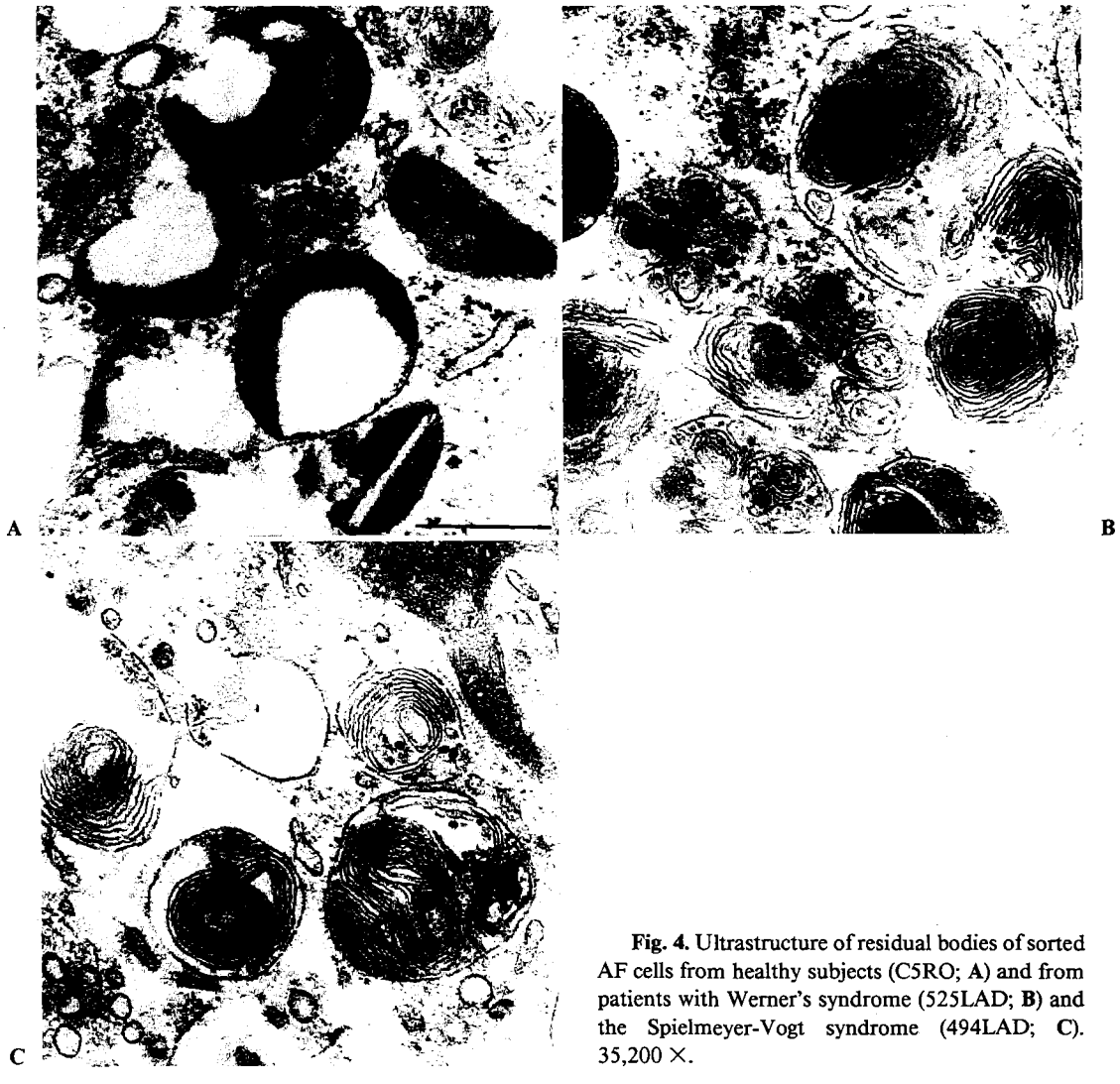

Fig. 4. Ultrastructure of residual bodies of sorted AF cells from healthy subjects (C5RO; **A**) and from patients with Werner's syndrome (525LAD; **B**) and the Spielmeyer-Vogt syndrome (494LAD; **C**). 35,200 ×.

many more of these structures in the AF cells relative to their NF counterparts. Strikingly, the AF cells from both of the cell strains from the patients contained many residual bodies with lamellar or vesicular structures (fig. 4B, C), while in the control cell strain homogeneous and 'empty' structures were prominent (fig.4A).

Vitamin E

The presence of vitamin E in the culture medium completely suppresses the accumulation of AF cells in a confluent culture of sorted NF cells (C5RO; fig. 5). Comparing cells after 3 weeks in confluence in the presence or absence of vitamin E the ultrastructure of these cells did not differ. Both cultures contained many cells with a high number of residual bodies (fig. 6).

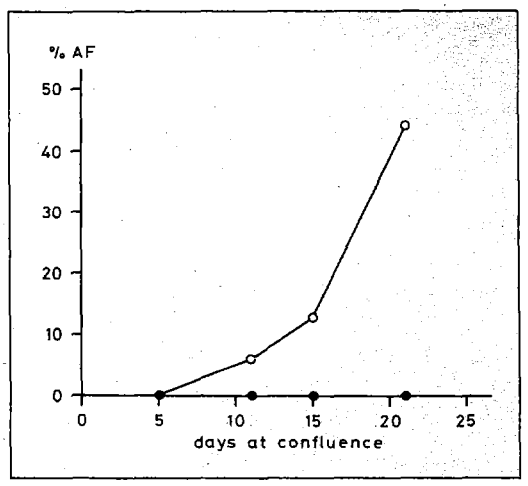

Fig. 5. AF during confluence of sorted NF cells (strain C5RO) in the presence (●) and in the absence of vitamin E (○). The serum used is from Boehringer (lot No. 66.3902.02). AF is expressed as percentage AF cells (% AF).

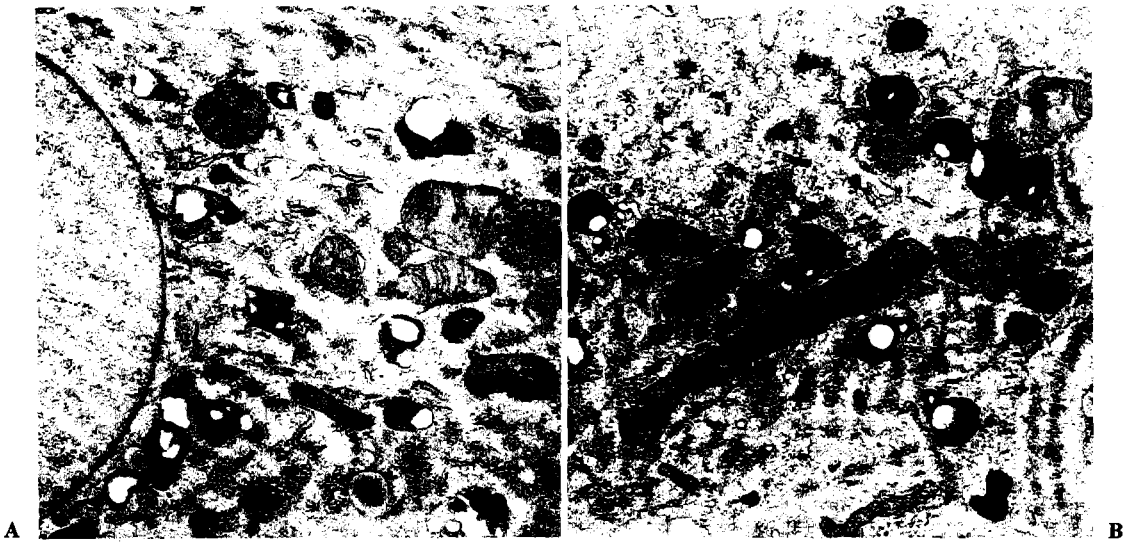

Fig. 6. Ultrastructure of sorted NF cells kept at confluence during 3 weeks in the presence of vitamin E (**A**) and in the absence of vitamin E (**B**). 9,900 ×.

Discussion

The early investigation of *Deamer and Gonzales* [3] left the question open as to whether cellular AF results from the reduced proliferative potential of in vitro aged cells or is a specific ageing phenomenon. *Jongkind* et al. [4] showed that there is a relationship between cellular AF and proliferative capacity, but they did not indicate the cause which gave rise to the AF cells. Using hamster embryo fibroblasts, *Raes and Remacle* [7] indicated that a slowly dividing class of cells emerged from a rapidly dividing class and that the reduced proliferative potential of the former may lead to the changes in the morphology, being a development from a slim, spindle-like cell towards a short, bulky appearance. *Rattan* et al. [8] showed that the AF of a culture increases during the whole of the course of in vitro ageing, while a confluent culture of their cells (4 weeks) did not lead to a substantial increase in AF.

The AF we measured in our cultures (fig. 1, 2) is expressed as a percentage AF cells [as defined in ref. 4] as well as the mean AF of all cells (defined in the Materials and Methods section). Both parameters lead to curves that resemble one another, although they are not identical. Mean AF is the parameter of choice as this one takes into account the fluorescence of all cells measured. The percentage of AF cells is merely included to enable comparison with data reported previously [4].

In continuously growing cultures an increase in mean AF is found when the labeling index of the same culture decreases (fig. 1, 2). Moreover, growth inhibition leads in all cell strains studied to an increase in the mean AF of the culture (fig. 3). Therefore, growth inhibition is the sole factor leading to the accumulation of AF cells. In continuously growing cultures some cell strains attain a high level of mean AF at an earlier point than others do (fig. 1, 2). Typically, both the cell strains from Werner's syndrome patients reached at PDL 18 the same mean AF value as did control cell strains after PDL 30. Also, the rate by which these mean AF values are attained is higher among the Werner's syndrome cell strains than in the control cell strains and the strains from the Spielmeyer-Vogt syndrome (table I). From these observations one can infer that the rate of accumulation of AF in a continuously growing culture could be used as a diagnostic criterion for Werner's syndrome. We also analyzed the *net* rate of accumulation of AF in growth-inhibited cultures. These *net* accumulation rates varied widely among the control cell strains (fig. 3). The *net* rates of the strains from Werner's syndrome and the Spielmeyer-Vogt syndrome remained within the range covered by the control strains (fig. 3). Therefore, the high accumulation rate in the cells from Werner's syndrome patients in a continuously growing culture can only be explained as a result of an early decrease in cellular proliferation rate. In this respect the cells from the Spielmeyer-Vogt syndrome are different from cells from Werner's syndrome (their maximum in vitro life span is about 38 PDL).

The normal cell strains studied exhibited in their sorted AF cells characteristics of in vitro aged cells, such as an increased number of residual bodies [4, 6]. The AF cells from the strains from the patients with Werner's syndrome and the Spielmeyer-Vogt syndrome also have a large number of residual bodies, but they contain in addition many multilamellar residual bodies.

The free radical theory of cellular ageing presumes lipid peroxidation to be the cause

of the increase in AF in cells during their in vitro ageing [5]. Vitamin E, a lipophilic-free radical scavenger, completely suppresses the increase in AF seen in the control culture of sorted NF cells. So, the cellular AF is caused by free radical reactions in the lipid compartments of the cell. As cellular AF is always confined to residual bodies [3], one would presume an inhibition of free radical reactions to lead to a decreased number of residual bodies in the cell. However, there is no difference in the morphology of vitamin E treated and in the control cells (fig. 6). So, it is concluded that cellular AF results from free radical reactions in the lipid compartments of the cell, but that the formation of residual bodies is not dependent upon this type of reaction.

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References

1 Brunk, U.T.; Collins, V.P.: Lysosomes and age pigments in cultured cells; in Sohal, Age pigments, pp. 243–264 (Elsevier/North-Holland Biomedical Press, Amsterdam 1981).

2 Cristofalo, V.T.; Scharf, B.B.: Cellular senescence and DNA synthesis. Expl Cell Res. *76:* 419–427 (1973).

3 Deamer, D.W.; Gonzales, J.: Autofluorescent structures in cultured Wi-38 cells. Archs biochem. Biophys. *165:* 421–426 (1974).

4 Jongkind, J.F.; Verkerk, A.; Visser, W.J.; Dongen, J.M. van: Isolation of autofluorescent 'aged' human fibroblasts by flow sorting. Morphology, enzyme activity and proliferative capacity. Expl Cell Res. *138:* 409–417 (1982).

5 Leibovitz, B.E.; Siegel, B.V.: Aspects of free radical reactions in biological systems: aging. J. Geront. *35:* 45–56 (1980).

6 Lipetz, J.; Christofalo, V.J.: Ultrastructural changes accompanying the aging of human diploid cells in culture. J. Ultrastruct. Res. *39:* 43–56 (1972).

7 Raes, M.; Remacle, J.: Ageing of hamster embryo fibroblasts as the result of both differentiation and stochastic mechanisms. Exp. Gerontol. *18:* 223–240 (1983).

8 Rattan, S.I.S.; Keeler, K.D.; Buchanan, J.H.; Holliday, R.: Autofluorescence as an index of ageing in human fibroblasts in culture. Biosci. Rep. *2:* 561–567 (1982).

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APPENDIX PAPER 2

REVERSIBLE INHIBITION OF DNA REPLICATION AND PROTEIN SYNTHESIS BY CUMENE HYDROPEROXIDE AND 4-HYDROXY-NONENAL IN HUMAN SKIN FIBROBLASTS

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REVERSIBLE INHIBITION OF DNA AND PROTEIN SYNTHESIS BY CUMENE HYDROPEROXIDE AND 4-HYDROXY-NONENAL

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SUMMARY

To test the possible role of lipid peroxidation in the process of *in vitro* ageing, human diploid skin fibroblasts were cultured with the lipophilic hydroperoxide cumene hydroperoxide (Chp) or the breakdown product of lipid peroxidation 4-hydroxy-2,3-*trans*-nonenal (HNE). Both compounds inhibited cellular DNA and protein synthesis in a dose-dependent way. Cells exposed to Chp or to HNE during growth inhibition recovered DNA and protein synthesis within 24 h upon removal of Chp or HNE from the culture medium. Continuously proliferating cells showed only a partial recovery of DNA and protein synthesis. Pre-culturing cells with the lipophilic free radical scavenger vitamin E did not abolish the effect of Chp upon DNA synthesis. Cellular levels of reduced glutathione (GSH) rose slightly during 1 week of culture with HNE, but remained unaltered with Chp. Neither ATP levels nor cellular energy charges were affected during culture with Chp or HNE. So, DNA synthesis is not impaired due to a shortage of nucleotides nor does GSH protect DNA synthesis against the effects of Chp or HNE. These results suggest that oxygen free-radical induced lipid peroxidation is not the cause of the irreversible loss of proliferation occurring during *in vitro* ageing.

Key words: Ageing; Oxygen free radicals; Glutathione; Cumene hydroperoxide; Hydroxy-nonenal

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INTRODUCTION

Human foetal fibroblasts have been found to lose their proliferative capacity during culture *in vitro* [1]. This finding is reproduced in a number of different systems [2–4] and is now accepted as the general phenomenon of *in vitro* ageing. Two types of theories have been put forward to explain this phenomenon; one assuming an underlying genetic programme [5], while the other invokes a randomly occurring damage as the basic cause. One of the main theories of the second type is the free-radical theory of ageing [6]. Free-radical reactions, for instance those arising upon the univalent reduction of oxygen during cellular oxygen metabolism [7], are presumed to damage the cell to the extent that proliferation ceases. Cellular oxygen consumption gives rise to several reactive oxygen species, including superoxide, hydrogen peroxide and hydroxyl radical or a related species [8–10], against which the cell has various defence mechanisms [11]. One of the major consequences of free-radical reactions in the cell is lipid peroxidation [12].

Hitherto, it remains to be seen whether lipid peroxidation is responsible for the irreversible loss of cellular proliferation during *in vitro* ageing. To investigate the possible effects of lipid peroxidation this process was imitated using two model compounds: the lipophilic hydroperoxide cumene hydroperoxide (Chp) [13–16], which serves as a model lipophilic peroxide, and a major breakdown product of lipid peroxides, 4-hydroxy-2,3-*trans*-nonenal (HNE) [17].

MATERIALS AND METHODS

Cells and culture

Diploid skin fibroblasts were derived from a healthy subject (C5RO: female, 30 years). Cells were cultured in Ham's F10 medium supplemented with 8% foetal calf serum (Flow Laboratories lot: 29101086) and antibiotics (100 U of penicillin and 100 μm of streptomycin/ml of culture medium). Cultures were subcultivated by 1 : 3 splits when the cultures reached confluency. In the experiments with vitamin E, cells were cultured with 10 μg vitamin E/ml of culture medium.

Growth-inhibited incubation system

Cells were seeded into holes of 24-well culture dishes (Costar 3524) and allowed to reach confluency and density-dependent inhibition of growth, which was ascertained by the complete abscence of mitoses. Thereupon, the culture medium was replenished and test compounds were added to the appropriate concentrations. Chp was suspended into phosphate buffered saline (PBS) and diluted with PBS to a final concentration of 0.2 mM. HNE was obtained by evaporation of the chloroform in which it was stored, dissolved into distilled water and diluted with PBS. Both test compounds were filter sterilised (0.22 μm Sterivex-GS, Millipore, Molsheim, France). All solutions of test compounds were prepared immediately before use. Media with the test compounds were replished after 3 and 6 days of culture. On the

7th day these cells were used to determine DNA and protein synthesis rates. On the same day these cells were compared with control cell cultures incubated under the same conditions without Chp or HNE with respect to GSH and adenine nucleotide contents. From the data reported previously [18] it can be concluded that both test compounds are detoxified by the cells in approximately 2 days. The levels of GSH and ATP and the rates of DNA and protein synthesis are determined in the presence of freshly added test compounds if not stated otherwise.

Incubation system with proliferating cells

Cells were seeded into petri dishes at a density of 1000 cells/ cm^2 and immediately thereafter the test compounds were added. After 3 and 6 days the culture medium with the test compounds was replenished. On the 7th day cells were trypsinised and resuspended in culture medium with the test compound and seeded again in petri dishes onto glass coverslips. After 24 h of culture DNA synthesis was determined as described for the growth inhibited cells.

Reversibility of the inhibition of DNA and protein synthesis

After 7 days of culture with Chp or HNE in the growth-inhibited or continuously proliferating system, cells were trypsinised and reseeded in culture medium without the test compounds. After 24 h DNA and protein synthesis rates were determined as described.

Assay for DNA synthesis

After 1 week of culture in the presence of either Chp or HNE, cells were plated onto glass coverslips in petri dishes at a density of 1000 cells/ cm^2 and 24 h-labelling indices were determined using [^{3}H]thymidine (Amersham, specific activity 52 Ci/ mmol) according to Cristofalo and Scharf [19].

Assay for protein synthesis

To determine protein synthesis rates cultures, being exposed during 1 week to Chp or HNE, were pulse labelled during 2 h with 10 μCi L-(4,5[^{3}H]leucine/ml of culture medium (Amersham, specific activity 128 Ci/mmol). The cultures were washed twice with ice-cold PBS and cells were solubilised with 0.25% Triton X-100 in distilled water. Upon addition of 1 mg of bovine serum albumin to each sample, trichloroacetic acid precipitates were prepared. The protein precipitates were washed once with 70% ethanol and dissolved in 0.1 ml of 3.5 N KOH. Radioactivity was determined with a Packard Tri-Carb liquid scintillation counter using dilumine as a scintillation solution.

Assay for glutathione

Levels of reduced glutathione (GSH) were determined in intact cells using a flow cytometric method, which has been published previously [20]. Briefly, cells, being cultured during 1 week with either Chp or HNE, were harvested by trypsinization

and collected by centrifugation (5 min at 200 g). They were resuspended in F10 culture medium without serum and incubated during 1 h at 37°C with the appropriate concentration of test compound. One sample was incubated during 1 h with 0.2 mM *N*-ethyl-maleimide as a control for spurious staining. Monobromobimane (Calbiochem) was added to all suspensions at a final concentration of 0.375 mM and staining was during 20 min at 37°C. A stock solution of monobromobimane of 50 mM was prepared in acetonitrile (Merck) and stored at 4°C in the dark. After staining fluorescence was recorded with a FACS II cell sorter (Becton and Dickinson) equipped with an argon ion laser being set at 360 nm excitation wavelength. Fluorescence above 420 nm was obtained using a KV 399 and a K 420 filter (Schott). The average fluorescence of the cell suspension was calculated. All GSH contents were expressed as a percentage relative to a control sample cultured without test compounds.

Assay for adenine nucleotides

Cells cultured during 7 days with either Chp or HNE were trypsinised and subsequently ATP, ADP and AMP levels were determined in neutralised perchloric acid extracts of the cell suspensions with a HPLC method [21]. Cellular energy charges were calculated from these data according to Atkinson [22]:

$$E = \frac{(ATP) + 1/2\,(ADP)}{(ATP) + (ADP) + (AMP)}$$

RESULTS AND DISCUSSION

This study was undertaken to unravel the effects of lipid peroxidation upon cells in culture and to determine its possible significance for the process of *in vitro* ageing. Both Chp, a model lopophilic peroxide, and HNE, a breakdown product of lipid peroxides, inhibited DNA synthesis in a dose-dependent way (Fig. 1). The ID_{50} values were 10 μm for Chp and 8 μm for HNE. The HNE doses required for inhibition are within the range reported by others for Ehrlich Ascites Tumour cells [23,24], guinea pig keratinocytes [25], neonatal hepatocytes and Yoshida Ascites hepatoma cells [26].

Supplementation of the culture with 10 μg of vitamin E/ ml of culture medium, a concentration sufficient to fully prevent the formation of cellular autofluorescence [27], did not prevent the Chp-mediated inhibition of DNA synthesis (Fig. 1). This result indicates that Chp affects DNA synthesis *via* a route which cannot be blocked by a lipophilic free-radical scavenger.

A few explanations for the inhibition of DNA synthesis by Chp and HNE are possible. For instance, cells may fall short of a substrate required for DNA synthesis or the enzymes and other proteins involved might be inactivated. The major

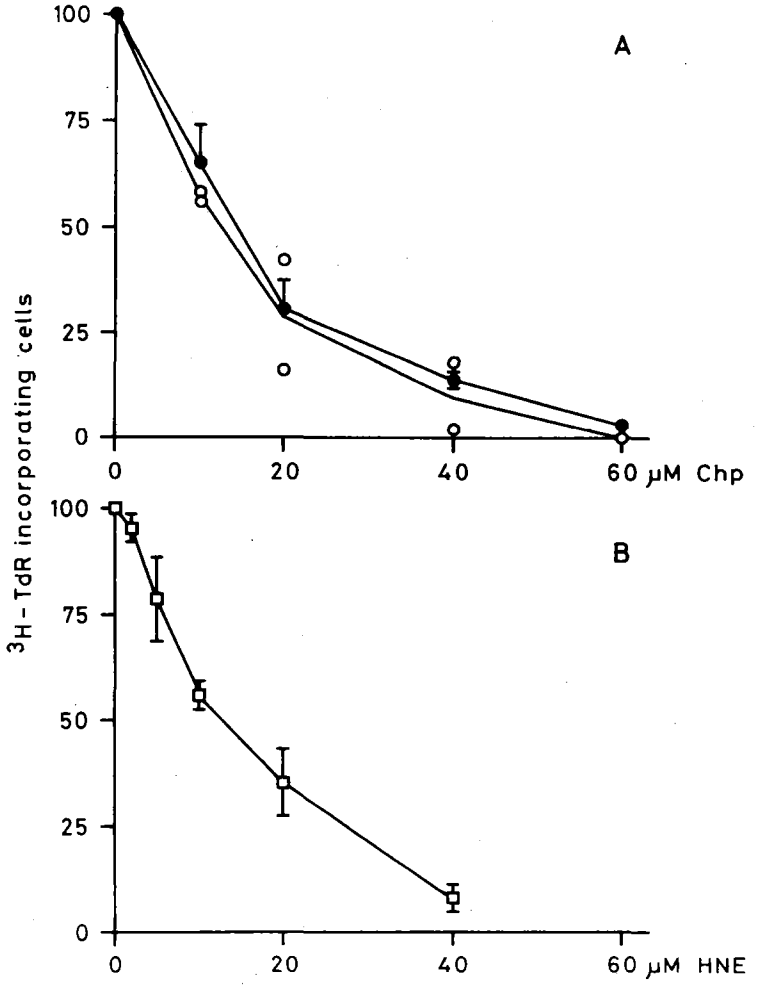

Fig. 1. DNA synthesis after 1 week of culture with a concentration series of Chp or HNE. The percentages of labelled nuclei in the control samples were normalised to 100% to allow for comparison between different experiments. Bars indicate S.E. from the mean as obtained from four independent experiments. Panel A: fibroblasts cultured without (●) or with 10 μg vitamin E/ ml of culture medium (○) before and during the experiment. Panel B: fibroblasts cultured with a concentration series of HNE.

substrates for DNA replication are deoxyribonucleotides and ATP [28]. During culture with Chp or HNE cells had no lowered level of ATP nor were cellular energy charges altered (Table I). A shift from nucleotide triphosphates to mono- and diphosphates was found in Ehrlich Ascites Tumour cells exposed to very high (1 mM) concentrations of 4-hydroxpentenal, a homologue of HNE [29]. In our sytem, where cells are cultured with 40 μm of HNE, this shift is not observed (Table I). In preliminary experiments it was found that neither Chp nor HNE affected the intracellular

TABLE I

THE EFFECTS OF Chp AND HNE UPON ATP CONTENTS AND CELLULAR ENERGY CHARGES.

Nucleotide	*Control* ($n = 6$) (*nmol/mg protein*)	*40 μm HNE* ($n = 3$)	*40 μM Chp* ($n = 3$)
AMP	1.0 ± 0.2	1.3 ± 0.1	1.0 ± 0.2
ADP	0.38 ± 0.13	0.45 ± 0.36	0.36 ± 0.60
ATP	45.0 ± 3.1	39.3 ± 3.1	40.8 ± 4.0
Energy charge	0.974	0.963	0.972

specific activity of radiolabelled thymidine (data not shown). So, the impairment of DNA synthesis is not due to a shortage of substrates.

