STUDIES ON THE DNA REPAIR CAPACITY OF ISOLATED NEURONAL, ASTROGLIAL AND OLIGODENDROGLIAL CELLS OF RAT BRAIN OF DIFFERENT AGES

A THESIS SUBMITTED TO THE UNIVERSITY OF HYDERABAD
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

BY

K. SUBRAHMANYAM

SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD-500 134
INDIA

JUNE 1989

ENROLMENT NO. : JL2139

To my parents

CERTIFICATE

School of Life Sciences

UNIVERSITY OF HYDERABAD

Hyderabad 500 134

India

Date: 5-6-1989

This is to certify that I, K. **SUBRAHMANYAM,** have carried out the research work embodied in the present thesis for the full period prescribed under Ph.D. Ordinances of the University of Hyderabad under the supervision of Prof. Kalluri Subba Rao.

I declare to the best of my knowledge that no part of this thesis was earlier submitted for the award of Research Degree of any other university.

Signature of the Supervisor

(Prof. K. Subba Rao)

Signature of the candidate

(K. Subrahmanyam)

Enrolment No: JL 2139.

DEAN

SCHOOL OF LIFE SCIENCES

ACKNOWLEDGEMENTS

It gives me great pleasure to express my deep sense of gratitude to Prof. Kalluri Subba Rao, M.Sc, Ph.D., A.I.C., for introducing me to the exciting field of DNA damage and repair. I am grateful to him for his constant discussions, suggestions and help without which I could not have succeeded in my doctoral work.

I am thankful to the Dean, School of Life Sciences for providing me with all the necessary facilities in carrying out this work.

It is a great pleasure to express my thanks, to my colleagues, Dr. K.V. Subba Rao, Dr. V. Prabhakar, Dr. (Mrs.) Usha Rani, Mr. J. Venugopal, Miss. E. Suvarchala, Miss D. Rebecca Prapurna, Mr. S.V. Rajagopal, Mr. P. Rameshkumar, Dr. A. Ramaseshu, and Mr. George Louis Kumar with whom I have shared my research career.

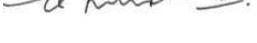
I owe my thanks to Dr. Ch. R. K. Murthy, Dr. M. Ramanadham, and Dr (Mrs.) Aparna Datta Gupta for their help and suggestions during the course of this study.

I extend my thanks to Dr. A. Pratapkumar, Mr. V.L. Raghavendra Rao, Mrs. Preeti bajaj and other friends who helped me in many ways.

I wish to thank non-teaching employees of the university for their help and cooperation.

I sincerely and gratefully acknowledge the financial assistance from I.C.M.R. and U.G.C., India.

Finally I extend my thanks to the authorities of the University of Hyderabad tor providing me with all the necessary facilities.



K. SUBRAHMANYAM

ABBREVIATIONS USED

AP	Apurinic or apyrimidinic
UV	Ultraviolet
UDS	Unscheduled DNA Synthesis
PBS	Phosphate buffered saline
MEM	Minimal Essential Medium
DPM	Disintegrations per minute
DNase	Deoxyribonuclease
HU	Hydroxyurea
TCA	Trichloroacetic acid
g	gram
xg	multiples of gravitational force
hr	hour
min	minutes
Ci	Curie
w/w	weight by weight
w/v	weight by volume

C O N T E N T S

Chapter I

General Introduction

CHAPTER **I**

GENERAL INTRODUCTION

Humans have been curious about the process of aging from the time they developed the ability for abstract thought. In the ancient times the legends of enormous longevity or immortality as a consequence of either natural cause or as a God's gift were popular. For example, Aurora obtained the gift of immortality for Tithonus, but omitted to obtain perpetual youth. Cadmus and his wife were given same opportunity. Even in the Hindu mythology, the existence of the creatures called "Devatas" who do not have mortality was mentioned.

The thirst to live for a long time is not a new ambition. Magic was the first of all applied to ward off both the diseases and old age. Then, attempts to alter the life span fell into the hands of sages, alchemists and quacks. The sage Wei Po-Yang (second century A.D.), perhaps, the originator of the "philosopher's stone", the supernatural fountain of youth, was one of the most popular. This idea played a significant role in the scientific history of European alchemists who tried to produce "elixir of life" (Comfort, 1963).

The lure of the immortality is so powerful that there are dozens of people in United states who have paid out large sums of money for their bodies to be deep-frozen after death, in the hope that there will one day be a medical technology capable of resur-

recting them. One such society is cryonics society of California which began freezing the dead bodies in 1967.

UNDERSTANDING THE PROCESS OF AGING

The first scientific study of biology of aging was that of August Welsmann, a German Zoologist (1889) about a century ago. He first made clear distinction between germ cells, which are transmitted from generation to generation and therefore immortal, and the somatic cells which in most higher organisms have finite lifespan. Until 1950s little work was carried out. A few historical observations were made by those of Pearl, Minot, Child **and** Carrel (cited in Comfort, 1963). In 1928 Pearl proposed the factors, which retard development or reduce metabolism, tend in many organisms to prevent or postpone senescence. Minot considered senescence as the decline of growth and it continues with morphogenesis. In 1925 Child showed that cell differentiation and senescence in planarian are reversible. Carrel (1912) described some experiments purporting to show that fibroblasts derived from chick heart tissue could be kept in an active state of division indefinitely. The importance of these experiments to gerontologists was the implication that, if the cells are removed from in vivo control, could divide and function normally for periods in excess of lifespan of the species. The inference would be that aging is not, per se the result of events occurring at the cellular level but would be as a result of functional decrement only in organized tissue. However, this concept **was**

disproved by the work of Hayflick and Moorhead (1961). They found that normal human fibroblasts underwent a fixed number of population doublings and then died. They postulated that manifestation of aging could be at cellular level and that the observations of Carrel's could be experimental artifact (Hayflick, 1976).

Since 1950s, research in the field of gerontology gained a strong impetus. Numerous experimental systems including rodents, fish, nematodes, insects, cultured human and chick cells, as well as simple organisms such as fungi and protozoa have been established. A variety of age related changes have been documented in these biological systems in different fields.

The process of aging and senescence has been defined in many ways by different investigators. Medawar (1952) suggested that senescence could be defined as

"that change of the bodily faculties and sensibilities and energies which accompanies aging, 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 deaths are in some degree accidental. No death is wholly 'natural', no one dies merely of the burden of the years."

Alex Comfort (1960) said aging is

"an increased liability to die, or an increasing loss of vigour, with increasing chronological age, or with the passage of the life cycle."

Strehler (1962) defined senescence as

"the changes which occur generally in the post reproductive

period and resulting in a decreased survival capacity on the part of the individual organism."

Aging process has been defined by Maynard Smith (1962) as "that which renders the individuals more susceptible as they grow older to the various factors, intrinsic or extrinsic, which may cause death."

Be that as it may, the functional deterioration at all levels of organization is the result of aging process.

There has been a tremendous theoretical work done on aging. It has been said that more than two hundred theories have been suggested at one time or another. Most of the contemporary theories which explain the process of aging fall into two main categories. According to one view, the programmed aging, "fundamentalist"s' view, "intrinsic" or controlled theories, the process of aging is genetically fixed. This view includes Weismann's theory of germ plasm (1889), aging clocks, codon restriction theory, chromatin reorganization theory and gerontogenes (Rattan and Clark, 1988). However, the absence of any clearly formulated and experimentally tenable predictions in these suggestions severely limits their usefulness (Rattan, 1985). In addition it is difficult to explain easily how simple environmental disturbances, such as temperature shift, treatment with various antioxidation agents can alter the strictly determined stable clock or program for aging (Holliday and Rattan, 1984; Holliday, 1986a; 1986b).

On the other hand, theories termed "epiphenomenalist's"

view, "extrinsic" or random or "stochastic" assume that the aging process results from the contingencies of living rather than from a programmed development. On this line, theories like somatic mutation, error catastrophe, free radical theory, post translational modification are proposed.

Somatic mutation theory was first proposed by Szilard (1959). He assumed that the elementary process in aging is an "aging hit" which destroys a chromosome of the somatic cell in the sense that it renders all genes carried on the chromosome inactive. A cell becomes ineffective either when two homologous chromosomes have each suffered a hit, or when one carries a hit and other carries a deleterious recessive allele. Other proponents of this were Harman (1962) and Medvedev (1964). Basic difficulties of this theory were discussed by Sacher (1968) and Maynard Smith (1962). At present this theory appears to be unsupportable because of lack of any evidence for mutations which inactivate whole chromosomes.

Orgel's error catastrophe theory (1963) relates the possible mechanisms of cellular aging with self propagation of errors in translational system. However, Medvedev (1980) considered that Orgel's idea was part of the general error theory which was proposed earlier. Numerous experiments have been performed to test the error catastrophe theory which have been reviewed by Schofield and Davies (1978) and by Harley *et al.* (1980). The majority of this evidence appears inconsistent with the error catastrophe theory.

Harman proposed first in 1956 and later reviewed recently (1988) that, free radicals might play a role in aging process by damaging important components of the cells such as membranes, proteins or DNA. There are few experiments, which reviewed by Harman (1988) showed that, by regulating free radicals the life expectancy of an organism can be modulated. If the free radicals are the cause of aging, either their production would increase with age or defence against them would decrease in function. There are few experiments showing the evidence that free radical generating reactions would increase with age. However, the experimental work has a number of loopholes, so unequivocal conclusions can not be drawn. Further, no conclusive evidence of reduced levels of free radical eradicating enzymes like superoxide dismutase and glutathione peroxidase, with age has been found (Rockstein, 1986).

Some species live longer and others live shorter means their survival capacity resides in their DNA. Thus DNA has become a distinct target for the studies on the mechanism of aging. An increasing amount of interest has been paid on metabolic functions closely associated with DNA viz, DNA content, gene expression, alterations in the chromatin, damage and repair of DNA (Rothstein, 1982). The theories of stochastic mechanism fail to explain the genetic basis of aging process. However, there are two theories which have genetic basis and at the same time explain the influence of environmental factors on the process of aging. They are: gene regulation theory of aging of Kanungo (1980) and DNA damage/repair hypothesis (Hart and Setlow, 1974;

Gensler and Bernstein, 1981; Lehmann, 1985 and Vijg and Knook, 1987).

DNA DAMAGE/REPAIR HYPOTHESIS

Fundamental to aging theories is the importance of genome integrity. Various physical, chemical environmental changes and cellular metabolites have been shown to damage cellular DNA. Studies on microorganisms, mammalian and plant cells have shown that DNA damage results in change in physiological processes like growth, division, transcription, cell death, mutation and induction of transformation (Hart and Trosko, 1976). Various DNA repair pathways exist in the cell to cope up with these damages.

DNA damage:

The cellular DNA *in vivo* can be damaged either spontaneously or by numerous physical and chemical agents which could be either exogenous or endogenous. The forms of DNA damage induced vary with the type of the damaging agent and also with the target site of the macromolecule. The most relevant forms of DNA damage with respect to aging would seem to be those induced by endogenous biochemical and physical reactions (Hart *et al.*, 1979). However, these forms of damage are less understood than those forms induced by model DNA damaging agents eg. radiation, alkylating agents etc.

The most frequent spontaneous DNA lesions are depurination, depyrimidination and conversion of cytosine to uracil (Lindahl,

1977). The rate of spontaneous depurination of duplex DNA at physiological pH 7.4 and ionic strength, is calculated to be about $k \simeq 3 \times 10^{-11}/S$ at 37°C. This corresponds in mammalian cells, to purine loss at a rate of 10,000 per cell generation (Lindahl, 1979). The depurination occurs by spontaneous hydrolysis of purine base by protonatlon of the base followed by direct cleavage of glycosyl bond (Zoltewicz et al., 1970). Pyrimidine nucleosides are considerably more stable than purine nucleosides and are hydrolysed at a rate of twenty fold slower than that of purines (Lindahl and Karlstram, 1973). The basic mechanism of depyrimidination is similar to depurination. It is estimated that in a long lived non-replicating cells like neurons about 10 purine bases are released from the DNA of a single cell during the life time of a human being, i.e., about three per cent of the total number of purines in DNA (Lindahl and Nyberg, 1972). The release of free bases leads to the formation of apurinic and apyrimidinic sites in DNA referred to as AP sites. The influence, if any, of the packing of DNA into nuclosomes and chromatin on the rate of spontaneous base loss has not been extensively studied. The deoxyribose at AP site exists in equilibrium between a closed furanose form and open aldehyde form. The 3'-phosphodiester bond associated with the aldehyde form is labile and can be hydrolysed by β-elimination resulting a nick in the DNA. The average life time of AP site is approximately 400 hours at physiological pH and temperature (Lindahl and Andersson, 1972). The cleavage of deoxyribose phosphate back bone is promoted by the presence of Mg and primary amines and polyamines

(Tammet al. , 1953; Lindahl and Andersson, 1972).

In the recent years increasing public awareness of environmental mutagens and carcinogens has led to an increased interest in the study of the mechanisms whereby genotoxic chemicals interact with and damage DNA. These agents can be divided into two groups: those requiring metabolic activation, eg. dimethyl nitrosamines, benzo(a)pyrenes, aromatic amines like N-2-acetyl-2-aminofluorene (AAF), mitomycin-C, aflatoxin B etc., and those direct acting without any metabolic activation, eg. alkylating agents like methyl nitrosourea (MNU) and methyl methane sulfonate (MMS).

Alkylating agents are electrophilic compounds and interact with nucleophilic sites in DNA. Some of them are monofunctional reacting with only one of the reactive centre in DNA and some of them are bifunctional which react with two reactive centres in DNA. These reactive centres include: N^1, N^3, N^6, and N^7 of adenine; N^1, N^2, N^3, N^7 and 0^6 of guanine; N^3, N^4 and 0^2 of cytosine; and N^3, 0^2 and 0^4 of thymine. The N^3 of adenine and N of guanine are particularly more reactive. Alkylation of oxygen in phosphodiester linkage results in phosphotriester (Roberts, 1978, Singer and Kusmierek, 1982). However, substitution on N^7 guanine appears not to be mutagenic, since the 7-alkylguanine can correctly pair with the DNA base cytosine during DNA replication (Ludlum, 1970). The reactivity of a given alkylating agent for a particular chemical group in DNA is roughly correlated with a constant S, often referred to as Swain-Scott constant (Swain and Scott, 1953). It is estimated that MNU

alkylates O^6-guanine 400 times more rapidly than MMS (Lawley, 1974). This alkylation has now been correlated with mutagenesis (Loveless, 1969), carcinogenesis (Goth and Rajewsky, 1974) and transcriptlonal errors (Gerchman and Ludlum, 1973).

There is yet another class of nonpolar compounds which are converted to reactive forms by a multiprotein complex, frequently referred to as cytochrome P-450 system. These include N-2-acetyl-2-aminofluorene (AAF), benzopyrenes, aflatoxlns, etc. AAF, originally used as an insecticide, is converted to a highly reactive acetoxy or ester form by an enzyme catalyzed process. These active forms react readily with C[8] and N[2] positions of guanine to yield acetoxy or acetyl derivatives (Kriek, 1972) which inturn alter the gene function by altering DNA conformation (Grunberger and Santella, 1982). Benzo(a)pyrenes, nonpolar polycyclic hydrocarbons, can be metabolized by P-450 system to highly reactive diol epoxide forms which react with 2-amino group of guanine. The exposure to such class of chemicals is prevalent from the sources of cigarette smoke and automobile exhaust fumes (Albert and Burns, 1977). Aflatoxins are the example of DNA damaging agents that have the origin as products of natural metabolism. The most potent carcinogen is aflatoxin B_1, which can be activated by P-450 system to the active oxide or epoxide derivative. The possible sites in the DNA are O or N of guanine and, N^1 and N^3 of adenine (Muench et al., 1983). Mitomycin-C is a potent antitumor antibiotic and causes cross-links in DNA, SOS response and sister chromatid exchanges. The activated form of the drug forms adduct at N^2 and 0° positions of guanine, and N^6

of adenine in DNA (Tomasz et al., 1986).

The major ultraviolet light (UV) damage is the formation of cyclobutane type pyrimidine dimers. The formation of dimer at or around 260 nm is a reversible process. Under normal conditions formation of the dimer is favoured (Setlow, 1968). However, in E. coli continuous irradiation at 254 nm does not yield pyrimidine dimers not more than seven per cent of the total thymine content (Radany et al., 1981). Theoretically there can be twelve isomeric forms of pyrimidine dimers. However, only four of them cis-syn, cis-anti, trans-syn and trans-anti are formed biologically in significant amounts. In B DNA, it is thought that only cis-syn form exists (Kittler and Lober, 1977). Formation of pyrimidine dimer is influenced by the nucleotide composition of the DNA (Setlow and Carrier, 1966). For example in the AT rich DNA, it is as follows TOT > COT > C<>C. Likewise in GC rich DNA the order is COT > TOT > COC at 280 nm. The steady state level of dimer formation is also influenced by the nature of nucleotide flanking at dimer site (Gordon and Haseltine, 1982). It has been recently reported that pyrimidine dimers occur in the nucleosome core DNA at a base periodicity of 10.3 and occur non-randomly (Gale and Smerdon, 1988).

Other photoproducts formed in DNA by UV irradiation are non-cyclobutane type pyrimidine adducts, pyrimidine-pyrimidine 6-4 lesions, pyrimidine hydrates and thymine glycols (Roberts, 1978). A good correlation between nonsense mutations and pyrimi-

dine-pyrimidine 6-4 lesions was observed (Haseltine, 1983). DNA damage can also result by the indirect action of UV. UV light at about 300 nm can sensitize some molecular species called photosensitizers (eg. psoralens) which in turn react with DNA forming cross-links. These photosensitizers have wide biological implications like study of the repair of inter-strand DNA cross-links and in the treatment of human diseases like psoriasis (Pathak et al., 1974).

Ionizing radiation can damage DNA by direct action, that is by causing ionizatlon within the DNA. For example the bases in the DNA can be damaged by ionizing radiation to form radical cations. The indirect action of ionization radiation is mediated by free radicals like hydrogen peroxide, hydrogen atoms, hydrated electrons and hydroxyl radicals, generated by the interaction of water and radiation in the biological system. The lesions formed by these two routes include single strand breaks, DNA-DNA cross-links, DNA-protein cross-links, alkali labile sites, double strand breaks and altered bases (Collins, 1987; Teoule, 1987).

Information about DNA damage at a higher level of organization of the DNA, i.e., at nucleosome and chromatin level, is scanty. It was reported that base damage by variety of chemicals occurs selectively in linker regions (Lieberman et al., 1979). Similarly, there is a non-random distribution of pyrimidine dimers in the core nucleosome DNA molecule (Gale and Smerdon, 1988). For convenience, various ways through which DNA can be damaged or altered are summarized in Table 1.

TABLE 1

VARIOUS TYPES OF DNA DAMAGE

I SPONTANEOUS DAMAGE	CAUSATIVE AGENTS/EVENTS
a) Mismatched base pairs	DNA replication and/or repair
b) Alterations in the structure of bases like tautomeric shifts and deamination.	
c) Loss of bases (depurination and depyrimidination)	
II DAMAGE DUE TO ENVIRONMENTAL FACTORS:	
a) Baseless sites	Alkylating agents, radiation, free radicals etc.
b) Cross-links	
c) Strand breaks and deletions	
d) Alkylation and bulky adducts	
e) Oxidative damage	

Information largely gathered from Friedberg (1985).

DNA repair pathways

The immediate cellular response to DNA damage is to repair its damaged DNA. Eukaryotic DNA repair is complex process involving multistep reactions catalysed by different enzymes, and has been extensively reviewed (Hart and Trosko, 1976; Lehmann and Karran, 1981; Friedberg, 1985; Bohr and Wassermann, 1988). There exists at least three general types of DNA repair processes: direct reversal of the damage, excision repair and postreplicational repair.

(a) Direct reversal of the damage: Enzymatic photoreactivation (EPR) is a highly specific and simple mechanism for the direct reversal of pyrimidine dimer to monomers. Historically, EPR was the first DNA repair mode to be discovered (Kelner, 1949). This enzyme has been detected, purified and characterized from a number of species including placental mammals (Rupert, 1975; Sancar and Sancar, 1988). DNA photolyase catalyzes the monomerization of cys-syn cyclobutane type of pyrimidine dimers utilizing the energy source generally between 300 and 500 nm. An enzyme substrate complex forms before the absorption of photoreactivating light. The precise wavelength utilized by the enzyme varies with the source of enzyme and also the chromophore composition. In general the enzyme contains two chromophores: one is FADH and the other is either a pterin or diazaflavin (Eker et al., 1987; Sancar and Sancar, 1988). DNA photolyase is assayed by restoration of transforming ability of DNA carrying a known genetic marker (Rupert, 1962); membrane binding assay

where only uncomplexed ^{3}H-DNA binds to the membrane (Madden et al., 1973); light dependent loss of pyrimidine dimers from DNA (Friedberg and Hanawalt, 1983); and by increased template activity for DNA polymerase (Piessens and Eker, 1975).

Another mode of simple reversal of damage is the repair of 0 -alkylguanine. This lesion has considerable biological importance in mammalian systems since it is implicated in mutagenesis (Loveless, 1969; Newbold et al., 1980). An enzyme activity called alkylguanine methyl transferase, has been found in various mammalian species including human, which transfers alkyl group of the DNA to cysteine residue of the enzyme molecule and thus becomes inactivated (Demple et al., 1982). The other enzyme which performs a simple reversal of the damage in mammals is the purine insertase. This enzyme, from human cultured fibroblasts has been found to insert purine base into depurinated DNA (Deutsch and Linn, 1979). The reversal of damage in DNA requires only a single gene product and would be relatively error free.

(b) Postreplicational repair: This mode of DNA repair was first proposed by Rupp and Howard-Flanders in prokaryotes (1968). According to this model, DNA damage like pyrimidine dimers interrupt DNA chain elongation during DNA synthesis, which then resumes beyond the damaged site, leaving a gap opposite to the damage that can be filled by recombination or de novo synthesis. This type of repair is monitored by pulse-chase experiments.

In prokaryotes both recombinational repair and translesion synthesis are very well characterized. Two equally possible

recombinational events can occur, one in which the dimer remains in the parental strand and the other in which the dimer is transferred to daughter strand (Cox and Lehman, 1987). It is hypothesized that in the translesion synthesis, the DNA polymerase I 'idles' at the lesion, adding and removing the nucleotides with the proofreading function of 3'-5' exonuclease activity. The DNA damage induces the 'SOS' response which somehow suppresses the 3'-5' exonuclease activity, thus allowing the synthesis continuously.

The two processes, i.e., recombinational events and translesion synthesis are studied to a lesser extent in higher eukaryotes. It was observed that recombination events do occur, but at a level significantly lower than those observed in E. coli (Waters and Regan, 1976; Fujiwara and Tatsumi, 1977; Lehmann and Kirk-Bell, 1978; Fornace, 1983). Evidence was presented for the occurrence of translesion synthesis in UV irradiated SV-40 virus infected in monkey kidney cells (Sarasin and Hanawalt, 1980). A specific model, the 'copy choice mechanism' was proposed for translesion synthesis in eukaryotes (Higgins et al., 1976). According to this model, one of the daughter strands becomes template for the second daughter strand at the damaged site.

(c)Excision repair (ER): It is assumed that excision repair pathway is the predominant one operated to repair cellular DNA damage (Teebor and Frenkel, 1983). This is the most extensively studied of all the DNA repair pathways. Although the mechanism of ER is not very well understood, some aspects are well studied

in prokaryotes. In contrast, this process is less clearly understood in eukaryotes (Lehmann and Karran, 1981; Friedberg, 1987; Sancar and Sancar, 1988). The progress that has been made in higher eukaryotes is to a large extent, the result of the discovery of human genetic disorders associated with DNA repair defects (Setlow, 1978; Hanawalt and Sarasin, 1986). A recent development in this field is the development of mutants in higher eukaryotes which are defective in one step or the other in ER pathway (Collins and Johnson, 1987).

The concept of excision repair was first put forward by Setlow and Carrier in 1964, which envisaged a four step process for the removal of damaged sections of DNA. According to this concept, after making incision at the damaged site by an endonuclease, the damaged section was removed by an exonuclease, the resulting gap was filled by DNA polymerase with opposite intact strand as template and finally the nick was ligated by DNA ligase. This basic principle remains same even today, but the details of the events turned out to be quite complicated.