As both HNE and Chp are capable of binding to thiol groups, these compounds might inactivate enzymes involved in DNA replication, which are critically dependent upon their thiol groups. This is the case, for instance, with DNA polymerase α [28]. Protein synthesis can also be impaired by inactivation of the sulfhydryl-dependent peptidyl-tRNA translocase [30]. Both Chp and HNE reduce cellular GSH levels in short-term incubations [18], but after 1 week of culture with Chp the cellular GSH level was restored and was even higher after culture with HNE (Fig. 2). Hence, a

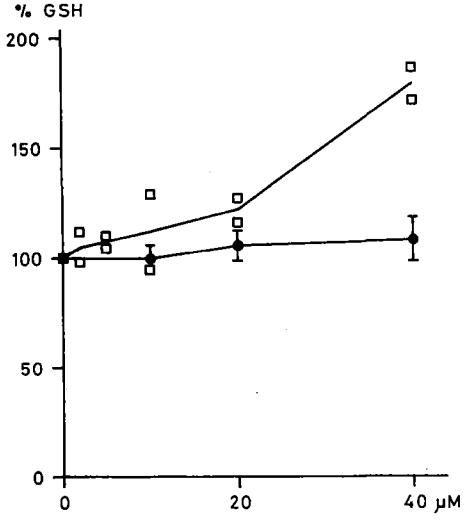

Fig. 2. GSH contents of cells after 1 week of culture with a concentration series of Chp (●) or HNE (□). 100% GSH is 20.0 ± 0.6 nmol GSH/mg protein. Bars indicate S.E. as obtained from seven independent experiments.

normal level of GSH does not protect DNA and protein synthesis against these agents, as has already been shown in Ehrlich Ascites Tumour cells [23].

Although a direct effect of Chp or HNE upon one of the enzymes involved in DNA replication is possible, a diminished level of DNA synthesis might also result from a impairment of cellular protein synthesis [26]. Chp and HNE caused a dose-dependent decrease in the rate of [^{3}H]leucine incorporation (Fig. 3) with ID_{50} values of 35 μm for Chp and 18 μm for HNE. Moreover, protein synthesis rates were restored to control levels within 24 h upon removal of Chp or HNE. So, in our experiments Chp and HNE exerted a stronger effect upon DNA synthesis than upon protein synthesis. Hence, it is not likely that DNA synthesis is impaired due to a shortage of necessary proteins.

If contact-inhibited cell cultures exposed to 20 μm Chp or HNE during 1 week were incubated for 24 h without the test compounds, cells recovered fully the DNA and protein synthesis capacity of control cells cultured without test compounds. Cells incubated during continuous proliferation with 20 μm Chp or HNE, however, recovered only partially (74–78% of control levels respectively) their DNA synthetic capacity during 24 h without test compound. So, continuously proliferating cells are more sensitive to the deleterious effects of Chp or HNE than cells rendered quiescent.

During the course of our experiments Krokan *et al.* reported the influence of HNE on the clone-forming ability of cultured human bronchial fibroblasts [31].

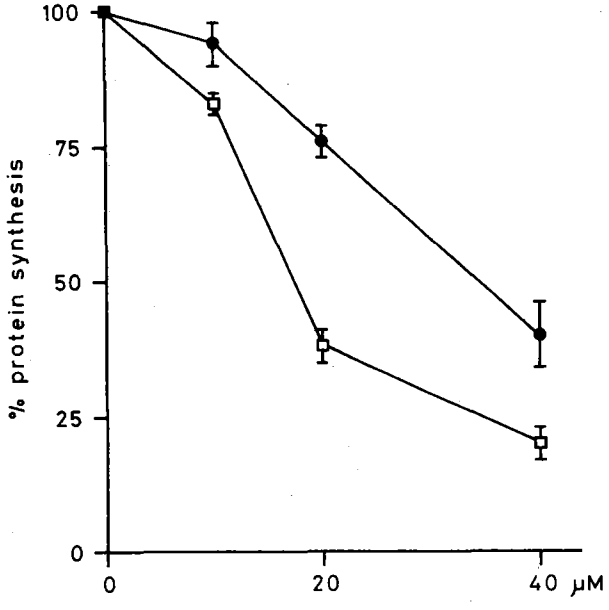

Fig. 3. Protein synthesis rates of cells after 1 week of culture with a concentration series of Chp (●) or HNE (□). Bars indicate S.E. from the mean as obtained from four independent experiments.

Proliferating cells being incubated for 1 hour with 30 μm of HNE lost entirely their capacity to form a clone during 8 days of culture without HNE. This indicated an irreversible influence of HNE on cellular proliferation. Our findings that cells can partially recover their DNA synthetic capacity drying 24 h of culture without Chp or HNE do not necessarily contradict the findings of Krokan *et al.* [31]. First, it cannot be excluded that cells with a slightly reduced level of DNA synthesis might be unable to form a full clone according to the criteria used by Krokan *et al.* [31]. Secondly, the ratio of HNE to cell protein used by Krokan *et al.* is much higher than the ratio used in this study. Our findings corroborate with results reported by Hornsby and Harris [32], who showed that Chp, although damaging DNA to some extent, is not detectably mutagenic. Hornsby and Harris conclude that oxidative damage is not a determinant of cellular senescence.

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REFERENCES

1 L. Hayflick and P.S. Moorhead, The serial cultivation of human diploid cell strains. *Exp. Cell. Res.*, *25* (1961) 585–621.
2 J.P. Jacobs, C.M. Jones and J.P. Baille, Characterisation of a human diploid cell strain designated MRC-5. *Nature*, *227* (1970) 168–170.
3 W.W. Nichols, D.G. Murphy, V.J. Cristofalo, L.H. Toji, A.E. Greene and S.A. Dwight, Characterisation of a new human diploid cell strain, IMR-90. *Science*, *196* (1977) 60–63.
4 M. Raes and J. Remacle, Ageing of hamster embryo fibroblasts as the result of both differentiation and stochastic mechanisms. *Exp. Gerontol.*, *18* (1983) 223–240.
5 D.L. Wilson, In M. Rockstein, M.L. Sussman and J. Chesky (eds.), *Theoretical Aspects of Aging*, Academic Press, New York, 1974.
6 D. Harman, Aging: A theory based on free radical radiation chemistry. *J. Gerontol.*, *11* (1956) 289–300.
7 B. Chance, H. Sies and A. Boveris, Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.*, *59* (1979) 527–605.
8 H. Nohl and D. Hegner, Do mitochondria produce oxygen radicals *in vivo*? *Eur. J. Biochem.*, *82* (1978) 563–567.
9 H. Sies and B. Chance, The steady state level of catalase compound I in isolated hemoglobin-free perfused rat liver. *FEBS Lett.*, *11* (1970) 172–176.
10 S.D. Aust, L.A. Morehouse and G.E. Thomas, Role of metals in oxygen radical reactions. *J. Free Rad. Biol. Med.*, *1* (1985) 3–25.

11 B.A. Freeman and B. D. Crapo, Biology of disease. Free radical and tissue injury. *Lab. Invest., 47* (1982) 412–426.
12 W.A. Pryor, The role of free radical reactions in biological systems. In W. A. Pryor (ed.), *Free radicals in biology*, Vol. 1, Academic Press, New York, 1976, pp. 1–49.
13 H. Sies and K.-H. Summer, Hydroperoxide metabolizing systems in rat liver. *Eur. J. Biochem. 57* (1975) 503–512.
14 H. Sies, C. Gerstenecker, H. Menzel and L. Flohe, Oxidation in the NADP system and release of GSSG from hemoglobin-free perfused rat liver during peroxidatic oxidation of glutathione by hydroperoxides. *FEBS Lett., 27* (1972) 171–175.
15 E. Chiarpotto, G. Poli, E. Albano, E. Gravela and M.U. Dianzani, Studies on fatty liver with isolated hepatocytes. III. Cumene hydroperoxide-induced change of several cell functions. *Exp. Mol. Pathol., 41* (1984) 191–201.
16 J. Shimura, F. Shimura and N. Hosoya, Functional disability of rat splenocytes provoked to lipid peroxidation by cumene peroxide. *Biochim. Biophys. Acta, 845* (1985) 43–47.
17 A. Benedetti, H. Esterbauer, M. Ferrali, R. Fulceri and M. Comporti, Evidence for aldehydes bound to liver microsomal protein following CCl_4 or $BrCCl_3$ poisoning. *Biochim. Biophys. Acta, 711* (1982) 345–356.
18 M. Poot, A. Verkerk, J.F. Koster, H. Esterbauer and J.F. Jongkind, Influence of cumene hydroperoxide and 4-hydroxy-nonenal on the glutathione metabolism during *in vitro* of human skin fibroblasts. *Eur. J. Biochem., 162* (1987) 287–291.
19 V.T. Cristofalo and B.B. Scharf, Cellular senescence and DNA synthesis, thymidine incorporation as a measure of population age in human diploid cells. *Exp. Cell. Res., 76* (1973) 419–427.
20 M. Poot, A. Verkerk, J.F. Koster and J.F. Jongkind, *De novo* synthesis of glutathione in human skin fibroblasts during *in vitro* ageing and in some metabolic diseases. *Biochim. Biophys. Acta, 883* (1986) 580–584.
21 E. Harmsen, P.Ph. De Tombe and J.W. De Jong, Simultaneous determination of myorcardial adenine mucleotides and creatinine phosphate by high-performance liquid chromatography. *J. Chromatogr., 230* (1982) 131–136.
22 D. E. Atkinson, The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. *Biochemistry, 7* (1968) 4030–4034.
23 E. Schauenstein and H. Esterbauer, In *Submolecular Biology in Cancer*, Ciba Foundation Symp. 67, Excerpta Medica, Amsterdam, 1979, pp. 225–244.
24 S. Hauptflorenz, H. Esterbauer, W. Moll, R. Pumpel, E. Schauenstein and B. Puschendorf, Effects of the lipidperoxidation product 4-hydroxy-nonenal and related aldehydes on proliferation and viability of cultured ehrlich ascites tumor cells. *Biochem. Pharmacol., 34* (1985) 3803–3809.
25 J.S. White and K.R. Rees, The mechanism of action of 4-hydroxynonenal in cell injury. *Chem.-Biol. Interact., 52* (1984) 233–241.
26 E. Wawra, H. Zollner, R.J. Schauer, H.M. Tillian and E. Schauenstein. The inhibitory effect of 4-hydroxynonenal on DNA-polymerases alpha and beta from rat liver and rapidly dividing Yoshida ascites hepatoma cells. *Cell Biol. Function, 4* (1986) 31–36.
27 M. Poot, W.J. Visser, A. Verkerk and J.F. Jongkind, Autofluorescence of human skin fibroblasts during growth inhibition and *in vitro* ageing, *Geronthology, 31* (1985) 158–165.
28 R. Sheinin, J. Humbert and R.E. Pearlman, Some aspects of eukaryotic DNA replication. *Annu. Rev. Biochem., 47* (1978) 277–316.
29 S. Seeber, B. Warnecke and W. Weser, Zur Wirkungsweise des 4-hydroxy-pentenals bei der Nukleinsaeurebiosynthese. *Z. Krebsforsch., 72* (1969) 137–143.
30 H.N. Munro, B.S. Baliga and A.W. Pronczuk, *In vitro* inhibition of peptide synthesis and GTP hydrolysis by cycloheximide and reversal of inhibition by glutathione. *Nature, 219* (1968) 944–946.
31 H. Krokan, R. C. Grafstrom, K. Sundqvist, H. Esterbauer and C. Harris, Cytotoxicity, thiol depletion and inhibition of 06-methylguanine-DNA methyltransferase by various aldhydes in cultured human bronchial fibroblasts. *Carcinogenesis, 6* (1985) 1755–1759.
32 P.J. Hornsby and S.E. Harris, Oxidative damage to DNA and replicative lifespan in adrenocortical cells. *Exp. Cell Res., 168* (1987) 203–217.

Appendix paper 3

Disturbance of cell proliferation by two model compounds of lipid peroxidation contradicts causative role in proliferative senescence

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Peter S. Rabinovitch and Holger Hoehn

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DISTURBANCE OF CELL PROLIFERATION BY TWO MODEL COMPOUNDS OF LIPID PEROXIDATION CONTRADICTS CAUSATIVE ROLE IN PROLIFERATIVE SENESCENCE

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Running head: Lipid peroxidation and cell proliferation

ABSTRACT

Cumene hydroperoxide (Chp), a lipophilic peroxide, and hydroxy-nonenal (HNE), a breakdown product of lipid peroxides, were used as model compounds to assess the effects of lipid peroxidation upon cell proliferation. Amniotic fluid fibroblast like (AFFL) cells and human diploid skin derived (HDFL) cells were cultured with the two model compounds and cell proliferation was assayed via BrdU-Hoechst flow cytometry. At low doses Chp elicited an accumulation of cells in the S and G2 phase, while at higher doses the fraction of non-proliferating cells increased as well. Low doses of HNE caused an accumulation of cells in the G1 and G2 phase, whereas an additional increase of cells in S phase and in the non-proliferating fraction was found at an elevated concentration. A delay of onset of proliferation was obtained with both Chp and HNE. Permanent arrests in the S, G2 and G1 compartment are provoked by Chp only when Chp was applied together with serum. HNE, to the contrary, elicited a permanent arrest in the G2 and the G1 compartment even if added to quiescent cell cultures. Additionally, HNE caused a combination of a prolongation of the G1 phase of the cell cycle and an arrest in this compartment, which is reminiscent of cell differentiation. HDFL cells were much more sensitive towards Chp than were AFFL cells, but both cell types showed similar sensitivities towards HNE. We conclude that lipophilic peroxides exert toxic effects upon cell proliferation distinct from the pattern elicited by aldehydic breakdown products of lipid peroxides. The pattern of cell cycle arrest induced by Chp and HNE makes it unlikely that Chp and HNE, or related products of lipid peroxidation, are responsible for the limitation of the proliferative lifespan of human fibroblasts in culture.

INTRODUCTION

In major clinical conditions like inflammation (Fantone and Ward, 1982), cancer (Troll and Wiesner, 1985), post-ischemic injury (McCord, 1985) and degenerative disease during aging (Harman, 1956; Halliwell, 1982; Balin, 1982; Melhorn and Cole, 1986) one-electron reduced species of oxygen (superoxide, hydrogen peroxide, hydroxyl radical) have been implicated to exert causative or exacerbating effects. These highly reactive species are able to attack and modify DNA (Freeman and Crapo, 1982; Filho and Meneghini, 1984; Sies, 1986; Teoule, 1987; Kasai et al., 1986; Frenkel and Chzran, 1987), proteins (Freeman and Crapo, 1982; Stadtman, 1986; Wolff et al., 1986; Vince and Dean, 1987) and lipids (Ursini et al., 1980; Halliwell, 1981; Comporti, 1985). In addition, cellular morphology (Thaw et al., 1983), calcium homeostasis (Bellomo et al., 1982), ATP levels and energy

charges (Spragg et al., 1985; Poot et al.,1988), and the glutathione system (Sies et al., 1972; Sies and Summer, 1975) may be affected by these species of reduced oxygen. The origin of all these disturbances of cell structure and function is not fully understood, but lipid peroxidation may be a major causative mechanism (Chance et al., 1979; Halliwell, 1981, Halliwell and Gutteridge, 1984; Comporti, 1985).

Lipid peroxidation involves the formation of lipophilic organic peroxides, which can decay into reactive aldehydes (Poli et al., 1985, Esterbauer et al., 1985, 1986, Comporti, 1985). The deleterious sequelae of this process, and the metabolism of its products, have been studied extensively using cumene hydroperoxide (Chp) as a model lipophilic organic peroxide (Sies et al. 1972, Sies and Summer, 1975; Hoegberg et al., 1975; Chiarpotto et al., 1984; Poot et al., 1987, 1988). A major breakdown product of lipophilic organic peroxides, 4-hydroxy-2,3-trans-nonenal (HNE) (Esterbauer et al., 1985, 1986), was found to cause a plethora of effects in cultured cells (Brambilla et al. 1986; Esterbauer et al., 1985, 1986, 1987; Poot et al. 1987, 1988). A most prominent effect of HNE is an impairment of cell proliferation (Hauptlorenz et al., 1985; Krokan et al. 1985, Poot et al., 1988). The latter effect is of particular interest in view of the free radical theory of ageing (Harman, 1956; Halliwell, 1981) and the possible role of lipid peroxidation in carcinogenesis (Krokan et al., 1985). We have, therefore, determined the effects of Chp and HNE upon cell proliferation with a novel, high resolution cell kinetic assay (BrdU-Hoechst flow cytometry).

MATERIALS AND METHODS

Test compounds

Chp was a kind gift of Shell Chemicals (Rotterdam, Netherlands). The compound was diluted with phosphate buffered saline (PBS) immediately before use. HNE was synthesized as described previously (Esterbauer and Weger, 1967). Stock solutions in dichloromethane (10 mg of HNE per ml) were prepared and stored until use at 4 ^{o}C. Aqueous solutions were prepared by evaporation of the dichloromethane and the HNE was dissolved into PBS. Its concentration was determined spectrophotometrically at 224 nm assuming a molar extinction coefficient of 13,600. Both test compounds were filter-sterilized (0.22 um, Sterivex-GS, Millipore).

Cell types and culture conditions

Papillary dermal human human diploid fibroblastlike (HDFL) cells were obtained from the skin of a healthy male donor of 7 years of age (Harper and Grove, 1979). Amniotic fluid fibroblast like (AFFL) cells (Hoehn and Salk, 1982) were from a terminated diagnostic mid-trimester pregnancy and were without detectable inherited metabolic deficiencies. All cells were cultured in Eagle's Minimal Essential Medium (MEM) (Gibco, Grand Island, NY) supplemented with 10 % of pretested and heat inactivated fetal calf serum (Flow Laboratories, lot nr.29101831).

Aliquots of confluent cultures were rendered quiescent by reducing the serum concentration to 0.1 % during 48 hours. Subsequently, cells were trypsinized and resuspended in MEM supplemented with 10 % fetal calf serum and 65 uM of each bromodeoxyuridine (BrdU) and deoxycytidine (CdR). Cells were plated into 80 cm^2 cell culture flasks (Nunc, Intermed, Denmark) at a density of 2000 to 2500 cells per cm^2. All experiments were carried out in incubators with sensor-regulated CO_2 and oxygen supply (Heraeus, Hanau, F.R.G.). CO_2 was kept constant at 5 % (vol/vol). An oxygen concentration of 5 % (vol/vol) was obtained using nitrogen to replace air. Literature data (Balin et al., 1978) and our own experience (M. Poot et al., manuscript in preparation) indicate that cells suffer an artificial arrest in the G2 phase of the cell cycle if cultured at ambient oxygen.

Cells were harvested by trypsinization from 22 to 106 hours after plating and stored at -20 ^{o}C in MEM supplemented with 10 % fetal calf serum and 10 % dimethylsulfoxide, which conserves cells without the loss of certain sub-populations, nor any other detrimental effects that might interfere with flow cytometry (Rabinovitch, 1983). Care was taken to avoid any exposure to light of short wavelengths during all stages of handling.

Cell staining and flow assay

After thawing, cell pellets were resuspended in staining buffer containing 1.2 µg Hoechst 33258 and 2.0 µg Ethidium Bromide per ml of buffer (Rabinovitch et al., 1988). Flow cytometric analysis was carried out with an epiillumination flow system of conventional design (ICP 22, Ortho Diagnostic Systems, Raritan, NJ). Two dimensional plots of BrdU-quenched Hoechst (blue) and the non-quenched ethidium bromide (red) fluorescence were collected by a PDP11/23 microcomputer (Digital Equipment Corp., Maynard, MA). Each assay comprised at least 60,000 cells, which gives a standard deviation in the compartment distributions of 0.40 %. By electronic framing, rotation and deconvolution, each of the three component cell cycles were obtained from the bivariate plot. Each histogram was then analysed by automated curve-fitting as described (Rabinovitch et al., 1988). The cell numbers in each cell cycle compartment were normalized to the percent of original cells by correction for the number of cell divisions. These data were then plotted semilogarithmically as a function of time after serum stimulation and fitted to a modified version of the Smith-Martin model (Smith and Martin, 1973) of exponential cell cycle compartment exit (Rabinovitch, 1983).

Univariate DNA flow cytometry was performed on samples stained during 30 minutes with the same staining buffer as above, containing only 2 µg DAPI (4,6'-Diamidino-2-phenylindole) per ml of buffer. Assays were performed with a similar ICP 22 instrument linked to a PDP 11 microcomputer. Data were stored and processed as described above.

RESULTS

Disturbance of cell cycle distribution by Chp and HNE

The BrdU-Hoechst method for cell cycle kinetics allows the distinction between cells in the first, second or third cell cycle after serum stimulation of quiescent cells. In figure 1 typical bivariate dot plots of HDFL cells stained with Hoechst 33258 (abscissa) and with ethidium bromide (ordinate) are shown. The quenching of Hoechst fluorescence by BrdU allows to discern cells that have incorporated the base analog during one, two or three rounds of DNA synthesis. The ethidium bromide fluorescence, not being quenched by incorporated BrdU, differentiates cells in the G1, S and G2 compartments within each of the three consecutive cell cycles. When serum stimulation was done in the presence of 10 uM of either Chp or HNE, the projections of the dot plots on the Hoechst axis clearly show that, as compared to controls, cells accumulate in the G2 phase of the first cycle and in the G1 phase of the second cycle (figure 1). The subtle, quantitative differences between the patterns obtained with Chp and HNE may be explained by differential sensitivity of the cells towards the two model compounds. On the other hand, Chp and HNE might provoke two qualitatively different patterns of cell cycle disturbance. In order to distinguish between these two possibilities, cells were incubated with a concentration series of Chp or HNE.

Figure 2 depicts the cell cycle distribution of quiescent HDFL cells stimulated to proliferation in the presence of a concentration series of Chp or HNE. The curves denote subsequently the proportion of cells in the G0/G1, S, G2 of the first cycle, and in the G1, S and G2 phase of the second cycle after serum stimulation, and so on. An increase in distance between two curves with increasing dose indicates an accumulation of cells in the particular cell cycle compartment. Thus, Chp causes an accumulation in S and G2 phase of the first cell cycle and in the G1 phase of the second cell cycle. At higher doses of Chp the fraction of G0/G1 cells, which fails to incorporate BrdU, inceases as well. Consequently, the fraction of cells in the second cell cycle decreases, as most cells remain locked into the first cycle. HNE to the contrary, elicits a clear rise in the fraction of cells retained in the G2 phase of the first cell cycle and in the G1 of the second cycle, while the fraction of cells in the S phase increases only slightly. The number of cells not responding to serum stimulation increases as well with increasing doses of HNE. These data reveal that the mechanism of inhibition of cell proliferation of the two model compounds is distinct, suggesting a different mode of action of lipophihlic peroxides and reactive aldehydes.

Cell kinetic effects of Chp and HNE

To analyse the cell kinetic pattern of disturbance of cell activation and cell cycling by Chp and HNE, quiescent cultures of HDFL and AFFL cells were stimulated to proliferation in the presence of 10 µM of either Chp or HNE and sampled from 22 to 106 hours after serum stimulation. At this concentration both test compounds inhibited incorporation of radiolabelled thymidine to some 50 % of control level (Poot et al., 1988). In the incubation system used here, half of the added Chp or HNE will be metabolised by the cells in approximately 20 days. The present experiment resolves minimal durations and transition probabilities for each cell cycle compartment, and determines the fraction of cells that is permanently arrested in a particular cell cycle compartment. Figure 3 shows histograms of cell cycle distributions obtained for each time point. The individual curves denote exit of cells from each compartment; the first being exit from G0, or release from quiescence, the second exit from S, the third exit from G2, and so on. If the linear part of the curve is extrapolated to 100 % cells, the intercepts give the mininmal duration of the cell cycle compartments. The plateaus on the right hand side to which the curves approach yield the fraction of cells irreversibly arrested in each particular compartment. From the exponential part of the curve the transition probability is computed. This parameter is subsequently used to calculate the mean duration of each cell cycle compartment (Rabinovitch, 1983).

The exit kinetic curves displayed in figure 3 show different patterns regarding the two model compounds and the two cell types used. Chp causes a slightly prolonged G0/G1 transit and a more shallow decay in the exponential part of the curves for the first cycle. The distance between the plateau for the S and the G2 compartment is higher than in the control panel. The fraction of cells refractive to serum stimulation (G0/G1), and the number of cells arrested in G1 of the second cycle rises as well, but to a lesser extent. These results are in keeping with the observations in figure 2A. HNE, to the contrary, provokes a clear delay in G0/G1 transit, and a rapid rate of exit from G0/G1 and S phase, but a diminished rate of exit from G2. Cells incubated with HNE also exhibit a larger fraction retained in G0/G1 and show an elevated arrest in the S and G2 phase of the first cycle and in the G1 phase of the second cycle. The pattern of perturbed cell proliferation over time provoked by Chp and HNE is clearly distinct, as was already inferred from the dose response curves (figure 2). AFFL cells display an only slightly perturbed pattern of proliferation if incubated with Chp, whereas their response to HNE is comparable to the response shown by HDFL cells.

Table 1 shows the minimal duration of each cell cycle compartment as computed from the curves of figure 3, whereas table 2 exhibits the mean durations of each compartment. In table 3 the fraction of cells arrested in each cell cycle

compartment as provoked by Chp and HNE is listed. These computed data further support the conclusions drawn from inspection of the curves. A rather subtle effect of HNE is a prolongation of the minimal duration of the G1 phase of the second cycle in HDFL and AFFL cells by 10.1 and 2.8 hours respectively. Chp, on the other hand, causes a minor increase in the minimal duration of the G2 phase and a concomitant decrease in the duration of the G1 phase of the next cycle.

Disturbance of cell proliferation by Chp and HNE is dependent upon cell cycle stage during exposure

The complex pattern of disturbed cell proliferation elicited by Chp and HNE might result from an effect provoked during a specific cell cycle stage. To analyse this point, cells were incubated with Chp or HNE during quiescence only, during early activation before onset of DNA replication, or permanently. This scheme allows to decide whether a particular type of disturbance of cell activation and cell cycling is reversible or not, and whether it requires expression of an event associated with G0/G1 traverse.