Depending upon the nature of incision event the ER has been classified into two types, viz, base excision repair and nucleotide excision repair. In the base excision repair the damage is recognized by a class of narrow specific enzymes called DNA glycosylases (Lindahl, 1979). These enzymes remove the damaged base, leaving an 'AP' site in the DNA. This AP site is recognized by an AP endonuclease which makes nick at the damaged site. In the nucleotide excision repair, a damage specific endonuclease makes nick at the damaged site. Examples of enzymes

or gene products involved in nucleotide excision repair are: UV specific endonucleases and uvrABC gene products in prokaryotes. It was shown with UV specific endonuclease of M.luteus that the enzyme removes dimer in a two step mechanism thus making the distinction between the two repair processes less clear (Haseltine et al. , 1980). For convenience various DNA repair pathways that exist in eukaryotes are presented in Fig 1.

Enzymes and other gene products involved in ER in prokaryotes have been identified, purified and characterized. eg. UV specific endonuclease from M.luteus, DNA glycosylases, AP endonucleases, uvrABC gene products. In contrast, the enzymology of ER in eukaryotes is less understood, eventhough some damage specific nucleases, AP endonucleases, DNA glycosylases, DNA polymerases, DNA ligase II, poly(ADPR)polymerase and five RAD gene products have been found to be involved in the repair of DNA damage in vitro (Lindahl, 1979; Lehmann and Karran, 1981; Friedberg, 1987; Sancar and Sancar, 1988).

Excision repair is measured by (Hart et al. , 1977): i) assaying for the loss of radiolabelled DNA-chemical adducts or pyrimidine dimers from DNA. ii) loss of endonuclease sensitive sites in DNA; iii)decrease in the binding of damage-DNA directed antibody molecules to DNA (Cornelius et al. , 1977; Poirier et al., 1977); iv) measuring the amount of unscheduled DNA synthesis either by autoradiography or scintillometry (Rasmussen and Painter, 1964; Stephen Hsia, 1987) or v) by determining the number of breaks resulting from the photolysis of DNA containing

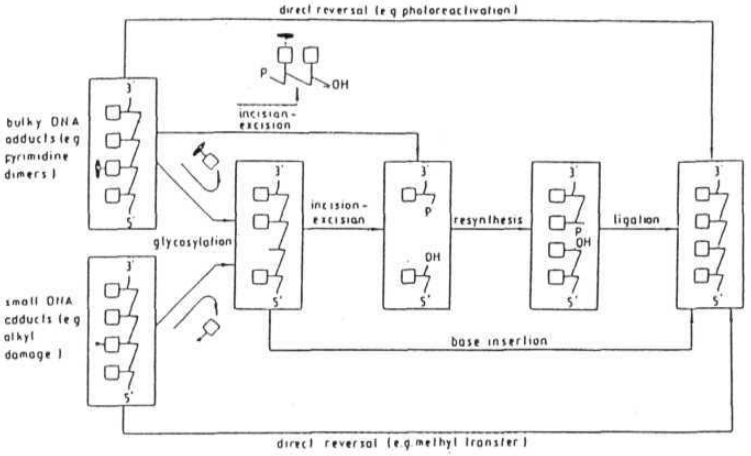


Fig 1. Schematic representation of different repair pathways (Vijg and Knook, 1987).

bromodeoxyuridine incorporated into DNA during repair (Regan et al., 1971).

On the basis of nucleotides added in the 'repair patch' during repair synthesis, it is frequently described as 'long' and 'short' patch repair modes. In mammalian cells it is thought that short patches are estimated to be about four nucleotides long and are thought to be associated with the damage caused by ionizing radiation and certain alkylating agents. On the other hand, long patch repair is estimated to be about 30 to 40 nucleotides and has been associated with the damage of bulky adducts like pyrimidine dlmers, AAF, aflatoxins and psoralens (Regan and Setlow, 1974a; Walker and Th'ng, 1982).

Various factors like nucleosome structure, functional state of chromatin and the state of differentiation are known to affect the ER. An early indication of DNA repair heterogeneity was recognized from the observation of a biphaslc time course for the removal of DNA lesions in cultured mammalian cells (Hanawalt et al., 1979; Kantor and Setlow, 1981), where a phase of rapid removal of pyrimidine dimers observed in the first few hours after UV irradiation was followed by a gradually decreasing rate, approaching plateau by 24 hours. It was usually assumed that DNA repair occurs uniformly throughout the genome. However, it has been established that DNA damage is processed more efficiently in some regions of the genome than in others. Lesion specific nucleases (Mansbridge and Hanawalt, 1983), and nucleases like staphylococcus nuclease, micrococcal nuclease and DNase I have been implicated to study the DNA repair heterogeneity (Smerdon

and Lieberman, 1978; Hanawalt et al., 1979). It is implied from these experiments that excision repair occurs preferentially in linker DNA, transcriptionally active regions and transcriptionally active strands (Bohr et al., 1986; Hanawalt, 1987). It has also been shown that preferential DNA damage repair sites are associated with nuclear matrix DNA and to hypomethylation (McCready and Cook, 1984; Mullenders et al., 1984; Bohr et al., 1986; Madhani et al., 1986; Harless and Hewitt, 1987). Many groups have compared the state of differentiation and the level of excision repair in cells in vivo and in culture. Most of the investigators found either complete absence or decreased levels of mitogen induced UDS in differentiated cells like muscle, neurons and retinal cells (Lehmann and Karran, 1981).

All these factors may account for the tissue specific, cell specific and animal specific differences in carcinogenic response to various DNA damaging agents.

The relationship between DNA repair and aging:

The idea that DNA damage might be the primary cause of aging was first proposed by Alexander (1967). The importance of maintenance of genome integrity and DNA damage/repair in relation to aging process has been extensively reviewed in later years (Hart and Trosko, 1976; Tice, 1978; Hart et al., 1979a; Hart and Modak, 1980; Gensler and Bernstein, 1981; Lehmann, 1985; Vijg and Knook, 1987).

Painter and Cleaver (1969) were the first to compare several

kinds of mammalian cells in culture with respect to DNA repair parameters. With the exception of Xeroderma pigmentosum (XP) patients, all kinds of cells showed UV induced unscheduled DNA synthesis (UDS). However, the cells of human origin showed this phenomenon to a greater extent when compared to rodent cell line. These investigations gave the credence to "DNA repair" as the longevity assurance system. According to this hypothesis, enzymatic systems have been developed during evolution that limit the rate at which deteriorative events take place. Therefore, the concept that the animal species with the most efficient DNA repair systems throughout its lifespan has the highest longevity, has emerged (Sacher, 1982).

Gensler and Bernstein (1981) hypothesized that aging is determined by the balance between the rate at which DNA damage occurs and the rate of DNA repair. According to them aging in unicellular organisms, characterized by decline in replicative capacity, occurs during asexual phase and rejuvenation or an increase in replicative capacity occurs following sexual reproduction, a phase when efficient DNA repair occurs (Martin, 1977). Multicellular organisms, which do not age in germ line in principle, are able to overcome DNA lesions in their germ line by recombinational repair occurring during synopsis of homologous chromosomes. Support for this hypothesis comes from substantial evidence that recombinational processes are effective in overcoming various DNA lesions like DNA cross-links induced by psoralens plus light (Cole *et al.*, 1978), X-ray induced double strand breaks (Krasin and Hutchinson, 1977), nitrous acid induced les-

ions (Nonn and Bernstein, 1977), mitomycin-C induced lesions (Holmes et al., 1980) and methylmethane sulfonate induced double strand breaks (Chlebowicz and Jachymczyk, 1979). Other means of protecting germ line DNA include: selection of most viable spermatozoa for fertilization (Bernstein, 1979) and lowered temperature of the external testis in mammals (Baltz et al., 1976). Despite of the above protections natural aging occurs in nucleus of ovum (Smith and Berg, 1976). Any organism which accumulates DNA lesions in germ line over the successive generations would extinct. On the other hand, aging of the somatic line may be caused primarily by accumulation of DNA damage resulting from a relaxed state of DNA repair (Gensler and Bernstein, 1981).

Vijg and Knook (1987) proposed the pleiotropic nature of DNA repair. According to this concept, DNA repair is beneficial in early life, in removing DNA damage more efficiently. As the organism ages DNA repair activities become error prone as a consequence of stress by the accumulated lesions in the DNA, and this is responsible for aging and cell death. An hypothetical schematic representation of the role of DNA damage/repair is presented in the Fig 2.

BRAIN, DNA REPAIR AND AGING

Although aging may be a generalized phenomenon throughout the mammalian body, attention has frequently been focused on such controlling and integrative organs like 'brain' and 'endo-

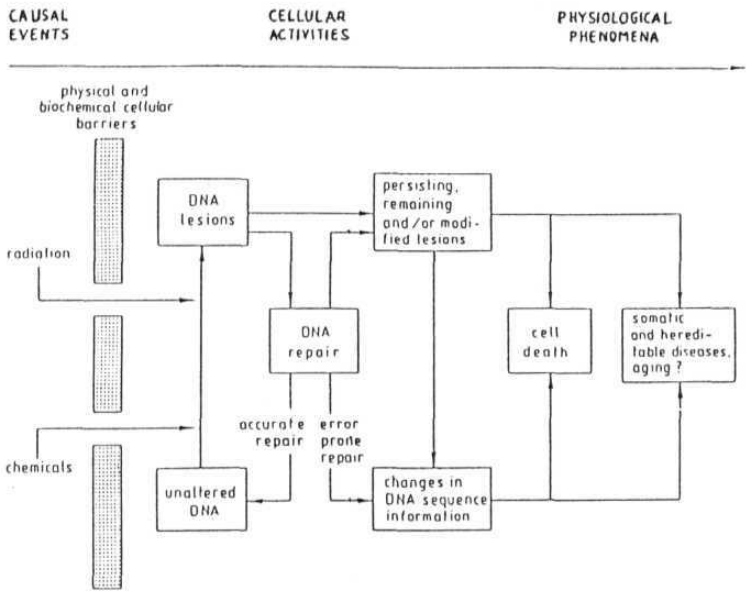


Fig 2. Hypothetical representation of the role of DNA repair in aging (Vijg and Knook, 1987)

crines' in which age dependent changes may have widespread consequences throughout the body. Many researchers interconnected the species lifespan and the brain's regulatory abilities. Friedenthal was the first to correlate the lifespan of a species and cephalization (cited in Frolkis, 1982). He regarded cephalization as the ratio of the brain mass to the protoplasm mass and he assumed that a definite parallelism exists between cephalization ratio and lifespan of a species. Sacher (1973) identified a significant correlation between brain weight and lifespan and he proposed that brain may play an important 'pacemaker' role in aging or it may be an 'organ of longevity' due to its unique role in adaptation to the external environment and in homeostatic integration of all organs of the body.

Although research on the brain started a century ago, the studies on biochemical changes during brain development and aging has gained considerable importance only in the last three decades. During this course of study, structural, morphological, neurochemical and physiological changes have been noted during aging of the brain, and reviewed extensively (Terry, 1980; Frolkis, 1982; Creasy and Rapoport, 1985).

The high metabolic rate of cerebral tissue probably results in high rate of production of DNA damaging free radicals from electron transport and other normal metabolic reactions (Slater, 1984). Also, brain transcribes a greater portion of its DNA than other non-cerebral tissues (Hahn and Laird, 1971). Further, neurons, once they form do not divide (Korr, 1980). DNA repair in these cells is not accessible by the postreplication mechanism

and the death of damaged neurons can not be remedied. Thus it is possible to expect that these cells should possess good DNA repair machinery to maintain the integrity of the genome. Although there are many reports on the occurrence of DNA repair in the adult brain, studies on DNA repair capacity in the aging brain are lacking. Presence of unscheduled DNA synthesis, X-ray and Y-ray induced strand break repair have been reported in brain either *Vn* vivo or in neuronal cells in vitro (Kimberlin et al., 1974; Adrian et al., 1975; Vilenchlk and Tretjak, 1977; Wintzerith et al., 1977; Lehmann and Karran, 1981). Recently, Kuenzle (1985) reviewed the enzymology of DNA repair in the brain. Some enzymes that are involved in DNA repair have been identified. These include: uracil DNA glycosylase, DNA polymerase 6, DNA ligase, poly(ADPR)polymerase, 0 -alkylguanine alkyl transferase, an exonuclease which acts on depurlnated DNA, acid and alkaline DNases (Subba Rao, 1986).

SCOPE OF THE PRESENT INVESTIGATION

For the last several years, this laboratory has been engaged on the metabolism of DNA in both chick and rat brain. During the course of these investigations, it became apparent that DNA content of the brain continued to increase even beyond the adult stages indicating that there is some cell proliferation occurring even in the old age, probably the glial cells. Further, it was observed that two DNases, one with acidic pH optimum and the other with alkaline pH optimum are possibly involved in DNA repair/replication. DNA polymerase β, an enzyme involved in DNA

repair showed highest activity during early stages of development, reached a low value in adult brain but once again showed a small peak during later stages of lifespan. Also, it was observed that β-polymerase from mouse neurons exhibits its function throughout the lifespan with the same fidelity (Subba Rao and Subba Rao, 1982; Subba Rao and Subba Rao, 1984; Subba Rao et al., 1985; Subba Rao and Subba Rao,1986).

It is well known that brain is composed of at least three distinct types of cells viz., neurons, astrocytes and oligodendroglial cells, with different proliferative schedules (Korr, 1980). It is therefore possible that DNA repair capacity of each cell type may vary from the other and also depending upon the age of the brain. In order to test the possible involvement of DNases and DNA polymerases at different ages of brain cells, the following studies have been undertaken.

Acid and Alkaline DNases were studied in three different cell types viz, neurons, astrocytes and oligodendrocytes. Enzymatic activities of acid and alkaline DNases were also localized in nuclear fraction, thereby indicating their role at genomic level. UV irradiated and depurinated DNA were also tested as substrates for acid and alkaline DNases respectively in order to substantiate their role in DNA repair. The results are discussed in chapter III.

DNA polymerase activities were measured in all three types of cells in the brain at different ages. An attempt has been made to distinguish α-polymerase and β-polymerase making use

of their specific inhibitors, aphidicolin and dideoxy thymidine 5'triphosphate (ddTTP) respectively. These results were further substantiated by making use of a α-polymerase specific inhibitor, a polysaccharide isolated from Physarum polycephalum. The results are presented and discussed in chapter IV.

The DNA repair synthesis was measured in the neuronal cells isolated from the rat brain of different ages, making use of hydroxyurea (HU) as a specific inhibitor of semiconservative replicative synthesis. These studies were also extended to another cell type, the spleen lymphocytes which have the capacity to proliferate on stimulation. These results which showed that adult neurons offer a good model for DNA repair studies are discussed in chapter V.

Finally, the isolated neuronal cells, from brains of different ages, were challenged with UV light (254 nm) to study the UV induced unscheduled DNA synthesis (UDS) both by scintillometry and autoradiography. Simultaneously the UV induced UDS was also measured in lymphocytes as a positive control system. These results which showed contrasting abilities of DNA repair in neurons and lymphocytes, are discussed in chapter V.

Chapter II

Materials and Methods

CHAPTER II

MATERIALS AND METHODS

Animals:

Rats of Wistar strain and of specific age (both sexes) were obtained from this University animal house. They were fed ad libitum on Hindustan level (New Delhi, India) 'Rat and mice feed' supplied by local dealers.

Chemicals:

Trypsin (Type I, from bovine pancreas), Soybean trypsin inhibitor (Type II S), highly polymerized calf thymus DNA, Trizma base and N-2-Hydroxy ethyl piperazine-N'-2'ethane sulfonic acid (HEPES) were purchased from Sigma Chemical Company, St. Louis, MO, USA. Hank's balanced salt mixture was purchased from Hi-Media, Bombay. Ficoll 400 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Nitex nylon screens of definite pore size were purchased from Small Parts Inc. Miami, Florida, USA. All other chemicals used were of analytical grade.

Pregnant rats:

For the studies with embryonic brain, timed pregnancies were achieved by placing proestrus female rats (2-3 months old) in cages overnight (from 4.00 P.M. to 9.30 A.M.) with males of the same strain. The presence of sperm in the vaginal smears was

taken as an indication of successful breeding, and the day was fixed as day zero of gestation.

Extraction of DNA:

DNA was extracted essentially according to the procedure of Schmidt and Thannhauser (1945) slightly modified as per the suggestion of Munro (1966).

To the 0.1 ml of homogenate 1.0 ml of ice cold 10% TCA was added and centrifuged after allowing to stand in ice for ten minutes, to remove acid soluble compounds. The precipitate was washed twice with 1.0 ml of 10% TCA. The final sediment was washed once with 1.0 ml of 95% ethanol and once with 1.0 ml of alcohol:ether (3:1 v/v) to remove any traces of lipids. The lipid free pellet was suspended in 0.5 ml of 1N KOH and incubated for two hours at 37°C, in order to hydrolyse RNA. DNA and proteins were sedimented by centrifugatlon, removing the hydrolysed RNA after the precipitation by adding 0.1 ml of 6 N HC1 and 1.0 ml of 10 % TCA and keeping in ice for 10 minutes. The final sediment was suspended in 1.0 ml of 5% TCA and boiled at 90°C for 15 minutes with occasional stirring. The sediment was washed once more with 1.0 ml of 5% TCA and both the supernatants were taken together for the estimation of DNA.

Estimation of DNA:

The DNA content was estimated by measuring the UV absorption of the final acid soluble fraction at 260 nm with reference to

proper blank and similarly treated DNA as standard.

Estimation of Proteins:

Protein content was determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as standard. To the 1.0 ml of water containing the protein sample, 5.0 ml of solution D was added, and mixed thoroughly. The solution D contained 2% sodium carbonate in 0.1 N NaOH, 1.0 ml of 2% sodium potassium tartrate and 1.0 ml of 1% $CuSO_4$ for 100 ml. After keeping at room temperature for 10 min, 0.5 ml of 1.0 N Folin's reagent was added and vortexed immediately. After keeping at room temperature for 30 min the developed blue colour was read at 670 nm against a proper blank and standard.

Preparation of Neuronal and astroglial enriched fractions:

Neuronal and astroglial cell enriched fractions were isolated essentially according to the procedure of Usha Rani *et al.* (1983) Rats were decapitated and the entire cerebral hemispheres were removed. Grey matter from the cerebral hemispheres (about 2g) was sliced into small pieces and incubated in isolation medium containing 0.1% trypsin at 37°C for 60 min. The isolation medium consisted of 8% glucose (w/v), 5% fructose (w/v) and 2% ficoll (w/v) in 10 mM KH_2PO_4-NaOH buffer, pH 6.0. The incubation was carried out for 30 min without trypsin in the case of 1 day old postnatal and 16th day embryonic brains. After the incubation, the trypsin containing medium was removed and an

equal amount of medium containing 0.1% soybean trypsin inhibitor was added and kept in ice for 5 min. The remaining procedure was carried out at 4°C. The medium was removed and the tissue was washed thrice with ice cold medium. The tissue was then passed through 105 um size nylon mesh with the help of flat bottomed glass rod. The tissue was kept moist with medium during this operation. The suspension thus obtained was filtered through 74 um and 53 um nylon mesh thrice successively.

The resulting crude cell suspension was centrifuged at 260 xg for 15 min. The supernatant was discarded and the cell enriched pellet which consisted of both neurons and astrocytes was suspended in 15 ml of 7% ficoll in medium and centrifuged at 270 xg for 10 min to obtain a crude neuronal pellet. The pellet was suspended in isolation medium and kept for further purification on gradients. The supernatant was diluted with medium in the ratio of 1: 1.125 and centrifuged at 1,100 xg for 10 min. The crude astrocyte enriched pellet thus obtained was suspended in 10 ml of medium for further purification. 5 ml portions of crude neuronal and astroglial suspensions were layered onto discontinuous ficoll gradients. The gradients consisted of 4 ml each of 28%, 22% and 10% ficoll (w/v) in the medium. In the case of neuronal enriched suspension, the suspension was directly layered on 22% ficoll, and 10% ficoll was omitted in the density gradient centrifugation step. The tubes were centrifuged at 7,800 xg for 10 min. The layers, at each interface were removed with a pasteur pipette. Neurons were obtained as a pellet in 28% ficoll gradient. Astrocytes were obtained as a layer on 22% ficoll

gradient. The interface between 22% and 10% consisted broken processes and debris, and was discarded. The cell suspensions were diluted with the isolation medium (without ficoll) to bring down the ficoll concentration to that of original isolation medium. For convenience a flow diagram of the procedure for isolation of neurons and astrocytes is presented in Fig 3.

Oligodendrocytes were prepared essentially by the method of Snyder et al. (1980) with a slight modification as follows. The isolation medium was Hank's balanced salt solution containing 25 mM HEPES, pH 7.2. The whole cerebral hemisphere from the rat brain was removed, made into small pieces, and incubated in 0.1% trypsln in medium for 30 min at 37°C. After incubation, the trypsin containing medium was removed and an equal amount of medium containing 0.1% soybean trypsin inhibitor was added and chilled on ice for 5 min. The tissue was washed three times with ice cold medium. This was done by centrifugation at 190 xg for 5 min. Then the tissue was placed on 105 µm nylon mesh stretched over a porcelain Hirsch funnel and by means of glass rod the tissue was gently squeezed with occasional moistening the tissue with medium. The cell suspension thus obtained passed three times through 74 µm and 53 µm nylon meshes successively. To the cell suspension obtained an equal volume of 70% sucrose (w/v in medium) was added. A 20 ml aliquot of this suspension was layered over a discontinuous sucrose gradient consisting of 8 ml of 53% sucrose (w/v in medium) and 5 ml of 45% sucrose (w/v in medium). The tubes were centrifuged at 3065 xg for 15 min. Oligodendrocytes were obtained in 53% gradient. This fraction was

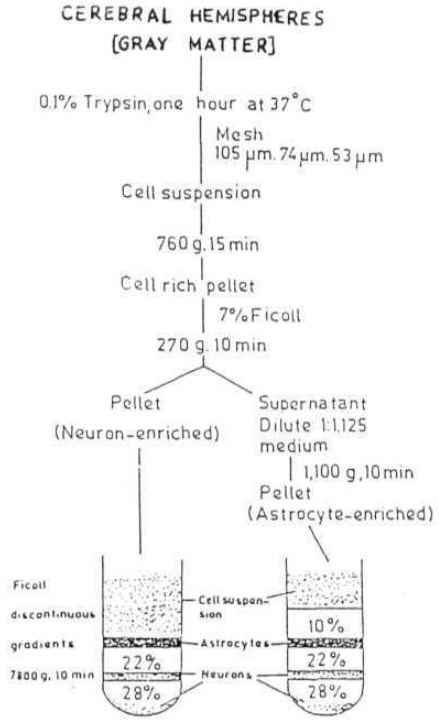


Fig 3. Flow diagram of the procedure for isolation neurons and astrocytes from the brain.

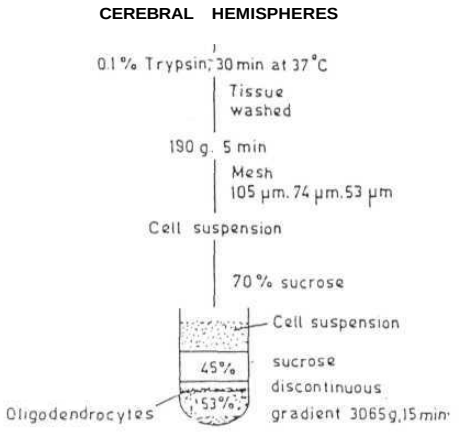


Fig 4. Flow diagram of the procedure for isolation of oligodendrocytes from the brain.

diluted five times with isolation medium and filtered through 25 um nylon mesh to trap capillary fragments. A flow diagram of the procedure for isolation of oligodendrocytes is presented in Fig 4

Aliquots of the cell suspensions isolated by the above procedures were taken for cell counting and viability test. The purity of the preparations was determined by phase contrast microscopy. The viability of the cells as judged by trypan blue exclusion was more than 85%. The purity of neuronal preparation was more than 90%, astrocyte fraction was greater than 70% and that of oligodendrocytes was more than 80%.

The isolated cell enriched fractions were well characterized both biochemically and morphologically in this laboratory earlier (Usha Rani, 1985).