Figure 4A and 4B shows the exit kinetics obtained with this incubation scheme for Chp and HNE, respectively. The pattern obtained by incubation with Chp during quiescence is similar to the pattern of the control incubation (Fig. 4A). If Chp is added to cells together with serum it provokes a delay in onset of proliferation of 11.6 hours and a prolongation of the G2 phase. The fraction of non-proliferating cells and the fraction arrested in the S, G2 and the G1 phase of the cell cycle increases as well. If Chp is present continuously during serum stimulation a similar delay of activation (12.2 hours), prolongation of the G2 phase and comparable arrest fractions are obtained. So, Chp does not require onset of DNA synthesis but acts already during early activation of cells.

The pattern provoked by HNE is distinct from the one caused by Chp (Fig. 4B). HNE caused minor increases in the fraction of not activated cells (G0/G1) and in the fraction of cells arrested in the S and G1 phase if supplied during early activation, but these fractions of arrested cells increase much more if HNE is present continuously. The arrest in the G2 phase increases already slightly if HNE is given during early activation, but rises more during continuous exposure. The duration of the G2 phase is not significantly prolonged upon exposure to HNE, but addition to quiescent cells causes a clear prolongation of the G1 phase of the second cycle. This prolongation is exacerbated if HNE is given together with serum and leads at continuous exposure to a 10.1 hour increase in the minimal duration of the G1 phase. This pattern reflects the induction of cell quiescence after one round of cell cycling, rather than arrest during cell cycling.

Disturbances of cell activation and cycling do not result from a synergism between BrdU and Chp or HNE

It can be envisaged that the effects upon cell activation and cell cycling of Chp and HNE obtained with the BrdU-Hoechst method are mediated incorporation of BrdU. To rule out this possibility, quiescent cells were serum stimulated in the presence of 10 µM Chp or HNE and a concentration series of BrdU ranging from 0 to 200 µM. After 72 hours of growth, cell numbers from three independent cultures were counted in quadruplicate in a hemocytometer and cell cycle distribution were recorded in duplicate via univariate DNA flow cytometry. If no synergism between BrdU and the test compounds occurs, the same cell numbers and the same cell cycle distribution regardless of BrdU concentration should be found.

The growth rate in the presence of either Chp or HNE was some 50 % of the control growth rate irrespective of the BrdU concentration used (results not shown). The cell cycle distributions also show constant G1 and G2 fractions at all BrdU concentrations tested (results not shown). We conclude that neither Chp nor HNE show synergistic effects with BrdU.

DISCUSSION

If free radical reactions can be shown to irreversibly affect cell proliferation, they are likely to be in some way involved in the limitation of the cellular proliferative lifespan (Hayflick and Moorhead, 1961: Jacobs et al., 1970; Martin et al., 1970; Nichols et al., 1977). This paper reports disturbances of cell activation and cell cycling induced by two model compounds of lipid peroxidation. Both Chp and HNE elicit a prolonged G0/G1 transit if applied together with serum. As to other effects, both model compounds are unexpectedly divergent. Chp causes permanent arrests of cells in cell cycle compartments only in the presence of serum, whereas HNE provokes an arrest in G2 even if applied to quiescent, serum deprived cells. In both quiescent and in serum stimulated cells, HNE elicits a prolongation of the G1 phase and an arrest in this compartment. This pattern indicates that HNE might cause proliferating cells to enter a quiescent state. Chp does not share this property with HNE. The mechanism by which both compounds exert their effects is clearly distinct. This indicates that lipophilic organic peroxides are toxic to cell proliferation independent of the putative formation of reactive aldehydes. Our results suggest that specific products of lipid peroxidation are in some way involved in the regulation of cellular proliferation and differentiation, as suggested in the literature (Cheeseman et al., 1986).

Some controversy exists as to the reversibility of the effects of biogenic aldehydes on cell proliferation and the importance of lipid peroxidation for the limitation of cellular proliferation. An irreversible reduction in cloning efficiency upon exposure to HNE has been reported (Krokan et al., 1985), but also a partially reversible decrease in incorporation of radiolabelled thymidine (Poot et al. 1988). The BrdU-Hoechst method allows us to see how cells proliferate during three consecutive cell cycles, and thus might enable us to resolve this controversy. Quiescent or growth stimulated cells incubated with HNE invariably show reduced rates of proliferation, mainly resulting from a delay of onset of proliferation and from an arrest in the G1 and the G2 compartments of the cell cycle. This is in keeping with the reduced clone forming ability reported (Krokan et al., 1985). In the present data set, HNE causes an irreversible arrest of cells in the G1 and in the G2 compartments of the cell cycle, which would be consistent with cells incorporating radiolabelled thymidine, but inconsistent with clone formation. This means that both methods are right and observe the same effect of HNE, but the contradictory conclusions derive from the differences in methodology. The present experiment clearly shows that HNE causes an irreversible inhibition of cell proliferation.

This result does not imply that the free radical theory of ageing is conclusively proven. During ageing in culture, cells exit from the G1 phase of the cell cycle (Yanishevsky

et al., 1974; Grove and Cristofalo, 1977; Gorman and Cristofalo, 1986), a pattern reminiscent of cell differentiation. Chp and HNE both cause an exit from the G1 phase, but in addition an exit from G2. Hence, if ageing of cells in culture were to result from lipid peroxidation, cells would have to accumulate in G2. Therefore, the exit from continuous cycling during serial propagation of cells in culture is unlikely to result from lipid peroxidation induced by oxygen free radicals.

REFERENCES

Balin, A.K., Goodman, D.G., Rasmussen, H. and Cristofalo, V.J. (1978) Oxygen sensitive stages of the cell cycle of human diploid cells, J.Cell Biol. 78, 390-400.

Balin, A.K. (1982) Testing the free radical theory of ageing, In: Testing the theories of ageing, Eds. R.S. Adelman and G.S. Roth, pp. 137-182, CRC Press Inc., Boca Raton.

Bellomo, G., Jewell, S.A., Thor, H. and Orrenius, S. (1982) Regulation of intracellular calcium compartmentation: Studies with isolated hepatocytes and t-butyl-hydroperoxide, Proc.Natl.Acad.Sci.USA 79, 6842-6846.

Brambilla, G., Sciaba, L., Faggin, P., Maura, A., Marinari, U.M., Ferro, M. and Esterbauer, H. (1986) Cytotoxicity, DNA fragmentation and sister-chromatid exchange in chinese hamster ovary cells exposed to the lipid peroxidation product 4-hydroxynonenal and homologous aldehydes, Mut.Res. 171, 169-176.

Chance, B., Sies, H. and Boveris, A. (1979) Hydroperoxide metabolism in mammalian organism, Physiol.Rev. 59, 527-605.

Cheeseman, K.H., Collins, M., Proudfoot, K., Slater, T.F., Burton, G.W., Webb, A.C. and Ingold, K.U. (1986) Studies on lipid peroxidation in normal and tumour tissues. The Novikoff rat liver tumour, Biochem.J. 235, 507-514.

Chiarpotto, E., Poli, G., Albano, E., Gravela, E. and Dianzani, M.U. (1984) Studies on fatty liver with isolated hepatocytes. III Cumene hydroperoxide-induced changes of several cell functions, Exp.Mol.Pathol. 41, 191-201.

Comporti, M. (1985) Biology of disease, lipid peroxidation and cellular damage in toxic liver injury, Lab.Invest. 53, 599-623.

Esterbauer, H. and Weger, W (1967) Ueber die wirkung von aldehyden auf gesunde und maligne zellen. 3. Mitteilung: Synthese von homologen 4-hydroxy-2-alkenalen, Monatsh.Chem. 98, 1994-2000.

Esterbauer, H., Zollner, H. and Lang, J. (1985) Metabolism of the lipid peroxidation product 4-hydroxynonenal by isolated hepatocytes and by liver cytosolic fractions Biochem.J. 228, 363-373.

Esterbauer, H., Benedetti, A., Lang, J., Fulceri, R., Fauler, G. and Comporti, M. (1986) Studies on the mechanism of formation of 4-hydroxynonenal during microsomal lipid peroxidation, Biochim.Biophys.Acta 876, 154-166.

Esterbauer, H., Juergens, G., Quehenberger, O. and Koller, E. (1987) Autoxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes, J.Lipid Res. 28, 495-509.

Fantone, J.C. and Ward, P.A. (1982) Role of oxygen-derived free radicals and metabolites in leukocyte-dependent inflammatory reactions, Am.J.Pathol. 107, 394-418.

Filho, A.C.M. and Meneghini, R. (1984) In vivo formation of single-strand breaks in DNA by hydrogen peroxide is mediated by the Haber-Weiss reaction, Biochim.Biophys.Acta 781, 56-63.

Freeman, B.A. and Crapo, J.D. (1982) Biology of disease, free radicals and tissue injury, Lab.Invest. 47, 412-426.

Frenkel, K. and Chrzan, K. (1987) Hydrogen peroxide formation and DNA base modification by tumor promoter-activated polymorphonuclear leukocytes, Carcinogenesis, 8, 455-460.

Gorman, S.D. and Cristofalo, V.J. (1986) Analysis of the G1 arrest position of senescent WI-38 cells by quinacrine dihydrochloride nuclear fluorescence. Evidence for a late G1 arrest, Exp.Cell Res. 167, 87-94.

Grove, G.L. and Cristofalo, V.J. (1977) Characterization of the cell cycle of cultured human diploid cells: effects of aging and hydrocortisone, J.Cell.Physiol. 90, 415-422.

Halliwell, B. and Gutteridge, J.M.C. (1984) Oxygen toxicity, oxygen radicals, transition metals and disease, Biochem.J. 219, 1-14.

Halliwell, B. (1981) Free radicals, oxygen toxicity and aging, In: Age Pigments, pp. 1-62, Ed. R.S. Sohal, Elsevier/North-Holland Biomedical Press, Amsterdam.

Harman, D. (1956) Aging: a theory based on free radical and radiation chemistry, J.Gerontol. 11, 298-300.

Harper, R.H. and Grove, G. (1979) Human skin fibroblasts derived from papillary and reticular dermis: differences in growth potential in vitro, Science 204, 526-527.

Hayflick, L. and Moorhead, P.S. (1961) The serial cultivation of human diploid cell strains, Exp.Cell Res. 25, 585-621.

Hauptlorenz, S., Esterbauer, H., Moll, W., Puempel, R., Schauenstein, E. and Puschendorf, B. (1985) Effects of the lipidperoxidation product 4-hydroxynonenal and related aldehydes on proliferation and viabiblity of cultured ehrlich ascites tumor cells, Biochem.Pharmacol. 34, 3803-3809.

Hoegberg, J., Orrenius, S. and O'Brien, P.J. (1975) Further studies on lipid-peroxide formation in isolated hepatocytes, Eur.J.Biochem. 59, 449-455.

Hoehn, H. and Salk, D. (1982) Morphological and biochemical heterogeneity of amniotic cells in culture, Methods in Cell Biol. 26, 11-34.

Jacobs, J.P., Jones, C.M. and Baille, J.P. (1970) Characterisation of a human diploid cell strain designated MRC-5, Nature 227, 168-179.

Kasai, H., Crain, P.F., Kuchino, Y., Nishimura, S. Ootsuyama and Tanooka, H. (1987) Formation of 8-hydroxyguanine moiety in cellular DNA by agents producing oxygen radicals and evidence for its repair, Carcinogenesis 7, 1849-1851.

Krokan, H., Grafstrom, R.C., Sundqvist, K., Esterbauer, H. and Harris, C.C. (1985) Cytotoxicity, thiol depletion and inhibition of O6-methylguanine-DNA methyltransferase by various aldehydes in cultured human bronchial fibrboblasts, Carcinogenesis 6, 1755-1759.

McCord, J.M. (1985) Oxygen-derived free eadicals in postischemic tissue injury, N.E.J.Med. 312, 159-163.

Martin, G.M., Sprague, C.A. and Epstein, C.J. (1970) Replicative lifespan of cultivated human cells. Effects of donor's age, tissue and genotype, Lab.Invest. 23, 86-92.

Melhorn, R.J. and Cole, G. (1985) The free radical theory of aging: A critical review, Adv.Free Radical Biol.Med. 1, 165-223.

Nichols, W.W., Murphy, D.G., Cristofalo, V.J., Toji, L.H., Greene, A.E. and Dwight, S.A. (1977) Characterisation of a new diploid cell strain, IMR-90, Science 196, 60-63.

Poli, G., Dianzani, M.U., Cheeseman, K.H., Slater, T.F., Lang, J. and Esterbauer, H. (1985) Separation and characterization of the aldehydic products of lipid peroxidation stimulated by carbon tetrachloride or ADP-iron in isolated rat hepatocytes and rat liver microsomal suspensions, Biochem.J. 227, 629-638.

Poot, M., Verkerk, A., Koster, J.F., Esterbauer, H. and Jongkind, J.F. (1987) Influence of cumene hydroperoxide and 4-hydroxy-nonenal on the glutathione metabolism during in vitro ageing of human skin fibroblasts, Eur.J.Biochem. 162, 287-291.

Poot, M., Verkerk, A., Koster, J.F., Esterbauer, H. and Jongkind, J.F. (1988) Reversible inhibition of DNA and protein synthesis by cumene hydroperoxide and 4-hydroxy-nonenal, Mech.Ageing Dev. 43, 1-9.

Rabinovitch, P.S. (1983) Regulation of human fibroblast growth by both noncycling cell fraction and transition probability is shown by growth in 5-bromodeoxyuridine followed by Hoechst 33258 flow cytometry, Proc.Natl.Acad.Sci.USA 80, 2951-2955.

Rabinovitch, P.S., Kubbies, M., Chen, Y.M., Schindler, D. and Hoehn, H. (1988) BrdU-Hoechst flow cytometry: A unique tool for quantitative cell cycle analysis, Exp.Cell Res. 174, 309-318.

Sies, H. (1986) Biochemistry of oxidative stress, Angew.Chem.Int.Ed.Engl. 25, 1058-1071.

Sies, H., Gerstenecker, C., Menzel, H. and Flohe, L. (1972) Oxidation in the NADP system and release of GSSG from hemoglobin-free perfused rat liver during peroxidatic oxidation of glutathione by hydroperoxides, FEBS Letters 27, 171-175.

Sies, H. and Summer, K.-H. (1975) Hydroperoxide-metabolizing systems in rat liver, Eur.J.Biochem. 57, 503-512.

Smith, J.A. and Martin, L. (1973) Do cells cycle ? Proc.Natl.Acad.Sci.USA 70, 1263-1267.

Spragg, R.G., Hinshaw, D.B., Hyslop, P.A., Schraufstatter, I.U. and Cochrane, C.G. (1985) Alterations in adenosine triphosphate and energy charge in cultured endothelial and P388D1 cells after oxidant injury, J.Clin.Invest. 76, 1471-1476.

Stadtman, E.R. (1986) Oxidation of proteins by mixed-function oxidation systems: implication in protein turnover, ageing and neutrophil function, Trends Biochem.Sci. 11, 11-12.

Téoule, R. (1987) Radiation-induced DNA damage and repair, Int.J.Radiat.Biol. 51, 573-589.

Thaw, H.H., Hamberg, H. and Brunk, U.T. (1983) Acute and irreversible injury following exposure of cultured cells to reactive oxygen metabolites, Eur.J.Cell Biol. 31, 46-54.

Troll, W. and Wiesner, R. (1985) The role of oxygen radicals as a possible machanism of tumor promotion, Annu.Rev.Pharmacol.Toxicol. 25, 509-528.

Ursini, F., Bindoli, A., Cavallini, L. and Maiorino, M. (1980) Hydrogen peroxide and singlet oxygen in lipid peroxidation, In: Biological and clinical aspects of superoxide and superoxide dismutase, pp.98-105, Eds. W.H. Bannister and J.V. Bannister, Elsevier/North-Holland, New York.

Vince, G.S. and Dean, R.T. (1987) Is enhanced free radical flux associated with increased intracellular proteolysis, FEBS Letters 216, 253-256.

Wolff, S.P., Garner, A. and Dean, R.T. (1986) Free radicals, lipids and protein degradation, Trends Biochem.Sci. 11, 27-31.

Yanishevsky, R., Mendelsohn, M.L., Mayal, B.H. and Cristofalo, V.J. (1974) Proliferative capacity and DNA content of aging human diploid cells in culture: A cytophotometric and autoradiographic analysis, J.Cell.Physiol. 84, 165-170.

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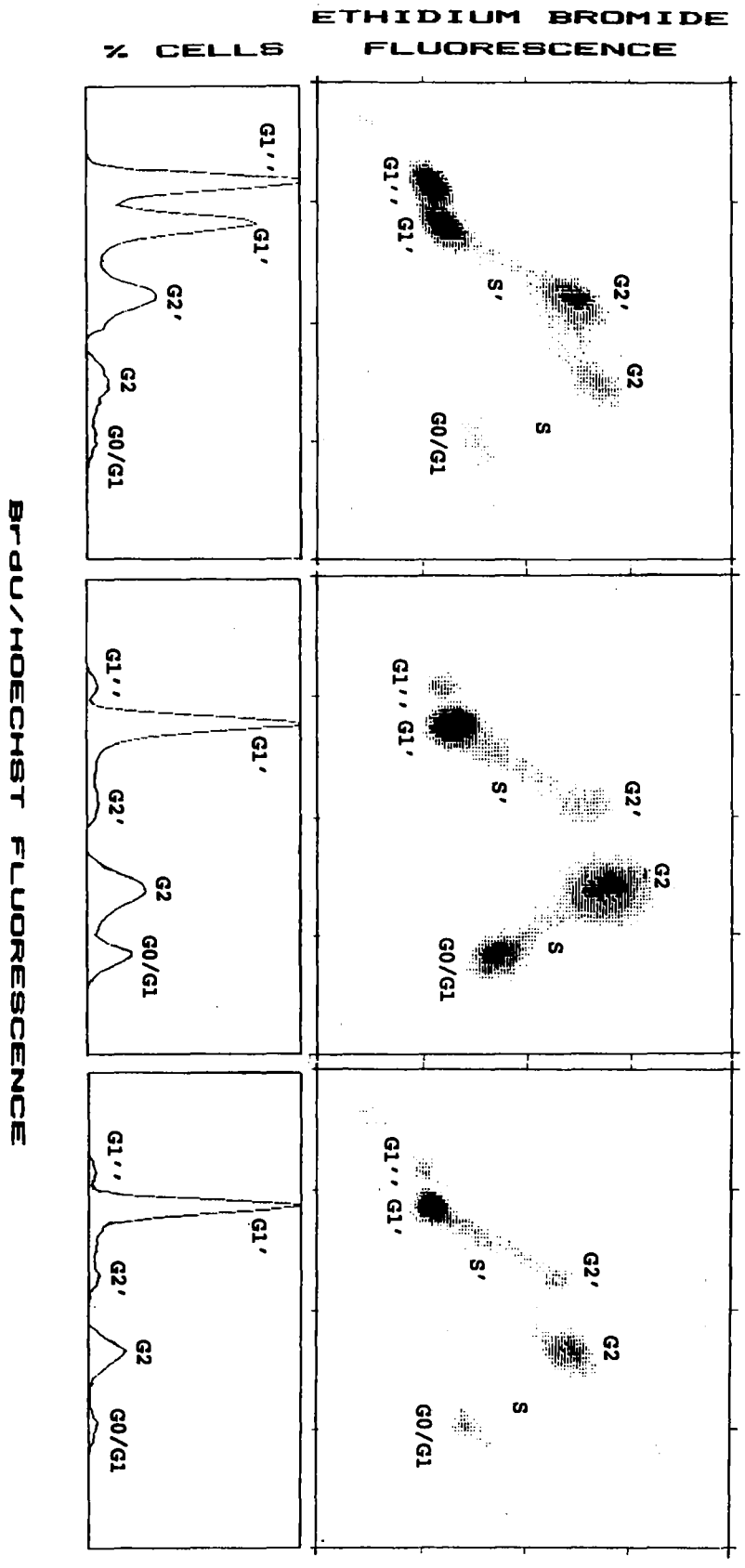
LEGENDS TO THE FIGURES

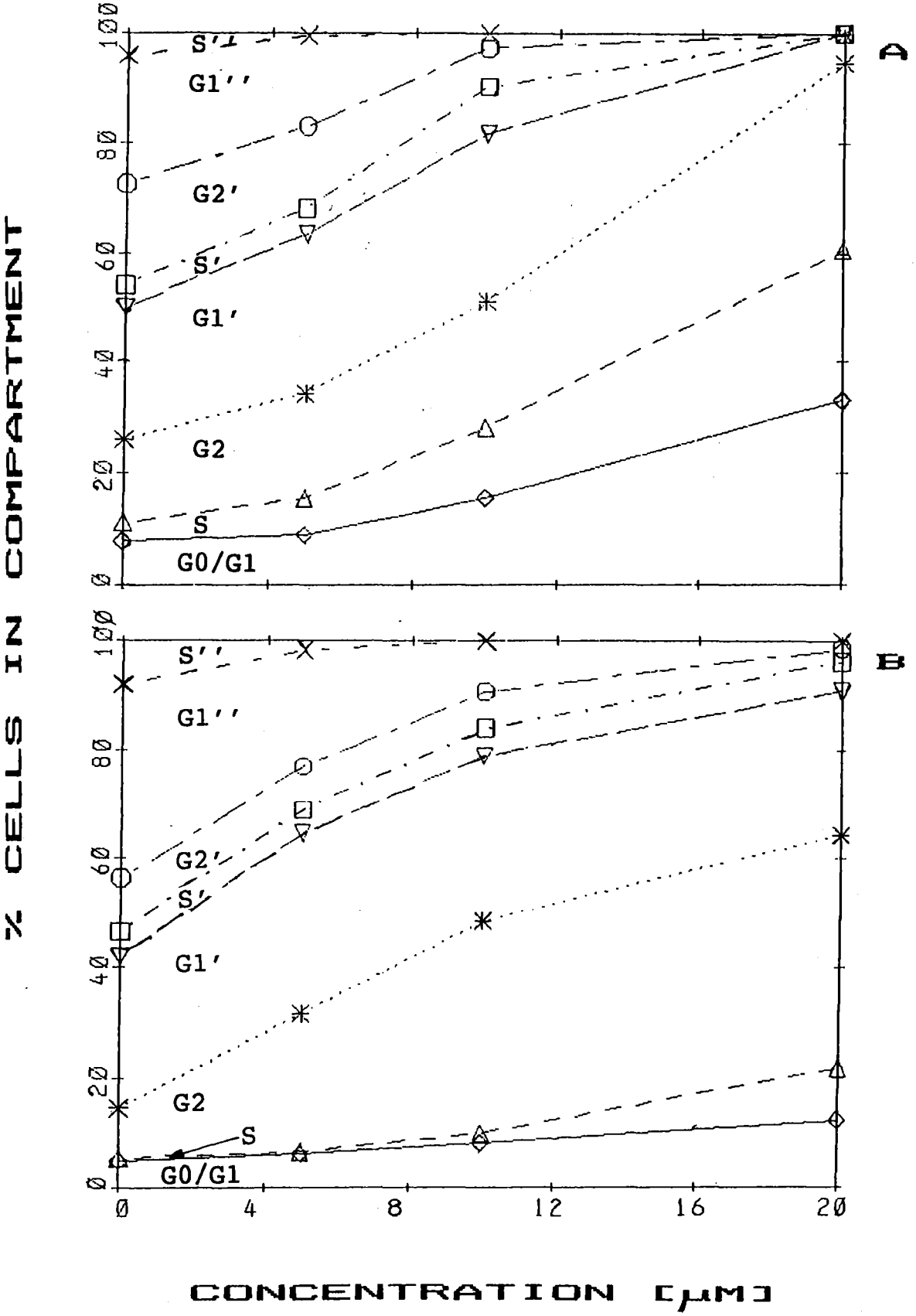
FIGURE 1: Bivariate distribution of HDFL cells cultured during 70 hours with Chp or HNE. Abscissa: BrdU-quenched Hoechst fluorescence and Ordinate: non-quenched ethidium bromide fluorescence. A, control; B, 10 µM Chp; C, 10 µM HNE. The compartments of the first cycle are labelled G0/G1, S and G2; of the second cycle G1′, S′ and G2′ and the third cycle G1′′, S′′ and G2′′.