Chapter III

Acid and Alkaline DNases in isolated cell enriched fractions from the rat brain at different ages

CHAPTER III

ACID AND ALKALINE DNases IN ISOLATED CELL ENRICHED FRACTIONS FROM THE RAT BRAIN AT DIFFERENT AGES

INTRODUCTION

Although deoxyribonucleases are assumed to be concerned with the degradation of DNA, a role in DNA replication and repair has been assigned to them (Lehman, 1967; Bernardi, 1968; Slor and Lev, 1971; Hanawalt et al., 1979; Lindahl, 1982; Linn, 1982).

Nucleases involved in DNA replication or repair were studied extensively in prokaryotes and in various organs of eukaryotes. For some reasons, the brain has been neglected in such studies, eventhough it has got prime importance in maintaining homoestasis in all body organs.

Sung (1968) was the first to report two distinct DNases from the brain, the acid and alkaline DNases which resemble DNase I and II respectively from the other organs. He separated the two DNases by using ammonium sulfate fractionation. One of them, the acid DNase acted optimally at pH 5.0, hydrolyzing preferentially native DNA and the other, the alkaline DNase, hydrolyzed denatured DNA, having optimum pH between pH 7.4 and 8.9. The latter enzyme was thought to be similar to a phosphodiesterase isolated earlier from lamb brain (Helay et al., 1963). Ratio of the alkaline DNase to acid DNase increased with age showing

higher levels of alkaline DNase in different regions of the brain. Later studies of Chanda *et al.* (1975) also revealed similar pattern.

Studies were also performed in this laboratory from human, rat and chick brains, in an attempt to identify the possible role of these DNases in DNA replication and repair and these results were reviewed recently (Subba Rao, 1986). During these studies an interesting phenomenon was observed. Acid DNase shows highest activity at a stage where rapid cellular proliferation occurs. Immediately after this replicative period the activity exhibits a precipitous decline. On the other hand, alkaline DNase also shows high activity during embryonic period but this activity increases with age. Similar type of results were obtained by Chanda et al.(1975) in rat cerebellum. This prompted this laboratory to speculate an intimate role for acid DNase to DNA replication or a process associated with it possibly postreplication repair, while for alkaline DNase in a process that assumes greater importance with age, probably DNA repair.

As it is well known that brain composed of different cell populations with different proliferative schedules, studies on these enzymes in different cell types should yield valuable information about their physiological role. Hence a study has been taken up to measure acid and alkaline DNases in different cell types viz, neurons, astrocytes and oligodendrocytes isolated from rat brain at different stages of lifespan. Further, in order to substantiate the role of these DNases in DNA replication/ repair, the activities were also measured in nuclear frac-

tions, using different types of substrates.

MATERIALS AND METHODS:

Highly polymerized calf thymus DNA, RNase were purchased from Sigma Chemical Company, St. Louis, MO, USA. ^{3}H-thymidine (18 Ci/mmole) was purchased from Bhabha Atomic Research Centre, Bombay, India. All other chemicals used were of analytical grade.

Neuronal, astroglial and oligodendroglial cell enriched fractions were prepared as described in the chapter II.

Isolation of Nuclei:

The nuclei were isolated from the isolated cell enriched fractions according to the procedure of Lovertrup-Rein and McEwen (1966).

The cell enriched fractions were homogenized by hand with fifteen up and down strokes. The homogenizing medium consisted of 0.32 M Sucrose, 1 mM $MgCl_2$ and 1 mM potassium phosphate pH 6.5. The homogenate was filtered through 80 um nylon mesh and centrifuged for 10 min at 850 xg. After the centrifugation the supernatant was discarded and the pellet was resuspended in the homogenization medium and centrifuged for 10 min at 850 xg. The sediment was washed once again in the homogenization medium and centrifuged at 600 xg for 10 min. The crude nuclear pellet thus obtained was suspended in 2.0 M sucrose containing 1 mM $MgCl_2$ and 1 mM potassium phosphate pH 6.5 and centrifuged for 45 min in

SW 60 rotor of the Beckman L 8-M centrifuge at 53,500 xg. The nuclear pellet thus obtained was washed once with 0.32 M sucrose.

The morphology of the different types of nuclear fractions resembled very well with that described by Lovertrup and McEwen (1966). Nuclei from neuronal enriched fractions displayed a pale chromatin network with a well shaped single nucleolus, often centrally located, contrasting with the light background of the nucleoplasm. Those isolated from astrocyte enriched fraction, were large, with faint nucleoplasm and contained two or more nucleoli. The nuclei from the oligodendrocytes were smaller, with densely stained chromatin and lightly stained peripheral nucleoli.

The contamination of the nuclear fraction with mitochondria and lysosomes was tested by assaying succinic dehydrogenase, a mitochondrial enzyme and acid phosphatase, a lysosomal marker enzyme.

Preparation of ^{3}H labelled DNA from E.coli:

E.coli A 19 cells were labelled with ^{3}H-thymidine according to Inga Mahler (1967). A 250 ml flask containing 50 ml of growth medium (1 g NH_4Cl, 0.5 g of NaCl, 3 g KH_2PO_4, 6 g of Na_2HPO_4, 1ml of 1 M $MgSO_4$, 0.1 ml of 1 M $CaCl_2$ and 1.5 g casamino acids per liter, after autoclaving, was supplemented with 10 ml 50% sterile glucose) was inoculated with E.coli A 19 and shaken overnight at 37°C. The overnight grown culture was added as inoculum to 500 ml of growth medium in a 2 liter flask and the incubation was con-

tinued for 2 hrs. (Cell density 2 X 10^8). Then 100 mg of deoxy guanosine and 1 mCi of labelled nucleoside, (methyl ^{3}H)-thymidine, were added to the medium. After further growth for 1 hr, the cells were chilled with crushed ice and harvested. DNA from the labelled cell pellet was isolated by the method of Marmur (1961).

2 to 3g of wet packed cells were washed with 50 ml of saline-EDTA consisting of 0.15 M NaCl and 0.1 M EDTA, pH 8.0. The final cell pellet was suspended in 25 ml of saline-EDTA and 10 mg of lysozyme was added and incubated at 37°C for 1hr. 2.0 ml of 25% sodium lauryl sulfate (SDS) was added and kept at 60°C for 10 min and then brought to room temperature. 5 M solution of sodium perchlorate was added to bring a final concentration of 1 M. The whole mixture was shaken with equal volume of chloroform:isoamyl alcohol (24:1) for 30 min and then centrifuged for 10 min at 5000 rpm. The upper aqueous phase containing the nucleic acids was taken out. To this 2 volumes of ethyl alcohol (95%) was added gently and the DNA was dissolved in 10 to 15 ml of saline-citrate (0.015 M NaCl and 0.0015 M trisodium citrate, pH 7.0). The concentration of saline-citrate was adjusted to 0.15 M NaCl and 0.015M citrate by adding required volume of concentrated saline-citrate (1.5 M NaCl and 0.15 M trisodium citrate, pH 7.0). Then an equal volume of chloroform:isoamyl alcohol (24:1) was added and was shaken for 15 min and then centrifuged for 5 min at 5000 rpm. Deproteination was carried out until little protein was seen at the interface. Two volumes of ethyl alcohol was added to the last aqueous phase containing DNA and the precipitate was dissolved in saline-citrate. Ribo-

nuclease was added at a concentration of 50 µg/ml and incubated at 37°C for 30 min. The whole digestion mixture was extracted with chloroform:isoamyl alcohol (24:1) and the DNA in the aqueous phase was precipitated with ethyl alcohol. The DNA was dissolved in 9.0 ml of dilute saline-citrate and 1.0 ml of acetate-EDTA consisting of 3.0 M acetate and 0.001 M EDTA, pH 7.0 was added. To this solution 0.54 volumes of isopropyl alcohol was added drop wise while stirring rapidly. The DNA was spooled and made free of acetate-EDTA by successive washing with increasing percentage of ethyl alcohol (70% to 90%).

The final DNA precipitate was dissolved in 10 mM Tris-HCl pH 8.0, containing 1 mM EDTA. 2 drops of chloroform were added and stored at 4°C. The specific activity of the isolated DNA was 7 x 10^6 DPM/mg of DNA.

Preparation of the substrates:

Heat denatured DNA was prepared by keeping the solution of E.coli ^{3}H-DNA at 100°C for 10 min and cooling rapidly in an ice bath (Sung, 1968).

Native UV irradiated DNA was prepared by irradiation of E.coli ^{3}H-DNA solution on ice at a dose of 2 x 10^4 J/m^2 at 254 nm using Philips TUV 8 15 W germicidal lamp.

Depurinated native DNA was prepared by heating solution of E.coli native ^{3}H-DNA in 10 mM citrate buffer, pH 4.0 containing 100 mM NaCl for 20 min at 70°C. After incubation, the solution was chilled immediately.

Assay procedure for DNase

Acid and alkaline DNases were assayed according to the procedure of Subba Rao and Subba Rao (1982) with a slight modification. E. coli ^{3}H-DNA was used as a substrate instead of calf thymus DNA.

Acid DNase:

The 60 ul of reaction mixture consisted of 10 ug of E. coli ^{3}H-DNA (70,000 DPM), native or UV irradiated, 0.1 M sodium acetate buffer, pH 5.1 and cell homogenate or nuclear preparation equivalent to 5-10 µg of DNA. At the end of 30 min of incubation at 37°C in a Dubnoff metabolic shaker water bath, 100 ug of highly polymerized calf thymus DNA was added as carrier and the reaction was terminated by adding 80 ul of 1.4 N PCA. After keeping in ice for 10 min, the tubes were centrifuged at 10,000 rpm for 10 min and the supernatants were transferred into scintillation vials containing 10 ml of Bray's mixture and counted for radioactivity in Beckman LS 1800 scintillation counter. The enzyme activity was expressed as µg of ^{3}H-DNA degraded in 30 min at 37°C.

Alkaline DNase:

The reaction mixture volume, method of assay and expression of activity and other details are the same as in the case of acid DNase, except that the reaction mixture consisted of either heat denatured or depurinated ^{3}H-DNA as the substrate and 0.05 M Tris-

HC1 buffer pH 8.25.

Acid Phosphatase:

Acid phosphatase activity was assayed according to the procedure of Henrickson and Clever (1972) with a slight modification. The activity was assayed by following the hydrolysis of p-nitrophenyl phosphate to p-nitrophenol. The final reaction mixture (0.9 ml) contained 3.3 mM p-nitrophenyl phosphate, 0.12 M acetate buffer, pH 5.5 and 50 to 100 µg of enzyme protein. After the incubation at 37°C for 1 hr the reaction was stopped by adding 2.25 ml of 0.1 N NaOH which also developed the yellow colour. The colour was read against appropriate blank and standards at 400 nm. Enzyme activity was expressed as umoles of p-nitrophenol liberated per hour.

Succinlc dehydrogenase:

Succinic dehydrogenase (SDH) activity was assayed by a combination of the procedures of Nachlas et al. (1960) and that of Susheela and Ramasarma (1971) with a few modifications.

In a final reaction mixture of 0.5 ml containing 40 mM phosphate buffer pH 7.6, 17.5 mM succinic acid (disodium salt) and 0.11 ml of colour reagent containing 0.1 ml of 3 mM 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) and 10 ul of 1% phenazine methosulfate (PMS). The reaction was started with the addition of 0.1-0.2 mg of protein. The substrate free reaction mixture substituted by buffer served as blank. After incubating at 37°C for 15 min, the reaction was

stopped by the addition of 1 ml of glacial acetic acid.

Glacial acetic acid stops the reaction by bringing down the pH of the reaction mixture. (PMS-INT) serves as the electron acceptor system during the dehydrogenation of succinate. The bright pink coloured formazan was eluted with 5.0 ml of toluene and read at 500 nm.

In order to get a standard curve, a final reaction mixture of 0.5 ml containing different concentrations of INT with 40 mM phosphate buffer, pH 7.6 with 10 ul of 1% PMS was taken along with 0.1 ml of 1% ascorbic acid as the reducing agent. After standing for 15 min, 1 ml of glacial acetic acid was added and the colour was eluted with 5.0 ml of toluene. The absorbance of reduced INT was measured at 500 nm.

The unit activity of SDH was expressed as umoles of INT reduced per hour.

RESULTS AND DISCUSSION:

Specific activities of acid and alkaline DNases in neurons, astrocytes and oligodendrocytes are presented in tables 4 and 5 respectively. As can be seen, at all the ages studied both the enzyme activities are present in nuclear fraction also. The percentage activities of acid DNase in nuclear fraction decreased with age in both neurons and astrocytes, and there was no change in oligodendrocytes (Tables 12, 13 and 14).

In all the cell types, the acid DNase activity decreased

from young to adult stage and there was no further decline of activities during old age both in homogenate and nuclear fractions. In astrocyte and oligodendroglial nuclear fractions, high specific activities of acid DNase were observed. Similar type of results were observed in the nuclear fractions when the activities of acid DNase were expressed per mg of DNA (Table 6). This may reflect its involvement in DNA replication in glial cells in the later stages of rat brain (glial proliferation) which is in line of our earlier observations. A similar type of correlation was observed between acid DNase activity, cell division and DNA synthesis by Alfrey and Mirskey (1952). A correlation between DNase II, which is considered as acid DNase and the capacity of certain tissues to divide was also demonstrated (Cordonnier and Bernard!, 1968).

There are two limitations to relate the acid DNase activity to DNA replication or repair process associated with it, possibly postreplication repair. Firstly, acid DNase was considered as an exclusively lysosomal enzyme (de Duve *et al.*, 1962). Secondly, acid DNase is optimally active at pH values between 4.5 and 5.5 in 0.15-0.2 M acetate buffer, which is non physiological. As it can be seen we have found significant activities of acid DNase in the nuclear fractions also (Tables 12, 13 and 14). We have also tested the possible contamination of the nuclear fraction isolated from the rat brain, with mitochondria and lysosomes by assaying succlnic dehydrogenase, a mitochondrial marker, and acid phosphatase, a marker for lysosomes. From the results presented in Table 2, it can be concluded that our nuclear fractions were

TABLE 2

RELATIVE DISTRIBUTION OF ACID PHOSPHATASE AND SUCCINIC DEHYDROGENASE IN THE ADULT RAT BRAIN

Subcellular fraction	Acid Phosphatase	Succinic dehydrogenase
Homogenate	100.0	100.0
Nuclei	0.41 $\pm$ 0.07	0.20 $\pm$ 0.03

Total activities in the homogenate were considered as 100% and the remaining values are expressed as percentage of these activities.

Values are mean of four individual experiments in the case of acid phosphatase and three experiments in the case of succinic dehydrogenase.

For other details of incubation conditions and expression of activities please see the text.

not contaminated with either mitochondria or lysosomes. The presence of acid DNase in nuclear fraction is further ascertained by the observed increased ratio of acid DNase to acid phosphatase from homogenate to nuclear fraction (Table 3). The presence of acid DNase in the nuclear fraction was also established in the nuclei of calf thymus, HeLa S3 cells, and neurons, astrocytes and oligodendroglial cells of chick brain (Slor and Lev, 1971, Stambolova et al., 1973). It was found that highly purified DNase II is active at neutral pH, provided appropriate conditions of lower ionic strength and divalent cations are used. It was also demonstrated that the purified enzyme shows more than 100% activity at pH 7.0 when compared with its activity at pH 4.5, and that these two activities are probably due to same protein molecule (Slor and Lev, 1972). The role of acid DNase in DNA replication was further substantiated by the experiments of Slor et al. (1973), where a 2 to 7 fold increase in the acid DNase II activity in S-phase HeLa S3 cells was noticed. However, no such induction was seen in the acid phosphatase activity which is a lysosomal enzyme.

All this data along with our present observation that the presence of high activities in the glial nuclear fractions in the later stages of lifespan support the contention that acid DNase is involved in DNA replication or to a process that is associated with it, possibly postreplication repair.

Alkaline DNase activity (Table 5) shows highest activity in oligodendrocytes followed by neurons, and astrocytes both in

TABLE 3

RELATIVE DISTRIBUTION OF ACID DNase AND ACID PHOSPHATASE IN THE ADULT RAT BRAIN

Subcellular fraction	Acid DNase (AD) (Total activity)	Acid Phosphatase (AP) (Total activity)	AD/AP	Increase in ratio
Homogenate	2656.0 + 314.0	283.0 + 7.5	9.4	1.0
Nuclei	124.3 ± 14.0	1.1 ± 0.2	105.3	11.2

Values are expressed as Mean + SD.

Number of experiments in each case are 4.

Starting material in each experiment was 1 g of grey matter of the brain.

Activities of acid DNase are expressed as ug of H-DNA degraded into acid soluble products per 30 min. Activities of acid phosphatase are expressed as umoles of p-nitrophenol liberated per 1 hr. For other details please see the text.

TABLE 4

SPECIFIC ACTIVITIES OF ACID DNase IN NEURONAL, ASTROGLIAL AND OLIGODENDROGLIAL FRACTIONS OF RAT BRAIN AT DIFFERENT AGES

Age and Subcellular fraction	Neurons	Astrocytes	Oligodendrocytes
Young:			
(1 day old)			
Homogenate	18.62 + 1.01	22.24 + 3.27	13.35 + 1.69
Nuclei	7.89 + 1.76	21.85 + 0.85	7.71 + 1.48
Adult:			
(6 months old)			
Homogenate	9.51 + 1.01*	9.65 + 1.14*	9.45 + 0.61+
Nuclei	2.19 + 0.35*	11.40 + 1.65*	7.46 + 0.58
Old:			
(>540 days old)			
Homogenate	11.48 + 1.21*#	11.94 + 0.50*#	10.47 + 0.56+#
Nuclei	2.79 + 0.48+	11.84 + 1.16*	8.17 + 0.78

Specific activities are expressed as μg of native ^{3}H-DNA degraded into acid soluble products/mg of protein/30 min.

For other details please see the text.

Values are expressed as mean + SD.

Number of experiments in each case are 4.

* These values are significantly different from the corresponding young values $p < 0.001$.

+ These values are significantly different from the corresponding young values $p < 0.05$.

These values are significantly different from the corresponding adult values $p < 0.05$.

TABLE 5

SPECIFIC ACTIVITIES OF ALKALINE DNase IN NEURONAL, ASTROGLIAL AND OLIGODENDROGLIAL FRACTIONS OF RAT BRAIN AT DIFFERENT AGES

Age and Subcellular fraction	Neurons	Astrocytes	Oligodendrocytes
Young:			
(1 day old)			
Homogenate	5.76 + 0.55	4.62 + 0.45	8.12 + 0.86
Nuclei	3.33 + 0.41	5.04 + 0.88	6.31 + 0.46
Adult:			
(6 months old)			
Homogenate	5.68 + 0.81	2.77 + 0.52^{+}	5.14 + 0.78^{+}
Nuclei	6.72 + 0.97*	4.99 + 0.59	4.08 + 0.21*
Old:			
(>540 days old)			
Homogenate	6.06 + 0.68	4.27 + 0.51$^{\#}$	5.94 + 0.68^{+}
Nuclei	6.47 + 0.58*	6.50 + 0.56$^{+\#}$	5.50 + 0.49$^{\#}$

Specific activities are expressed as ug of denatured ^{3}H-DNA degraded into acid soluble products/mg of protein/30 min.

For other details please see the text.

Values are expressed as mean $\pm$ SD.

Number of experiments in each case are 4.

* These values are significantly different from the corresponding young values $p < 0.001$.

$^{+}$ These values are significantly different from the corresponding young values $p < 0.05$.

$^{\#}$ These values are significantly different from the corresponding adult values $p < 0.01$.

TABLE 6

ACTIVITIES OF ACID DNase EXPRESSED PER mg OF DNA IN NUCLEI OF VARIOUS CELL ENRICHED FRACTIONS OF RAT BRAIN AT DIFFERENT AGES

Age	Neurons	Astrocytes	Oligodendrocytes
Young (1 day old)	45.14 + 9.77	122.03 + 7.18	42.97 + 7.31
Adult (6 months old)	25.03 + 5.19*	127.22 + 11.15	47.52 + 5.77
Old (> 540 days old)	30.73 + 6.00*	134.95 + 16.34	47.43 + 4.90

Activities are expressed as pg of native ^{3}H-DNA degraded into acid soluble products/mg of DNA/30 min.

For other details please see the text.

Values are expressed as mean + SD.

Number of experiments in each case are 4.

* These values are significantly different from the corresponding young values $p < 0.05$.

TABLE 7

ACTIVITIES OF ALKALINE DNase EXPRESSED PER mg OF DNA IN NUCLEI OF VARIODS CELL ENRICHED FRACTIONS OF RAT BRAIN AT DIFFERENT AGES

Age	Neurons	Astrocytes	Oligodendrocytes
Young (1 day old)	19.26 + 2.87	28.30 + 5.96	35.49 + 1.62
Adult (6 months old)	71.91 + 8.72*	56.06 + 7.21+	26.04 + 3.34+
Old (>540 days old)	71.28 + 10.03*	76.49 + 9.29*	31.94 + 3.49

Activities are expressed as ug of denatured H-DNA degraded into acid soluble products/mg of DNA/30 min.

For other details please see the text.

Values are expressed as mean $\pm$ SD.

Number of experiments in each case are 4.

* These values are significantly different from the corresponding young values $p < 0.001$.

+ These values are significantly different from the corresponding young values $p < 0.01$.

homogenate and nuclear fractions in young rat brain. However, in adult and old, the specific activity of alkaline DNase is more in neurons followed by oligodendrocytes and astrocytes. In neurons the specific activity of alkaline DNase does not change with age in homogenate. However, it is significantly more in adult and old brain nuclear fractions as compared to young, suggesting its role in DNA repair. On the other hand, in oligodendroglial nuclear fractions, the alkaline DNase decreased from young to adult stage and again increased in the old age. When the values are expressed per mg of DNA, the activities increased from young to adult stage in the nuclear fractions, and there was no further increase in old age (Table 7). These results substantiate for a role of alkaline DNase in DNA repair.

Recently two DNases with alkaline pH optimum, having a role in DNA repair have been reported in the rat brain. It was suggested that neocortex AP endonuclease, having a pH optimum of 7.4, attacks AP-DNA and hydrolyzes the phosphodiester bond that is immediate neighbour of the AP site on its 5' side, producing 3'-0H and 5'-phosphate ends and thus providing a primer for DNA polymerase β. It was also shown that another exodeoxyribonuclease with a pH optimum of 8.4, DNase B III, removes AP sites from the AP endonuclease nicked DNA and that this promotes more efficient DNA repair synthesis by DNA polymerase β (Ivanov et al., 1983; 1988).

It is to be mentioned here that at all the ages studied acid DNase showed similar activities towards both native and UV irradiated native DNA (Table 8 and 9) and this prompted us to rename

TABLE 8

SPECIFIC ACTIVITIES OF UV DNase IN NEURONAL, ASTROGLIAL AND OLIGODENDROGLIAL FRACTIONS OF RAT BRAIN AT DIFFERENT AGES

Age and Subcellular fraction	Neurons	Astrocytes	Oligodendrocytes
Young:			
(1 day old)			
Homogenate	17.33 + 2.50	21.54 + 3.38	12.15 + 1.48
Nuclei	7.63 + 1.66	19.77 + 0.99	6.74 + 1.15
Adult:			
(6 months old)			
Homogenate	7.94 + 0.64*	9.29 + 0.88*	8.71 + 0.59+
Nuclei	1.86 + 0.14*	10.99 + 1.87*	6.69 + 0.56
Old:			
(>540 days old)			
Homogenate	10.91 + 1.17+#	11.46 + 0.54*#	9.51 + 0.58+
Nuclei	2.33 + 0.55*	10.22 + 1.04*	7.59 + 0.83

Specific activities are expressed as ug of UV irradiated ^{3}H-DNA degraded into acid soluble products/mg of protein/30 min.

For other details please see the text.

Values are expressed mean + SD.

Number of experiments in each case are 4.

* These values are significantly different from the corresponding young values $p < 0.001$.

+ These values are significantly different from the corresponding young values $p < 0.05$.

These values are significantly different from the corresponding adult values $p < 0.01$

TABLE 9

ACTIVITIES OF UV DNase EXPRESSED PER mg OF DNA IN NDCLEI ISOLATED FROM VARIOUS CELL ENRICHED FRACTIONS OF RAT BRAIN AT DIFFERENT AGES

Age	Neurons	Astrocytes	Oligodendrocytes
Young: (1 day old)	43.68 + 9.11	112.32 + 5.05	37.47 + 4.55
Adult: (6 months old)	21.23 + 2.75*	124.32 + 5.54+	42.61 + 5.68
Old: (>540 days old)	25.77 + 7.18+	116.45 + 15.06	43.96 + 5.11

Activities are expressed as µg of UV irradiated ^{3}H-DNA degraded into acid soluble products/mg of DNA/min.

For other details please see the text.

Values are expressed as mean + SD.

Number of experiments in each case are 4.

* This value is significantly different from the corresponding young value $p < 0.01$.

+ These values are significantly different from the corresponding young values $p < 0.05$.

TABLE 10

SPECIFIC ACTIVITIES OF AP DNase IN NEURONAL, ASTROGLIAL AND OLIGODENDROGLIAL FRACTIONS OF RAT BRAIN AT DIFFERENT AGES

Age and Subcellular fraction	Neurons	Astrocytes	Oligodendrocytes
Young:			
(1 day old)			
Homogenate	2.12 + 0.18	2.42 + 0.53	6.98 + 1.21
Nuclei	2.76 + 0.60	2.06 + 0.45	5.19 + 0.94
Adult:			
(6 months old)			
Homogenate	4.52 + 0.52*	2.63 + 0.59	4.45 + 0.57+
Nuclei	4.98 + 0.52+	4.52 + 0.67*	3.39 + 0.42+
Old:			
(>540 days old)			
Homogenate	4.44 + 0.61*	3.44 + 0.54+	5.13 + 0.77+
Nuclei	4.91 + 0.45+	5.14 + 0.74*	4.80 + 0.42#

Specific activities are expressed as µg of depurinated H-DNA degraded into acid soluble products/mg of protein/30 min.

For other details please see the text.

Values are expressed as mean + SD.

Number of experiments in each case are 4.

* These values are significantly different from the corresponding young values $p < 0.001$.

+ These values are significantly different from the corresponding young values $p < 0.05$.

This value is significantly different from the corresponding adult value $p < 0.01$.

TABLE 11

ACTIVITIES OF AP DNase EXPRESSED PER mg OF DNA IN NUCLEI ISOLATED FROM VARIOUS CELL ENRICHED FRACTIONS OF RAT BRAIN AT DIFFERENT AGES

Age	Neurons	Astrocytes	Oligodendrocytes
Young: (1 day old)	15.87 + 3.64	11.64 ± 3.09	29.09 + 6.16
Adult: (6 months old)	54.92 + 5.54*	51.00 + 8.52*	21.74 + 4.41
Old: (>540 days old)	54.09 + 8.07*	65.02 + 7.03*#	27.83 + 2.39

Activities are expressed as pg of depurinated ^{3}H-DNA degraded into acid soluble products/mg of DNA/30 min.

For other details please see the text.

Values are expressed as mean + SD.

Number of experiments in each case are 4.

* These values are significantly different from the corresponding young values $p < 0.001$.

This value is significantly different from the corresponding value at adult stage $p < 0.05$.

TABLE 12

PERCENTAGE ACTIVITIES OF DNases IN NEURONAL NUCLEI ISOLATED FROM THE RAT BRAIN AT DIFFERENT AGES

Age	Acid DNase	Alkaline DNase	UV DNase	AP DNase
Young (1 day old)	23.3 + 3.0	24.3 + 1.6	32.9 + 6.0	73.8 + 11.7
Adult (6 months old)	10.1 + 2.1*	9.9 + 1.4*	47.3 + 7.4+	47.1 + 11.0+
Old (>540 days old)	9.9 + 1.5*	8.8 + 2.5*	46.9 + 10.8	48.8 + 9.3+

Values are expressed as mean of percentage activities + SD.

These values are recalculated from the activities per mg of DNA.

For other details please see the text.

* These values are significantly different from the corresponding young values $p < 0.001$.

+ These values are significantly different from the corresponding young values $p < 0.05$.

TABLE 13

PERCENTAGE ACTIVITIES OF DNases IN ASTROGLIAL NUCLEI ISOLATED FROM THE RAT BRAIN AT DIFFERENT AGES

Age	Acid DNase	Alkaline DNase	UV DNase	AP DNase
Young (1 day old)	32.9 + 5.7	33.2 + 3.8	36.1 + 5.7	28.9 + 5.9
Adult (6 months old)	17.5 + 1.4[+]	17.8 + 1.6[*]	27.3 + 4.8	26.2 + 5.1
Old (>540 days old)	20.7 + 1.7[+]	18.6 + 1.3[*]	33.1 + 3.2	38.1 + 3.4[#]

Values are expressed as mean of percentage activities + SD.

These values are recalculated from the activities per mg of DNA.

For other details please see the text.

[*] These values are significantly different from the corresponding young values $p < 0.001$.

[+] These values are significantly different from the corresponding young values $p < 0.01$.

[#] This value is significantly different from the corresponding young and adult values $p < 0.05$.

TABLE 14

PERCENTAGE ACTIVITIES OF DNases IN OLIGODENDROGLIAL NDCLEI ISOLATED FROM THE RAT BRAIN AT DIFFERENT AGES

Age	Acid DNase	Alkaline DNase	UV DNase	AP DNase
Young (1 day old)	30.1+5.5	29.3+4.1	40.3+6.3	38.7+6.0
Adult (6 months old)	34.1 + 3.4	32.3 + 0.9	34.4 + 7.3	32.9 + 6.9
Old (>540 days old)	29.8 + 4.7	30.8 + 4.3	36.0 + 8.5	36.7 + 9.6

Values are expressed as mean of percentage activities + SD.
These values are recalculated from the activities per mg of DNA.
For other details please see the text.

this so called acid DNase as UV DNase. However, only the purified protein should yield more information and such work is progressing in this laboratory. Similarly the alkaline DNase found to attack depurinated DNA also and this activity is quite high in adult and old ages (Tables 10 and 11). Therefore, this activity is renamed as AP DNase and attempts to isolate this enzyme in pure form are going on.

Chapter IV

DNA Polymerases in isolated cell enriched fractions from the rat brain at different ages

CHAPTER IV

DNA POLYMERASES IN ISOLATED CELL ENRICHED FRACTIONS FROM THE RAT BRAIN AT DIFFERENT AGES

INTRODUCTION:

Previous studies from this laboratory have shown that the DNA content of both rat (Subba Rao and Subba Rao, 1982) and chick brains (Shrivastaw and Subba Rao, 1975) goes up significantly in the later stages of lifespan and this was attributed to one or more of the following possibilities a) replication of glial cells b) an increase in the intracellular DNA not necessarily connected with the replication process but including the DNA repair. Further, when the activity of DNA polymerase (mostly DNA polymerase 3) was examined, it showed a corresponding peak at stages when increased DNA content was observed (Subba Rao and Subba Rao, 1984). This pattern of changes led us to believe that brain probably retains good repair capacity throughout the life-span.

We have extended these studies to isolated cell enriched fractions. It is generally accepted that brain consists of three distinct types of cells viz, neurons, astrocytes and oligodendro-glia, all with different replicative schedules (Korr, 1980). It is therefore, possible that DNA polymerase in these cell types may vary depending upon the age of the brain. We have measured

the DNA polymerase activity in these cell types isolated from the rat brain at different stages of the lifespan. Effort was also made to distinguish between a and β polymerase activity making use of specific inhibitors.

MATERIALS AND METHODS:

Highly polymerized calf thymus DNA, dATP, dGTP, and dTTP were purchased from Sigma Chemical Company, St. Louis, MO, USA. Dideoxythymidine 5'-triphosphate was from P.L. Biochemicals Inc., Wisconsin, USA and (methyl-^{3}H) dTTP (sp. act., 46 Ci/mmole) was from Radiochemical Centre, Amersham, England. Aphidicolin was a gift from Dr. A.H. Todd of I.C.I.Ltd., U.K. Polysaccharide purified from Physarum polycephalum was a kind gift from M. Shioda, University of Tokyo, Japan. All other chemicals used were of analytical grade.

Neuronal, astroglial and oligodendrogllal cell enriched fractions were isolated as described in the chapter **II.**

Preparation of the Enzyme extract:

The homogenates of isolated cell enriched fractions were prepared by homogenizing the isolated cell pellets in homogenization medium, which consisted of 0.02 M Tris-HCl buffer, pH 7.5, 0.1 mM β-mercaptoethanol, 1 mM $MgCl_2$, 0.1 mM EDTA, 5% glycerol, 1% Triton X-100, and 0.5 M KC1. After the homogenization the samples were kept at zero to 4°C for one hour to aid the extraction of the polymerases from the nuclei and centrifuged at 100,000 xg for 1hr. The clear supernatant thus obtained was used

as the enzyme source of DNA polymerase. An aliquot of the supernatant was taken for the protein estimation according to Lowry's method (1951).

DNA polymerase assay:

The reaction mixture contained in a total volume of 50 ul: 40 mM Tris-HCl pH 8.0, 1 mM β-mercaptoethanol, 7.5 mM $MgCl_2$, 4 mM ATP, 5 ug of 'activated' DNA (Loeb.,1969), 0.1 mM each dATP, dGTP, dCTP and 25 uM of dTTP (1 uCi). Incubation was carried out at 37°C for 20 min. At the end of incubation, 0.4 mg of DNA was added as carrier and the reaction was stopped by adding 2 ml of cold 10% TCA. The samples were kept in ice for 10 min and centrifuged at 4000 rpm for 5 min. The precipitate thus obtained was washed thrice with 5% cold TCA and twice with 95% ethanol. The precipitate after washing, was dissolved in 0.1 ml of 0.05 M NaOH and aliquots were taken into scintillation vials containing 10 ml of Bray's mixture and were counted in Beckman LS 1800 liquid scintillation counter. The enzyme activity was linear up to 30 µg of enzyme protein. Specific activity was expressed as picomoles of dTMP incorporated into activated DNA per mg of protein per hour.

In order to distinguish the activity into α and β polymerase, specific inhibitors were used. Aphidicolin was added in the reaction mixture at a concentration of 50 uM with a simultaneous reduction of dCTP concentration to 5 uM. ddTTP was added at a concentration of 1 mM. Polysaccharide isolated from the slime mold Physarum polycepahalum, (Shioda and Murakami-Muro-

fushi, 1987) was added at concentration of 0.5 mg/ml. Further increase in concentrations of these inhibitors had not resulted any increase in the extent of inhibition.

BESUTLS AND DISCUSSION:

The specific activities of DNA polymerase in neuronal, astroglial and oligodendroglial cells obtained from rat brains of various ages are presented in Fig 5. It may be noted that at all the ages studied, neuronal cells possessed the highest polymerase activity. The activity in these cells decreased from 16th day of embryonic life to adult life (225 days), but no further decrease occurred thereafter. Eventhough there was a slight increase in the old age (>540 days), it is not significant. These values compare well with those already reported by Waser et al. (1979) and by Subba Rao et al. (1985). In both astrocytes and oligodendrocyte fractions the activity steadily decreased with age starting from a high level at the neonatal stage.

It is intriguing that cerebral neurons, with no capacity to divide during postnatal life, should exhibit higher activities of DNA polymerase at all the stages of lifespan as compared with the other cell types, viz, astrocytes and oligodendrocytes which are the cells known to retain their replicative capacity to a significant extent throughout life. It is known that there are at least three distinct DNA polymerases in mammalian cells; polymerase α,β and γ (Weissbach et al., 1975 and Scovassi et al., 1980). In the brain also similar type of diversity of DNA poly-

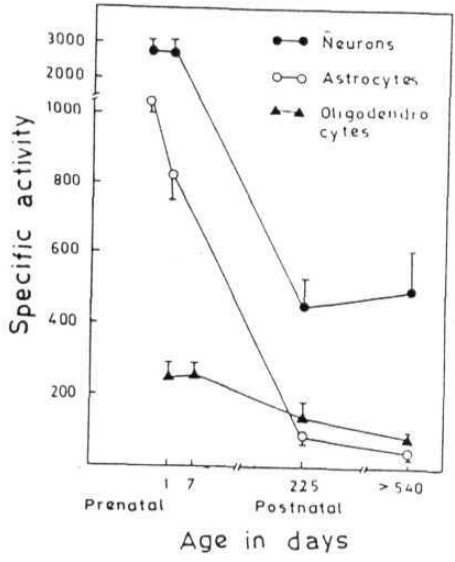


Fig 5. DNA polymerase in neuronal, astroglial and oligodendroglial cells of developing and aging rat brain. Each point represents the average of 4 at 16th day gestation and 6 experiments at all other ages, with bars indicating the variation.

merases was noticed. Studies from different laboratories have shown that the activities of polymerase α and β could be distinguished by using specific inhibitors (Edenberg et al., 1978; Ikegami et al., 1978; ; Ohashi et al., 1978; van der Vliet and Kwant 1978; Waqar et al., 1978). Thus, α-polymerase can be inhibited specifically by aphidicolin and β-polymerase can be inhibited by ddTTP. These inhibitors have been implicated in a number of studies to define a role for these polymerases. In a number of studies DNA polymerase β has been postulated to have a role in the repair of DNA (Waser et al., 1979; Pedrali-Noy and Spadari, 1980; Seki et al., 1980; Giulotto and Mondello, 1981; Spadari et al., 1982). Studies on the DNA polymerase a has produced conflicting results. Several studies implicated a role for polymerase a in the repair process (Berger et al., 1979; Ciarrocchi et al., 1979; Hanaoka et al., 1979; Snyder and Regan, 1982) while other studies assigned no such role (Pedrali-Noy and Spadari, 1980; Seki et al., 1980 and Spadari et al., 1982). Recent studies using different inhibitors suggested that both polymerases are involved in DNA repair depending upon the damaging agent used and the dosage applied (Miller and Chinault, 1982; Miller and Lui, 1982; Cleaver, 1983; Dresler and Lieberman, 1983). The precise physiological role of polymerase γ which is shown to be similar to mitochondrial DNA polymerase is not yet clear (Hubscher, et al., 1977).

Therefore, we have made use of these two inhibitors to distinguish DNA polymerase a and B in different cell types. The effect of aphidicolin and ddTTP in neurons, astrocytes and

oligodendrocytes are presented in Tables 15, 16 and 17 respectively. A summary of the average percentage inhibition is presented in Table 18.

As it can be seen, in neurons the DNA polymerase activity at all the postnatal ages was inhibited more than 90% by ddTTP, with aphidicolin exerting no inhibition at all at 225 and >540 days of age. During the embryonic stages, however, ddTTP inhibited the activity by 70% only, while aphidicolin did so by 9%. These results are taken to indicate that at all the postnatal ages, most of the DNA polymerase activity in neurons is of the β-type. The picture was, however, different in the case of astrocytes and oligodendrocytes. In astrocytes, the maximum inhibition (95%) of activity by ddTTP was observed at 1 day old postnatal, pointing to the fact that at this stage the polymerase present is almost exclusively of the B-type. At the other ages the inhibition varied from 48 to 78% with a marginal effect by aphidicolin at the 16th day of gestation and 225 days. From the pattern of the inhibition by the two inhibitors in the oligodendrocyte fraction, it can probably concluded that while B -polymerase is the predominant one throughout the postnatal life, some amount of α-polymerase also seems to be present at all the ages studied. There were few oligodendrocytes in the prenatal brain and therefore could not be isolated.

In view of the claimed specificities of the two inhibitors ddTTP and aphidicolin towards DNA polymerase β and α, respectively, it can normally be expected that the sum total of inhibition by ddTTP and aphidicolin should reach close to 100%. How-

TABLE 15

EFFECT OP APBIDICOLIN AND ddTTP ON DNA POLYMERASE ACTIVITY IN NEURONS OF RAT BRAIN AT DIFFERENT AGES

Age	Control	ddTTP	% inhibition	Aphidicolin	% inhibition
16th day (Prenatal) (4)	2767.56 + 294.34	825.03 + 142.08	70.18	2506.38 + 61.40	9.44
1 day (Postnatal) (6)	2713.00 + 341.80	210.03 + 108.00	92.25	2630.00 + 345.80	3.00
Adult (225 days) (6)	449.23 + 81.47	34.55 + 9 2 . 3 0 16.31		531.43 + 171.57	
Old (>540 days) (6)	500.52 + 110.89	35.37 + 14.75	93.03	522.02 + 137.00	

Activities are expressed as picomoles of TMP incorporated into activated DNA/mg of protein/hr.

Values are expressed as Mean + SD.

Numbers in parentheses represent number of experiments carried out.

For other details please see the text.

TABLE 16

EFFECT OF APHIDICOLIN AND ddTTP ON DNA POLYMERASE ACTIVITY IN ASTROCYTES OF RAT BRAIN AT DIFFERENT AGES

Age	Control	ddTTP	% inhibition	Aphidicolin	% inhibition
16th day (Prenatal) (4)	1476.84 + 360.75	442.46 + 164.49	70.04	1265.04 + 342.92	14.24
1 day (Postnatal) (6)	823.30 + 76.90	42.35 + 25.90	94.85	806.42 + 111.11	2.05
Adult (225 days) (6)	75.47 + 15.62	39.28 + 12.42	47.90	65.37 + 17.12	**13.46**
Old (>540 days) (6)	37.52 + 11.92	18.70 + 8.32	50.16	44.75 + 15.91	

Activities are expressed as picomoles of TMP incorporated into activated DNA/mg of protein/hr.

Values are expressed as Mean + SD.

Numbers in parentheses represent number of experiments carried out.

For other details please see the text.

TABLE 17

EFFECT OF APHIDICOLIN AND ddTTP ON DNA POLYMERASE ACTIVITY IN OLIGODENDROCYTES OF RAT BRAIN AT DIFFERENT AGES

Age	Control	ddTTP	% inhibition	Aphidicolin	% inhibition
1 day (Postnatal) (6)	245.10 + 53.19	91.51 + 10.35	62.60	205.00 + 44.30	16.36
Adult (225 days) (6)	144.43 + 42.30	28.10 + 10.70	80.50	137.42 + 41.80	4.80
Old (>540 days) (6)	78.80 + 16.30	44.20 + 18.35	43.90	74.63 + 28.68	5.29

Activities are expressed as picomoles of TMP incorporated into activated DNA/mg of protein/hr.

Values are expressed as Mean + SD.

Numbers in parentheses represent number of experiments carried out.

For other details please see the text.

TABLE 18

EXTENT OF INHIBITION BY ddTTP AND APHIDICOLIN IN DIFFERENT CELL TYPES IN THE RAT BRAIN OF DIFFERENT AGES

Cell type and inhibitor	Age	16th day of prenatal	1 day postnatal	225 days postnatal	>540 days postnatal
Neurons:					
ddTTP		70.2	92.3	92.3	93.0
Aphidicolin		9.4	3.0	Nil	Nil
Astrocytes:					
ddTTP		70.0	94.9	47.9	50.2
Aphidicolin		14.3	2.0	13.5	Nil
Oligodendrocytes:					
ddTTP			62.6	80.5	43.9
Aphidicolin			16.4	4.8	5.3

Values represent average percentage of inhibition.

Number of experiments carried out are 4 at 16th day prenatal **and** 6 at all other ages.

For other details please see the text.

ever, this is not the case particularly, in glial fraction, as can be noticed in Table 18. This could be due to two reasons: l)the α-polymerase in crude extracts may not be so sensitive towards aphidicolin as it is reported to be in studies with pure a-polymerase enzyme (Ikegami et al., 1978). 2)the molecular species of a-polymerase present in the glial cells may be only marginally susceptible to aphidicolin. Indeed, there are reports in literature claiming the presence of multiple forms of DNA polymerase a in HeLa cells (Pedrali-Noy and Weissbach, 1977), calf thymus (Hesslewood et al., 1978) and rat liver (Holmes et al., 1974). On the other hand, the sensitivity of β-polymerase present in crude extracts towards ddTTP can not be suspected since at some ages profound inhibition by this inhibitor was observed. It thus appears that aphidicolin is not a good indicator to assess the levels of a-polymerase in the crude extracts as is the case in the present experiments.

Recently a polysaccharide isolated from the slime mold Physarum polycephalum was found to inhibit a-polymerase specifically with no effect on β-polymerase (Shioda and Murakami-Murofushi,1987). We have therefore, studied the effect of this polysaccharide on the DNA polymerase activity in isolated cell enriched fractions from the rat brain of different ages. The results are presented in Table 19. It is clear from the results that polysaccharide inhibited the DNA polymerase activity by about 25% in astrocytes at all the ages studied, while this inhibition ranged from 30 to 50% in the oligodendroglial fraction. These results suggest that at all the postnatal stages,

TABLE 19

EXTENT OP INHIBITION BY POLYSACCHARIDE ISOLATED FROM PHYSARUM POLYCEPHALUM ON DNA POLYMERASE ACTIVITY IN NEURONAL, ASTROGLIAL AND OLIGODENDROGLIAL CELLS OF RAT BRAIN OF DIFFERENT AGES.

Age	Neurons	Astrocytes	Oligodendrocytes
Young	50.5 + 4.7	25.8 + 4.9	32.5 + 5.8
(1 day old)	(6)	(4)	(4)
Adult	31.7 + 5.0	27.7 + 6.6	53.5 + 5.3
(6 Months old)	(4)	(4)	(4)
Old	23.5 + 2.0	27.7 + 3.0	32.3 + 3.0
(> 540 days old)	(4)	(5)	(4)

Values represent Mean of percentage inhibition + SD.

Numbers in parentheses represent number of experiments carried out.

Actual values (expressed as picomoles of TMP incorporated into activated DNA/mg protein/hr.) for neurons at young, adult and old ages are 2713, 449 and 500; for astrocytes 823, 75 and 37; for oligodendrocytes are 245, 144 and 78 respectively.

For other details please see the text.

about 25 to 50% of the DNA polymerase activity in the glial fraction is of α-type. It is surprising to see that polysaccharide inhibited DNA polymerase activity in neurons at all the three postnatal ages studied and this inhibition decreased with age (50% in young, 31% in adult and 23% in old). These results are not in complete agreement with our above mentioned inhibition studies with ddTTP and aphidicolin (Table 18). This could be due to marginal susceptibility of 8 -polymerase of neuronal cells to polysaccharide. Further work is necessary.

In any event, these results do indicate that rat cerebral neurons possess significant amounts of DNA polymerase activity both in adult and old life and that the enzyme is almost exclusively of β-type. On the other hand, the glial cells at adult and old stages of life seem to possess other type(s) of DNA polymerase in addition to the predominant β-polymerase. This appears to be in line with the known proliferative capacity of the glial cells in later stages of lifespan.

Chapter V

Unscheduled DNA Synthesis in isolated Neurons and Lymphocytes of rat of different ages.

CHAPTER V

UNSCHEDULED DNA SYNTHESIS IN ISOLATED NEURONS AND LYMPHOCYTES OF RAT OF DIFFERENT AGES

INTRODUCTION

Eversince the observations of Hart and Setlow (1974) that DNA repair capacity of a species is directly related to its maximum lifespan, much attention has been paid on DNA repair studies in the field of gerontology. Although a voluminous data have been accumulatd, a direct evidence for DNA repair as the primary determinant of aging is lacking. Evidences have been presented that DNA repair capacity declines with age in human fibroblasts (Hart and Setlow, 1976) and human peripheral blood leukocytes (Lambert et al., 1979). However, some dissenting reports do exist for the above observations (Hall et al., 1982 and Mayer et al., 1986).

Most of the investigations cited above had been carried out either in fibroblasts or in lymphocytes. A close correlation between DNA repair capacity and the proliferative status of a cell has been well established. It was demonstrated that the DNA repair activity is more in actively proliferating fibroblasts (Dowens et al., 1982 and Volberg et al., 1984) and mitogen stimulated lymphocytes (Llcastro and Walford, 1985) when compared to the resting cells of the same lineage. Since the proliferative capacity of both lymphocytes (Walford, 1969) and fibroblasts

(Schneider *et al.*, 1979) declines with age of the donor it is unclear whether the decrease in DNA repair capacity with age is due to decreased proliferative capacity or it is independent of proliferative status. For such studies probably a postmitotic tissue like brain is well suited. Further, repair of DNA damage in neuronal cells is of prime importance in maintaining the functional integrity of the genome since the chances of corrective DNA replicative synthesis are virtually nil in neurons.