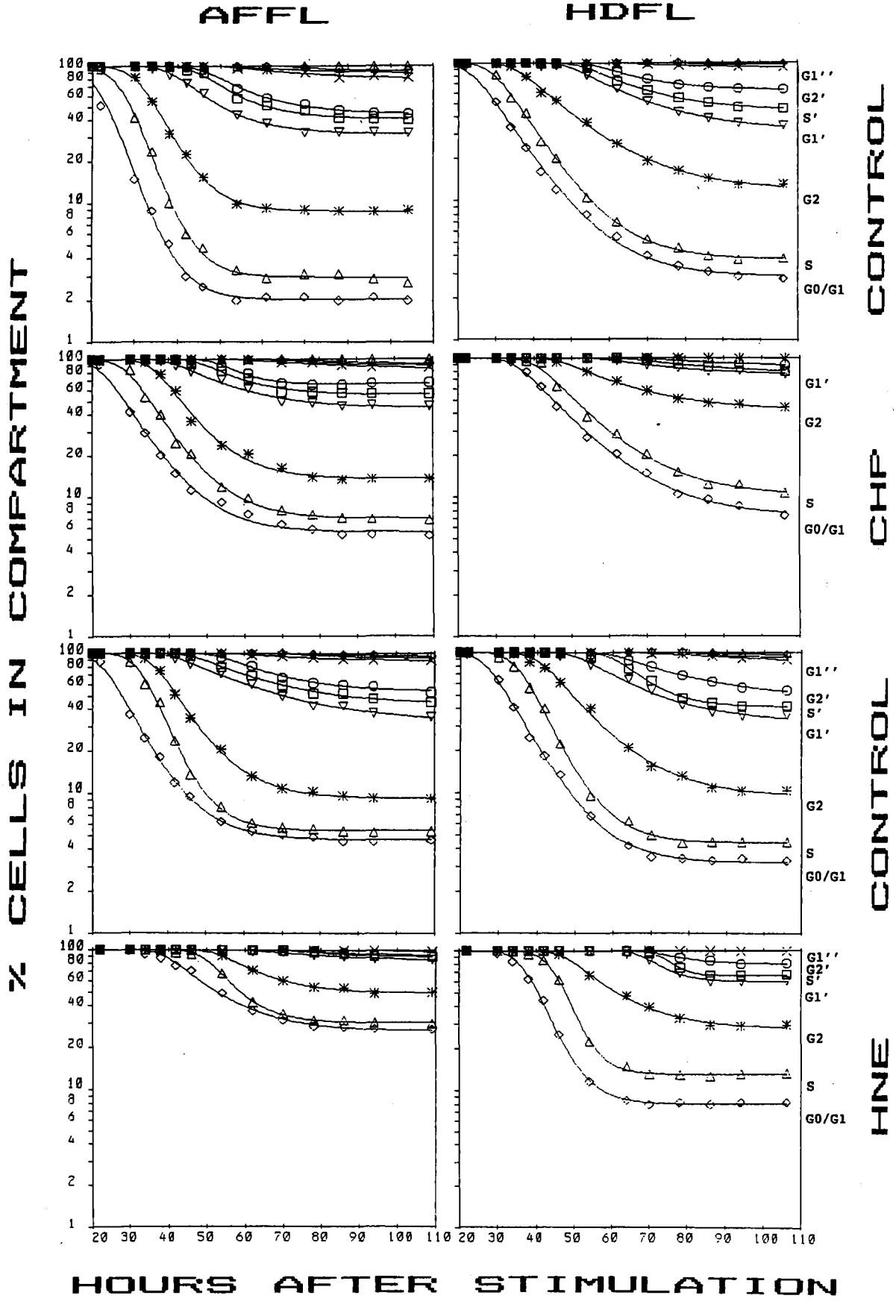
FIGURE 2: Cell cycle distribution of quiescent HDFL activated in the presence of a concentration series of Chp (A) or HNE (B) and grown during 72 hours. The subsequent curves denote G0/G1, S, G2, G1′, S′ compartment and so on. Parallel curves indicate no accumulation of cells in a particular cell cycle compartment and increasing distance between two curves denotes accumulation of cells with increasing concentration of the model compound.

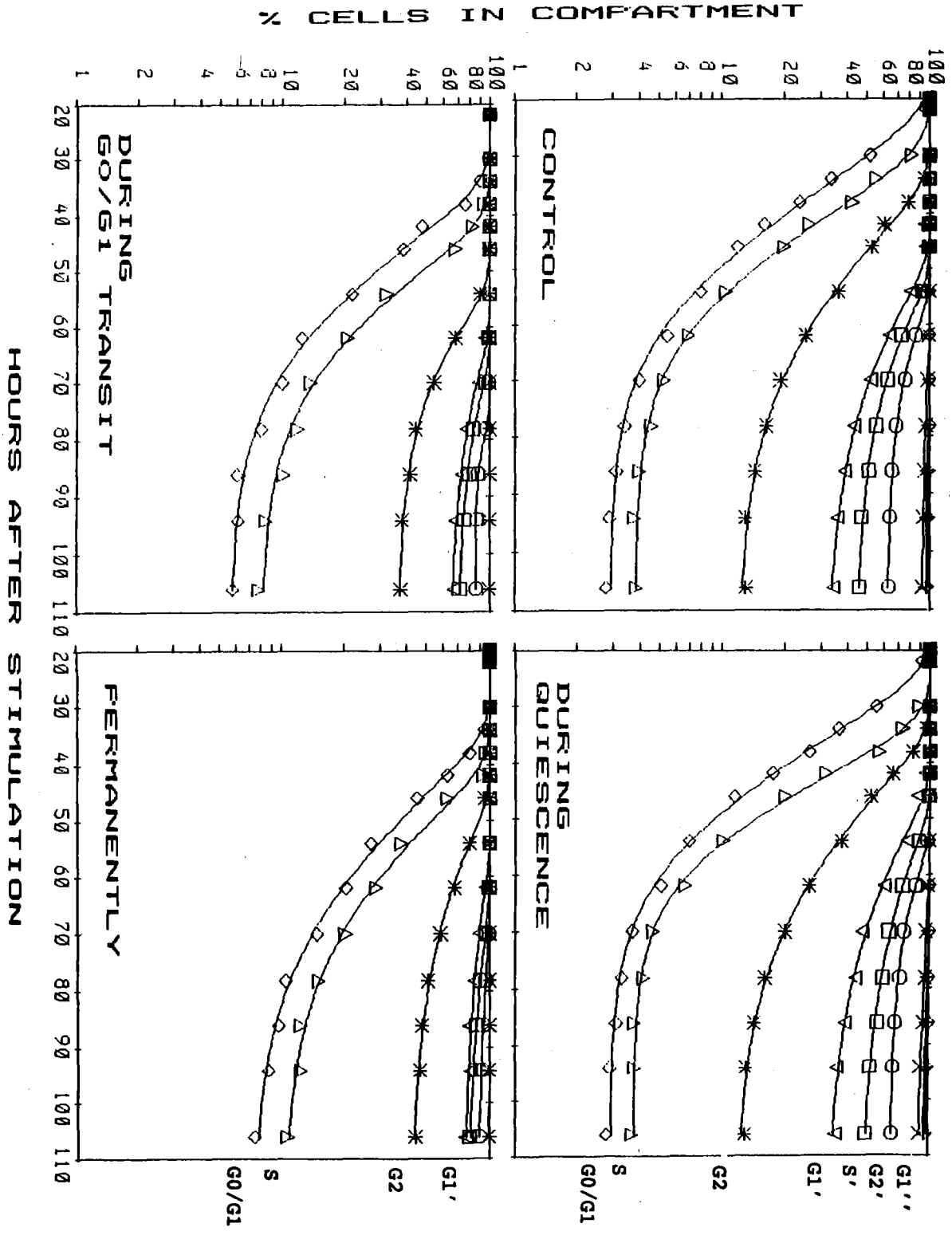
FIGURE 3: Exit kinetics of AFFL (left column) and HDFL (right column) cells exposed to Chp or HNE. The subsequent curves denote exit from G0/G1 (quiescence), S, G2, G1′, S′, and so on.

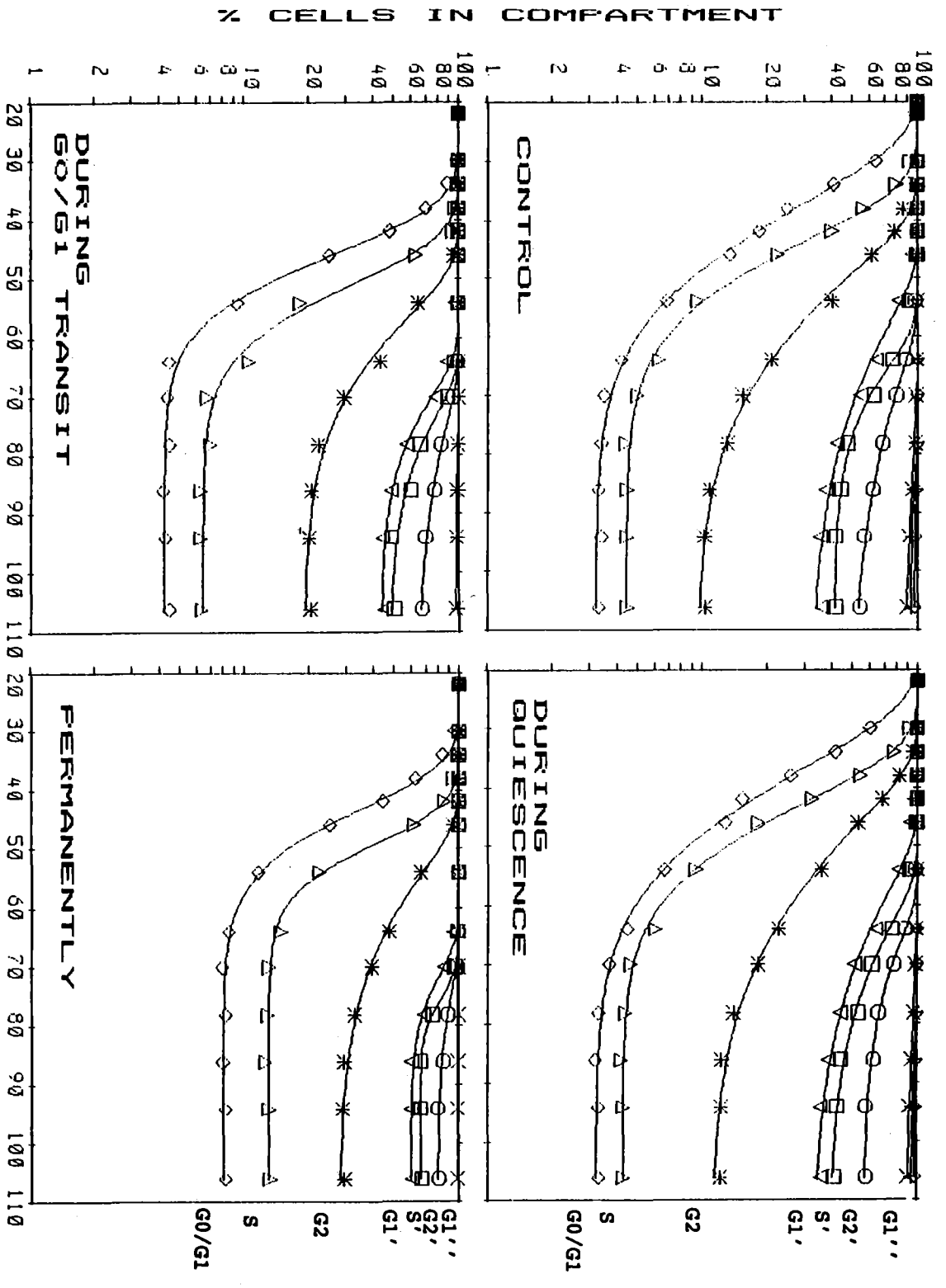
FIGURE 4: Exit kinetics of HDFL cells cultured with Chp (Panel A) or with HNE (Panel B) during quiescence, during the first 22 hours of activation or permanently. Curves see legend to figure 3.


CONTROL
CHP
HNE
ETHIDIUM BROMIDE FLUORESCENCE
% CELLS
BrdU/HOECHST FLUORESCENCE
G1'', G1'
S'
G2'
G2
S
G0/G1


A
B
S'1
G1''
G2'
S'
G1'
G2
S
G0/G1
S''
G1''
G2'
S'
G1'
G2
S
G0/G1
100
80
60
40
20
0
0
4
8
12
16
20
% CELLS IN COMPARTMENT
CONCENTRATION [µM]


AFFL
HDFL
% CELLS IN COMPARTMENT
CONTROL
CHP
CONTROL
HNE
G1''
G2'
S'
G1'
G2
S
G0/G1
HOURS AFTER STIMULATION


CUMENE HYDROPEROXIDE
% CELLS IN COMPARTMENT
CONTROL
DURING G0/G1 TRANSIT
DURING QUIESCENCE
PERMANENTLY
G0/G1
S
G2
G1'
S'
G2'
G1''
HOURS AFTER STIMULATION


% CELLS IN COMPARTMENT
HYDROXY-NONENAL
CONTROL
DURING G0/G1 TRANSIT
DURING QUIESCENCE
PERMANENTLY
HOURS AFTER STIMULATION
G0/G1
S
G2
G1'
S'
G2'
G1''

ADDENDUM TO APPENDIX PAPER 3

Table 4: Minimal duration of cell cycle compartments in hours

Treatment with Chp

AFFL cells

sample	cell cycle compartment			
	G0/G1	S	G2	G1'
control	17.7+/-1.5	5.5+/-0.2	4.6+/-0.8	6.6+/-0.4
during quiescence	18.7+/-1.1	3.4+/-0.7	9.1+/-0.3	5.4+/-0.8
during G0/G1 transit	22.0+/-0.2	9.0+/-0.5	4.1+/-0.8	8.9+/-0.8
permanently	21.0+/-1.0	6.7+/-0.1	7.2+/-1.0	5.5+/-1.3

HDFL cells

control	21.9+/-0.6	5.9+/-0.4	6.0+/-0.5	14.5+/-1.0
during quiescence	23.1+/-0.4	8.3+/-0.4	3.5+/-0.4	10.1+/-1.6
during G0/G1 transit	33.5+/-0.6	6.7+/-0.8	12.9+/-0.6	9.8+/-1.3
permanently	34.1+/-0.6	5.4+/-0.9	6.4+/-0.7	11.1+/-1.3

Treatment with HNE

AFFL cells

sample	cell cycle compartment			
	G0/G1	S	G2	G1'
control	21.2+/-0.5	9.9+/-0.4	3.7+/-0.4	5.7+/-1.3
during quiescence	23.1+/-0.4	7.6+/-0.5	5.7+/-0.4	5.1+/-1.7
during G0/G1 transit	36.7+/-0.7	8.6+/-0.3	5.2+/-0.9	4.7+/-2.2
permanently	38.2+/-0.9	10.5+/-0.6	3.6+/-1.0	9.3+/-1.0

HDFL cells

control	25.3+/-0.4	8.2+/-0.4	5.9+/-0.7	10.3+/-1.6
during quiescence	25.0+/-0.5	7.6+/-0.5	3.7+/-0.5	13.4+/-1.4
during G0/G1 transit	36.8+/-0.4	5.4+/-1.0	5.9+/-1.5	15.5+/-1.1
permanently	35.3+/-0.3	7.6+/-0.4	3.4+/-1.1	20.8+/-1.2

Table 5: Mean duration of cell cycle compartments (hrs)

Treatment with Chp

AFFL cells

sample	cell cycle compartment			
	G0/G1	S	G2	G1'
control	22.6+/-0.1	10.5+/-0.2	5.2+/-0.2	10.4+/-0.4
during quiescence	23.4+/-0.2	6.7+/-0.2	9.2+/-0.2	10.9+/-0.6
during G0/G1 transit	27.6+/-0.2	6.8+/-0.3	7.2+/-0.5	9.7+/-1.5
permanently	27.2+/-0.3	6.7+/-0.5	7.3+/-1.4	9.7+/-2.0

HDFL cells

control	29.0+/-0.1	6.4+/-0.4	9.5+/-0.5	16.4+/-1.2
during quiescence	29.9+/-0.1	9.2+/-0.2	7.8+/-0.3	12.5+/-1.5
during G0/G1 transit	41.3+/-0.1	6.7+/-0.4	11.9+/-0.7	12.8+/-2.3
permanently	43.5+/-0.3	5.7+/-0.7	10.1+/-1.2	14.6+/-2.9

Treatment with HNE

AFFL cells

sample	cell cycle compartment			
	G0/G1	S	G2	G1′
control	26.3+/-0.2	12.1+/-0.4	5.5+/-0.5	16.0+/-1.6
during quiescence	27.3+/-0.1	7.8+/-0.3	7.0+/-0.6	18.9+/-2.0
during G0/G1 transit	41.5+/-9.2	9.2+/-0.3	5.8+/-0.3	18.7+/-1.1
permanently	46.6+/-0.6	11.9+/-0.8	15.2+/-0.4	18.4+/-0.9

HDFL cells

control	31.1+/-0.1	9.2+/-0.1	6.0+/-0.4	13.7+/-1.8
during quiescence	30.8+/-0.1	8.4+/-0.1	7.8+/-0.3	15.7+/-1.5
during G0/G1 transit	40.4+/-0.1	8.5+/-0.3	9.3+/-0.9	15.9+/-2.0
permanently	39.1+/-0.1	8.4+/-0.2	9.0+/-0.8	16.4+/-1.5

Table 6: Percentage of cells arrested in cell cycle compartments

Treatment with Chp

AFFL cells

sample	cell cycle compartment			
	G0/G1	S	G2	G1'
control	2.0+/-0.1	1.0+/-0.1	5.7+/-0.3	23.0+/-0.4
during quiescence	2.6+/-0.1	1.5+/-0.1	5.2+/-0.1	19.5+/-0.7
during G0/G1 transit	2.4+/-0.1	0.6+/-0.1	6.4+/-0.4	27.1+/-0.7
permanently	5.7+/-0.2	1.4+/-0.1	6.7+/-0.5	30.5+/-1.4

HDFL cells

control	2.9+/-0.1	0.9+/-0.1	8.2+/-0.4	19.4+/-1.3
during quiescence	2.9+/-0.1	0.9+/-0.1	8.2+/-0.3	21.7+/-2.0
during G0/G1 transit	5.7+/-0.2	2.4+/-0.4	28.4+/-0.6	28.6+/-2.6
permanently	7.2+/-0.3	3.1+/-0.7	31.1+/-1.0	32.4+/-3.1

Treatment with HNE

AFFL cells

sample	cell cycle compartment			
	G0/G1	S	G2	G1'
control	4.6+/-0.1	0.8+/-0.1	3.9+/-0.1	22.3+/-2.0
during quiescence	4.2+/-0.1	0.4+/-0.1	7.6+/-0.2	28.7+/-3.2
during G0/G1 transit	17.0+/-0.4	2.3+/-0.2	17.1+/-0.4	36.8+/-1.9
permanently	26.7+/-0.8	4.0+/-0.4	19.0+/-0.9	31.2+/-0.6

HDFL cells

control	3.2+/-0.1	1.3+/-0.1	5.1+/-0.4	22.5+/-2.1
during quiescence	3.2+/-0.1	1.1+/-0.1	6.9+/-0.3	21.3+/-1.8
during G0/G1 transit	4.3+/-0.1	2.2+/-0.3	12.5+/-1.0	24.1+/-1.8
permanently	8.0+/-0.1	5.1+/-0.2	14.6+/-0.9	32.1+/-1.2

APPENDIX PAPER 4

ACCUMULATION OF A HIGH MOLECULAR WEIGHT GLYCOPROTEIN DURING IN VITRO AGEING AND CONTACT INHIBITION OF GROWTH

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ACCUMULATION OF A HIGH MOLECULAR WEIGHT GLYCOPROTEIN DURING *IN VITRO* AGEING AND CONTACT INHIBITION OF GROWTH

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SUMMARY

A 240 000 molecular weight protein was found to accumulate in sorted autofluorescent (AF) cells, and during growth inhibition and *in vitro* ageing of cultures of human skin fibroblasts. Vitamin E, a lipophilic free radical scavenger which suppressed completely the formation of cellular autofluorescence, did not affect the accumulation of this protein. So, this accumulation is not related to cellular autofluorescence and lipid peroxidation, the major cause of this autofluorescence. This protein was also found in cells from a patient with the Spielmeyer-Vogt syndrome with a high percentage of maximal lifespan (MLS), while it was completely absent from all cells of a patient with Werner's syndrome.

On two-dimensional gel electrophoresis the protein showed a heterogenous acidic isoelectric point (IEP) of around 5.3. Neuraminidase treatment caused the IEP of this protein to shift towards a less acidic pH value (5.85). Upon differential centrifugation of a cell homogenate the protein was found to be located in the microsomal pellet and the cytosol. Chromatography on gelatin-sepharose revealed that the protein was not fibronectin. It is concluded that in human skin fibroblasts a high molecular weight glycoprotein accumulates as a result of impaired proliferation and that this accumulation is not related to cellular lipid peroxidation.

Key words: Autofluorescence; Werner's syndrome; Spielmeyer-Vogt syndrome; Human fibroblasts; Ageing

INTRODUCTION

During *in vitro* ageing of a cell culture there is an accumulation of autofluorescent (AF) cells [1–4]. The accumulation of these AF cells is due to free radical reactions, as vitamin

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Abbreviations: AF, autofluorescent; 2D, two-dimentional; IEP, isolectric point; MLS, maximum lifespan; PBS, phosphate buffered saline; TCA, trichloric acetic acid.

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E, a lipophilic free radical scavenger, completely prevents this accumulation [4]. Free radical reactions can result in cross-linking of cellular proteins [5]. Among the many changes cells undergo during *in vitro* ageing is the accumulation of altered proteins [6].

Here we report the results of an investigation into the changes in the protein patterns of human skin fibroblasts during *in vitro* ageing. We used a 5–10% gradient SDS-polyacrylamide gel electrophoresis system, which is capable of visualising with reasonable resolution high molecular weight proteins, to compare the protein patterns of control fibroblasts of different *in vitro* ages and of cells from patients with the syndromes of Werner and of Spielmeyer-Vogt. The effect of contact inhibition of growth and the complete suppression of lipophilic free radical reactions by vitamin E upon these protein patterns was investigated. In growth inhibited and in *in vitro* aged cells a protein was seen that was absent from continuously dividing cells.

The isoelectric point (IEP) of this protein was determined using two-dimensional (2D) gel electrophoresis. Neuraminidase incubation was used to determine whether this protein contained neuraminic acid residues. The subcellular localisation of this protein was determined using differential centrifugation of a cell homoganate. Finally, with the aid of a gelatin-sepharose column it was investigated whether the protein was fibronectin.

MATERIALS AND METHODS

Cells and culture

Diploid skin fibroblasts were derived from two healthy persons (C5RO: female, 30 years; 79 RD 200: male, 5 years), a patient with the Spielmeyer-Vogt syndrome (494 LAD: male, 5 years) and a patient with Werner's syndrome (525 LAD: male, 27 years). All cell strains were cultured in Ham's F10 medium supplemented with 8% foetal calf serum (Sera Lab lot No. 101107) and antibiotics (100 μg of streptomycin and 100 U of penicillin/ml of culture medium). Cultures were routinely checked for mycoplasma contamination and found not to be contaminated. The *in vitro* ageing process of these cells has been investigated using [^{3}H] thyminidime labeling as published previously [4]. The maximal lifespan (MLS) of the cultures has as well been determined [4]. A low percentage MLS has been defined as less than 85% of MLS completed, while more than 90% of MLS completed is coined as a high percentage MLS. Cultures were subcultivated by 1:3 splits, when the cultures reached confluence.

In some cases cells from C5RO were cultivated in the presence of 10 μg of D,L-α-tocopherol (Sigma)/ ml of culture medium as described previously [4].

Contact inhibition of growth

Cells were kept confluent for 3 weeks in 80-cm^2 glass culture flasks. Culture media were replenished twice a week.

Flow cytometry and cell sorting

Cells were harvested by trypsinization just before cell sorting. They were resuspended

in culture medium containing 8% foetal calf serum. Flow cytometry and sorting of AF and non-AF cells were done using a FACS II cell sorter (Becton and Dickinson) equipped with an argon ion laser set at 457 nm exitation wavelength as described previously [2].

Radioactive labeling of proteins

Cellular proteins were labelled overnight with L-[^{35}S] methionine (Amersham; spec. act. 1420 Ci/mmol; 20 μCi/ml of culture medium). Cells were harvested by tripsinization, washed twice with phosphate buffered saline (PBS) and lysed with 0.25% Triton X-100. After determination of radioactivity, using liquid scintillation counting, the samples were diluted with SDS-sample buffer (see below) to obtain a radioactivity of 25 000 cpm/μl. The radioactive culture medium was harvested and proteins were precipitated with trichloric acetic acid (TCA) at a final concentration of 10%. The precipitate was obtained by centrifugation and as well dissolved in 0.25% Triton X-100, after which radioactivity was determined and SDS-sample buffer was added to obtain 25 000 cpm/μl.

Gel electrophoresis, staining and fluorography

Gel Electrophoresis was essentially done according to the method of O'Farrell [7]. However, a stacking gel of 3% was used, while the separation gel was made as a gradient of 5–10% acrylamide. The β-mercaptoethanol in the lysis buffer was replaced by 0.1% dithiothreitol (Calbiochem). In the lysis buffer Triton X-100 was used instead of Nonidet P-40. The agarose in the 2D system was substituted by acrylamide, as agarose caused spurious staining with the silver staining method used. The slab gels were 1.5 mm thick. 14 cm wide and 12 cm long. Stacking gels were run at 15 mA/gel and the separating gels were 20 mA/gel. After the run gels were fixed and silver stained according to Morrissey [8]. Gels with radioactively labeled proteins were fixed and prepared for fluorography according to Pulleyblank and Booth [9].

Cell fractionation

Cells were fractionated by a modified version of the differential centrifugation method of Berthet and De Duve [10]. About 2×10^6 cells were harvested by trypsinisation and washed twice with PBS. The final pellet was resuspended in 2 ml of homogenisation buffer (250 mM sucrose; 3 mM imidazole-HCl (pH 7.4); 1 mM Na_4 EDTA) and homogenised at 4°C by 15 strokes in a Potter-Elvejhem apparatus at 1300 rev./min. From this crude homogenate cellular debris and unbroken cells were removed by centrifugation at 100 *g* for 10 min. The supernatant was centrifuged at 2000 *g* for 10 min to yield a nuclear pellet. This supernatant was centrifuged with an L5 Spinco centrifuge (Beckman) in an SW 50.1 swinging bucket rotor at 10 000 *g* for 60 min. This yields a pellet essentially consisting of mitochondria and lysosomes and a supernatant containing microsomes and cytosol. This supernatant was ultimately centrifuged at 108 000 *g* for 60 min in the same centrifuge and rotor, yielding to a microsomal pellet and the cytosol. The pellets were resuspended in 0.25% Triton X-100. Protein contents of the crude homogenate and the fractions were determined using fluorescamine as described previously [2]. Five micro-

grams of protein sample was diluted with SDS-sample buffer and applied to a lane of a gel.

Concanavalin-A-sepharose chromatography

A sample of 100 μg protein of a post 10 000 *g* supernatant (see above) of an homogenate of C5RO cells, kept at confluence for 3 weeks, was subjected to concanavalin-A-sepharose chromatography as described by Verheijen *et al.* [11].

Neuraminidase treatment

A 100-μg sample of the same supernatant as used for the concanavalin-A-sepharose chromatography was incubated with neuraminidase. The pH of the sample was brought to 5.0 with HCl and 0.08 U of neuraminidase (type VI, Sigma) was added. This mixture was incubated during 3 h at 37°C. Thereafter urea was added to a final concentration of 8 M and this mixture was stored at −20°C. The next day the sample was subjected to 2D gel electrophoresis as described above.

Gelatin-sepharose chromatography

A gelatin-sepharose column was prepared using CNBr activated sepharose (Pharmacia) according to the procedure described by the manufacturer. Denatured collagen (gelatin) from UCB-RPL (Leuven, Belgium) was coupled to the sepharose (approx. 1 ml bed vol). After extensive blocking and washing this column was used to isolate fibronectin from the serum fo a healthy donor (male, 29 years) according to Chandrasekhar *et al.* [12]. This fibronectin was used as a reference in the SDS-polyacrylamide gel electrophoresis. A 100-μg sample of the supernatant mentioned above was applied to the gelatin-sepharose column and eluted according to Chandrasekhar *et al.* [12]. All the fractions were precipitated with TCA, redissolved in 0.25% Triton X-100 and processed for gel electrophoresis as described above.

RESULTS

Cell biology

Analysing the protein patterns of sorted AF and non-AF cells we found a protein of apparently 240 000 molecular weight in AF cells, which was absent from non-AF cells (Fig. 1, lanes 1 and 2). This protein was always present in the protein patterns of both the control cells strains, when the cells had a high % MLS completed, while it was absent from the cells with a low % MLS completed (Fig. 1, lanes 7 and 8). The protein was faintly visible in the pattern of continuously proliferating cells of an intermediate % MLS completed, while it strongly accumulated in cells subjected to growth inhibition (Fig. 2A). The protein is synthesized in both proliferating and in contact inhibited cells as is revealed by fluorography of a gel run with [^{35}S] methionine labelled cells (Fig. 2B). The presence or absence in the culture medium of vitamin E, a lipophilic free radical scavenger, did not influence the accumulation of this protein (Fig. 2C).

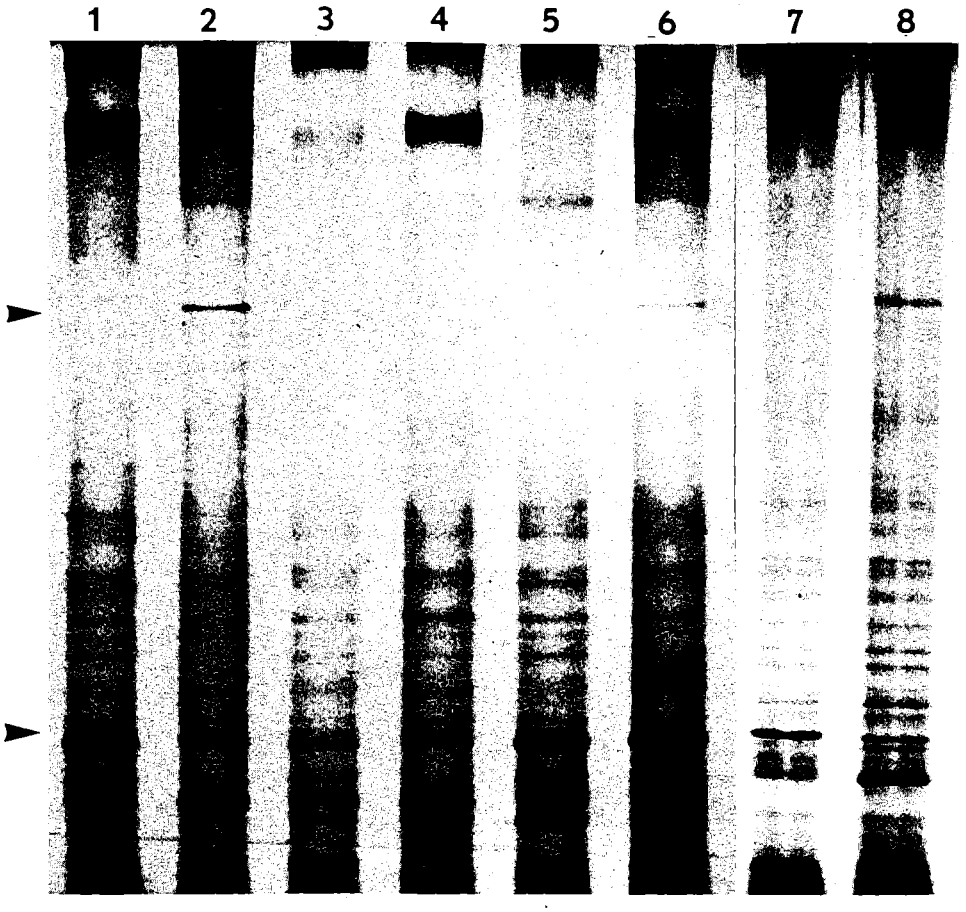

Fig. 1. SDS-PAGE of total cell homogenates from continuously proliferating cultures. *Lane 1:* sorted non-AF cells of C5RO (% MLS = 84). *Lane 2:* sorted AF cells of C5RO (% MLS = 84). *Lane 3:* young culture of Werner's syndrome (% MLS = 85). *Lane 4:* old culture of Werner's syndrome (% MLS = 100, terminal). *Lane 5:* young culture of Spielmeyer-Vogt syndrome (% MLS = 66). *Lane 6:* old culture of Spielmeyer-Vogt syndrome (% MLS = 95). *Lane 7:* young culture of C5RO (% MLS = 57). *Lane 8:* old culture of C5RO (% MLS = 95). Arrows indicate the positions of the molecular weight markers; top arrow: 220 000 and bottom arrow: 67 000 molecular weight.

The protein was present in cells with a high % MLS completed from a patient with the syndrome of Spielmeyer-Vogt (Fig. 1, lane 6), while it was absent from cells of the same patient, which had a low % MLS completed (Fig. 1, lane 5). The protein was never present in cells from a patient with Werner's syndrome (Fig. 1, lanes 3 and 4).

Biochemical characteristics of the protein

To obtain more information about this protein some of its biochemical characteristics were determined. The subcellular localisation of the protein was analysed using differential centrifugation of a cell homogenate from cells kept at confluence for 3 weeks.

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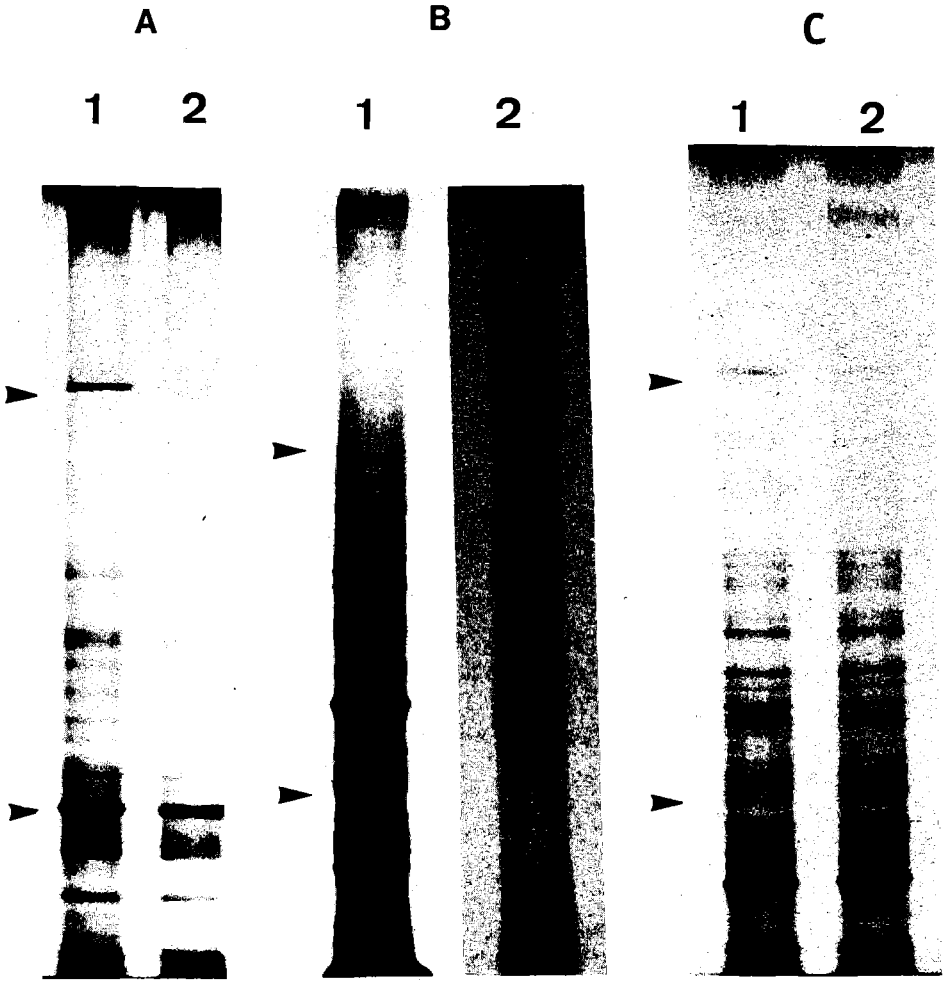


Fig. 2. SDS-PAGE of growth inhibited (A), [^{35}S] methionine labeled (B) and vitamin E treated cells (C5RO) (C). (A) *Lane 1:* cells at confluence for 3 weeks. *Lane 2:* same cells, continuously proliferating. (B) *Lane 1:* [^{35}S] methionine labeled growth inhibited cells. *Lane 2:* [^{35}S] methionine labeled continuously proliferating cells. (C) *Lane 1:* cells cultured at confluence for 3 weeks in the presence of 10 μg vitamin E/ml of culture medium. *Lane 2:* cells cultured in the same way in the absence of vitamin E. Arrows indicate the positions of the molecular weight marker; top arrow: 220 000 and bottom arrow: 67 000 molecular weight.

The protein was present in the crude homogenate and was most prominent in the microsomal fraction and in the cytosol (Fig. 3, lanes 1, 4 and 5). The protein was completely absent from the nuclear fraction (Fig. 3, lane 2) and only faintly discernible in the fraction containing mitochondria and lysosomes (Fig. 3, lane 3). It is inferred, that the protein is located in the microsomes as a soluble protein of which part can be liberated into the cytosol by the homogenisation procedure used.

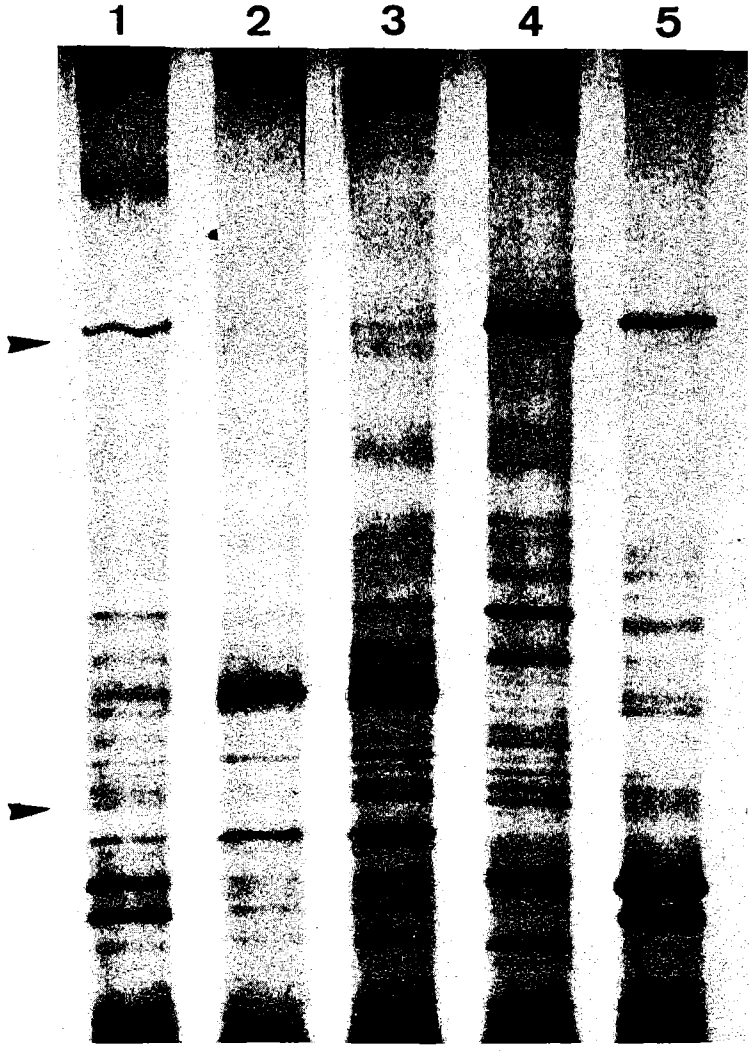
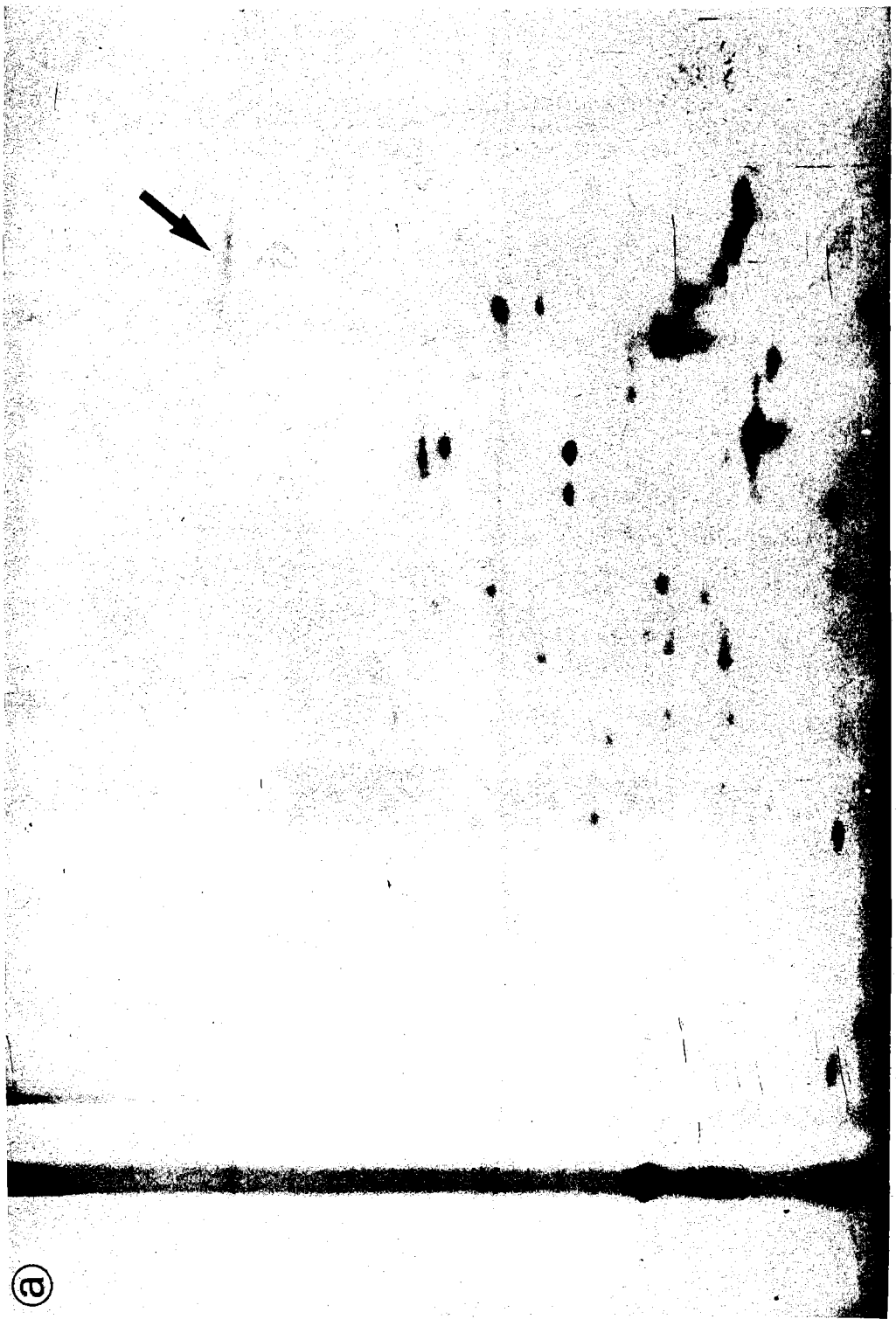


Fig. 3. SDS-PAGE of the fractions obtained by differential centrifugation of a cell homogenate of C5RO kept at confluence for 3 weeks. *Lane 1:* crude homogenate. *Lane 2:* nuclear pellet. *Lane 3:* lysosomes and mitochondria. *Lane 4:* microsomes. *Lane 5:* cytosol. Arrows indicate the positions of the molecular weight markers; top arrow: 220 000 and bottom arrow: 67 000 molecular weight.

On 2D gel electrophoresis the protein had an apparent isoelectric point of approximately 5.3 (Fig. 4A). This isoelectric point was heterogenous, what can be interpreted as an indication of the presence of charged residues on the surface of the protein. Treatment of a fraction of the homogenate with neuraminidase caused the protein to shift from an isoelectric point of 5.3 to 5.85 (Fig. 4 B). This strongly indicates that the protein contained at least one neuraminic acid residue. As the protein after this treatment still has an acidic isoelectric point it seems to contain a considerable number of acidic amino acid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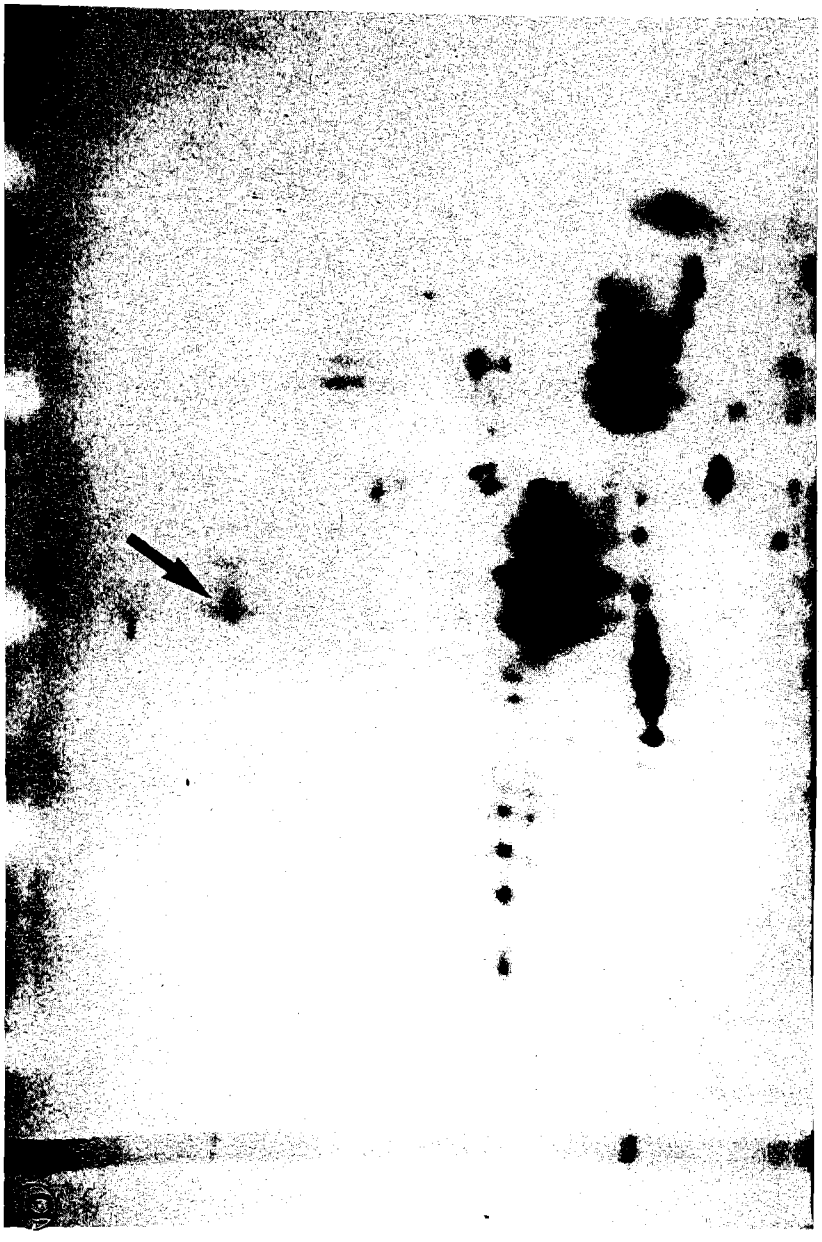

Fig. 4. 2D gel electrophoresis before and after neuraminidase treatment of a post 10 000 *g* supernatant of C5RO cells kept at confluence for 3 weeks. (A) Without neuraminidase treatment. The lane on the left shows the SDS-PAGE pattern of this fraction. (B) After neuraniminidase treatment. The lane on the left shows the SDS-PAGE pattern of this fraction. Arrows indicate the position of the 240 000 dalton protein.

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residues. Containing neuraminic acid residues the protein is clearly a glycoprotein, but it failed to bind to a Concanavalin-A sepharose column (results not shown). This behaviour reveals that the protein contains no exposed mannosyl or glucosyl residues. The major cellular protein of approximately 240 000 molecular weight, which contains neuraminic

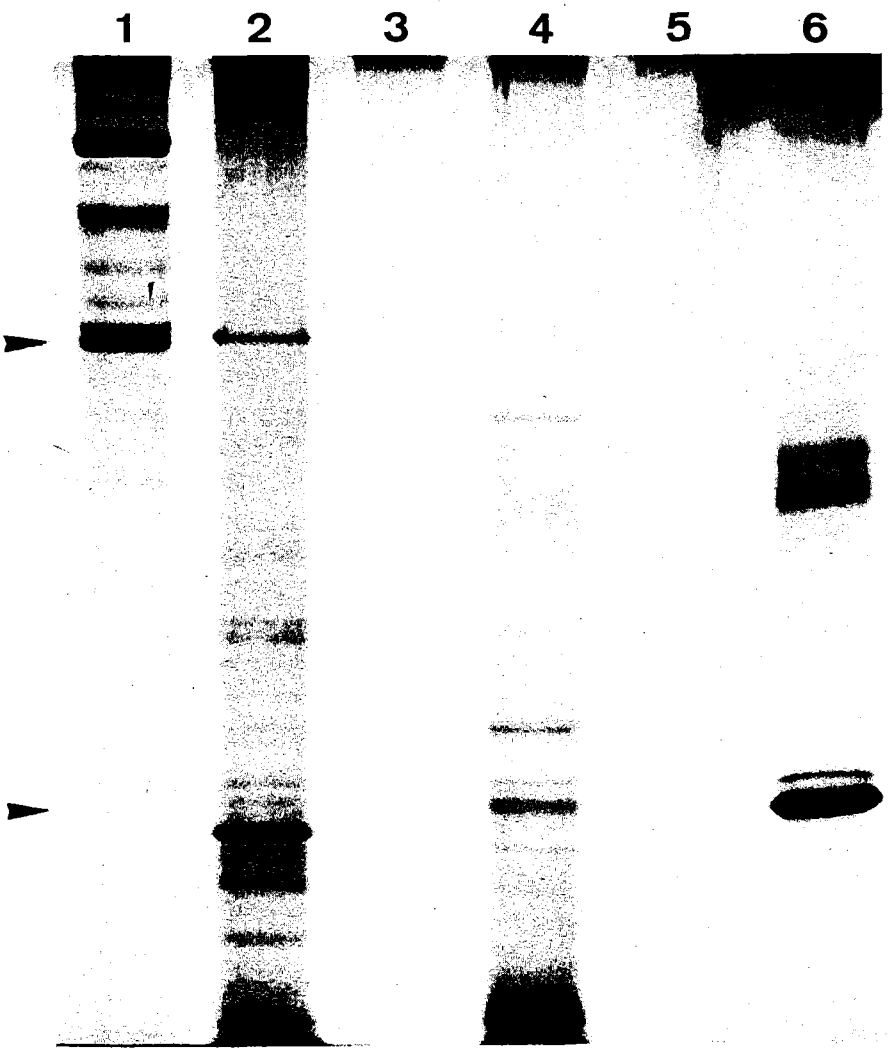

Fig. 5. SDS-PAGE of fractions obtained by chromatography on gelatin-sepharose of a post 10 000 *g* supernatant of cells from C5RO kept at confluence for 3 weeks. *Lane 1:* purified human serum fibronectin. *Lane 2:* the crude homogenate as applied onto the column. *Lane 3:* run through fraction. *Lane 4:* wash with first column volume. *Lane 5:* wash with first column volume. *Lane 6:* elution fraction. Arrows indicate the positions of the molecular weight markers; top arrow: 220 000 and bottom arrow: 67 000 molecular weight.

acid residues is fibronectin. To test this hypothesis a gelatin-sepharose column was prepared and subsequently used to purify fibronectin from human serum. In a second experiment the column was used to analyse a fraction of a cell homogenate. The purified fibronectin and the run-through, wash and elution fraction of the homogenate were then analysed by SDS-PAGE. It is clear that the 240 000 molecular weight protein (Fig. 5, lane 2) did not migrate in exactly the same way as did the purified fibronectin (Fig. 5, lane 1). Furthermore, the 240 000 molecular weight protein appeared only in the first wash fraction of the column (Fig. 5, lane 4), indicating that the 240 000 molecular weight protein did not interact with the gelatin-sepharose. The cellular homogenate, indeed, contains proteins with an apparent molecular weight of 72 000 and 85 000 binding to the gelatin-sepharose (Fig. 5, lane 6), which are presumably breakdown products of fibronectin [13].