The best known type of DNA repair in mammalian cells is the excision repair. Excision repair can be induced by different DNA damaging agents like ultraviolet rays, X-rays, alkylating agents etc. (discussed in detail in chapter I). Ultraviolet light (254 nm) may represent an excellent model agent for inducing DNA repair. It is directly acting, primarily absorbed by nucleic acids and induces long patch repair which appears to be primarily error free (Regan and Setlow, 1974a and Hanawalt *et al.*, 1979). Hence, a study has been taken to measure UV induced unscheduled DNA synthesis in isolated neurons of rat brain of different ages. Lymphocytes isolated from rat spleen of the same age groups have been taken as a positive control system for such studies.

MATEBIALS AND METHODS

Phosphate buffered saline and RPMI-1640 (developed in Roswell Park Memorial Institute) were purchased from Hi-Media, Bombay, India. Minimal essential medium (Eagle) and foetal calf

serum were obtained from Gibco Laboratories, New York. Penicillin and streptomycin sulfate were purchased from Sigma Chemical Company, St. Louis, MO, USA. ^{3}H-thymidine was obtained from Bhabha Atomic Research Centre, Bombay. NTB-2 autoradiography emulsion was from Eastman Kodak, USA. Germicidal lamps (15 W TUV 8) were purchased from Philips, Netherlands. All other chemicals used were of analytical grade.

Neuronal cells were isolated as described in chapter II.

Isolation of Lymphocytes

Lymphocytes from the rat spleen were isolated essentially as described by Licastro and Walford (1985). Rats were killed by mild ether anaesthesia. Spleens were removed aseptically and placed in cold RPMI-1640 supplemented with 100 units/ml of penicillin and 100 µg/ml of streptomycin. The spleen was minced and pressed gently through a sterile stainless steel mesh with arterial forceps fitted with steel brushes. The cell suspension was allowed to stand for 5 min at room temperature. The clumps which were settled to the bottom were discarded and the cell suspension was centrifuged at 400 xg for 7 min. Erythrocytes were lysed with 0.85% ammonium chloride and the nucleated lymphocytes were collected by centrifugation. The cells were washed three times with cold RPMI-1640. The final cell pellet was suspended in RPMI-1640 supplemented with 5% foetal calf serum at a density of 2 X 10^{6} cells/ml. Cell viability as judged by trypan blue exclusion test was about 90%.

UV irradiation:

The isolated neuronal cells were washed once with phosphate buffered saline (PBS, pH 7.4) and suspended in minimal essential medium (MEM, Hank's salts, pH 7.4) supplemented with 100 units/ml of penicillin and 100 µg/ml of streptomycin, at a density of 1 X 10 cells/ml. The cell suspension was spread as a thin layer in 55 mm plastic petridish and irradiated at a dose of 20 J/m or 40 J/m^2 at 254 nm using Philips TUV 8, 15 W germicidal lamp. The intensity of the lamp was monitored with a Black-Ray UV meter Model J-225. All other manipulations after UV irradiation were performed in the red dim light in order to eliminate the possible occurrence of photoreactivation. After the irradiation the cells were collected by centrifugation and suspended in fresh MEM. Control cells received the same treatment except the UV irradiation. Lymphocytes were irradiated in similar manner except that the suspension medium was RPMI-1640.

Measurement of DNA synthesis:

DNA synthesis was measured by the incorporation of H-thymidine into DNA in vitro. To the cell suspension H-thymidine was added at a concentration of 10 µCi/ml and incubated at 37°C for 2 hrs in the dark. After the incubation, the cells were collected by centrifugation and the DNA was extracted as described in the chapter II. The DNA synthesis was expressed as disintegrations per minute (DPM) of H-thymidine incorporated into one ug of DNA per 2 hrs at 37°C.

Measurement of unscheduled DNA synthesis:

Unscheduled DNA synthesis (UDS) was measured by the incorporation of ^{3}H-thymidine into DNA *in vitro* in the presence of 5 mM hydroxyurea (HU). Unscheduled DNA synthesis was expressed as DPM of ^{3}H-thymidine incorporated into one microgram of DNA per 2 hrs at 37°C. Unscheduled DNA synthesis was also measured by autoradiography.

Autoradiography:

Autoradiography was performed according to the procedure of Cleaver and Thomas (1981) with slight modification. After the incubation, the cells were collected by centrifugation and washed extensively with PBS (pH 7.4). Then the cells were fixed with *2%* acetic acid and a smear of cells was prepared on a glass slide. The slides were rinced once with 50% alcohol, once with 70% alcohol and once with 90% alcohol. Then the slides were air dried and coated with 1:1 diluted NTB 2 emulsion (Kodak) and kept in light tight box at 4°C. After six months of exposure the slides were developed with Kodak D 19 developer and fixed with acid fixer. The slides were observed under phase contrast microscope.

RESULTS AND DISCUSSION:

The results of ^{3}H-thymidine incorporation into neurons are presented in Table 20. As can be seen, notable amounts of ^{3}H-thymidine were found to be incorporated into neuronal DNA at all the ages studied although the amount of incorporation in 1 day

old neurons was ten times more than that at adult and old stages.

Hydroxyurea (HU) has been implicated by various investigators to distinguish between semiconservative DNA replication and repair replication. It has been shown that HU inhibits thymidine incorporation into DNA without affecting RNA and protein syntheses (Young et al. , 1964; Young and Hodas, 1964). However, HU is known to inhibit scheduled DNA synthesis but not unscheduled DNA synthesis (Young and Hodas, 1964; Cleaver, 1969; Vilenchik and Tretjak, 1977). When HU was present in the medium at a concentration of 5 mM, about 80% of the thymidine incorporation was abolished in 1 day old neurons while no such effect could be observed in neurons isolated from adult and old rat brains. (Table 20) These results demonstrate that the thymidine incorporation into adult and old neurons is only due to unscheduled DNA synthesis (DNA repair) and therefore isolated neuronal cells from the adult brain should offer a good model system for a study of DNA repair without the interference of DNA replication process. The basal excision repair capacity (UDS) in neurons decreased significantly from young to adult stage but remained at the same level even in old age.

On the other hand, the thymidine incorporation into lymphocytes was inhibited by more than 90% in young and old lymphocytes and by about 80% in adult lymphocytes pointing the fact that there is persistence of DNA replicative activity in these cells at all ages (Table 21). The results also indicate that while the

TABLE 20

EFFECT OF HYDROXYUREA OH ^{3}H-THYMIDINE INCORPORATION INTO NEDRONAL DNA OF YOUNG, ADULT AND OLD RAT BRAINS

Age	Control DPM x 10^{-3}/mg DNA	+ HU DPM x 10^{-3}/mg DNA	% inhibition
Young (1 day old)	289 +87 (4)	60+2 (8)	79
Adult (6 months old)	28+4 (8)	31+4 (4)	
Old (> 540 day old)	29+3 (8)	29+2 (4)	

Values are expressed as Mean + SD.

Number in parentheses represents the number of experiments carried out.

For other details please see the text.

Adult and old values are significantly different from the corresponding young values $p < 0.001$.

TABLE 21

EFFECT OF HYDROXYUREA ON ^{3}H-THYMIDINE INCORPORATION INTO DNA OF LYMPHOCYTES OF YOUNG, ADULT AND OLD RAT SPLEENS

Age	Control DPM x 10^{-3}/mg DNA	+ HU DPM x 10^{-3}/mg DNA	% inhibition
Young (1 day old)	1490 +40	60 + 11	96
Adult (6 months old)	274 + 23*	57 + 11	79
Old (> 540 day old)	447 + 103*+	42 ± 5+	91

Values are expressed as mean + SD.

Number of experiments in each case are 4.

For other details please see the text.

* These values are significantly different from corresponding young values $p < 0.001$.

+ These values are significantly different from corresponding adult values $p < 0.05$.

DNA replicative activity is slightly higher in old lymphocytes as compared to the adult, the basal DNA repair capacity (UDS) actually decreased with age - thus pointing a contrasting picture from that of neurons.

The effect of UV (254 nm) on DNA synthesis in neurons and lymphocytes are presented in Tables 22 and 23 respectively. It is clear from the results that UV inhibited DNA synthesis in all the cases and this inhibition is dose dependent. A similar dose dependent reduction in DNA replication has also been observed in other mammalian cells (Rasmussen and Painter, 1964; Park and Cleaver, 1979; Griffiths and Ling, 1985). This reduction appears to be due to at least two factors; blockage of DNA fork progression by DNA lesions produced by UV (Dahle et al., 1980) and inhibition of replication initiation (Kaufmann et al., 1980). It is also clear from the results that the inhibition of DNA synthesis by UV in lymphocytes does not change significantly with age of the donor suggesting that the effect of UV on DNA synthesis is not age dependent. Also it can clearly be observed that the effect of UV on DNA synthesis is more pronounced in neurons than lymphocytes.

The results of UV induced UDS in neurons are presented in Table 24. It may be seen that a significant increase in the UDS was observed in both young and adult neurons with the two doses tested. However, old neurons did not exhibit any ability to respond to UV challenge at 20 J/m^2 but a marginal induction could be seen at 40 J/m^2 ($p < 0.05$). In order to test the possibility that neuronal cells isolated from the old rat brain may require

TABLE 22

EFFECT OF UV ON DNA SYNTHESIS IN NEURONS OF YOUNG **RAT BRAIN**

Control	20 J/m^2	% inhibition	40 J/m^2	% inhibition
289 + 87	184 + 33	36	116 + 42*	60

Values are expressed as mean of DPM x 10^3 of ^{3}H-thymidine incorporated/mg DNA + SD.

Number of experiments in each case are 4.

For other details please see the text.

* This value is significantly different from control value $p < 0.05$.

TABLE 23

EFFECT OF UV OH DNA SYNTHESIS IN LYMPHOCYTES OF RAT SPLEEN AT DIFFERENT AGES

Age	Control	20 J/m^2	% inhibition	40 J/m^2	% inhibition
Young (1 day old)	1490 + 340	1269 + 235	15	1183 + 74	21
Adult (6 months old)	274 + 9	225 + 50	17	190 + 51	30
old (>540 days old)	447 + 103	407 + 91	9	328 + 107	26

Values are expressed as mean of DPM x 10 of H-thymidine incorporated/mg DNA + SD.

Number of experiments in each case are 4.

For other details please see the text.

more time (more than 2 hrs of incubation) to respond to UV, we have extended the incubation time up to 6 hrs. Even then, old neurons did not show any significant response to the challenge of UV light at a dose of 20 J/m^2 (Table 25).

The picture is somewhat different in lymphocytes which are known to have the capacity to proliferate on stimulation (Table 26). These cells responded to UV challenge through a marked increase in UDS at all the stages of llfespan studied. Further, UV induced UDS (UV/cont) increased with age suggesting that UV induced UDS may be dependent upon the replicative potential but not the replicative schedule of the cell.

Autoradiography experiments showed significant number of grains (4 to 6) in both neurons and lymphocytes at all the ages. These grains may represent the basal DNA repair synthesis in these cells. When the cells were irradiated with UV this number was found to be increased significantly (12 to 15) in lymphocytes at all the ages. However, significant increase in the number of grains was observed in neurons only at young and adult (6 to 8) stages of llfespan (Fig 6 and 7). These results substantiate our biochemical observations. It has also been observed in these experiments that the percentage of the neuronal cells with grains is more in young (about 70%) when compared to adult and old (about 20%).

It is understood from the above results that the effect of UV in neuronal cells, especially in the old life is quite limited while UV induced UDS is more in lymphocytes and it increased with

TABLE 24

UV INDUCED UNSCHEDULED DNA SYNTHESIS IN NEURONS OP RAT BRAIN AT DIFFERENT AGES

Age	Control	20 J/m^2	UV/Cont.	40 J/m^2	UV/Cont.
Young (1 day old)	61 +2 (8)	71 + 6* (8)	1.16	91 + 21$^+$ (3)	1.49
Adult (6 months old)	28 + 4 (8)	35 + 2 * (8)	1.25	33 + 2$^+$ (4)	1.18
Old (>540 days old)	29 + 3 (8)	30 + 4 (8)	1.03	34 + 3$^+$ (3)	**1.17**

Values are expressed as mean of DPM x 10 of H-thymidine incorporated/mg of DNA + SD.

Number in parentheses represents the number of experiments carried out.

For other details please see the text.

* These values are significantly different from the corresponding control values $p < 0.001$.

+ These values are significantly different from the corresponding control values $p < 0.05$.

TABLE 25

TIME COURSE OF UV INDUCED UNSCHEDULED DNA SYNTHESIS IN NEURONS OF RAT BRAIN AT DIFFERENT AGES

Age	Hours after irradiation	Control	20 J/m^2	UV/Cont.
Young	2	61 + 2	71 + 6 *	1.16
(1 day old)	4	83 + 2	93 + 2	1.12
	6	96 + 3	114 + 6$^+$	1.19
Adult	2	28 + 4	35 + 2 *	1.25
(6 months old)	4	34 + 3	44 + 2$^+$	1.29
	6	42 + 3	48 + 2$^\#$	1.14
Old	2	29 + 3	30 + 4	1.03
(>540 days old)	4	37 + 2	39 + 1	1.05
	6	49 + 5	52 + 7	1.06

Values are expressed as mean of DPM x 10^{-3} of ^{3}H-thymidine incorporated/mg of DNA + SD.

Number of experiments are 8 at 2 hrs and 4 at 4 hrs and 6 hrs.

For other details please see the text.

* These values are significantly different from the corresponding control values $p < 0.001$.

+ These values are significantly different from the corresponding control values $p < 0.01$.

This value is significantly different from the corresponding control value $p < 0.05$.

TABLE 26

UV INDUCED UNSCHEDULED DNA SYNTHESIS IN LYMPHOCYTES OF RAT SPLEEN AT DIFFERENT AGES

Age	Control	20 J/m^2	UV/Cont.	40 J/m^2	UV/Cont.
Young (1 day old)	69 + 11	104 + 19*	1.50	112 + 27*	1.62
Adult (6 months old)	57 +11	90 + 7$^+$	1.58	85 + 3$^+$	1.49
Old (>540 days old)	42+5	100 + 2$^\#$	2.33	96 + 12$^\#$	2.29

Values are expressed as mean of DPM x 10 of H-thymidine incorporated/mg DNA + SD.

Number in parentheses represents the number of experiments carried out.

For other details please see the text.

* These values are significantly different from the corresponding control values $p < 0.05$.

$^+$ These values are significantly different from the corresponding control values $p < 0.01$.

$^\#$ These values are significantly different from the corresponding control values $p < 0.001$.

Fig 6: UV induced unscheduled DNA synthesis in Neurons of rat brain at different ages.

a, d and g control of young, adult and old respectively

b, e and h 20 J/m of young, adult and old respectively

c, f and i 40 J/m of young, adult and old respectively

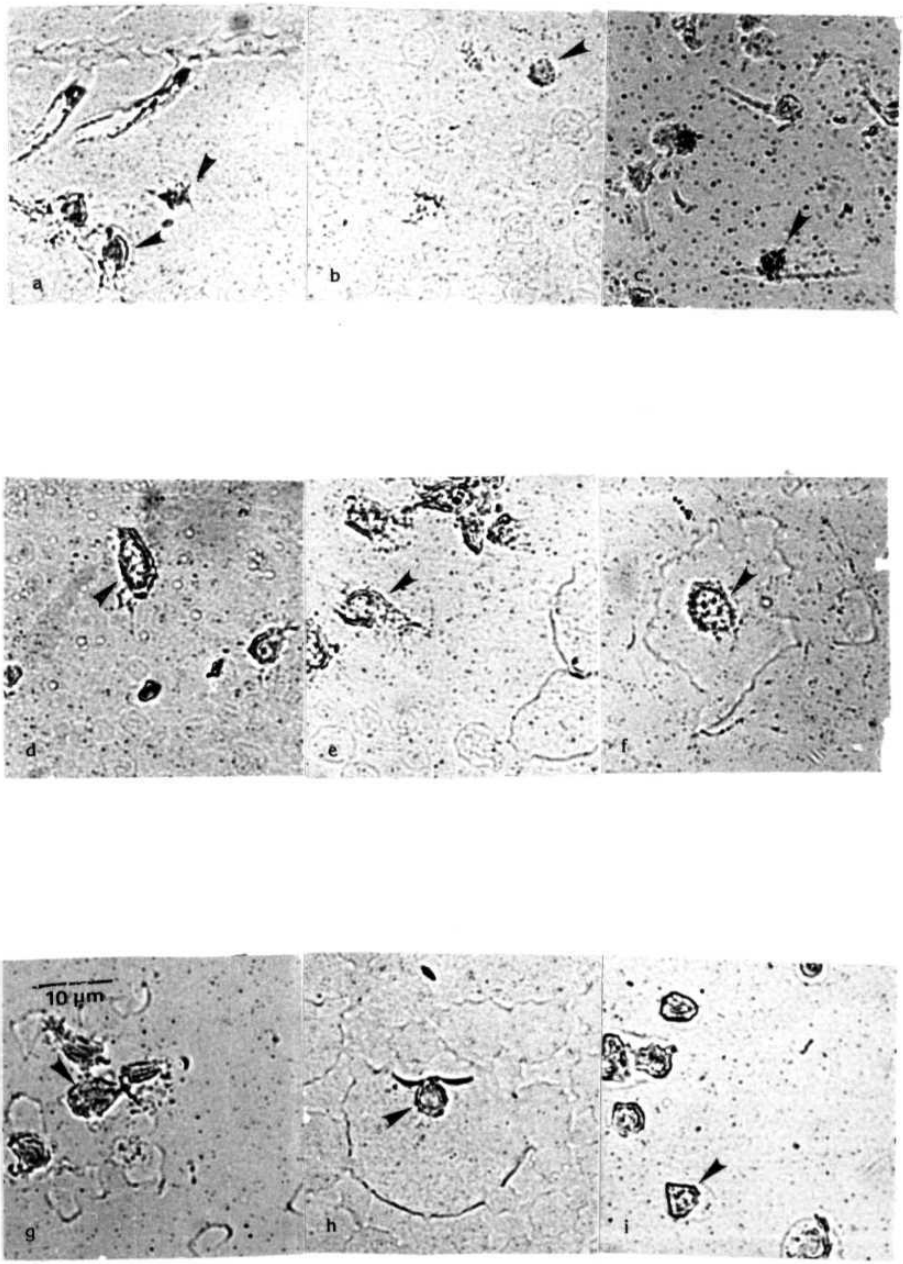
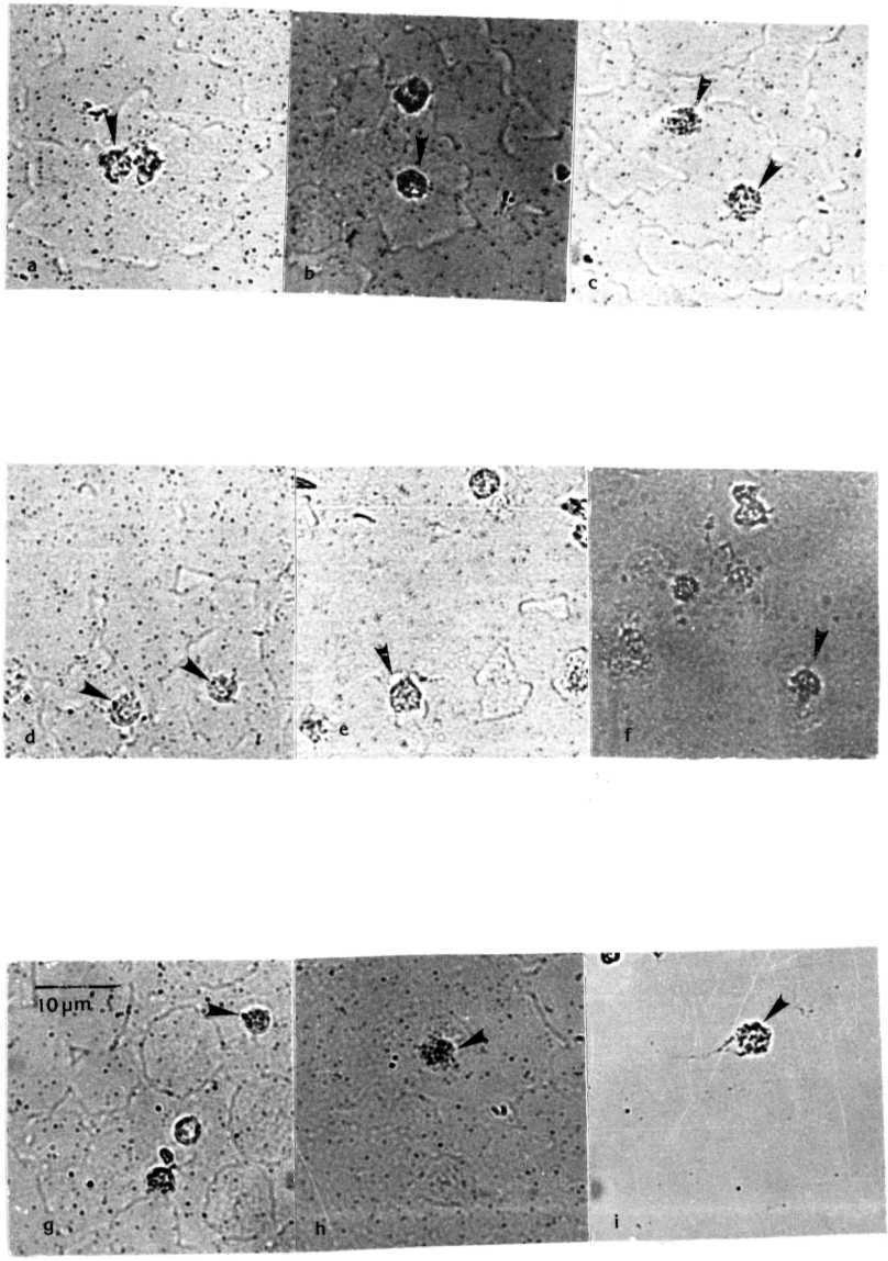

a
b
d
e
f
10 µm
g
h
i

Fig 7: UV induced unscheduled DNA synthesis in lympocytes of rat spleen at different ages.

a, d and g control of young, adult and old respectively

b, e and h 20 J/m^2 of young, adult and old respectively

c, f and i 40 J/m^2 of young, adult and old respectively


a
b
c
d
e
f
10 µm
g
h
i

age. A similar type of differences have been observed earlier by other workers between postmitotic and proliferating cells (Gibson and D'Ambrosio, 1982; de Sousa et al., 1986).

Age related changes in DNA repair capacity may result from age dependent differences in thymidine uptake, precursor pool size, patch size and conformation of the chromatin. However, it has been observed that thymidine uptake, precursor pool size and patch size do not change with age (Hart et al., 1979b; Hall et al., 1984; Hasegawa et al., 1984 Nette et al., 1984; Vijg et al., 1984). Further, it has been shown that nucleotide excision repair synthesis is independent of perturbations of deoxy nucleoside triphophate pool size (Katz and Sirover, 1987). On the other hand, the effect of age related changes in chromatin on DNA repair capacity remain speculative (Medvedev, 1984).

Nevertheless, these results do indicate that the basal DNA repair capacity (UDS) in neuronal cells does not change from adult to old stage of lifespan of rat which is in line with the observations on the changes of DNA repair enzymes (Chapter III and IV).

Chapter VI

General Discussion

CHAPTER VI

GENERAL DISCUSSION

Among the several theories that explain the process of aging, the theories that explain this process on the genetic basis have been attracted by many investigators. DNA damage/ repair hypothesis is the one among such theories. According to this hypothesis the greater the DNA repair capacity of a species or a cell, the longer its lifespan.

The first studies on DNA repair with relevance to the aging process were those of Painter and Cleaver (1969) who compared DNA repair capacities of several kinds of cultured mammalian cells. They found a greater extent of UV induced UDS in the cells of human origin when compared to that of rodent. Later, Hart and Setlow (1974) observed a strong log-linear correlation between the DNA repair capacity and maximum lifespan in skin flbroblasts of seven mammalian species. These experiments gave the credence to the 'DNA repair' as the longevity assurance system. This concept continues to enjoy the attention of several scientists all over the world and excellent reviews have appeared on this subject (Hart and Trosko, 1976; Hart *et al.*, 1979a; Gensler and Bernstein, 1981; Lehmann, 1985; Vijg and Knook, 1987.)

The studies of DNA damage and repair assume greater importance in a postmitotic tissue like brain because - neurons do not possess the mitotic ability and the brain has high metabolic

rate. Further, the brain brings about homeostatic integration of all the bodily organs. So the repair potential of neuron should be a good reflection of the functional state of all other organs. Therefore, it is our belief that the amount of DNA damage that may occur in an aging neuron of a species and its ability to repair such damage might play a critical role in determining the maximum lifespan of that species.

If the DNA repair capacity plays any significant role in the process of aging, one would generally expect that the following possibilities exist:a) an age related unrepaired DNA lesions should accumulate either linearly or exponentially, b) the rate of aging process should itself be modified by the changes in the levels of DNA damaging agents. In other words, DNA damaging agents should accelerate the aging process, c) the human genetic disorders of premature aging should show low levels of DNA repair capacity and d) the DNA repair capacity should decrease with age in a species, and species with different longevities could be expected to have different DNA repair capabilities or both.

a)Age related occurrence of unrepaired DNA lesions: Various types of unrepaired DNA lesions like DNA single strand and double-strand breaks, DNA cross-links and chromosomal aberrations have been reported to be accumulated with age. Price et al. (1971) studied the template activity using calf thymus DNA polymerase in ethanol fixed sections of brain, heart and liver tissues from four month and thirty five month old mice. The calf thymus DNA polymerase requires denatured DNA at least twenty nucleotides length and free 3'-OH end for its activity. They concluded from

their results that DNA single-strand breaks accumulate with age more in brain cells than in liver or heart cells. They also observed the presence of more acid sensitive regions in all the aged cells (Modak and Price, 1971).

Massie et al. (1972) observed, more single- and double-strand breaks as a function of age in rat liver cells. Similarly Karran and Ormerod (1973) observed lower molecular weight DNA isolated from twenty eight day old rat striated muscle when compared to one day old.

Chetsanga et al. (1977) showed that brain DNA isolated from six month old rat showed a single band on alkaline sucrose gradient corresponding the MW of 120 X 10^6 daltons, while DNA from thirty month old brain showed four peaks corresponding to MW of 140 X 10^6, 70 X 10^6, 15 X 10^6 and 3 X 10^6. The sensitivity of isolated DNA to S_1-nuclease digestion, an enzyme specific to single strand regions, increases from two per cent in 6-15 month old, to ten per cent by 20 month old, and to fourteen per cent by 30 months. Similar type of results were obtained by Murthy et al. (1976) both with naked and chromatin bound DNA.

Cutler (1976) observed an accumulation in DNA-protein cross-links with age in rodent liver tissue, and at a rate approximately proportional to lifespan in two rodent species. Similarly, Acharya (1972) found increase in the frequency of DNA-protein-RNA complexes in the rat brain and liver with age. The decline in the percentage of DNA that can be readily isolated from aged rat tissue (Amici et al., 1974) and increase in melting

temperature of DNA isolated from aged organs support the formation of increased DNA-protein cross-links with age (von Hahn and Fritz, 1966; our unpublished data).

On the other hand, Ono et al. (1976) examining DNA isolated from aging mouse liver, spleen, thymus and cerebellum, could not detect any differences except in liver. Dean and Cutler (1978) also failed to observe any significant age dependent increase of single-stranded regions in the DNA extracted from mouse liver nuclei. These discrepancies in the literature have to be clarified further. This could be due to differences in experimental design, age of the animals and tissue specificity.

b)Acceleration of aging process by exposure to DNA damaging agents: Alexander and Connell (1960) observed, for the first time, the effect of DNA damaging agents on the lifespan. They observed that chlorambucil, a nitrogen mustard compound, myleran and an alkylating agent when given at one half the LD_{50} dose shortened the lifespan of mice. A dose dependent lifespan shortening effect had occurred when the rats were treated with myleran (Dunjic, 1964). Ohno and Nagai (1978) showed that 7,12-dimethyl-benzanthracene (DMBA) induced accelerated aging in mice. The mean lifespan of mice was reduced from 608 to 297 days and this aging process appeared to be natural. Similar life shortening effect was observed in mice when their diet was supplemented with AAF (Kodell et al., 1980).

Lindop and Rotblat (1962) observed life shortening effect with single X-ray dose in mice. After postmortem examination

they concluded that this accelerated aging process is natural. Lamb and Maynard Smith (1969) hypothesized that radiation shortens life span by accelerating aging process in a dose dependent manner.

On the other hand, Alexander (1967) and, Stevenson and Curtis (1961) could not observe any such effect with mutagens.

c)DNA repair capacity and human genetic disorders of premature aging: Martin (1978) conducted an exhaustive analysis of 2336 mendelian characteristics and inherited diseases in human, listed in Mc Kusick•s 1974 catalogue. Based on 21 phenotypes which include premature greying of hair, diabetes mellitus, presenile dementia, increased neoplasms possibly associated with senescence, he selected 10 syndromes which represent premature aging. These include: Down's syndrome, Werner's syndrome, Cockayne's syndrome, progeria, ataxia telangiectasia, Seip's syndrome, cervical lipodysplasia, Klinefelter's syndrome, Turner's syndrome and myotonic dystrophy. However, defects in DNA repair were studied only in a few of these disorders.

Progeria or Hutchinson-Gilford syndrome was described in 1886 by Hutchinson and Gilford. The mean lifespan in the recorded cases was calculated to be 13.4 years (Rosenbloom and Debusk, 1971). Epstein et al. (1973) observed a marked decrease in the ability to repair X-ray induced single-strand breaks in progeric cells and attributed this defect to shortened lifespan of the cells in vitro. Brown et al. (1976) observed similar type of defect in mid passage progeric fibroblasts. Rainbow and Howes

(1977) using host cell reactivation as an assay of DNA repair after Y-irradiation, have also reported that progeric cells show defective capacity to rejoin single-strand breaks. However, Regan and Setlow (1974b) observed minimal difference in the repair capacity between normal and progeric fibroblasts. Bradley et al. (1976) observed no defect in rejoining single-strand breaks in progeric flbroblast cell line. These differences in these observations could be perhaps attributed to different strains used by investigators and the existence of complementation groups (Ted Brown, 1979),

Werner's syndrome was first described by Werner in 1904. The mean age of survival is 47 years. Fujiwara et al. (1977) have reported normal levels of repair of UV and X-ray irradiation damage in five Werner's syndrome cell strains. However, they showed a slower rate of DNA elongation.

Cockayne's syndrome was reported by Cockayne in 1936. It has been reported that fibroblasts from Cockayne's patients exhibit increased sensitivity to UV irradiation with abnormal thymine dimer excision (Schmickel et al., 1977). Further, Cockayne's cells showed reduced ability to repair DNA damage in UV irradiated infecting adenovirus (Day et al., 1981), elevated levels of UV induced sister chromatid exchange (Marshal et al., 1980) and UV induced mutations (Arlett, 1980).

Patients with Down's syndrome (trisomy 21) have features in common with premature aging. The life expectancy of patients with this syndrome was only 26 per cent at the age of 10 years

and only 8 per cent of the patients survive to the age of 40. Down's syndrome cells were sensitive to radiation and high frequency of X-ray and UV light induced chromosome aberrations were observed in leukocytes (Lambert et al., 1976). Similarly Otsuka et al.(1985) observed hypersensitivity to ionizing radiation in cultured cells of the patients. A dose dependent incidence of bleomycin induced chromosomal aberrations were reported in lymphocytes of Down's patients, in culture.

Ataxia-Telangiectasia (AT), a neurodegenerative syndrome was first documented in 1941 by Louis-Bar. AT fibroblasts have normal sensitivity to UV light and excise UV photoproducts but were killed more rapidly than normal cells by Y-irradiation (Taylor et al., 1975). Defective Y-induced strand-break repair was observed in AT fibroblasts (Vincent et al., 1975; Paterson et al., 1976). At least four complementation groups of AT were observed and some of them exhibit anomalous DNA (Paterson et al., 1982).

Bloom's syndrome is a rare autosomal recessive disorder associated with a greatly increased cancer frequency. The cells from these patients have been shown to possess defective DNA ligase I (Chan et al., 1987; Wills and Lindahl, 1987).

Although Xeroderma pigmentosum (XP) and Alzheimer's disease (AD) are not classified as premature aging disorders, XP patients develop signs of aged skin, abnormalities of internal organs and changes in chromatin and nucleus comparable to those observed in the aged, while AD, with an incidence of more than 5 per cent

among individuals over 65 years age in some populations is a major concomitant of aging (Kidson and Chen, 1986; Puvion-Dutilleul and Sarasin,1989). Both fibroblasts and lymphocytes of XP patients possessed little or no UV induced UDS (Cleaver, 1968; Burk, 1971). XP cells were also shown to have reduced levels of photoreactivating enzyme levels (Sutherland et al., 1975), reduced levels of carcinogen induced UDS (Stich et al., 1973), defect in postreplication repair of UV induced damage (Lehmann et al., 1975). At least nine complementation groups have been recognised with various degrees of deficiency in DNA repair capacity. A class of AP endonuclease has been reported to be missing in XP group D cells (Kuhnlein et al., 1978). Restoration of DNA repair to the normal levels has been observed either by microinjection of crude extracts of DNA repair enzymes or gene products involved in DNA repair, and by cell fusion of the cells of the different complementation groups (Hoeijmakers, 1987). A number of studies concerning the response of cells from AD patients to DNA damaging agents have been carried out. Increased levels of sister chromatid exchanges in the presence of raitomycln-C (Fischman et al., 1984), increased susceptibility of the cells to DNA damaging agents (Tarone et al., 1983 Scudiero et al., 1986) and increased sensitivity of the cells to ionizing radiation (Kidson et al., 1983; Robbins et al., 1983) were observed from AD patients. Using UDS and alkaline elution assays, defective DNA repair was demonstrated in AD fibroblasts (Li and Kaminskas, 1985; Robison and Bradley, 1985).

Eventhough the aforesaid syndromes can not be equated to

true aging in vivo, they do have some aspects in common with normal aging. It should be remembered that even in the normal population aging is not characterized by common characteristics in all the individuals. It can be tentatively concluded that these syndromes appear to lack in some aspects of DNA repair capability. However, some discordance exists in this aspect.

d)DNA repair capacity in relation to aging process and species maximum life span: It is highly difficult to come to a conclusion that DNA repair capacity of an organism or a cell decreases or increases with age because of so much contradictory data exist in the literature. From the investigations performed on rats either by whole body γ-irradiation, or back skin UV irradiation, and using different carcinogens, it was concluded that there is a gradual decline in the UDS in many of the organs with age (Niedermuller, 1980, 1982). Methylnitrosourea induced UDS was found to decline with age in the bone marrow cells in vivo in five different strains of mice (Bond and Singh, 1987). Similarly a decline in UV induced DNA repair synthesis in human peripheral blood leukocytes (Lambert et al., 1979), lymphocytes (Lezhava et al., 1979; Kovacs, et al., 1984), rat hepatocytes (Plesko and Richardson, 1984), rat fibroblasts (Kempf et al., 1984), bleomycin induced UDS in rat hepatocytes (Kennah et al., 1985); and γ-ray induced UDS in human peripheral blood lymphocytes (Licastro et al., 1982) was observed from old donors. Nette et al. (1984) reported a decline in UV induced UDS but not the percentage of the cells responding to UV in human epidermal cells with donor's age.

On the contrary, no such age associated decline in DNA repair capacity could be observed by other investigators in different cell types either in human or in other species (Goldstein, 1971; Ishikawa et al., 1978; Hennis et al., 1981; Hall et al., 1982; Liu et al., 1982; Turner et al., 1982). Kutlaca et al. (1982), by clonal technique, observed no change in UV sensitivity in human lymphocytes with donor's age. However, a two fold increase in sensitivity towards X-irradiation was observed. Similarly an absence of decline in average DNA repair capacity with age was observed in postmitotic tissues or cells like granular layer of neurons from beagle dogs (Wheeler and Lett, 1974) and mice cerebellum (Ono and Okada, 1978).

Also, conflicting results do exist about cellular aging in vitro. A decline in UV induced UDS was observed in human fibroblasts (Bowman et al., 1976; Hart and Setlow, 1976) and X-ray induced DNA repair in late passage human cells (Suzuki, et al., 1980). In contrast no such decline with age in average DNA repair capacity *La* vitro could be noticed by other workers (Painter et al., 1973; Clarkson and Painter, 1974; Dell'Orco and Whittle., 1978).

Hart and Setlow (1974) suggested that the DNA repair capacity of a species may be the primary determining factor in fixing the maximum lifespan of that species after observing a strong log-linear correlation between UV induced DNA repair capacity in skin fibroblasts in culture and maximum lifespan of seven placental mammalian species: shrew, mouse, rat, hamster, cow, ele-

phant and man. Subsequently, Hart et al. (1979b) suggested a correlation between maximum lifespan in two taxanomically close related species. They reported 2.2 fold greater rate of UV induced DNA repair synthesis using the technique of bromodeoxyuridine photolysis assay, in the cells of whitefooted mouse, Peromyscus leucopus than those of the house mouse, Mus musculus, whose lifespan is 2.5 times lesser than the former. Similar type of correlation was reported by Paffenholz (1978) who studied UV induced UDS in embryonic fibroblasts in three inbred strains of mice with different mean lifespan: 900, 600 and 300 days. Hall et al. (1984) reported correlation between UV induced DNA synthesis and species maximum lifespan both in lymphocytes and fibroblasts only in closely related species. Maslansky and Williams (1985) reported a correlation between potential species lifespan and both UV induced DNA repair synthesis and the proportion of the hepatocytes responded in culture. It was observed by Licastro and Walford (1985) that UV induced UDS declines more quickly in short-lived than long-lived strains of mice. The data of Francis et al. (1981) on 21 mammalian species suggested only a slight linear correlation between maximum lifespan and UV induced DNA repair synthesis.

However, Kato et al. (1980) studying on the UV induced UDS in fibroblasts in 31 mammalian species reported no such correlation. Similar type of results were observed by Woodhead et al. (1980) in three species of cold blooded vertebrates. Further, no difference was observed in DNA repair capacity of the cells in a pair of congenic mice with different longevities (Collier, 1982).

Studies on DNA repair capacity with aging at enzymatic level are scanty. Barton and Wang (1975) observed a decrease in DNA polymerase g in spleen and mice with age, while no such decrease occurred in DNA polymerase a. Similar type of results were obtained by Muller et al. (1980) in bone marrow cells and in mouse liver during aging (Fry et al., 1984).

The present investigation is an attempt to provide some data regarding the levels of some enzymes, possibly involved in DNA repair in a non-dividing cell like neuron. We have however noticed constant levels of DNA repair enzymes in the rat brain from adult life onwards, an observation in line with the findings of Chiu and Sung (1972). Attempts have been made to correlate the species maximum lifespan and the levels of 0 -alkylguanine alkyl transferase, a repair enzyme, in liver and articular chondrocytes (Hall et al., 1985; Woodhead et al., 1985; Lipman et al., 1987). However, no age related decrease in the levels of this enzyme was observed. Contradicting results have been reported regarding the fidelity of DNA polymerase in different cell types with age, some groups reporting a decreased fidelity (Murray and Holliday, 1981; Krauss and Linn, 1982) while other groups reporting no such change (Fry et al., 1981; Silber et al., 1985 and Subba Rao et al., 1985). The present results also showed that significant amounts of UV induced UDS have been observed in both adult and old neurons (at both 20 J/m^2 and 40 J/m^2). However, neurons isolated from the old rat brain exhibited UV induced UDS only at higher dose (40 J/m^2). Neverthe-

less, it must be borne in mind that UV irradiation is the only one kind of damage and it is possible that neurons may respond to different types of damage to varying degrees.

It is generally assumed that DNA repair synthesis, measured as UDS reflects the removal of pyrimidine dimers. However, Lohman et al. (1976) showed that inter species difference in DNA repair synthesis can be misleading, by demonstrating that UV irradiated hamster cells showed DNA repair synthesis but not the removal of pyrimidine dimers. In contrast, human cells were able to remove pyrimidine dimers. Recently Vijg et al. (1984) have shown that human cells were able to remove pyrimidine dimers faster than the rodent cells eventhough there was not much difference in dimer removal over a period of 24 hours. DNA excision repair capacity in vivo was found to be different from in vitro. It was recently found that rat epithelial cells in vivo remove UV induced pyrimidine dimers much faster than the same cells in vitro do (Vijg and Knook, 1987). DNA repair capacity within the cell may vary with the stage of cell cycle. A temporal expression of DNA repair was demonstrated in human cells (Gupta and Sirover, 1980, 1981). Further, there exist different DNA repair pathways and it is likely that different species utilize different DNA repair mechanisms to repair their damaged DNA. Moreover, it is becoming increasingly clear that the repair may proceed at certain discrete sites of DNA involving some kind of specificity as to which damage is to be repaired first (Bohr and Wassermann, 1988). All these factors might account to the differences observed by different investigators.

Thus the interspecies difference in repair capacity is more complicated than one might assume. Before coming to any realistic conclusions it is essential to assess the DNA repair activities in different tissues and organs, and different species with age. It is also important to study the frequency and nature of DNA repair pathway by a variety of assay methods.

Be as it may, it is the conclusion of this investigator that for correlating DNA repair capacity with the length of lifespan, one may have to use a non-mitotic cell like neuron for model studies. Such a cell exhibits the true DNA repair capacity not only in itself but also of that in other tissues and even the whole organism. It is therefore important that interspecies comparisons must be made with such model systems but not with cells which have a basic potential to replicate. This might well be the direction of further studies.

General Summary

GENERAL SUMMARY

1. DNA repair enzymes like acid DNase (UV DNase), alkaline DNase (AP DNase) and DNA polymerase β were studied in different cell enriched fractions (neurons, astrocytes and oligodendrocytes) of rat brain at different ages (1 day, 6 months and >540 days old). The presence of the two DNases in the nuclear fractions was also examined.

2. Significant amounts of DNase activities were observed in the nuclear fractions suggesting their involvement in DNA repair and/or replication.

3. Acid DNase (UV DNase) decreased with age in neurons. However, high specific activities were observed in glial cells in the adult and old stages of lifespan suggesting its role in a process linked with cell replication.

4. Alkaline DNase (AP DNase) increased in neurons, and did not change in glial cells with age indicating a role for this enzyme in DNA repair.

5. Significant amount of DNA polymerase was present in neurons at all the postnatal ages studied. Most of this activity was found to be of β-type, a repair enzyme.

6. The basal DNA repair capacity of neurons isolated from the rat brain at different ages was studied. The influence of UV (254 nm) on the DNA synthesis and unscheduled DNA synthesis (UDS) in these cells were also examined. Spleenic lymphocytes

isolated from the rats of same age groups were taken as positive control system for such studies.

7. Notable amounts of ^{3}H-thymidine incorporation were observed in neurons and this incorporation decreased from young to adult life.
8. The incorporation of ^{3}H-thymidine into neurons at adult and old stages of lifespan was not inhibited by hydroxyurea, suggesting that this incorporation was due to unscheduled DNA synthesis only.
9. In contrast, ^{3}H-thymidine incorporation into lymphocytes was inhibited by hydroxyurea at all the ages studied. Thus this observation offers neuronal cells isolated from the adult rat brain as a good model system for the study of DNA repair.
10. The basal UDS (DNA repair synthesis) did not decrease in neurons from adult to old stages of the lifespan of rat. This observation is in line of our studies on DNA repair enzymes.
11. UV (254 nm) inhibited the DNA synthesis only in young neurons. On the other hand, the DNA synthesis in lymphocytes was inhibited by UV at all the ages studied. These results suggest that the effect of UV on DNA synthesis is not age dependent. It has also been observed that the effect of UV on DNA synthesis is more pronounced in neurons than in lymphocytes, the cells which are known to have replicative potential on mitogen stimulation.

12. Significant UV induced DNA repair was observed in young as well as adult neurons at both doses studied (20 J/m and 40 J/m). However, neurons isolated from the old rat brain showed some UV induced UDS only at higher dose (40 J/m^2).

13. Lymphocytes exhibited significant amounts of UV induced UDS at all the ages studied and this capacity did not decrease with age suggesting that UV induced DNA repair capacity may be dependent on the replicative potential of the cell.

14. Autoradiography experiments showed significant number of grains in both neurons and lymphocytes at all the three ages studied. When the cells were irradiated with UV this number was found to be increased significantly in lymphocytes at all ages. However, a significant increase in the number of grains was observed in neurons only at young and adult stages of lifespan. These results confirm our biochemical results.

15. It is concluded that the basal DNA repair capacity of neuron remains somewhat constant throughout adult and old stages of lifespan. Their response to UV challenge is quite limited but nevertheless it exists. It is this responsiveness to various types of DNA damage that might act as a good pointer for the lifespan of the given species.

References

REFERENCES

Acharya, P.V.N. (1972) John Hopkins Med. J. Suppl. 1: 254.

Adrian, E.K, Cameron, I.L. and Hoage, T.R. (1975) J Cell Biol. 67: 3a.

Albert, R.E. and Burns, F.J. (1977) in **Origins of human cancer,** (eds. H.H. Hiatt, J.D. Watson and J.A. Winston) p 289, Cold Spring Harbor Lab, New York.

Alexander, P and Connell, D.I. (1960) Radiation Res. 12: 38.

Alexander, P (1967) Symp. Soc. Exp. Biol. **21:** 29.

Alfrey, V. and Mirsky, A.E. (1952) J. Gen. Physiol. 36: 227.

Amici, D., Gianfranceschi, G.L., Marsili, G. and Michetti. L. (1974) Experlentia 30: 633.

Arlett, C.F. (1980) in **Progress in Environmental mutagenesis** (ed. M. Alacevic) pp 161, Elsevier, Amsterdam.

Baltz, R.H., Bingham, P.M. and Drake, J.W. (1976) Proc. Natl. Acad. Sci. 73: 1269.

Barton, R.W. and Wang, W.K. (1975) Mech. Ageing Dev. 4: 123.

Berger, N.A., Kurohara, K.K., Petzold, S.J. and Sikorski, G. (1979) Biochem. Biophys. Res. Commun. 89: 218.

Bernardi, G. (1968) Advances in Enzymolgy 29: 165.

Bernstein, C. (1979) Perspect. Biol. Med. **22:** 539.

Bohr, V.A., Okumoto, D.S., Ho, L. and Hanawalt, P.C. (1986) J. Biol. Chem. **261:** 16666.

Bohr, V.A. and Wassermann, K. (1988) Trends Biochem. Sci. 13: 429.

Bond, S.L. and Singh, S.M. (1987) Mech. Ageing Dev. 41: 177.

Bowman, P.D., Meek, R.L. and Daniel, C.W. (1976) Mech. Ageing Dev. 5: 251.

Bradley, M.O. Erickson, L.C. and Kohn, K.W. (1976) Mutation Res. 37: 279.

Brown, W.T. Epstein, J. and Little, J.B. (1976) Exp. Cell Res. 97: 291.

Burk, P.G., Lutzner, M.A., Clarke, D.D. and Robbins, J.H. (1971)

J. Lab Clin. Med. **77:** 759.

Carrel, A. (1912) J. Exp. Med. 15: 516.

Chan, J.Y.H., Becker, F.F., German, J. and Ray, J.H. (1987) Nature 325: 357.

Chanda, R., Woodward, D.J. and Griffin, W.S.T. (1975) J. Neurochem. **24:** 723.

Chetsanga, C.J.M., Tuttle, M., Jacobini, A. and Johnson, C. (1977) Biochim. Biophys. Acta **474:** 180.

Chiu, J.F. and Sung, S.C (1972) Biochim. Biophys. Acta **269:** 364.

Chlebowicz, E. and Jachymczyk, W.J. (1979) Mol. Gen. Genet. **167:** 279.

Ciarrocchi, G., Jose, J.G. and Linn, S. (1979) Nucl. Acids Res. 7: 1205.

Clarkson, J.M. and Painter, R.B. (1974) Mutation Res. **23:** 107.

Cleaver, J.E. (1968) Nature **218:** 652.

Cleaver, J.E. (1969) Radiation Res. **37:** 334.

Cleaver, J.E. (1983) Biochim. Biophys Acta **739:** 301.

Cleaver, J.E. and Thomas, G.H. (1981) in **DNA repair: A Laboratory manual of research procedures** Vol. 1, Part B,(eds. E.C. Friedberg. and P.C. Hanawalt) pp 277, Dekker, New York.

Cole, R.S., Sinden, R.R., Yoakum, G.H. and Broyles, S. (1978) in **DNA repair mechanisms** (eds: P.C. Hanawalt., E.C. Friedberg., and F.C. Cox) pp 287., Academic Press., New York.

Collier, I.E., Popp, D.M., Lee, W.H. and Regan, J.D. (1982) Mech. Ageing Dev. **19:** 141.