DISCUSSION

During *in vitro* ageing the protein content of cells increases as a result of a decrease in the rate of breakdown of a certain class of proteins [14] and the emergence of a subpopulation of cells that do not at all turn over their proteins [15]. This might lead to the accumulation of defected proteins in the cells during *in vitro* ageing. It has been suggested, that errors in protein synthesis could yield to this accumulation of defected proteins [16]. Hitherto, no positive evidence to this has been found [17–19]. Another mechanism by which an accumulation of defected proteins might occur is post-translational modification. Deamidation of glutaminyl and asparaginyl residues [20], reactions of proteins with glucose [21] and cross-linking of proteins by free radical reactions [5] have been proposed. The last one of these modifications is easily accessible to analysis with the use of large pore poly-acrylamide gel electrophoresis systems, as these can detect the high molecular weight products that arise from cross-linking of smaller proteins.

In our study we used such a large pore poly-acrylamide gel electrophoresis system and we found a remarkable difference in the protein patterns of cells with different *in vitro* ages. Sorted AF cells from a control cell culture, which display characteristics of *in vitro* aged cells [2], contained a 240 000 molecular weight protein, which was absent from sorted non-AF cells out of the same culture. The presence of this protein might be the result of the diminished proliferative capacity of AF cells or the result of the free radical reactions that gave rise to the cellular autofluorescence [4]. Growth inhibition studies with young control cells revealed that the protein was present at a higher concentration in the homogenates of growth inhibited cells vs. their continuously dividing counterparts. From experiments with [^{35}S]methionine labelled cells in contact inhibited and in continuously growing cultures it is evident, that this protein is synthesized equally well in both types of cell cultures. Vitamin E, a lipophilic free radical scavenger, did not interfere with the accumulation of this protein in cells from growth inhibited cultures. So, free radical reactions yielding to protein cross-linking agents, are not involved in the accumulation of this protein. One is to conclude that this protein accumulates in cells with a high

% MLS completed as a response to the diminished proliferative capacity of these cells. The accumulation of this protein can only be explaineed as the result of a change in the pattern of protein breakdown or processing in cells with a high % MLS completed, as there is no specific difference in the synthesis rate of this protein in proliferating *vs.* growth inhibited cells.

Cells from a patient with the syndrome of Spielmeyer-Vogt were to contain the protein when these cells reached a high % MLS completed, while the protein was absent from cells of the same patient with a low % MLS completed. The cells from a patient with Werner's syndrome never contained this protein. Whether this peculiarity constitutes a means to diagnose Werner's syndrome can only be decided upon after an extension of these experiments with cells from other patients with Werner's syndrome.

In order to understand the nature of this protein, some of its biochemical characteristics were investigated. Its subcellular localisation was determined using differential centrifugation of a cell homogenate. The protein appeared in the microsomal pellet and in the cytosol, what indicates that protein is located as a soluble protein inside the endoplasmic reticulum or the Golgi vesicles. Upon 2D gel electrophoresis the protein was found to exhibit a heterogenous, acidic IEP. Its subcellular localisation and its IEP prompt us to presume the protein to be a glycoprotein. The protein contained neuraminic acid residues. This can explain fully the heterogeneity of the IEP of the protein, but our data (an IEP of 5.85 after extensive neuraminidase treatment) do not rule out that the peptide backbone of the protein contains acidic amino acid residues. The protein does not contain exposed mannosyl residues, as it did not bind to a concanavalin-A sepharose column. This makes it likely that the protein belongs to a class of proteins with complex N-linked oligosaccharides or to a class with 0-linked oligosaccharides. Our data do not permit us to distinguish between these two possibilities.

It might be possible that the 240 000 molecular weight glycoprotein has as yet not been described, but it is as well feasible that we were characterising an already known protein. For instance, fibronectin has a molecular weight of approximately 240 000, it contains neuraminic acid residues [22] and it has an acidic IEP [23]. What makes it even more attractive to suggest that this protein is fibronectin are the data showing that fibronectin of *in vitro* aged fibroblasts has a diminished collagen binding capacity [12]. Moreover, the fibronectin deposition pattern in the exoskeleton of *in vitro* aged is different from the one found with young cells [24] and, finally, the fibronectin of *in vitro* aged cells displays different molecular properties as did the fibronectin from young cells [25]. Our 240 000 molecular weight glycoprotein did not migrate in exactly the same way on SDS-poly-acrylamide gel electrophoresis as did the human plasma fibronectin, which might be explained assuming slight molecular differences between the two forms of the same protein [13]. Both forms of fibronectin, however, strongly bind to gelatin-sepharose, while our protein failed to do so. Henceforth, one has to conclude that the 240 000 molecular weight glycoprotein is not fibronectin.

During the course of our experiments Wang published results obtained with an antiserum directed against *in vitro* aged human fibroblasts [26]. This antiserum precipitated

a 57 000 molecular weight and a high molecular weight protein uniquely present in growth inhibited and in *in vitro* aged cells. It is possible that the high molecular weight protein described by Wang is the same as our 240 000 molecular weight, glycoprotein.

Others [27] published a report in which they described two polypeptides in terminally non-dividing cells which were absent from continuously proliferating cells. Given the molecular weight of these polypeptides (44 000) it is evident that these do not at all bear any resemblance to our 240 000 dalton protein. Finally, plasma membranes were found to contain glycoproteins with aspargine-linked oligosaccharides, which were found to be involved in contact inhibition of growth [28]. The relevance of these glycoproteins to the process of *in vitro* ageing has as yet not been shown.

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REFERENCES

1 D.W. Deamer and J. Gonzales, Autofluorescent structures in cultured WI-38 cells. *Arch. Biochem. Biophys., 165* (1974) 421-426.
2 J.F. Jongkind, A. Verkerk, W.J. Visser and J.M. van Dongen, Isolation of autofluorescent "aged" human fibroblasts by flow sorting. Morphology, enzyme activity and proliferative capacity. *Exp. Cell. Res., 138* (1982) 409–417.
3 S.I.S. Rattan, K.D. Keeler, J.H. Buchanan and R. Holliday, Autofluorescence as an index of ageing in human fibroblasts in culture. *Biosci. Rep., 2* (1982) 561–567.
4 M. Poot, W.J. Visser, A. Verkerk and J.F. Jongkind, Autofluorescence of human skin fibroblasts during growth inhibition and *in vitro* ageing. *Gerontology, 31* (1985) 158–165.
5 B.E. Leibovitz and B.V. Siegel, Aspects of free radical reactions in biological systems: Aging. *J. Gerontol., 35* (1980) 45–56.
6 R.C. Adelman, Macromolecular metabolism during aging. In C.E. Finch and L. Hayflick (eds.), *Handbook of the Biology of Aging*, Van Norstrand Reinhold, New York, 1977, pp. 63–72.
7 P.H. O'Farrell, High-resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem., 250* (1975) 4007–4021.
8 J.H. Morrissey, Silver stain for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. *Anal. Biochem., 117* (1981) 307–310.
9 D.E. Pulleyblank and G.M. Booth, Improved methods for the fluorographic detection of weak-emitting radioisotopes in agarose and acrylamide gel electrophoresis media. *J. Biomed. Biophys. Methods, 4* (1981) 339–346.
10 J. Berthet and C. De Duve, Tissue fractionation studies. 1. The existence of a mitochondria-linked enzymically inactive form of acid phosphatase in rat-liver tissue. *Biochem. J., 50* (1951) 174–181.
11 F. Verheijen, R. Brossmer and H. Galjaard. Purification of acid galactosidase from bovine testis: Evidence for an enzyme complex. *Biochem. Biophys. Res. Commun., 108* (1982) 868–875.
12 S. Chandrasekhar, J.A. Sorrentino and A.J.T. Millis, Interaction of fibronectin with collagen: Age- specific defect in the biological activity of human fibroblast fibronectin. *Proc. Natl. Acad. Sci. USA, 80* (1983) 4747–4751.

13 T. Vartio and A. Vaheri, Fibronectin: chains of domains with diversified functions. *Trends Biochem. Sci., 8* (1983) 442–444.
14 A.A. Okada and J.F. Dice, Altered degradation of intracellular proteins in ageing human fibroblasts. *Mech. Ageing Dev., 26* (1984) 341–356.
15 J.S. Amenta and S.C. Brocher, Studies on protein turnover in cell culture: Evidence for the existence of a specific proteolytic state and its relation to cellular proliferation and cellular senescence. *Acta Biol. Med. Germ., 40* (1981) 1215–1226.
16 L.E. Orgel, The maintenance of the accuracy of protein synthesis and its relevance to ageing. *Proc. Natl. Acad. Sci. USA, 49* (1963) 517–521.
17 C.B. Harley, J.W. Pollard, J.W. Chamberlain, C.P. Stanner and S. Goldstein, Protein synthetic errors do not increase during aging of cultured human fibroblasts. *Proc. Natl. Acad. Sci. USA, 77* (1980) 1885–1889.
18 N. Mori, K. Hiruta, Y. Funatsu and S. Goto, Codon recognition fidelity of ribosomes at the first and second positions does not decrease during aging. *Mech. Ageing Dev., 22* (1983) 1–10.
19 M.L. Van Keuren, C.R. Merril and D. Goldman, Protein variations associated with *in vitro* catastrophe hypothesis. *J. Gerontol., 38* (1983) 645–652.
20 A.B. Robinson, Evolution and the distribution of glutaminyl and asparaginyl residues in proteins. *Proc. Natl. Acad. Sci. USA, 71* (1974) 885–888.
21 S. Pongor, P.C. Ulrich, F.A. Bencsath and A. Cerami, Aging of proteins: Isolation and identification of a fluorescent chromophore from the reaction of polypeptides with glucose. *Proc. Natl. Acad. Sci. USA, 81* (1984) 2684–2688.
22 K.M. Yamada, Cell surface interactions with extracellular materials. *Annu. Rev. Biochem., 52* (1983) 761–799.
23 D.F. Mosher and L.T. Furcht, Fibronectin: Review of its structure and possible functions. *J. Invest. Dermatol., 77* (1981) 175–180.
24 G.F. Edick and A.J.T. Millis, Fibronectin distribution on the surface of young and old human fibroblasts. *Mech. Ageing Dev., 27* (1984) 249–256.
25 J.A. Sorrentino and A.J.T. Millis, Structural comparisons of fibronectins isolated from early and late passage cells. *Mech. Ageing Dev., 28* (1984) 83–97.
26 E. Wang, A 57 000-mol-wt protein uniquely present in nonproliferating cells and senescent human fibroblasts. *J. Cell Biol., 100* (1985) 545–551.
27 D.W. Lincoln II, K.I. Braunschweiger, W.R. Braunschweiger and J.R. Smith. The two-demensional polypeptide profile of terminally non-dividing human diploid cells. *Exp. Cell Res., 154* (1984) 134–146.
28 R.J. Wieser, R. Heck and F. Oesch, Involvement of plasma membrane glycoproteins in the contact-dependent inhibition of growth of human fibroblasts. *Exp. Cell Res., 158* (1985) 493–499.

APPENDIX PAPER 5

GLUTATHIONE CONTENT OF CULTURED HUMAN FIBROBLASTS DURING IN VITRO AGEING

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GLUTATHIONE CONTENT OF CULTURED HUMAN FIBROBLASTS DURING *IN VITRO* AGEING

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SUMMARY

The glutathione level of cultured human fibroblasts was determined with a micro-modification of a spectrophotometric glutathione cycling method. There was a slight increase in reduced glutathione (GSH) content during *in vitro* ageing of normal human fibroblasts. Fibroblasts from patients with Werner's syndrome or ceroid lipofuscinosis (Spielmeyer-Vogt syndrome) and healthy individuals exhibited similar patterns of GSH levels during *in vitro* ageing.

The GSH content of non-proliferating confluent cultures of normal fibroblasts and of proliferating normal fibroblasts was identical. Moreover, autofluorescent "aged" cells isolated by cell sorting did not differ in GSH content from the non-autofluorescent cells in the same culture. It was concluded that the GSH content does not play a role in *in vitro* ageing, nor in the accumulation of autofluorescent material in human skin fibroblasts.

Key words: Ageing; Human fibroblasts; Glutathione; Autofluorescence; Werner's syndrome; Spielmeyer-Vogt syndrome

INTRODUCTION

During *in vitro* ageing, cultured cells undergo many changes [1]. One of these is an increase in the number of autofluorescent (AF) cells [2,3]. When AF-cells were isolated from proliferating cultures by cell sorting they showed a reduced proliferative capacity and exhibited other characteristics of *in vitro* aged cells [4]. The increase in autofluorescence could be caused by increased lipid peroxidation, subsequent formation of malondialdehyde, and the appearance of fluorescent cross-linked proteins [5,6].

As reduced glutathione (GSH) is involved in the detoxification of oxygen-derived radicals and lipid peroxides [7], cellular glutathione content might be a factor in the

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appearance of AF cells during *in vitro* ageing. In this respect, the glutathione content in one strain of cultured human lung fibroblasts (IMR 90) was studied and found to be decreased in *in vitro* aged cells [8].

In this study we investigated the glutathione content of three different strains of normal human skin fibroblasts during *in vitro* ageing. Moreover, we compared the glutathione content of AF cells, isolated by cell sorting, with non-AF cells from the same culture. The effect of growth inhibition on glutathione content was also investigated. Finally, we studied the glutathione content of cultured fibroblasts from patients with ageing syndromes, such as Werner's syndrome and Spielmeyer-Vogt syndrome.

MATERIALS AND METHODS

Cells and culture

Diploid skin fibroblasts were derived from three healthy subjects (C5RO: female, 30 years; 79RD200: male, 5 years; 80RD174: male, 33 years). The patient material consisted of diploid fibroblasts from two patients with Spielmeyer-Vogt syndrome (494 LAD: male, 5 years; 75RD 97: female, 25 years) and from two patients suffering from Werner's syndrome (525 LAD: male, 27 years; 531LAD: male, 30 years).

All cell strains were cultured in Ham's F10 medium supplemented with 8% foetal calf serum and antibiotics (100 μg of streptomycin and 100 U of penicillin per ml of culture medium). For the normal strains serum from Boehringer (lot no. 66.3902.02) was used, and for the disease strains serum from Sera Lab (lot no. 101107). Cultures were subcultivated by 1:3 splits, when the cultures reached confluence. The population doubling level (PDL) was calculated assuming one passage to be 1.58 PDL (LOG3/LOG2).

Growth inhibition

Confluent cells were grown during 3 weeks on glass coverslips (diameter 24 mm) in holes of plastic culture trays (Costar 3524). Culture media were replenished twice a week. Control cells were harvested 3 or 4 days after subculture.

Flow cytometry and cell sorting

Three or 4 days after subculture the cells were harvested by trypsinization just before cell sorting. They were resuspended in culture medium containing 8% foetal calf serum. Flow cytometry and cell sorting were carried out using a FACS II cell sorter (Becton and Dickinson) equipped with an argon ion laser set at 457 nm excitation wavelength as described previously [4].

Cell extraction and protein assay

Two or 3 days after subculture cells were harvested by trypsinization. The day before harvesting the medium was replenished. Cell suspensions were washed twice with saline by centrifugation. The packed cell pellet was extracted by adding 100 μl of phosphate-buffered saline (approx. 10^6 cells/ml). After triple freezing and thawing, the cell debris

was precipitated by centrifugation (6400 *g*) in a microcentrifuge (Eppendorf 5414) at 4°C. With the saline extraction glutatione was solubilized completely in "young" and "old" cells, for in the resuspended pellets no glutathione could be detected. The precipitate was again extracted with 100 µl of 0.25% Triton X-100 in water to determine remaining protein not extracted with saline (approx. 20%). The protein in the supernatants was measured with fluorescamine as described previously [4].

Glutathione microassay

The total glutathione (GSH + GSSG) content of the saline extracts was determined by a micromodification of the spectrophotometric cycling method of Brehe and Burch [9]. For the measurement of GSSG only, this cycling procedure was preceded by treating the cell extracts with 2-vinylpyridine [10]. Ten microliters of dilutions of the saline cell extract were added to 10 µl of a buffered DTNB (5,5′-dithiobis-(2-nitrobenzoic acid) solution (0.3 mM DTNB, 110 mM Na_2HPO_4, 40 mM NaH_2PO_4, 15 mM Na_4-EDTA, 0.04% bovine serum albumin (BSA), pH 7.2). To this mixture 10 µl of a GSH cycling reagent were added. The freshly prepared cycling reagent consisted of glutathione reductase (EC 1.6.4.2, Boehringer, 1.2 IU/ml), 2 mM NADPH, imidazole-HCl 50 mM, pH 7.2, 1 mM Na_4-EDTA, and 0.02% BSA. Before use the ammonium sulphate was removed from the glutathione reductase by centrifugation (3 min at 6400 *g*) and the enzyme was resuspended in saline.

All incubations were done in duplicate in a Fisher isotemp dry bath at 20°C. After 30 min the incubations were stopped by placing the test tubes in a melting ice bath. Spectrophotometric measurements were done immediately upon incubation using a microspectrophotometer [11]. The glutathione assay is linear within the range of 0.2–5 µM in the extracts. Therefore the saline cell extracts have to be diluted so as to contain a glutathione concentration in this range. To determine the GSSG content, 60 µl of the saline extract were preincubated with 2 µl of 2-vinylpyridine for 1 h at room temperature to scavenge the GSH [10]. This preincubated extract was then assayed according to the procedure outlined above for total glutathione.

In each determination known amounts of GSSG (diluted from a 10 mM stock in 0.01 M HCl) were included as internal standards. This stock solution was stable at −70°C for at least 6 months. When freshly prepared GSH was used as a standard no difference was found with respect to GSSG in the cycling procedure (GSSG = 2GSH). GSH and GSSG contents of the extracts were expressed as nmol GSH or GSSG per mg of total (saline + Triton X-100) extractable protein. The significance of change in our data was tested using the rank correlation test of Spearman [13].

RESULTS

Glutathione in ageing cells

All normal strains studied displayed a similar pattern with respect to their GSH content during *in vitro* ageing (Fig. 1). The GSH content remained almost constant during

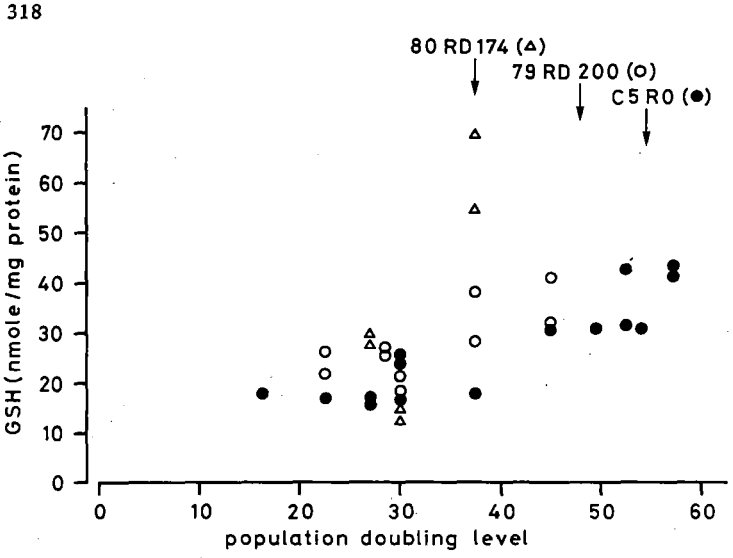

Fig. 1. GSH content of three normal cell strains during *in vitro* ageing. Arrows indicate the maximum PDL of the strains as determined in a separate experiment. Each point corresponds to the mean of a duplicate determination in a cell extract.

the first three-quarters of the *in vitro* lifespan and then increased towards an elevated level. This increase was significant with C5RO ($p < 0.001$) and 79RD200 ($p < 0.1$) but not significant with 80RD174 ($p > 0.1$). Both the cell strains derived from patients suffering from Spielmeyer-Vogt syndrome displayed a pattern similar to that of control strains (Fig. 2A) (75RD97: $p < 0.01$; 494LAD: $p > 0.1$). The cells from the patients

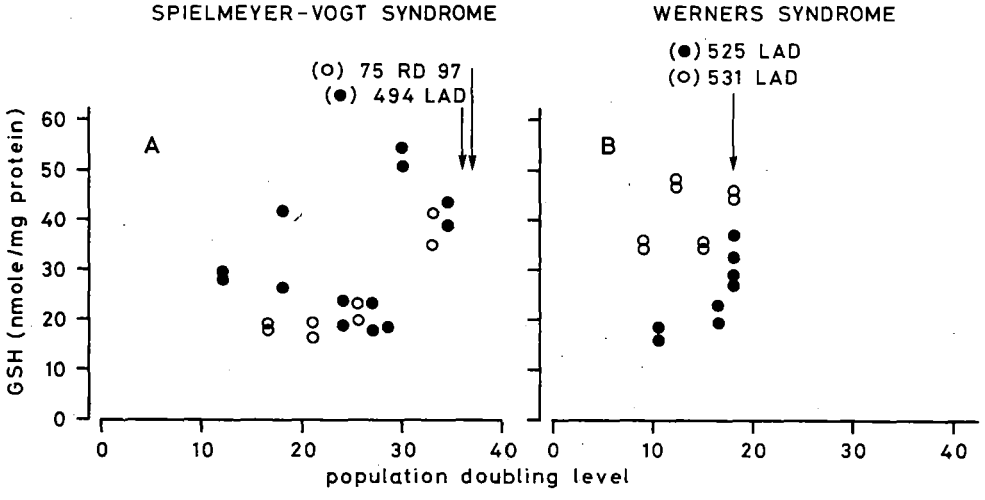

Fig. 2. GSH content of cell strains from patients with Spielmeyer-Vogt syndrome (A) and Werner's syndrome (B) during *in vitro* ageing. Arrows indicate the maximum PDL of the strains as determined in a separate experiment. Each point corresponds to the mean of a duplicate determination in a cell extract.

with Werner's syndrome (Fig. 2B) ceased to proliferate at PDL 18. In one of the cell strains was an increase in GSH content as in the normal fibroblasts (525LAD: $p < 0.001$), while in the other cell strain no significant (531LAD: $p > 0.1$) change was found.

The GSSG content of the cell strains during *in vitro* ageing varied between 0.3 and 9 nmol per mg protein. Only in the case of 79RD200 and 75RD97 there was a slight but significant increase in GSSG content (Figs. 3 and 4).

Glutathione in confluent cultures of fibroblasts

Cells of strain C5RO (PDL 51) were kept confluent for 3 weeks. In two separate experiments there was a slight decrease in GSH content relative to the proliferating controls (confluent: 28.7, 27.7; proliferating: 36.6, 32.0 nmol GSH per mg protein).

There was no change in the GSSG content (confluent: 1.50, 1.70; proliferating: 1.50, 1.60 nmol GSSG per mg protein).

Glutathione in sorted autofluorescent cells

AF-cells and non-AF-cells were sorted from a population of C5RO cells (PDL 51). Glutathione content was determined in two separate experiments.

There was no difference in the GSH levels between AF- and non-AF-cells (AF: 28.1, 22.9; non-AF: 24.8, 27.6 nmol GSH per mg protein).

For GSSG there were also no differences between AF- and non-AF-cells (AF: 0.74, 0.32; non-AF: 0.62, 0.31 nmol GSSG per mg protein).

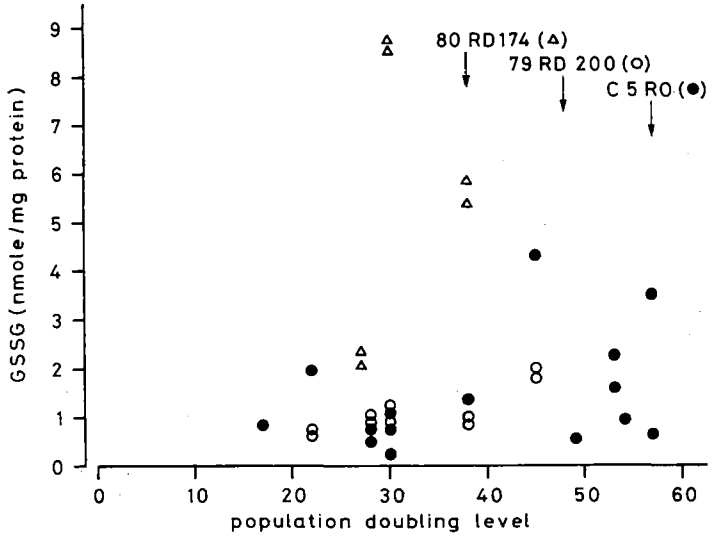

Fig. 3. GSSG content of three normal cell strains during *in vitro* ageing. Arrows indicate the maximum PDL of each of the strains as determined in a separate experiment. Each point corresponds to the mean of a duplicate determination in a cell extract.

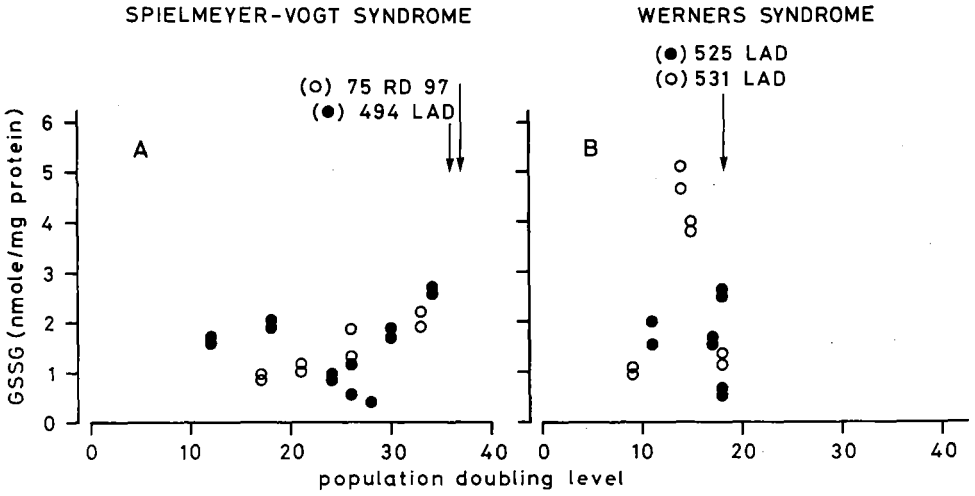

Fig. 4. GSSG content of cell strains from patients with Spielmeyer-Vogt syndrome (A) and Werner's syndrome (B) during *in vitro* ageing. Arrows indicate the maximum PDL of each of the strains as determined in a separate experiment. Each point corresponds to the mean of a duplicate determination in a cell extract.