Collins, A. (1987) Int. J. Radiation Biol. 51: 971.

Collins, A. and Johnson, R.T. (1987) J. Cell Sci. Suppl. 6: 61.

Comfort, A. (1960) in **The biology of Aging** (ed. B.L. Strehler), pp 3, Am. Inst. Biol. Sci. Washington.

Comfort, A. (1963) in **Ageing: the biology of Senescence** pp 1, Routledge Kegan Paul Ltd., London.

Cordonnier, C. and Bernardi, G. (1968) Can. J. Biochem. **46:** 989.

Cornelius, J.J. Rommelaere, J., Urbain, J. and Erreru, M. (1977) Phtochem. Photobiol. **26:** 241.

Cox, M.M. and Lehman, I.R. (1987) Ann. Rev. Biochem. 56: 229.

Creasy, H and Rapoport S.I. (1985) Ann. Neurol. **17:** 2.

Cutler, R.G. (1976) 29th Annual Meeting of Gerontological Scociety., Abstract, New York.

Dahle, D., Griffiths, T.D. and Carpenter, J.G. (1980) Photochem. Photobiol. **32:** 157.

Day, R.S., Ziolkowski, C.H.J. and Di Mattina, M. (1981) Photochem. Photobiol. **34:** 603.

Dean, R.G. and Cutler, R.G. (1978) Exp. Gerontol. **13:** 287.

De Duve, C., Wattiaux, R. and Bauduin. P. (1962) Advances in Ezymology **24:** 291.

De Sousa, J., De Boni, U. and Cinader, B. (1986) Mech. Ageing Dev. 36: 1.

Demple, B., Jacobsson, A., Olsson, M., Robbins, P. and Lindahl, T. (1982) J. Biol. Chem. **257:** 13776.

Deutsch, W.A. and Linn, S. (1979) Proc. Natl. Acad. Sci.(USA), 76: 141.

Dell'Orco, R.T. and Whittle, W.L. (1978) Mech. Ageing Dev. 8: 209.

Dowens, C.S., Johnson, R.T. and Collins, R.S. (1982) Exp. Cell Res. **142:** 47.

Dresler, S.L. and Lieberman, M.W. (1983) J. Biol. Chem. **258:** 9990.

Dunjic, A. (1964) Nature **203:** 887.

Edenberg, H.J., Anderson, S. and Depamphilis, M.L. (1978) J. Biol. Chem. **253:** 3273.

Eker, A.P.M., Hessels, J.K.C. and Yesui, A. (1987) in **DNA repair Workshop** p III, 2., Noordwijkerhout, Netherlands.

Epstein, J., Williams, J.R. and Little, J.B. (1973) Proc. Natl. Acad. Sci. **70:** 977.

Fischman, H.K., Reisberg, B., Albu, P., Ferris, S.H. and Rainer, J.D. (1984) Biol. Psychiat. 19: 319.

Fornace, A.J.Jr. (1983) Nature **304:** 552.

Francis, A.A., Lee, W.H. and Regan, J.D, (1981) Mech. Ageing Dev. 16: 181.

Friedberg, E.C. and Hanawalt, P.C. (1983) in **DNA repair - a laboratory manual of research procedures,** Vol 2, Dekker, New York.

Friedberg, E.C. (1985) in **DNA repair** (ed, E.C. Friedberg) W.H. Freeman and Company, New York.

Friedberg, E.C. (1987) J. Cell Sci. Suppl. 6: 1.

Frolkis, V.V (1982) in **Aging and life prolonging processes.** pp 33,Springr Verlog, Wein New York.

Fry, M., Loeb, L.A. and Martin, G.M. (1981) J. Cell Physiolo. **106:** 435.

Fry, M., Silber, J., Loeb, L.A. and Maratin, G.M. (1984) J. Cell Physiol. **118:** 225.

Fujiwara, Y., Higashikawa, T. and Tatsumi, M. (1977) J. Cell Physiol. 92: 365.

Fujiwara, Y. and Tatsumi, M. (1977) Mutation Res. 43: 279.

Gale, J.M. and Smerdon, M.J. (1988) J. Mol. Bio. 204: 949.

Gensler, H.L. and Bernstein, H. (1981) Quart. Rev. Biol. 56: 279.

Gerchman, L.L. and Ludlum, D.B. (1973) Blochim. Biophys. Acta **308:** 310.

Gibson, R.E. and D'Ambrosio, S.M. (1982) Photochem. Photobiol. 35: 181.

Giulotto, E. and Mondello, C. (1981) Biochem. Biophys. Res. Commun. 99: 1287.

Goldstein, S. (1971) Proc. Soc. Exp. Biol. Med. 137: 730.

Gordon, L.K. and Haseltine, W.A. (1982) Radiation Res. 89: 99.

Goth, R. and Rajewsky, M.F. (1974) Z. Krebsforsch. **82:** 37.

Griffiths, T.D. and Ling, S.Y. (1985) Biochim. Biophys. Acta **826:** 121.

Grunberger, D. and Santella, R.M. (1982) in **Mechanisms of chemical carcinogenesis** (eds. C.C. Harris and P.A. Cerutti) pp 155, Alan R. Liss, New York.

Gupta, P.K. and Sirover, M.A. (1980) Mutation. Res. 72: 273.

Gupta, P.K. and Sirover, M.A. (1981) Chem. Biol. Interactions **36:** 19.

Hahn, W.E. and Laird, C.D. (1971) Scince **173:** 158.

Hall, J., Bresil, H. and Montesano. R. (1985) Carcinogenesis 6: 209.

Hall, J.D., Almy, R.E. and Sherer, K.L. (1982) Exp. Cell Res. **139:** 351.

Hall, K.Y., Hart, R.W., Benirschke, A.K. and Walford, R.L. (1984) Mech. Ageing Dev. 24: 163.

Hanaoka, F. , Kato, H., Ikegami, S., Ohashi, M. and Yamada, M. (1979) Biochem. Biophys. Res. Commun. 87: 575.

Hanawalt, P.C., Cooper, P.K., Ganesan, A.K. and Smith, C.A. (1979) Ann. Rev. Biochem. 48: 783.

Hanawalt, P.C. and Sarasin, A. (1986) Trend. Genet. 2: 124.

Hanawalt, P.C. (1987) Environ. Health Sci. 76: 9.

Harless, J. and Hewitt, R.R. (1987) Mutation Res. 183: 177.

Harley, C.B., Pollard, J.W., Chamberlain, J.W., Stanners, C.P. and Goldstein, S. (1980) Proc. Natl. Acad. Sci. 77: 1855.

Harman, D. (1962) Radiation Res. 16: 753.

Harman, D. (1988) Mol. Cell Biochem. 84: 155.

Hart, R.W. and Setlow, R.B. (1974) Proc. Natl. Acad. Sci. 71: 2169.

Hart, R.W. and Setlow, R.B. (1976) Mech. Ageing Dev. 5: 67.

Hart, R.W. and Trosko, J.E. (1976) in **Interdisciplinary topics in Gerontology,** Vol 10. (ed. R.G. Cutler) pp 134, Karger, Basel.

Hart, R.W., Hays, S., Brash, D., Daniel, F.B., Davis, M.T. and Lewis, N.J. (1977) Ann. N. Y. Acad. Sci. **298:** 141.

Hart, R.W., D'Ambrosio, S.M., Ng, K.J. and Modak, S.P. (1979a) Mech. Ageing Dev. 9: 203.

Hart, R.W., Sacher, G.A. and Hoskins, T.L. (1979b) J. Gerontol. 34: 808.

Hart, R.W. and Modak, S.P. (1980) Adv. Expt. Med. and Biol. **129:** 123.

Hasegawa, N., Hanaoka, F. and Yamada, M. (1984) Mech. Ageing Dev. **25:** 297.

Haseltine, W.A., Gordon, L.K., Linden, C.P., Grafstrom, R.H., Sharper, N.L. and Grossman, L. (1980) Nature **285:** 634.

Haseltine, W.A. (1983) in **Cellular responses to DNA damage** (eds. E.C. Friedberg and B.A. Bridges) pp 3, Alan R. Liss, New York.

Hayflick, L. and Moorhead, P.S. (1961) Exp. Cell Res. 25: 585.

Hayflick, L. (1976) New Eng. J. Med. **295:** 1302.

Healy, J.W., Stollar, D., Simon, M.I. and Levine, L. (1963) Arch. Biochem. Biophys. **103:** 461.

Hennis, H.L., Braid, H.L. and Rusell, V.A. (1981) Mech. Ageing Dev.**16:** 355.

Henrickson, P.A. and Clever, U. (1972) J. Insect Physiol. 18: 1981.

Hesslewood, I.P., Holmes, A.M., Wakeling, W.F. and Johnston, I.R. (1978) Eur. J. Biochem. 84: 123.

Hlggins, N.P., Kato, K. and Strauss, B. (1976) J Mol. Biol. **101:** 417.

Hoeijmakers, J.A. (1987) J. Cell Sci. Suppl. 6: 111.

Holliday, R. and Rattan, S.I.S. (1984) Monogr. Dev. Biol. **17:** 115.

Holliday, R. (1986a) in **Sandoz Lectures: Dimensions in Aging** pp 21, Academic Press.

Holliday, R. (1986b) Exp. Cell Res. **166:** 543.

Holmes, A.M., Hesslewood, I.P. and Johnston, I.R.(1974) Eur. J. Biochem. 43: 487.

Holmes, C.E., Schneider, S., Bernstein, C. and Bernstein, H. (1980) Virology **103:** 299.

Hubscher, U., Kuenzle, C.C. Spadari, S (1977) Eur. J. Biochem. 81: 249.

Ikegami, S., Taguchi, T., Ohashi, M., Oguro, M., Nagano, H. and Mano, Y. (1978) Nature **275: 458.**

Inga Mahler. (1967) in **Methods in Enzymology.** Vol 12, Part A, p 693.

Ishikawa, T., Takayama, S. and Kitagawa, T. (1978) J. Natl. Cancer Inst. **61:** 1101.

Ivanov, V.A. Gaziev, A.I. and Tretyak, T.M. (1983) Eur. J. Biochem. **137:** 517.

Ivanov, V.A., Tretyak, T.M. and Afonin, Y.N. (1988) Eur. J. Biochem. **172:** 155.

Kantor, G.J. and Setlow, R.B. (1981) Cancer Res. 41: 819.

Kanungo, M.S.(1980) in **Biochemistry of Aging** pp 262, Academic Press, London.

Karran, P. and Ormerod, M.G. (1973) Bichim. Biophys. Acta **299:** 54.

Kato, H., Harada, M., Tsuchiya, K. and Moriwaki, K. (1980) Jpn. J. Genet. 55: 163.

Katz, E.J. and Sirover, M.A. (1987) Mutation Res. **183:** 249.

Kaufmann, W.K., Cleaver, J.E. and Painter, R.B. (1980) Biochim. Biophys. Acta **608:** 191.

Kelner, A. (1949) Proc. Natl. Acad. Sci. 35: 73.

Kempf, C., Schmitt, M., Danse, J.M. and Kempf, J. (1984) Mech. Ageing Dev. 26: 183.

Kennah II, H.E., Coetzee, M.L. and Ove, P. (1985) Mech. Ageing Dev. **29:** 283.

Kidson, C., Chen, P., Imray, F.P. and Gipps, E. (1983) in **Cellular responses to DNA damage** (eds. E.C. Friedberg and B.A. Bridges) pp 721, Alan R. Liss., New York.

Kidson, C. and Chen, P. (1986) Prog. Brain Res. 70: 291.

Kimberlin, R N., Shirt D.B and Collis S.C (1974) J. Neurochem. **23:** 241.

Kittler, L. and Lober, G. (1977) Photochem. Phtobiol. Rev. 2: 39.

Kodell, R.L., Farmer, J.H. and Littlefield, N.A. (1980) J. Environ. Pathol. Toxicol. 3: 69.

Korr, H. (1980) in **Proliferation of different Cell types in the brain.** Springer Verlag, Heidelberg, pp 55.

Kovacs, E., Weber, W. and Muller, Hj. (1984) Mutation Res. **131:** 231.

Krasin, F. and Hutchinson, F. (1977) J. Mol. Biol. **116:** 81.

Krauss, S.W. and Linn, S. (1982) Biochem. **21:** 1002.

Kriek, E (1972) Cancer Res. **32:** 2042.

Kuenzle, C.C. (1985) Brain Res. Rev. **10:** 231.

Kuhnlein, V., Lee, B., Penhoet, E.E. and Linn, S. (1978) Nucl. Acids Res. 5: 951.

Kutlaca, R., Seshadri, R. and Morley, A.A. (1982) Mech. Ageing Dev. **19:** 97.

Lamb, M.J. and Maynard Smith, J. (1969) Radiation Res. 40: 450.

Lambert, B., Hansson, K., Bui, T.H., Funes-Cravioto, F., Linsten, J., Holmberg, M. and Strausmanis, R. (1976) Ann. Hum. Genet. 39: 293.

Lambert, B., Ringborg, U. and Skoog, L. (1979) Cancer Res. 39: 2792.

Lawley, P.D. (1974) in **Molecular and environmental aspects of mutagenesis** (eds. L. Prakash., F. Sherman., M. Miller., C. Lawrence and H. Taber.) pp 17, Springerfield, Illinois.

Lehman, I.R. (1967) Ann. Rev. Biochem. 36: 645.

Lehmann, A.R. and Kirk-Bell, S. (1978) Photochem. Photobiol. 27: 297.

Lehmann, A.R., Kirk-Bell, S., Arlett, C.F., Paterson, M.C., Lohman, P.H.M, de Weerd-Kastelein, E.A. and Bootsma, D.C. (1975) Proc. Natl. Acd. Sci. 72: 219.

Lehmann, A.R. and Karran, P. (1981) Int. Rev. Cytol. 72: **101.**

Lehmann, A.R. (1985) in **Age related factors on carcinogenesis** (eds. A. Likhachev., V. Anisimov. and R. Montesano.) IARC Scientific Publication No. 58, Lyon.

Lezhava, T.A., Prokofieva, V.V. and Mikhelson, V.M. (1979) Tisitilogia **11:** 2792.

Li, J.C. and Kaminskas, E. (1985) Biochem. Biophys. Res. Commun. **129:** 733.

Licastro, F., Franceschi, C., Chiricolo, M., Battelli, M.G., Tabacchi, P., Cenci, M., Barboni, F. and Pallenzona, D. (1982) Carcinogenesis 3: 45.

Licastro, F. and Walford, R.L. (1985) Mech. Ageing Dev. 31: 171.

Lieberman, M.W., Smerdon, M.J., Tlsty, T.D. and Oleson. F.B. (1979) in **Environmental carcinogenesis** (eds. P. Emmelot and E. Kriek) pp 345, Elsevier/North-Holland Biomedical Press, Amsterdam.

Lindahl, T. and Andersson, A. (1972) Biochem. **11:** 3618.

Lindahl, T. and Nyberg, B. (1972) Biochem. **11:** 3610.

Lindahl, T. and Karlstram, 0. (1973) Biochem. **12:** 5151.

Lindahl, T. (1977) in **DNA repair processes** (eds. W.W. Nicholas and D.G. Murphy) pp 225, Symposia Specialist Inc, Miami.

Lindahl, T. (1979) Prog. Nucl. Acid Res. Mol. Biol. **22:** 135.

Lindahl, T. (1982) Ann. Rev. Biochem. 51: 61.

Lindop, P.J. and Rotblot, J. (1962) in **Biological Aspects of Aging** (ed. N.W. Schock) pp 216, Columbia University Press, New York.

Linn, S. (1982) in **Nucleases** (eds. S.M. Linn. and R.J. Roberts.) pp 59, CSH Laboratory Publication, New York.

Lipman, J.M., Sokoloff, L. and Setlow, R.B. (1987) Mech. Ageing Dev. 40: 205.

Liu, S.C., Parsons, C.S. and Hanawalt, P.C. (1982) J. Invest. Dermatol. 78: 330.

Leob, L.A. (1969) J. Biol. Chem. **244:** 1672.

Lohman, P.H.M., Paterson, M.C., Zelle, B. and Reynolds, R.J. (1976) Mutation Res. 46: 138.

Loveless, A. (1969) Nature **223:** 206.

Lovtrup-Rein, H. and Mc Ewen, B.S. (1966) J. Cell Biol. 30: 405.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. **193:** 265.

Ludlum, D.B. (1970) Biochim. Biophys. Acta **213:** 142.

Madden, J.J., Werbin, H. and Denson, J. (1973) Photochem. Photobiol. 18: 441.

Madhani, H.D., Bohr, V.A. and Hanawalt, P.C. (1986) Cell 45: **417.**

Mansbridge, J.N. and Hanawalt, P.C. (1983) in **Cellular Responses to DNA damage** (eds. E.C. Friedberg and B.R. Bridges.) pp 195, Alan R. Liss Inc., New York.

Marmur, J. (1961) J. Mol. Biol. 3: 208.

Marshall, R.R., Arlett, C.F., Harcourt, S.A. and Broughton, B.C. (1980) Mutation Res. 69: 107.

Martin, R. (1977) in **Molecular Human cytogenetics** ICN-UCLA Symposium on molecular and cellular biology. (eds. D.E. Commings and C.F. Fox.) **pp** 355, Academic Press, New York.

Martin, G.M. (1978) in **Genetic effects on Aging** (eds.D. Bergsma. and D.E. Harrison.) pp 5, Liss, New York.

Maslansky, C.J. and Williams, G.M. (1985) Mech. Ageing Dev. **29:** 191.

Massie, H.R., Baird, M.B. and Nicolosi, R.J. (1972) Arch. Biochem. Biophys. **153:** 736.

Mayer, P.J., Bradley M.O. and Nichols, W.W. (1986) Exp. Cell Res. **166:** 497.

Maynard Smith, J. (1962) Proc. R. Soc. Lond., Ser. B **157:** 115.

McCready, S.J. and Cook, P.R. (1984) J. Cell Sci. 70: 189.

Medewar, P.B. (1952) in **An unsolved problem of biology** reprinted in the 'The uniqueness of the Individual, pp 44, Methuen, London.

Medvedev, Z.A. (1964) Adv. Gerontol. Res. 1: 181.

Medvedev, Z.A. (1980) Mech. Ageing Dev. **14:** 1.

Medvedev, Z.A. (1984) Mech. Ageing Dev. 28: 139.

Miller, M.R. and Chinault, D.N. (1982) J. Biol. Chem. **257:** 46.

Miller, M.R. and Lui, L.R. (1982) Biochem. Biophys. Res. Commun. **108:** 1676.

Modak, S.P. and Price, G.B. (1971) Exp. Cell Res. 65: 289.

Muench, K.F., Misra, R.P. and Humayun, M.Z. (1983) Proc. Natl. Acad. Sci. 80: 6.

Mullenders, L.H.F., van Kestern, A.C., Bussman, C.J.M., van Zeeland, A.A. and Natarajan, A.T. (1984) Mutation Res. **141:** 75.

Muller, W.E.G., Zahn, R.K., Geursten, W. and Mensch, N. (1980) Mech. Ageing Dev. **13:** 119.

Munro, H.N.C. (1966) in **Methods in Biochemical Analysis** Vol. 14, (ed. D. Glick) pp 113, Interscience Publication, New York.

Murray, V. and Holliday, R. (1981) J. Mol. Biol. **146:** 55.

Murthy, M.R., Bharucha, A.D., Roux-Murthy, H., Jocob, J. and Ranjekar, P.K. (1976) in **Neuropsychopharmacology** (eds. P. Deniker, C. Radowco-Thomas. and A. Villeneuve) pp 1615, Pregmon, Oxford.

Nachlas, M.M., Margulies, S.I. and Seligman, A.M. (1960) J. Biol. Chem. **235:** 499.

Nette, E.G., Xi, Y.P., Sun, Y.K., Andrews, A.D. and King, D.W. (1984) Mech. Ageing Dev. **24:** 283.

Newbold, R.F., Warren, W., Medcalf, A.S.C. and Amos, J. (1980) Nature **283:** 596.

Niedermuller, H., Hofecker, G. and Kment, A. (1980) in **DNA repair and late effects** (eds. H. Altamann, E. Riklis and H. Slor.) pp 15, NRCN Publication, Isrel.

Niedermuller, H. (1982) Mech. Ageing Dev. 19: 259.

Nonn, E. and Bernstein, C. (1977) J. Mol. Biol. **116:** 31.

Ohashi, M., Taguchi, T. and Ikegami, S. (1978) Biochem. Biophys. Res. Commun. 82: 1084.

Ohno, S. and Nagai, Y. (1978) in Genetic Effects of **Aging** (eds. D. Bergsma, D.E. Harrison and N.W. Paul) pp 501, Alan R. Liss, New York.

Ono, T., Okada, S. and Sugahara, T. (1976) Exp. Gerontol. 11: 127.

Ono, T. and Okada, S. (1978) Int. J. Radiation Biol. 33: 403.

Orgel, L.E. (1963) Proc. Natl. Acad. Sci. **49:** 517.

Otsuka, F., Tarone, R.E., Seguin, L.R. and Robbins, J.H. (1985) J. Neurol. Sci. 69: 103.

Paffenholz, V. (1978) Mech. Ageing Dev. 7: **131.**

Painter, R.B. and Cleaver, J.E. (1969) Radiation Res. 37: 451.

Painter, R.B., Clarkson, J.M. and Young, B.R. (1973) Radiation Res. 56: 560.

Park, S.D. and Cleaver, J.E. (1979) Proc. Natl. Acd. Sci. 76: 3927.

Paterson, M.C., Smith, B.P., Lohman, P.H.M., Anderson, A.K. and Flschman, L. (1976) Nature **260:** 444.

Paterson, M.C, Smith, P.J., Bech-Hansen, N.T., Smith, B.P. and Middlestadt, M.V. (1982) in **Ataxia-telangiectasia: a cellular and molecular link between cancer, neuropatbalogy and immune deficiency** (eds. B.A. Bridges and D.G. Harnden) pp 271, John Wiley and Sons, Chichester.

Pathak, M.A., Kramer, D.M. and Fltzpatrick, T.B. (1974) in **Sunlight and man** (eds. M.A. Pathak., L.C. Harber., M. Seiji and A. Kukita) pp 335, Tokyo University Press.

Pedrali-Noy, G. and Weissbach, A. (1977) Biochim. Biophys. Acta 77: 70.

Pedrali-Noy, G. and Spadari, S. (1980) Mutation Res. 70: 389.

Piessens, J.P. and Eker, A.P.M. (1975) FEBS Lett. 50: 125.

Plesko, M.M. and Richardson, A. (1984) Biochem. Biophys. Res. Commun. **118:** 730.

Poirier, M.C., Yuspa, S.H., Weinstein, I.B. and Blobstein, S. (1977) Nature **270:** 186.

Price, G.B., Modak, S.P. and Makinodan, T. (1971) Science **171:** 917.

Puvion-Dutilleul, F. and Sarasin, A. (1989) Mutation Res. **219:** 57.

Radany, E.H., Love, J.D. and Friedberg, E.C. (1981) in **Chromosome damage and repair** (eds. E. Seeberg and K. Kleppe) pp 91, Plenum Press, New York-
Rainbow, A.J. and Howes, M. (1977) Biochim. Biophys. Acta **77:** 714.

Rasmussen, R.E. and Painter, R. B. (1964) Nature 203: 1360.

Rattan, S.I.S. (1985) Bioessays 2: 226.

Rattan, S.I.S. and Clark, B.F.C. (1988) Trends Biotech. 6: 58.

Regan, J.D., Setlow, R.B. and Ley R.D. (1971) Proc. Natl. Acad. Sci. 68: 708.

Regan, J.D. and Setlow, R.B. (1974a) Cancer Res. 34: 3318.

Regan, J.D. and Setlow, R.B. (1974b) Biochem. Biophys. Res. Commun. 59: 858.

Robbins, J.H., Otsuka, F., Tarone, R.E., Bromback, R.A., Moshell, A.N., Nee, L.E., Ganges, M.B. and Cayeux, S.J. (1983) Lancet 1: 468.

Roberts, J.J. (1978) Adv. Radiation Biol. 7: 211.

Robison, S.H. and Bradley, W. G. (1985) in **Senile dementia of the Alzheimer type** (eds. J.H. Hutton and A.D. Kenny) pp 205, Alan R. Liss, New York.