DISCUSSION

For the microspectrophotometric determination of glutathione in cultured human fibroblasts, using the method of Brehe and Burch [9] extended by Griffith [10], some technical problems had to be solved. The different steps in the procedure were carefully checked. Since sonication at neutral pH [8] leads to an oxidation of 10–15% of GSH we chose a freeze–thawing procedure which extracted the GSH quantitatively without losses.

Since protein and non-glutathione soluble thiol groups interfered less than 2% in our assay we omitted the sulfosalicyclic acid precipitation step used by Takahashi and Zeydel [8] and we included an appropriate tissue control without glutathione reductase in the reagent. In the GSSG determinations we also included fully processed standards since the 2-vinylpyridine inhibited the cycling velocity by 20–25%, contrary to what has been stated by Griffith [10].

In this respect it is noteworthy that the methods used by Takahashi and Zeydel [8] on one strain of human embryonic lung fibroblasts (IMR 90) could lead to a slight difference with our values. The large decrease in GSH which they observed in homogenates of old cells, however, cannot be explained by the methodological differences. These discrepancies with our results must be due to the different origin of the cells, since in our experiments the glutathione levels in three different cell strains of normal human skin fibroblasts did not decrease at the end of their proliferative lifespan.

Recently, the influence of culture conditions on GSH levels was investigated by Post *et al.* [12]. They stressed the effect of post-subculture time on GSH content of the cells. This GSH content increased sharply 24 h after passage and decreased thereafter

to the level before subculture. Our cells were harvested 48–72 h after subculture, and although we cannot exclude the possibility that this effect also plays a role in our experiments, our GSH levels are far below the 24 h peak value observed by Post *et al.* and our levels are similar to their steady-state values a few days after subculture.

The GSH content during *in vitro* ageing of skin fibroblasts from patients with Werner's syndrome or Spielmeyer-Vogt syndrome did not differ from the pattern of normal human skin fibroblasts. In all fibroblast strains no decrease in GSH content was found towards the end of the *in vitro* lifespan. Therefore there is no evidence that cellular GSH content plays a role in these ageing syndromes. In general these data suggest that total cellular GSH contents are not correlated with the process of *in vitro* ageing of human skin fibroblasts.

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REFERENCES

1 L. Hayflick, The cellular basis for biological aging. In C.E. Finch and L. Hayflick (eds.), *Handbook of the Biology of Aging*, Van Nostrand Reinhold, New York, 1977, pp. 159–186.
2 S.I.S. Rattan, K.D. Keeler, J.H. Buchanan and R. Holliday, Autofluorescence as an index of ageing in human fibroblasts in culture. *Biosci. Rep., 2* (1982) 561–567.
3 D.W. Deamer and J. Gonzales, Autofluorescent structures in cultured Wi-38 cells. *Arch. Biochem. Biophys., 165* (1974) 421–426.
4 J.F. Jongkind, A. Verkerk, W.J. Visser and J.M. van Dongen, Isolation of autofluorescent "aged" human fibroblasts by flow sorting. Morphology, enzyme activity and proliferative capacity. *Exp. Cell Res., 138* (1982) 409–417.
5 A. Tappel, B. Fletcher and D. Deamer, Effect of antioxidants and nutrients on lipid peroxidation, fluorescent products and aging parameters in the mouse. *J. Gerontol., 28* (1973) 415–424.
6 K.S. Chio and A.L. Tappel, Synthesis and characterization of the fluorescent products derived from malonaldehyde and amino acids. *Biochemistry, 8* (1969) 2821–2827.
7 B.E. Leibovitz and B.V. Siegel, Aspects of free radical reactions in biological systems: Aging. *J. Gerontol., 35* (1980) 45–56.
8 S. Takahashi and M. Zeydel, γ-Glutamyl transpeptidase and glutathione in aging IMR-90 fibroblasts and in differentiating 3T3L1 preadipocytes. *Arch. Biochem. Biophys., 214* (1982) 260–267.
9 J.E. Brehe and H.B. Burch, Enzymatic assay for glutathione. *Anal. Biochem., 74* (1976) 189–197.
10 O.W. Griffith, Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem., 106* (1980) 207–212.
11 J.E. de Josselin de Jong, P.J.M. Hartwijk, H.P. van de Giessen and H. Galjaard, A microspectrophotometer for rapid absorption measurements in 10-μl cuvettes. *Anal. Biochem., 106* (1980) 397–401.
12 G.B. Post, D.A. Keller, K.A. Connor and D.B. Menzel, Effects of culture conditions on glutathione content in A549 cells. *Biochem. Biophys. Res. Commun., 114* (1983) 737–742.
13 H. de Jonge, *Inleiding tot de Medische Statistiek*, Deel 1, [*Verhandeling van het Nederlands Institut voor Praeventieve Geneeskunde XLI*], Leiden, 1963, pp. 305–310.

APPENDIX PAPER 6

DE NOVO SYNTHESIS OF GLUTATHIONE DURING IN VITRO AGEING AND IN SOME METABOLIC DISEASES AS MEASURED BY A FLOW CYTOMETRIC METHOD

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De novo synthesis of glutathione in human fibroblasts during in vitro ageing and in some metabolic diseases as measured by a flow cytometric method

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Key words: Aging; Glutathione, Glutathione synthetase; Enzyme deficiency; Inborn error; (Human fibroblast)

A flow cytometric method to determine cellular GSH contents has been developed. This method is fast and simple and enables the determination of GSH contents in intact cells. Results obtained with the new method correlate well with the results obtained by a specific biochemical assay for GSH ($r = 0.9984$; $n = 7$). The method has been used to determine GSH recovery rates in cultured fibroblasts from healthy subjects and from patients with Werner's syndrome, Spielmeyer-Vogt syndrome and Fanconi's anemia. No obvious differences in GSH recovery rates were observed. GSH recovery rates were also not affected after in vitro ageing. Experiments with cells deficient in GSH synthetase revealed that the observed GSH recovery is exclusively due to de novo synthesis.

Introduction

One of the possible causes of ageing is presumed to be related to free radical reactions [1]. These reactions are also thought to be the underlying cause of conditions such as Werner's syndrome [2], Spielmeyer-Vogt syndrome [3] and Fanconi's anemia [4]. One of the functions of GSH is to defend the cell against free radical reactions [5]. The GSH contents of fibroblasts from healthy persons [6,7] and from patients with some of these syndromes remain constant during in vitro ageing [7]. Using the GSH stability test of Beutler [8], Sass showed old erythrocytes to have a reduced rate of GSH recovery [9]. To establish whether an impaired turnover of GSH is involved in normal in vitro ageing and in the syndromes mentioned we assayed for GSH recovery rates. Previously published methods for the determination of GSH are either insensitive [10] or laborious [11]. Therefore, we modified the flow cytometric technique of Durand and Olive [12] by introducing an *N*-ethylmaleimide blank and using monobromobimane as a fluorescent probe [13].

Materials

Diploid skin fibroblasts were derived from two healthy subjects (C5RO: female, 30 years; 80 RD 143: male, 30 years). The patient material consisted of diploid fibroblasts from a patient with Fanconi's anemia (JdW: male, 17 years), a patient with Werner's syndrome (525 LAD: male, 27 years) and a patient with Spielmeyer-Vogt syndrome (494 LAD: male, 5 years). The cell strain deficient in GSH synthetase [14] is the strain GM 3878 from the Human Genetic Mutant Cell Repository of the Institute for Medical Research (Camden, NJ, U.S.A.).

All cell strains, except for GM 3878 and the

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strain from the patient with Fanconi's anemia, were cultured in Ham's F10 medium supplemented with 8% fetal calf serum (Flow Laboratories) and antibiotics (100 U penicillin and 100 μg streptomycin per ml culture medium). The other two cell strains were cultured with Dulbecco's modified minimal essential medium supplemented in the same way as the F10. Cultures were subcultivated by 1 : 3 splits when the cultures reached confluency. The population doubling level was calculated assuming one passage to be equivalent to 1.58 population doubling (log 3/log 2).

Methods

Determination of GSH

Flow cytometric method. Diamide (diazenedicarboxylic acid bis(*N*,*N*-dimethylamide)) was obtained from Calbiochem. The diamide stock solution was freshly prepared in phosphate-buffered saline. The 50 mM monobromobimane stock solution was prepared in acetonitrile and stored at 4°C in the dark. As monobromobimane decomposes rapidly in aqueous solutions exposed to light, it is added to the F10 medium within 1 min prior to staining. Cells were harvested by trypsinisation and stained as suspensions for 20 min at 37°C with monobromobimane at a final concentration of 37.5 μM. After staining the cell suspensions were placed in an ice bath and assayed immediately. Assays were done with a FACS II cell sorter (Becton and Dickinson) equipped with an argon ion laser set at 360 nm excitation wavelength. Fluorescence above 420 nm was obtained using a KV 399 and a K 420 filter (Schott). The alignment of the instrument was optimised using fluorescent microspheres (Polysciences: £17458). The average fluorescence of the cell population was calculated.

Biochemical method. GSH and GSSG were assayed in homogenates and in culture medium using a biochemical assay as described previously [7]. This assay is an enzymic cycling method for total glutathione as described by Brehe and Burch [11]. To determine GSSG alone, samples were preincubated with 2-vinylpyridine according to Griffith [15]. Significance of difference was determined using student's two-tailed *t*-test.

Results and Discussion

Flow cytometric determination of GSH

In order to develop a flow cytometric assay for GSH we incubated cell suspensions with monobromobimane. To correct for staining due to protein thiol groups, a negative blank sample was introduced. This control consisted of a cell suspension incubated with 200 μM *N*-ethylmaleimide. Cells incubated with a concentration series of *N*-ethylmaleimide (0.01–1 mM) showed a strong decrease in fluorescence when stained with monobromobimane. Fluorescence reached a plateau of 10% of control fluorescence at 200 μM *N*-ethylmaleimide. Protein thiol groups account for 2.7% of cellular thiol [16] and the fluorescence of unstained cells amounts of 8% of that of stained cells. So, the 10% residual fluorescence found with 200 μM *N*-ethylmaleimide results from cellular autofluorescence and staining of protein thiol. Furthermore, *N*-ethylmaleimide completely suppressed GSH-specific fluorescence, without affecting protein thiol fluorescence [16].

In order to calibrate the method, cell suspensions with different GSH contents were assayed by both the biochemical and the flow cytometric method. These cell suspensions with different GSH contents were obtained by incubating the cells with various concentrations of cumene hydroperoxide. Both methods yield similar results ($r = 0.9984$, $n = 7$). Hence, the flow cytometric method gives essentially the same information as the biochemical assay, but in a much faster and less laborious way.

GSH recovery rates

This new method has been used to determine cellular GSH contents after depletion with diamide [17]. Cells were harvested by trypsinisation and collected by centrifugation (5 min at 200 × *g*). They were resuspended in F10 culture medium without serum. Incubating the cells for 40 min with 0.5 mM diamide at 37°C results in a depletion of GSH from these cells (Fig. 1). After depletion, cells were collected and resuspended in the F10 medium without serum or diamide in order to measure GSH recovery. Fig. 1 shows the GSH recovery as obtained with cell cultures of different in vitro age from two control cell strains. In one

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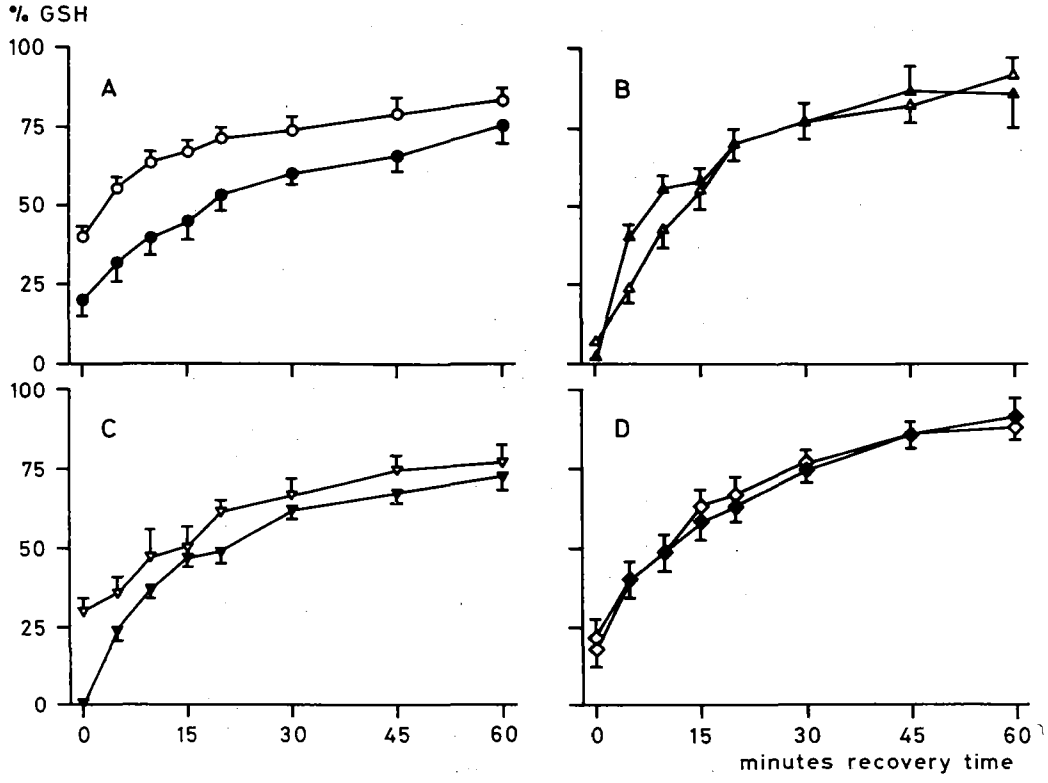

Fig. 1. GSH recovery after depletion with diamide of cells of differing in vitro age. The 100% value is the GSH content of the corresponding cells not treated with diamide. Zero time is taken as the moment at which diamide is removed from the cells and recovery is started. Bars indicate standard errors. (A) Control strain C5R0: (○) 'young' (population doubling level 33–39) and (●) 'old' (population doubling level 59–60) cells. Number of experiments is six for 'young' and four for 'old' cells. 100% GSH corresponds to 20.4 ± 2.2 nmol GSH per mg protein. In the presence of diamide, 'young' cells contained 3% and 'old' cells 0% of their initial GSH level. Time points from 0 to 45 min yield statistically significant differences between 'young' and 'old'. (B) Control strain 80 RD 143: (△) 'young' (population doubling level 24–28) and (▲) 'old' (population doubling level 46–50) cells. Number of experiments is five. 100% GSH corresponds to 23.6 ± 1.9 nmol GSH per mg protein. In the presence of diamide, 'young' cells contained 1% and 'old' cells 0% of their initial GSH level. No statistically significant difference was found. (C) Cells from a patient with Werner's syndrome (strain 525 LAD), (▽) 'young' (population doubling level 16) and (▼) 'old' (population doubling level 18, terminal). Number of experiments is four. 100% GSH corresponds to 22.2 ± 1.7 nmol GSH per mg protein. In the presence of diamide, 'young' and 'old' cells contained 0% of their initial GSH level. No statistically significant difference was found. (D) Cells from a patient with Spielmeyer-Vogt syndrome (strain 494 LAD), (◇) 'young' (population doubling level 17–23) and (◆) 'old' (population doubling level 37–43). Number of experiments is five. 100% GSH corresponds to 22.8 ± 2.1 nmol GSH per mg protein. In the presence of diamide, 'young' cells contained 4% and 'old' cells 2% of their initial GSH level. No statistically significant difference was found.

case (C5R0) a faster GSH recovery was found in 'young' relative to 'old' cells. However, another cell stain (80 RD 143) showed no significant differences in GSH recovery. So, contrary to the findings with erythrocytes [9], a decrease in GSH recovery is not a phenomenon that is linked with in vitro ageing of human skin fibroblasts.

Fibroblasts from patients with Werner's syndrome typically show a reduced growth rate and chromosomal instability [18,19]. It is suggested that this could result from an impaired defence against free radical reactions. GSH contents [7] and the activities of superoxide dismutase, catalase and glutathione peroxidase were within the normal range [2]. Neither the addition of free radical scavengers to the culture medium nor

lowered oxygen tension [20] could abolish the impaired growth rate and the chromosomal instability found in these cells. As Fig. 1C shows, GSH recovery is within the range covered by the control cell strains, and also, GSH recovery is not altered upon in vitro ageing of these mutant cells. Our findings and those reported in the literature do not point towards an impaired defence against free radical reactions as the underlying cause of the described phenomena.

Patients having Spielmeyer-Vogt syndrome (neuronal ceroid lipofuscinosis) showed a decreased defence against free radicals in the cerebrospinal fluid [3]. Furthermore, lipofuscin is stored in these patients. Lipofuscin is considered to be the final product of lipid peroxidation and protein cross-linking due to free radical reactions [21,22]. In cultured human skin fibroblasts from these patients a normal GSH content was found [7]. From fig. 1D it is evident that GSH recovery is also within the range covered by the control cell strains and that this recovery is not changed upon in vitro ageing of these mutant cells. Hence, a defect in the GSH system of cultured Spielmeyer-Vogt fibroblasts is not likely.

In erythrocytes from patients with Fanconi's anemia a decrease in activity of superoxide dismutase [4] and an increase in the rate of generation of superoxide was found [23]. With cultured fibroblasts, elevated levels of chromosome aberrations but normal levels of sister chromatid exchanges have been reproted [24]. These mutant fibroblasts have a normal GSH content and their GSH recovery is not distinct from that of control cells, as is evidenced in Fig. 2. These findings do not indicate a defect in the GSH system in cultured fibroblasts from patients with this syndrome.

After 40 min incubation with diamide, cells were devoid of GSH (Fig. 2), but showed no elevation of GSSG (1.6 ± 0.4 nmol GSSG/mg protein in diamide-treated cells vs. 1.5 ± 0.7 nmol GSSG/mg protein in controls). Assays in the culture medium indicated that the GSSG formed intracellularly due to the presence of diamide is extruded from the cells (11.5 ± 0.3 nmol GSSG/mg protein in diamide-treated cells vs. 0.6 ± 0.3 nmol GSSG/mg protein in controls). This is in line with the results obtained with isolated

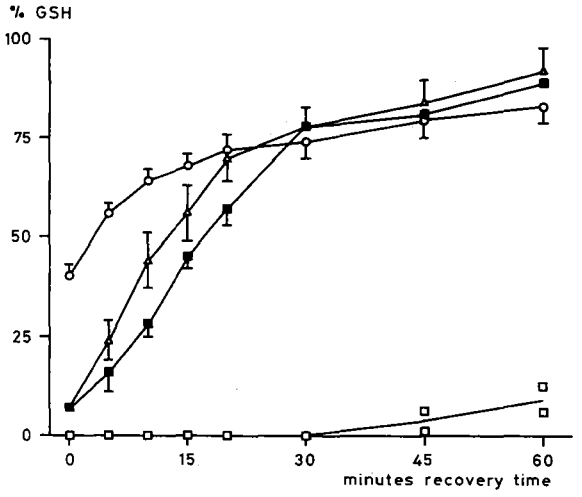

Fig. 2. GSH recovery after depletion with diamide of cells from a patient with Fanconi's anemia (■), from a 'young' culture of two control cell strains (○, △) and from the glutathione synthetase-deficient cell strain GM 3878 (□). Bars indicate standard errors as obtained from four experiments (Fanconi's anemia) and two experiments (GM 3878). Zero time is taken as the moment at which diamide is removed from the cells and recovery is started. 100% value is the GSH content of the corresponding untreated cells. The 100% GSH level is 12.0 ± 0.4 nmol GSH per mg protein for the GM 3878 cells and 18.9 ± 2.1 nmol GSH per mg protein for the cells from the patient with Fanconi's anemia. No statistically significant difference was found between the control and the Fanconi's anemia cell strain.

hepatocytes by Sies et al. [25]. From this we conclude that the recovery of the initial GSH content results from the de novo synthesis of GSH. This is substantiated by the results obtained with a cell strain with a severely diminished activity of GSH synthetase [14]. During the first 30 min following GSH depletion no intracellular GSH could be detected (Fig. 2), while during longer incubations only a small part of the GSH was recovered. This can be explained as resulting from the 4.4% residual activity of the glutathione synthetase in these cells. Hence, the GSH recovery assayed by this method is solely due to de novo synthesis of GSH. Our data indicate that the rate of de novo synthesis of GSH is not lowered in the syndromes of Werner or Spielmeyer-Vogt, nor in Fanconi's anemia. The hypothesis that a defect in the defence against cellular free radical reactions is the cause of in vitro ageing is not substantiated by this study.

After submission of this manuscript Treumer and Valet published a method for the determination of GSH [16]. They used *o*-phthaldialdehyde, which gives a GSH-specific fluorescence. This method is also a powerful flow cytometric assay for GSH.

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References

1 Harman, D. (1956) J. Gerontol. 11, 298–300
2 Marklund, S., Nordensson, I. and Beck, O. (1981) J. Gerontol. 36, 405–409
3 Gutteridge, J.M.C., Halliwell, B., Rowley, D.A. and Westermark, T. (1982) Lancet ii, 459–463
4 Joenje, H., Frants, R.R., Arwert, F., De Bruin, G.J.M., Kostense, P.J., Van de Kamp, J.J.P., De Koning, J. and Eriksson, A.W. (1979) Scand. J. Clin. Lab. Invest. 39, 759–764
5 Meister, A. (1984) Fed. Proc. 43, 3031–3042
6 Mbemba, F., Houbion, A., Raes, M. and Remacle, J. (1985) Biochim. Biophys. Acta 838, 211–220
7 Poot, M., Verkerk, A. and Jongkind, J.F. (1984) Mech. Ageing Dev. 27, 315–321
8 Beutler, E. (1957) J. Lab. Clin. Med. 49, 84–95
9 Sass, M.D. (1966) Clin. Chim. Acta 15, 1–6
10 Beutler, E., Duron, O. and Kelly, B.M. (1963) J. Lab. Clin. Med. 61, 882–888
11 Brehe, J.E. and Burch, H.B. (1976) Anal. Biochem. 74, 189–197
12 Durand, R.E. and Olive, P.L. (1983) Radiat. Res. 95, 456–470
13 Kosower, N.S., Newton, G.L. and Ranney, H.M. (1979) Proc. Natl. Acad. Sci. USA 76, 3382–3386
14 Spielberg, S.P., Kramer, L.I., Goodman, S.I., Butler, J., Tietze, F., Quinn, P. and Schulman, J.D. (1977) J. Pediatr. 91, 237–241
15 Griffith, O.W. (1980) Anal. Biochem. 106, 207–212
16 Treumer, J. and Valet, G. (1986) Exp. Cell Res. 163, 518–524
17 Kosower, E.W., Correa, W., Kinon, B.J. and Kosower, N.S. (1972) Biochim. Biophys. Acta 264, 39–44
18 Salk, D. (1982) Hum. Genet. 62, 1–15
19 Thompson, K.V.A. and Holliday, R. (1983) Gerontology 29, 73–82
20 Salk, D., Au, K., Hoehn, H. and Martin, G.M. (1981) Hum. Genet. 57, 269–275
21 Koster, J.F. and Slee, R.G. (1980) Biochim. Biophys. Acta 620, 489–499
22 Dillard, C.J. and Tappel, A.L. (1971) Lipids 6, 715–721
23 Scarpa, M., Rigo, A., Momo, F., Asacchi, G., Novelli, G. and Dallapiccola, B. (1985) Biochem. Biophys. Res. Commun. 130, 127–132
24 Kato, H. and Stich, H.F. (1976) Nature 260, 447–448
25 Sies, H., Gertsenecker, C., Menzel, H. and Flohe, L. (1972) FEBS Lett. 27, 171–175

APPENDIX PAPER 7

INFLUENCE OF CUMENE HYDROPEROXIDE AND 4-HYDROXYNONENAL ON THE GLUTATHIONE METABOLISM DURING IN VITRO AGEING OF HUMAN SKIN FIBROBLASTS

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Influence of cumene hydroperoxide and 4-hydroxynonenal on the glutathione metabolism during *in vitro* ageing of human skin fibroblasts

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Cumene hydroperoxide (Chp) and 4-hydroxynonenal (HNE) were used to investigate the effect of peroxidative challenge upon the glutathione (GSH) metabolism of human skin fibroblasts. Cellular GSH contents decreased during short-term incubations with Chp and oxidised glutathione (GSSG) was formed concomitantly. During longer incubations the GSH level was restored and the substrate flux through the pentose phosphate shunt increased. So in the presence of hydroperoxides the GSH level is maintained by reduction of GSSG.

HNE caused a strong decrease in cellular GSH contents. Prolonged incubation with HNE lead to a rise in GSH contents above the basal level. The flux through the pentose phosphate shunt did not change during exposure to HNE. Hence, during incubation with HNE the cell maintains its GSH content by *de novo* synthesis of GSH. This conclusion is further substantiated by the findings with a cell strain deficient in GSH synthetase. These cells survived if incubated with Chp but not if exposed to HNE.

GSH contents of normal cells from phase II (young) cultures and from phase III (aged) cultures responded similarly to Chp during short-term incubations and during a week of culture with the test compound. The flux through the pentose phosphate shunt rose much more in phase III than in phase II cells when incubated with the same concentration series of Chp. We conclude that during *in vitro* ageing the amount of NADPH needed to maintain cellular GSH levels in the presence of hydroperoxides increases, while the capacity to respond to such a challenge is not affected.

The phenomenon of cells ageing in culture was first described by Hayflick and Moorhead [1] and has been reproduced in many different systems [2–4]. To explain this phenomenon two types of theory have been put forward: one invokes an underlying genetic programme [5] while the other presumes stochastically arising damage to be the basic cause. One of the main hypotheses of the second type is the free-radical theory of ageing [6]. Free-radical reactions, for instance those arising upon the one-electron reduction of oxygen [7], are presumed to damage the cell. One of the major effects of free-radical reactions in the cell is lipid peroxidation [8]. Cellular aerobic metabolism gives rise to several species of reactive oxygen, including superoxide, hydroperoxides and hydroxyl radicals [9, 10], against which the cell has defence mechanisms such as the glutathione system [11]. During *in vitro* ageing the level of GSH remains almost constant [12, 13]. While GSH is equally available in all cell organelles, glutathione reductase and glutathione peroxidase activities decrease considerably during *in vitro* ageing [13]. On the other hand, the rate of *de novo* synthesis of GSH is not altered upon *in vitro* ageing [14].

Cumene hydroperoxide (Chp) turned out to be a suitable model compound with which to study the effects of hydroperoxides [15–19]. Chp is easily taken up by cells and is not metabolised by catalase [16]. Chp is transformed by glutathione peroxidase to the corresponding alcohol and GSH is concomitantly converted to GSSG. Therefore, Chp can be used to test the glutathione redox cycle and the metabolic circuitry connected with it. The lipophilicity of Chp allows its use as an initiator of lipid peroxidation [17], which in turn gives rise to diffusable products, among which 4-hydroxy-2,3-*trans*-nonenal (HNE) is a major one [20]. The latter compound can be conjugated to GSH by glutathione *S*-transferase to form a stable adduct [21, 22]. As no GSSG is formed during this reaction HNE can be used to analyse the system of *de novo* synthesis of glutathione.

MATERIALS AND METHODS

Cells and culture

Diploid skin fibroblasts were derived from a healthy person (C5RO: female, 30 years) and are referred to as normal cells. The glutathione-synthetase-deficient cell strain was GM 3878 from the Human Genetic Mutant Cell Repository of the Institute for Medical Research (Camden, New Jersey, USA) and has been described in detail [23]. The activity of

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Abbreviations. Chp, cumene hydroperoxide; HNE, 4-hydroxy-2,3-*trans*-nonenal; GSH, glutathione; GSSG, oxidised glutathione.

Enzymes. Glutathione peroxidase (EC 1.11.1.9); glutathione reductase (EC 1.6.4.2); glutathione synthetase (EC 6.3.2.3); glutathione *S*-transferase (EC 2.5.1.18).

glutathione synthetase of this cell strain is 4.4% that of normal cells [23].

The normal cell strain (C5RO) was cultured in Ham's F10 medium supplemented with 8% foetal calf serum (Flow Laboratories, lot 29101086) and antibiotics (100 U penicillin and 100 μg streptomycin/ml culture medium). The GM 3878 cells were cultured in Dulbecco's modified minimal essential medium supplemented as the F10 medium. Cultures were subcultivated by 1:3 splits when the cultures reached confluency. The population doubling level (PDL) was calculated assuming one passage to be equivalent to 1.58 PDL. Cultures are referred to as phase II (young) when they had completed less than two-thirds of their *in vitro* lifespan as was determined in a previous experiment [24]. Phase III (aged) cells are defined as cells having completed at least 90% of their *in vitro* lifespan. In the experiments with vitamin E, cells were cultured with 10 μg vitamin E/ml culture medium

Incubation system

Cells were seeded into holes of 24-well culture dishes (Costar 3524) and allowed to reach confluency and density-dependent inhibition of growth, which was ascertained by the complete absence of mitoses. Thereupon the culture medium was replenished and test compounds were added to the appropriate concentrations. Chp was suspended into phosphate-buffered saline ($NaCl/P_i$) and diluted with $NaCl/P_i$ to a final concentration of 0.2 mM. HNE was synthesized as described previously [25] and obtained by evaporation of the chlorophorm in which it was stored, dissolved into distilled water and diluted with $NaCl/P_i$. Both test compounds were filter-sterilised (0.22 μm Sterivex-GS, Millipore). All solutions of test compounds were prepared immediately before use. Media with the test compounds were replenished every three days. The GM 3878 cells were cultured in Dulbecco's minimal essential medium and some C5RO cells were also cultured in this medium to provide an appropriate control for effects of culture medium. One week after the initiation of the cultures with the test compounds the cells were analysed using the assays described below.

Assays for glutathione

GSH and GSSG were determined in $NaCl/P_i$ homogenates of cells and in incubation medium according to the method described previously [12]. In intact cells GSH contents were determined using a newly developed flow cytometric assay based upon monobromobimane as thiol-staining reagent [14]. Cells were harvested by trypsinization and collected by centrifugation (5 min at $200 \times g$). They were resuspended in F10 culture medium without serum and incubated during 20 min at 37 °C with the appropriate concentration of test compound. One sample was incubated with 200 μM *N*-ethylmaleimide during 1 h as a control for spurious staining. Monobromobimane was added to all suspensions at a final concentration of 37.5 μM and staining was for 20 min at 37 °C. A monobromobimane (Calbiochem) stock solution of 50 mM was prepared in acetonitrile (Merck) and stored at 4 °C in the dark. After staining fluorescence was recorded with a FACS II cell sorter (Becton and Dickinson) equipped with an argon ion laser set at 360 nm excitation wavelength. Fluorescence above 420 nm was obtained using a KV 399 and a K 420 filter (Schott). The average fluorescence of the cell population was calculated. All GSH contents are expressed as a percentage relative to a control cultured without test compounds.

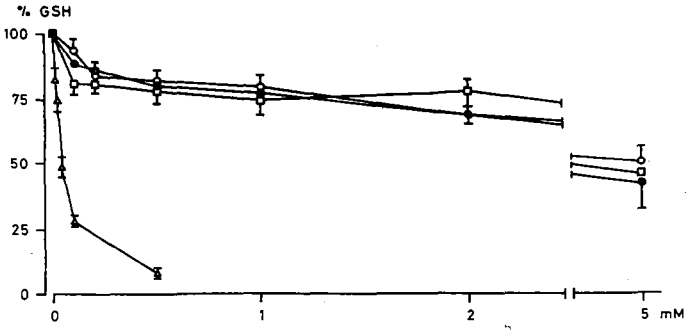

Fig. 1. *GSH contents of cells after a short-term incubation with a concentration series of Chp or HNE.* All incubations were during 15 min at 37 °C in F10 culture medium with 200000 cells in 200 μl. Incubations with Chp: phase II normal cells (●) ($n = 7$), phase III normal cells (○) ($n = 4$) and phase II cells from the glutathione-synthetase-deficient cell strain GM 3878 (□) ($n = 4$). Incubation with HNE: phase II normal cells (△) ($n = 4$). All values are percentages relative to samples not incubated with test compound. Bars indicate standard errors of the mean

Assay for pentose phosphate shunt

The flux through the pentose phosphate shunt was determined using radioactively labelled glucose. Briefly, cells cultured with either Chp or HNE were washed once with $NaCl/P_i$ and then incubated during 2 h with culture medium containing the appropriate concentration of test compound and 0.5 μCi [1-^{14}C]glucose (Amersham, specific activity 55 Ci/mol)/ml culture medium or 0.5 μCi [6-^{14}C]glucose (Amersham, specific activity 55 Ci/mol)/ml culture medium. The carbon dioxide liberated by the cells from the radiolabelled glucose was collected with a Millipore AP25 filter, which was saturated with 100 μl 3.5 M KOH. After incubation the medium was acidified by adding sulfuric acid to a final concentration of 0.3 M. After 1 h additional incubation the filters were put into scintillation vials to which 0.5 ml distilled water was added. By vigorous stirring the filter was suspended. After adding Instagel (Packard) to each vial radioactivity was determined with a liquid scintillation analyser (Packard).

The rate of NADPH generation due to the flux of glucose through the pentose phosphate shunt was calculated by subtracting the carbon dioxide liberated from C-6-labelled glucose from the carbon dioxide obtained from C-1-labelled glucose. This calculation mode is essentially according to the method developed by Reitzer et al. [26]. This method is valid in our system, as essentially all radiolabelled glucose is converted to lactate and pyruvate.

Protein determination

Protein was determined with the fluorescamine reagent according Jongkind et al. [27].

RESULTS

GSH contents

To analyse short-term effects of both Chp and HNE upon cellular GSH contents cells were incubated during 15 min at 37 °C in F10 culture medium with a concentration series of either Chp or HNE. From Fig. 1 it is evident that GSH contents of phase II (young) and phase III (aged) normal

Table 1. *Glutathione content of cells cultured during one week with a concentration series of Chp*
Cells were cultured during one week with Chp and culture media were replenished on days 0,3 and 6; GSH contents were assayed for on day 7. All GSH contents are relative to controls not incubated with Chp and are expressed as means plus standard errors of the mean. The 100% GSH level was 20.0 ± 0.6 nmol GSH/mg protein for phase II cells and 31.4 ± 1.1 nmol GSH/mg protein for phase III cells

Cell	Concentration of Chp	GSH content
	μM	%
Phase II	0	100
	10	96 ± 2
	20	107 ± 11
	40	106 ± 8
Phase III	0	100
	10	92 ± 11
	20	98 ± 13
	40	102 ± 15

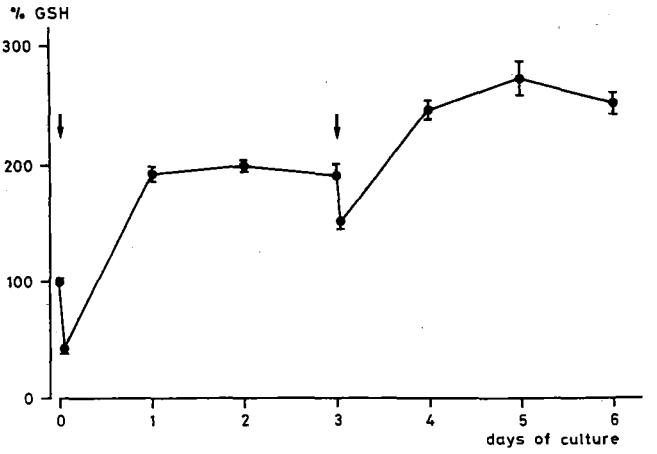

Fig. 2. *GSH content of normal cells cultured during one week with 40 μM HNE.* Arrows indicate points where fresh medium with HNE is added. All values are percentages relative a sample not incubated with HNE. Bars indicate standard errors of the mean from an experiment in triplicate

cells and of cells with a strongly diminished GSH synthetase activity (GM 3878) responded in exactly the same way to Chp. Cells cultured with vitamin E showed a similar response towards Chp as did normal cells (results not shown). So the GSH contents of all cell strains tested responded similarly towards Chp. Fig. 1 also displays the GSH depletion caused by HNE in the same incubation system as used with Chp. The GSH depletion with HNE is noteably more pronounced than that obtained with Chp.

To investigate the long-term effects of Chp and HNE, cells were cultured with these compounds during one week in a growth-inhibited (confluent) system. All normal cells survived during one week in concentrations up to 40 μM Chp or HNE, as was revealed by microscopical examination and protein determination. Cells from the glutathione-synthetase-deficient cell strain (GM 3878) survived in Chp as well as normal cells, but dit not survive in HNE at concentrations above 20 μM.

Table 1 shows cellular GSH contents as found after one week of culture with Chp. In all cell cultures tested a constant intracellular GSH level was found. In order to analyse long-term effects of HNE cells were cultured during a week with 40 μM HNE. In contrast to the results obtained with CHP, normal cells incubated with 40 μM HNE showed a 90% rise in GSH level after one addition of HNE and a 170% rise in GSH level after two additions of HNE (Fig. 2).

Cells exposed to 1 mM Chp in the absence of glucose showed a large extracellular accumulation of GSSG (Table 2). The intracellular GSSG content, however, was not significantly affected. Cells incubated with 0.5 mM HNE displayed a 92% decrease in GSH content without an intracellular or extracellular accumulation of GSSG (Table 2).

NADPH generation

To analyse the turnover of glutathione in the glutathione redox cycle during incubation with Chp and HNE we determined the velocity of the NADPH-generating pentose phosphate shunt. Fig. 3 shows the flux through the pentose phosphate shunt as obtained from cells cultured with Chp. The basal flux of glucose through the pentose phosphate shunt in the absence of Chp in phase III (aged) cells is 21% of the

Table 2. *Formation and efflux of GSSG during incubation with Chp or HNE in the absence of glucose*
Phase II normal cells were incubated during 15 min at 37°C with 1 mM Chp in phosphate-buffered saline. Values are from three independent experiments and expressed as means plus standard error of the mean

Compound	GSH or GSSH content after treatment with		
	control	1 mM Chp	0.5 mM HNE
	nmol/mg protein		
GSH	20.0 ± 0.6	7.2 ± 0.9	1.6 ± 0.4
GSSG			
Intracellular	1.50 ± 0.71	0.90 ± 0.31	0.72 ± 0.42
Extracellular	0.58 ± 0.26	7.73 ± 0.27	0.53 ± 0.25

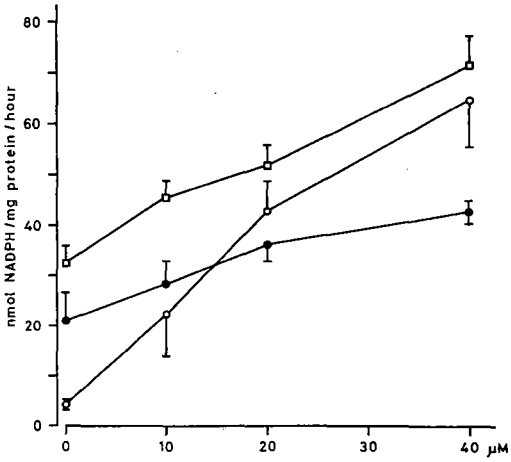

Fig. 3. *Glucose flux through the pentose phosphate shunt in cells cultured with Chp.* Phase II normal cells (●) (n = 4), phase III normal cells (○) (n = 3) and phase II cells from the glutathione-synthetase-deficient cell strain GM 3878 (□) (n = 4). Bars indicate standard errors of the mean

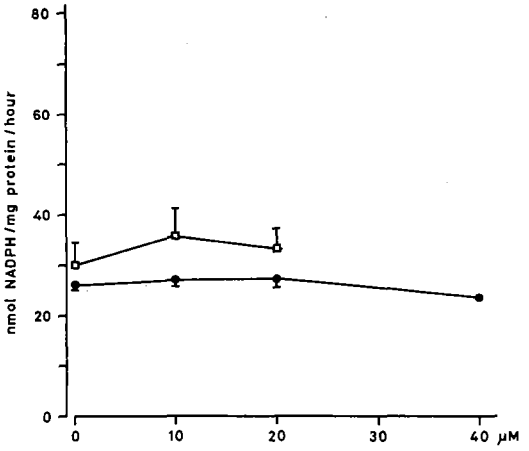

Fig. 4. *Glucose flux through the pentose phosphate shunt in cells cultured with HNE.* Phase II normal cells (●) (n = 3) and phase II cells from the glutathione-synthetase-deficient cell strain GM 3878 (□) (n = 3). Bars indicate standard errors of the mean

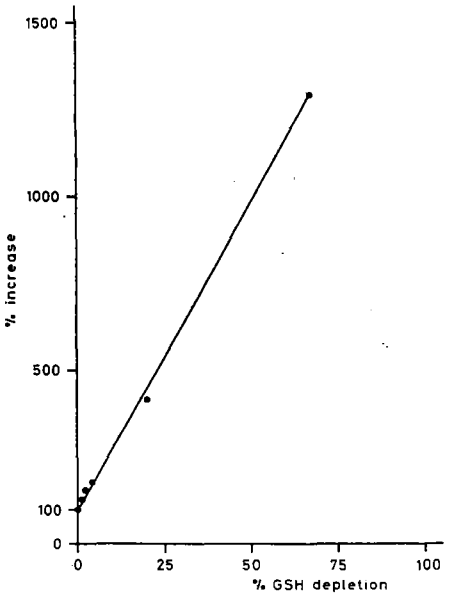

Fig. 5. *Correlation between rise in glucose flux through the pentose phosphate shunt and percentage of GSH depletion.* Control cells were incubated during 15 min with a concentration series of diamide, which leads to the formation of GSSG. Linear regression after five independent experiments (each of them in triplicate) r = 0.9993. The 100% value of flux through the pentose phosphate shunt is 21.0 ± 5.6 nmol NADPH mg protein^{-1} h^{-1} for cells containing 20.0 ± 0.6 nmol GSH mg protein^{-1}

flux in phase II (young) cells. Both phase II and phase III normal cells exhibit a rise in flux through the pentose phosphate shunt in response to increasing concentrations of Chp. In phase III cells this rise is more pronounced than in phase II cells. Cells cultured with vitamin E followed the same pattern as their counterparts not cultured with the free radical scavenger (results not shown). The cells with a stongly diminished activity of GSH synthetase (GM 3878) responded differently from Chp with respect to the flux through the pentose phosphate shunt. The basal level of flux through the pentose phosphate shunt is 60% higher than in normal cells. The rise in flux through the pentose phosphate shunt in GM 3878 cells is slightly, but not significantly, more than in phase II normal cells.

In contrast to the results obtained with Chp, incubation with HNE did not lead to any rise in the flux through the pentose phosphate shunt (Fig. 4). The response of the flux through the pentose phosphate shunt to various levels of GSH depletion is shown in Fig. 5. This flux rises linearly up to a GSH depletion of 68% (regression coefficient r = 0.9993; n = 5).

DISCUSSION

This study was undertaken to see to what extent and by which mechanisms human skin fibroblasts remain capable of maintaining GSH levels in the presence of hydroperoxides or products of lipid peroxidation. Although cellular GSH contents remain constant during *in vitro* ageing [12, 13], it is possible that the turnover of GSH changes and that the capability to maintain GSH levels during exposure to peroxides decreases. The cellular GSH level is maintained by two pathways: either *de novo* synthesis from amino acids or reduction of GSSG. In principle the cell can operate both systems simultaneously. The reductive pathway to maintain GSH levels requires NADPH, which in turn has to be replenished by the pentose phosphate shunt.

Chp causes GSH depletion (Fig. 1) and a concomitant rise in the flux through the pentose phosphate shunt (Fig. 3). Even more so, the flux through the pentose phosphate shunt correlates linearly with the level of GSH depletion (Fig. 5) [28]. Along with GSH depletion Chp also causes the formation of GSSG (Table 2). This prompts the question of whether the rise through the pentose phosphate shunt responds to GSH depletion or to the formation of GSSG. HNE also causes a decrease in GSH level (Fig. 1), but without concomitant formation of GSSG (Table 2). The flux through the pentose phosphate shunt is not affected by HNE. Hence, the flux through the pentose phosphate shunt rises only in response to an increase in the formation of GSSG.

The rise in flux through the pentose phosphate shunt in cells with a strongly diminished activity of glutathione synthetase exposed to a concentration series of Chp did not differ significantly from that in normal cells (Fig. 3). Therefore, the contribution of *de novo* synthesis of glutathione is negligible under these conditions. Hence, we conclude that the cell maintains its GSH content during exposure to hydroperoxides by reducing the GSSG formed.

Cells incubated with HNE showed a strong decrease in GSH content during a short incubation (Fig. 1), but an elevated GSH level during a week of culture with the test compound (Fig. 2). Incubation with HNE did not lead to formation of GSSG (Table 2) nor to a rise in the flux through the pentose phosphate shunt (Fig. 4). So the elevated GSH level after culture with HNE can only result from *de novo* synthesis of GSH. This means that cells exposed to compounds that can be conjugated to GSH, like the aldehydes resulting from lipid peroxidation, maintain GSH levels by *de novo* synthesis of GSH. Furthermore, this is strongly substantiated by the finding that GSH-synthetase-deficient cells did not survive in low concentrations of HNE.

Fig. 3 shows a much stonger rise in flux through the pentose phosphate shunt in phase III than in phase II cells when incubated with 40 μM Chp. This means that in phase III cells much more NADPH is being consumed during incubation with Chp than is needed to reduce the GSSG formed. A possible explanation for this result could be that during *in vitro* ageing impaired enzyme activities might accumulate.

Those might have been changed such that they consume NADPH without concomitant reduction of GSH. Evidence in favour of this explanation is reported for glutathione reductase [29, 30]. These authors showed that glutathione reductase can be modified into an NADPH dehydrogenase not possessing GSSG-reducing activity. Such a modified enzyme might be responsible for the increased rate of NADPH consumption found in our system. The accumulation of such a modified enzyme might lead to a changed coupling of metabolic systems during *in vitro* ageing.

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REFERENCES

1. Hayflick, L. & Moorhead, P. S. (1961) *Exp. Cell Res. 25*, 585–621.
2. Jacobs, J. P., Jones, C. M. & Baille, J. P. (1970) *Nature (Lond.) 227*, 168–170.
3. Nichols, W. W., Murphy, D. G., Cristofalo, V. J., Toji, L. H., Greene, A. E. & Dwight, S. A. (1977) *Science (Wash. DC) 196*, 60–63.
4. Raes, M. & Remacle, J. (1983) *Exp. Gerontol. 18*, 223–240.
5. Wilson, D. L. (1974) *Theoretical aspects of ageing* (Rockstein, M., Sussman, M. L. & Chesky, J., eds) Academic Press, New York.
6. Harman, D. (1956) *J. Gerontol. 11*, 289–300.
7. Chance, B., Sies, H. & Boveris, A. (1979) *Phys. Rev. 59*, 527–605.
8. Pryor W. A. (1976) in *Free radicals in biology* (Pryor, W. A., ed.) vol. 1, pp. 1–49, Academic Press, New York.
9. Nohl, H. & Hegner, D. (1978) *Eur. J. Biochem. 82*, 563–567.
10. Sies, H. & Chance, B. (1970) *FEBS Lett. 11*, 172–176.
11. Freeman, A. B. & Crapo, J. D. (1982) *Lab. Invest. 47*, 412–426.
12. Poot, M., Verkerk, A. & Jongkind, J. F. (1984) *Mech. Ageing Dev. 27*, 315–321.
13. Mbemba, F., Houbion, A., Raes, M. & Remacle, J. (1985) *Biochim. Biophys. Acta 838*, 211–220.
14. Poot, M., Verkerk, A., Koster, J. F. & Jongkind, J. F. (1986) *Biochim. Biophys. Acta 883*, 580–584.
15. Sies, H., Gerstenecker, C., Menzel, H. & Flohe, L. (1972) *FEBS Lett. 27*, 171–175.
16. Sies, H. & Summer, K.-H. (1975) *Eur. J. Biochem. 57*, 503–512.
17. Högberg, J., Orrenius, S. & O'Brien, P. J. (1975) *Eur. J. Biochem. 59*, 449–455.
18. Chiarpotto, E., Poli, G., Albano, E., Gravela, E. & Dianzani, M. U. (1984) *Exp. Mol. Pathol. 41*, 191–201.
19. Shimura, J., Shimura, F. & Hosoya, N. (1985) *Biochim. Biophys. Acta 845*, 43–47.
20. Benedetti, A., Esterbauer, H., Ferrali, M., Fulceri, R. & Comporti, M. (1982) *Biochim. Biophys. Acta 711*, 345–356.
21. Cadenas, E., Muller, A., Brigelius, R., Esterbauer, H. & Sies, H. (1983) *Biochem. J. 214*, 479–487.
22. Esterbauer, H., Zollner, H. & Lang, J. (1985) *Biochem. J. 228*, 363–373.
23. Spielberg, S. P., Kramer, L. I., Goodman, S. I., Butler, J., Tietze, F., Quinn, P. & Schulman, J. D. (1977) *J. Pediatr. 91*, 237–241.
24. Poot, M., Verkerk, A., Visser, J. W. & Jongkind, J. F. (1985) *Gerontology 31*, 158–165.
25. Esterbauer, H. & Wegner, W. (1976) *Monatsh. Chem. 98*, 1994–2000.
26. Reitzer, L. J., Wice, B. M. & Kenell, D. (1979) *J. Biol. Chem. 254*, 2669–2676.
27. Jongkind, J. F., Verkerk, A., Visser, W. J. & Van Dongen, J. M. (1982) *Exp. Cell Res. 138*, 409–417.
28. Hosada, S. & Nakamura, W. (1970) *Biochim. Biophys. Acta 222*, 53–64.
29. Carlberg, I., Sahlman, L. & Mannervik, B. (1985) *FEBS Lett. 180*, 102–106.
30. Carlberg, I. & Mannervik, B. (1986) *J. Biol. Chem. 261*, 1629–1635.

Chapter 6

CURRICULUM VITAE

May 4th, 1972: Completion of secondary education, HBS-B Certificate

May 4th, 1976: Completion of candidate studies in biology

December 4th, 1979: Completion of studies in Biology
Secondary subject: Botany (Phytopathology)
Primary subject: Metabolic Pharmacology/ Toxicology
(DNA repair as a test to detect DNA alkylating agents)
Primary subject: Cellular biochemistry
(Regulation of the cell cycle and differentiation in Physarum polycephalum (Schwein.)

Febraury 1st, 1980 to November 1st, 1981: Appointment in the Department of Chemical Cytology at the University of Nijmegen (Chairman Prof.Dr. Ch.M.A. Kuyper), to do research into the regulation of cell proliferation in cultured bovine liver cells with Dr. F. Wanka. Educational activity as assistent teacher in Cell Biology and assistent coordinator of the undergraduate course in Cellular Biochemistry.

November 1st, 1981 to April 1 st, 1986: Appointment in the Department of Cell Biology and Genetics of the Erasmus University Rotterdam (Chairman: Prof.Dr. H. Galjaard). Research into the possible role free radical reactions might play in the causation of ageing of cultured cells. This work was carried out under the authority of Prof.Dr. J.F. Jongkind and supported by Prof.Dr. J.F. Koster (Department of Biochemistry). Educational activities in Cell Biology, Histology and Immunology.

From August 1st of 1986 on, the writer works in the Department of Human Genetics of the University of Wuerzburg (Bavaria State, F.R.G.). Research is concerned with disturbed cell proliferation in human genetic phenotypes (Fanconi's anemia, Bloom's syndrome) or induced by species of reactive oxygen and their metabolic products. Teaching in the "Pathology of Oxygen Free Radicals", and the "Application of Flow Cytometry in Biology and Medicine".