Rocksteln, M. (1986) Chem. Eng. News Vol 64, August, 11th., pp 26.

Rosenbloom, A.L. and Debusk, F.L. (1971) Am. Heart J. 83: 287.

Rothstein, M. (1982) in **Biochemical approaches to Aging** pp 132, Academic Press, London.

Rupert, C.S. (1962) J. Gen. Physiol. 45: 703.

Rupert, C.S. (1975) in **Molecular Mechanisms of repair of DNA** (eds. P.C. Hanawalt and R.B.Setlow) part A,pp 73, Plenum, New

York.

Rupp, W.D and Howard-Flanders, P. (1968) J. Mol. Biol. **31:** 291.

Sacher, G.A. (1968) Exp. Gerontol. 3: 265.

Sacher, G.A. (1973) Proceedings of the IX International Congress of Anthropological and Ethnological Science, Chicago.

Sacher, G.A. (1982) Perspect. Biol. Med. 25: 339.

Sancar, A. and Sancar, G.B. (1988) Ann. Rev. Biochem. 57: 29.

Sarasin, A.R. and Hanawalt, P.C. (1980) J. Mol. Biol. **138:** 299.

Schmickel, R.D., Chu, E.H.Y., Trosko, J.E. and Chang, C.C. (1977) Pediatrics **60:** 135.

Schmidt, G. and Thannhauser, S.J. (1945) J. Biol. Chem. **161:** 83.

Schneider, E.L., Sternberg, H., Tice, R.R., Senula, G.C. Kram, D., Smith, J.R. and Bynum, G. (1979) Mechem. Ageing Dev. 9: 313.

Schofield, J.D. and Davies, I. (1978) in **Text book of Geriatric Medicine and Gerontology** (ed. J.C. Broklehurst) pp 37, Churchill Livingstone, New York.

Scovassi, A.I., Plevani, P. and Bertazzoni, U. (1980) Trends Bioche. Sci. 5: 335.

Scudiero, D.A., Polinsky, R.J., Brumback, R.A., Tarone, R.E., Nee, L.E. and Robbins, J.H. (1986) Mutation Res. 159: 125.

Seki, S., Oda, T. and Ohashi, M. (1980) Biochim. Biophys. Acta **610:** 413.

Setlow, R.B. and Carrier, W.L. (1964) Proc. Natl. Acad. Sci. 51: 226.

Setlow, R.B. and Carrier, W.L. (1966) J. Mol. Biol. **17:** 237.

Setlow, R.B. (1968) Prog. Nucl. Acid Res. Mol. Biol. 8: 257.

Setlow, R.B. (1978) Nature **271:** 713.

Shioda, M. and Murakami-Murofushi, K. (1987) Biochem. Biophys. Res. Commun. **146:** 61.

Shrivastaw, K.P. and Subba Rao, K. (1975) J. Neurochem. 45: 1273.

Silber, J.S., Fry, M., Martin, G.M. and Loeb, L.A. (1985) J. Biol. Chem. **260:** 1304.

Singer, B. and Kusmierek, J.T. (1982) Ann. Rev. Biochem. 52: 655.

Slater, T.F. (1984) Biochem. J. **222:** 1.

Slor, H. and Lev, T. (1971) Biochem. J. **123:** 993.

Slor, H. and Lev, T. (1972) J. Biol. Chem. **247:** 2926.

Slor, H., Bustan, H. and Lev, T. (1973) Biochem. Biophys. Res. Commun. 52: 556.

Smerdon, M.J. and Lieberman, M.W. (1978) Proc. Natl. Acad. Sci. 75: 4238.

Smith, G.F. and Berg, J.M. (1976) in **Down's Anomaly** pp 258, Churchill Livingstone, New York.

Snyder, D.S., Raine, C.S., Farooq, M. and Norton, W.T. (1980) J. Neurochem. **34:** 1614.

Snyder, R.D. and Regan, J.D. (1982) Biochim. Biophys. Acta. **697:** 229.

Spadari, S., Sala, F. and Pedrali-Noy, G. (1982) Trends Biochem. Sci. 7: 29.

Stabolova, M.A., Cox, D. and Mathias, A.P. (1973) Biochem. J. **136:** 685.

Stephen Hsia, M.T. (1987) Cell Biol. Toxicol. 3: 127.

Stevenson, K.G. and Curtis, H.J. (1961) Radiation Res. 15: 774.

Stich, H.F., San, R.H.C. and Kanojoe, Y. (1973) Mutation Res. 17: 127.

Strehler, B.L. (1962) in **Time, Cell and aging** Academic Press, New York.

Subba Rao, K.V. and Subba Rao, K. (1982) Mech. Ageing Dev. 18: 225.

Subba Rao, K.V. and Subba Rao, K. (1984) Biochem. Intl. 9: 391.

Subba Rao, K., Martin, G.M. and Loeb, L.A. (1985) J. Neurochem. 45: 1273.

Subba Rao, K. (1986) in **Bole of BNA and DNA in Brain Function** (eds. A. Giuditta., B.B. Kaplan. and C. Zomzely Neurath.) pp 224, Martin Nijhoff Publication, Boston.

Sung, S.C. (1968) J. Neurochem. 15: 477.

Susheela, L. and Ramasarma, T. (1971) Biochim. Biophys. Acta **242:** 532.

Sutherland, B.M., Rice, M. and Wagner, E.K. (1975) Proc. Natl.

Acad. Sci. **12:** 103.

Suzuki, F., Watanabe, E. and Horikawa, M. (1980) Exp. Cell Res. **127:** 299.

Swain, C.C. and Scott, C.B. (1953) J. Am. Chem. Soc. **75:** 141.

Szilard, L. (1959) Nature **184:** 958.

Tamm, C., Shapiro, H.S., Lipschitz, R. and Chargaff, E. (1953) J Biol. Chem. **203:** 673.

Tarone, R.E., Scudiero, R., Brumback, A., Polinsky, R., Nee, L.E., Clatterbuck, B.E. and Robbins, J.H. (1983) J. Cell Biochem. Suppl. 7B: 209.

Taylor, A.M.R., Harnden, D.G., Arlett, C.F., Harcourt, S.A., Lehmann, A.R., Stevens, S. and Bridges, B.A. (1975) Nature **258:** 427.

Ted Brown, W. (1979) Mech. Ageing Dev. 9: 325.

Teebor, G.W. and Frenkel, K. (1983) in **Advances in cancer Research** (eds. G. Klein and S. Weinhouse) Vol 38, pp 23, Academic Press, London.

Teoule, R. (1987) Int. J. Radiation Biol. 51: 573.

Terry, R.D. (1980) Mech. Ageing Dev. 14: 191.

Tice, R.R. (1978) in **The genetics of Aging** (ed. E.L. Schneider) pp 53, Plenum Press, New York.

Tomasz, M., Chowdary, D., Lipman, R., Shimotakahara, S., Veiro, D., Walker, V. and Verdine, G.L. (1986) Proc. Natl. Acad. Sci. **83:** 6702.

Turner, D.R., Griffith, V.C. and Morley, A.A. (1982) Mech. Ageing Dev. 19: 325.

Usha Rani, B., Indrapal Singh, N., Ray, A. and Subba Rao, K. (1983) J. Neurosci. Res. 10: 101.

Usha Rani, B. (1985) in **Isolation and some biochemical studies of various cell enriched fractions from young and old chick brain.** a Ph.D. thesis submitted to the University of Hyderabad, India.

Van der Vliet, P.C. and Kwant, M.M. (1978) Nature **276:** 532.

Vijg, J., Mullart, E., van der Schans, G.P., Lohman, P.H.M. and Knook, D.L. (1984) Mutation Res. **132:** 129.

Vijg, J. and Knook, D.L. (1987) J. Am. Geriat. Soc. **35:** 532.

Vilenchik, M.M. and Tretjak, T.M. (1977) J. Neurochem. 29: 1159.

Vincent, R.A.Jr., Sheridan, R.B. and Huang, P.C. (1975) Mutation Res. 33: 357.

Volberg, I.M., Lee, K.A. and Sirover, M.A. (1984) Cancer Res. **44:** 2377.

von Hahn, H.P. and Fritz, E. (1966) Gerontologia 12: 237.

Walford, R.L. (1969) in **The immunological Theory of Aging,** Copenhagen.

Walker, I.G. and Th'ng, J.P.H. (1982) Mutation Res. **105:** 277.

Waqar, M.A., Evans, M.J. and Huberman, J.A. (1978) Nucl. Acids Res. 5: 1933.

Waser, J., Hubscher, U., Kuenzle, C.C. and Spadari, S. (1979) Eur. J. Biochem. 97: 361.

Waters, R. and Regan, J.D. (1976) Biochem. Biophys. Res. Commn. 72: 803.

Weismann, A. (1989) in **Essays upon heredity and kindered bilogical problems** Vol. 1, Clarenden Press, Oxford.

Weissbach, A., Baltimore, D., Bollum, E. and Gallo, R.C. (1975) Science **190:** 401.

Wheeler, K.T. and Lett, J.T. (1974) Proc. Natl. Acad. Sci. 71: 1862.

Wills, A.E. and Lindahl, T. (1987) Nature 325: 355.

Wintzerith, M., Wittendorp, E., Rechenmann, R.V. and Mandel, P. (1977) J. Neurosci. Res. 3: 217.

Woodhead, A.D., Setlow, R.B. and Grist, E. (1980) Exp. Gerontol. **15:** 301.

Woodhead, A.D., Merry, B.J., Cao, E.H., Holehan, A.M., Grist, E. and Carlson, C. (1985) J. Natl. Cancer Inst. 75: 1141.

Young, C.W. and Hodas, S. (1964) Science **146:** 1172.

Young, C.W., Hodass, S. and Femelly, J.J. (1964) Proc. Am. Assoc. Cancer Res. 5: 71.

Zoltewicz, J.A., Clark, F.O., Sharpless, T.W. and Grahe, G. (1970) J. Am. Chem. Sco. 92: 1741.

Vol. 16, No. 6, June 1988 BIOCHEMISTRY INTERNATIONAL
Pages 1111-1118

ON THE TYPE OF DNA POLYMERASE ACTIVITY IN NEURONAL, ASTROGLIAL, AND OLIGODENDROGLIAL CELL FRACTIONS FROM YOUNG, ADULT, AND OLD RAT BRAINS

K. Subrahmanyam and Kalluri Subba Rao

School of Life Sciences, University nf Hyderabad
Hyderabad - 500 134, India

Received December 23, 1987

Summary: DNA polymerase activity in isolated neuronal, astroglial, and oligodendroglial cell-enriched fractions from rat brains of different ages was measured. Attempts were made to distinguish the total activity into β and α polymerase types making use of inhibitors like ddTTP and aphidicolin. The results indicate that at all the ages studied (16th day embryonic and 1, 225, and >540 days postnatal), neurons possess the highest polymerase activity in comparison with other types of cells. Further, throughout the postnatal life the polymerase present in neuronal cells is of the 6 type and this activity remains fairly constant from adult to old age. In contrast, both astroglial and oligodendroglial cells at adult and old stages of life appear to possess other type(s) of polymerase activity in addition to the predominant β polymerase. It is inferred that neurons, being postmitotic, are equipped with efficient DNA-repair machinery throughout their life span.

Introduction

For the past few years, we have been engaged in a study of enzymes involved in the DNA-repair process in brain (1-6). During the course of these studies, it became apparent that two DNases, one with acidic pH optimum and the other with alkaline pH optimum, are possibly involved in the DNA-repair process in brain (2). In addition, it is already known that DNA polymerase β is a repair enzyme in neurons (7). Earlier experiments from this laboratory concerning the changes in the activity of DNA polymerase in developing, adult, and old rat brains showed that polymerase β, while showing highest activity during early stages of development, reaches a low value in adult brain but once again shows **a** peak, although small, in old brain (5). This pattern of changes in DNA polymerase β activity led us to believe that brain probably retains good repair capacity even in old age.

However, brain is composed of at least three distinct types of cells viz., neurons, astroglia, and oligodendroglia all with different replicative schedules (8). Therefore it is possible that the DNA-repair capacities of these cell types may vary depending upon the age of the brain. We have therefore taken up a study to measure the DNA polymerase β activity in isolated neuronal, astroglial, and oligodendroglial cell fractions from rat brains of different ages. It is shown here that adult and

0158-5231/88/061111-07$01.00/0

old neurons contain DNA polymerase activity at a significant level and that the polymerase is almost exclusively of the 6 type. On the other hand, astroglia and oligodendroglia from adult and aging brains exhibit not only β polymerase activity but also that of other type(s).

Materials and Methods

Ficoll 400 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Highly polymerized calf thymus DNA, dATP, dGTP, dCTP, TTP, trypsin, soybean trypsin inhibitor, and HEPES were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A.; 2',3' Dideoxythymidine 5'-triphosphate (ddTTP) came from P.L.Biochemicals Inc., Wisconsin, USA and [methyl- H] dTTP (sp.act, 46 Ci/mmole), from Radiochemical Centre, Amersham, England. Aphidicolin was a gift from Dr.A.H.Todd of I.C.I.Ltd., U.K. All other chemicals used were of analytical grade.

Preparation of cell-enriched fractions: Neuronal-and astroglial-enriched fractions from the grey matter region of rat cerebrum were isolated as per the procedure of Usha Rani et al.(9). While the oligodendroglial cell-enriched fraction was prepared essentially according to the procedure described by Snyder et al.(10) with a slight modification which is, in brief, as follows: The isolation medium contained Hanks' balanced salt solution (11) with 25 mM HEPES, pH 7.2. The cell suspension obtained as described by Usha Rani et al.(9) was diluted with an equal amount of 70% sucrose (w/v) in medium and layered over a discontinuous gradient of 53% and 43% sucrose in medium. The tubes were centrifuged at 3013 xg for 15 min. The 53% sucrose layer containing oligodendroglia was collected, diluted five fold with medium, and passed through 25 y nylon mesh to trap blood capillaries. The suspension was centrifuged at 5000 rpm for 10 min and the oligodendroglial cells were obtained as a pellet. All cell isolation procedures were carried out at 4°C.

Preparation of Homogenate: The resultant cell pellets were homogenized in Tris-HCl buffer, pH 7.5, containing 0.1 mM β-mercaptoethanol, 1 mM $MgCl_2$, 0.1 mM EDTA, 5% glycerol, 1% Triton X-100, and 0.5 M KCl. The samples were kept in ice for 1 hr to aid extraction of the DNA polymerase enzymes. The homogenate was centrifuged at 100,000 xg for 1 hr, and the particle-free supernatant was used as enzyme source. An aliquot of the supernatant was taken for the protein estimation according to Lowry's method (12).

DNA polymerase assay: The reaction mixture (50 y l) contained 40 mM Tris-HCl (pH 8.0), 1 mM β-mercaptoethanol, 8 mM $MgCl_2$, 4 mM ATP, 5 µg of 'activated' calf thymus DNA (13), 100 yM each of dATP, dGTP, and dCTP, 25y M TTP (1 pCi), and 20-30 yg of enzyme protein. Incubation was carried out at 37°C for 20 min. During our preliminary experiments, it was observed that the activity was linear upto an enzyme protein concentration of 40 y g and 60 min of incubation time. At the end of the incubation, 0.4 mg of highly polymerized calf thymus DNA was added as carrier, and the reaction was stopped by adding 1.0 ml of 10% TCA. The samples were kept in ice for 15 min and centrifuged at 4000 rpm for 5 min. The precipitate was washed thrice with cold 5% TCA and thrice with 95% ethanol. The final precipitate was dissolved in 0.2 ml of 0.1 N NaOH, and aliquots were taken into vials containing 10 ml of Bray's mixture and counted in a Beckman LS 1800 liquid scintillation counter. Specific activity of DNA polymerase was expressed as picomoles of TMP incorporated into DNA per mg of protein per hr.

Results and Discussion

The specific activities of DNA polymerase in neuronal, astroglial, and oligodendroglial cells obtained from rat brains of various ages are presented in Fig.1. It may be noted that at all the ages studied, neuronal cells possessed the highest polymerase activity. The activity in these cells decreased from the 16th day of embryonic life to adult life (225 days), but no further decrease occurred thereafter, even in old age. These values compare well with those already reported by Waser et al. (7) and by Subba Rao et al. (28). However, in both astrocyte and oligodendrocyte fractions the activity steadily decreased with age starting from a high level at the neonatal stage.

It is intriguing that cerebral neurons, with no capacity to divide during postnatal life, should exhibit higher activities of DNA polymerase at all the stages of life span as compared with the other cell types, viz., astrocytes and oligodendrocytes, which are cells known to retain their replicative capacity to a significant extent throughout life. Mammalian cells are known to possess at least three distinct DNA polymerases - polymerase a, polymerase (3, and polymerase *y* (14-16). It is generally

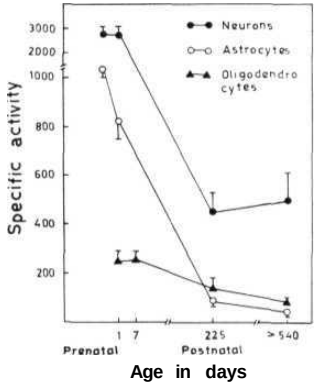


Fig. 1. DNA polymerase in neuronal, astroglial, and oiigadendrogliai cells of developing and aging rat brains. For details regarding the isolation of cell fractions and assay procedure, please see **text.** Each point represents the average of 4 at 16th day of gestation and 6 experiments at other ages, with the bars indicating the variation.

considered that polymerase α is involved in replicative DNA synthesis, while B polymerase is attributed with the function of DNA-repair synthesis. The function of polymerase *y* ,which is similar to mitochondrial polymerase, is not yet clearly understood (17-22).

Since, in the experiments described above, the total DNA polymerase activity in 100,000 xg supernatant of cells was estimated, the possibility exists that the type of DNA polymerase that is present at a given age may vary from one cell type to the other. In an effort to check this possibility we tried to distinguish the a and B polymerase activities by making use of already established specific inhibitors of a polymerase (aphidicolin) and β polymerase (ddTTP). It is supposed in these experiments that the extent of activities inhibited by aphidicolin and ddTTP are due to α and β polymerase, respectively. The results are summarized in Table 1.

At the concentrations indicated the inhibitors were found to exert their maximum inhibition. Further increase in the concentration of inhibitors had no effect. As can be seen, in neurons the DNA polymerase activity at all the postnatal ages was inhibited more than 90% by ddTTP, with aphidicolin exerting no inhibition at all at 225 and 540 days of age. During the embryonic stages, however, ddTTP inhibited the activity by 70% only, while aphidicolin did so by 9%. The effects of above inhibitors were also checked when the polymerase activity was assayed under separate optimal conditions fora and β polymerases as reported by Nagasaka and Yoshida (23). However, this protocol has not changed the above pattern of inhibitions. These results are taken to indicate that at all the postnatal ages, most of the DNA polymerase activity in neurons is of the β type. The picture was, however, different in the case of astrocytes and oligodendrocytes. In astrocytes the maximum inhibition of the activity by ddTTP was noticed at 1-day postnatal (95%), pointing to the fact that at this stage the polymerase present is almost exclusively of the 8 type. At other ages the inhibition varied from 48 to 70% with a marginal but significant effect by aphidicolin at the 16th day of gestation and at 225 days. There were few oligodendrocytes in the prenatal brain and therefore could not be isolated. From the pattern of inhibition of the polymerase activity by the two inhibitors in this cell fraction, it. can probably be concluded that while 8 polymerase is the predominant one throughout the postnatal life, some amount of a polymerase also seems to be present at all the stages.

In view of the claimed specificities of the two inhibitors ddTTP and aphidicolin towards DNA polymerase B and a ,respectively (22), it can normally be expected that the sum total of inhibition by ddTTP and aphidicolin should reach close to 100%. However, this was not the case, particularly in glial cell fractions, as can

Table 1: Extent of inhibition by ddTTP and aphidicolin of DNA polymerase activity in neuronal, astroglial and oligodendroglial cells from rat brains of different ages.

Cell type and inhibitor \ Age	16th day of gestation	1 day postnatal	225 days	> 540 days
Neurons:				
ddTTP	70.2	92.3	92.3	93.0
Aphidicolin	9.4	3.0	Nil	Nil
Astroglia:				
ddTTP	70.0	94.9	47.9	50.2
Aphidicolin	14.3	2.0	13.5	Nil
Oligodendroglia:				
ddTTP		62.6	80.5	43.9
Aphidicolin		16.4	4.8	5.3

For details regarding the reaction mixture and expression of polymerase activity, see text. Values represent average percentage inhibition. Number of experiments carried out are as indicated in Fig.1. ddTTP (1 mM) and aphidicolin (50 µM with a simultaneous reduction of dCTP concentration to 5µM) were added to the reaction mixture. Control values at any given age were taken as 100%. For example, the control values for polymerase activity in neurons at the 16th day of gestation, 1, 225, and >540 days postnatal were 2767, 2713, 449, and 500 picomoles of TMP incorporated/mg protein/hr respectively. The figures at the same ages for the astroglial fraction were 1476, 823, 75, and 57, respectively. The figures for the oligodendroqlia fraction at 1, 225, and > 540 days postnatal were 245, 144, and 78, respectively.

be seen from Table 1. This could be due to two reasons: 1) The inhibition of α polymerase in crude extracts by aphidicolin is not so potent as it is reported to be in studies with pure a polymerase enzyme (24). 2) The molecular species of α polymerase present in the glial cells may be only marginally susceptible to aphidicolin. Indeed, there are reports in literature claiming the presence of multiple forms of DNA polymerase a in calf thymus (25), HeLa cells (26), **and** rat **liver (27). On** the other **hand,** the susceptibility of β polymerase present in crude extracts to

ddTTP can not be suspected since at some ages profound inhibition by this inhibitor is observed. It thus appears that conclusions may have to be drawn more on the basis of ddTTP inhibition rather than the aphidicolin inhibition when one is dealing with non purified enzyme extracts, as is the case in the present experiments.

In any event, the present report,which is the first of its kind, does indicate that rat cerebral neurons possess significant amount of DNA polymerase activity both in adult and old life and that the enzyme is almost exclusively of the 6 type. In contrast, glial cells (both astrocytes and oligodendrocytes) at various stages of adult and old life seem to have other type(s) of DNA polymerase in addition to the predominant B polymerase. This appears to be in line with the known replicative capacity of glial cells during later stages of life span (8). The maintenance of significant amounts of β polymerase activity in neurons throughout life underlines the importance of the DNA-repair process in these non-dividing cells. The recent observations that β polymerase from mouse neurons carries out its function throughout life with the same fidelity (28), along with our present study, pointedly indicate that neuronal cells are equipped with the necessary machinery to carry out efficient DNA-repair throughout their life span.

References

1. Shrivastaw, K.P. and Subba Rao, K. (1975) J. Neurochem. 25, 861-865.
2. Subba Rao, K.V. and Subba Rao, K. (1982) Mech. Aging. Dev. 18, 225-238.
3. Subba Rao, K.V. and Subba Rao, K. (1982) J. Biosci. 4, 139-144.
4. Subba Rao, K.V. and Subba Rao, K. (1982) J. Biosci. 4, 391-400.
5. Subba Rao, K.V. and Subba Rao, K. (1984) Biochem. Intl. 9, 391-397.
6. Subba Rao, K.V. and Subba Rao, K. (1986) Biochem. Arch. 2, 45-52.
7. Waser, J., Hubscher, U., Kuenzle, C.C. and Spadari, S. (1979) Eur. J. Biochem. 97, 361-368.
8. Korr, H (1980) in Proliferation of different cell types in the brain, pp 6-10, Springer Verlag.
9. Usha Rani, B., Indrapal Singh, N., Ray, A. and Subba Rao, K. (1983) J. Neurosci. Res. 10, 101-105.
10. Snyder, D.C., Raine, C.S., Farooq, M. and Norton, W.J. (1980) J. Neurochem. 34, 1614-1621.
11. Hanks, J. and Wallace, R.E. (1949) Proc. Soc. Exp. Biol. Med. 71, 196-200.
12. Lowry, O.H., Rosenbrough, N.S., Farr, A.L. and Randall, R.J. (1951) J. Biol.Chem. 193, 265-275.
13. Loeb, L.A. (1969) J. Biol. Chem. 244, 1672-1687.
14. Chiu, J.F. and Sung, S.C. (1971) Biochim. Biophys. Acta. 246, 44-50.
15. Weissbach, A., Baltimore, D., Bollum,E. and Gallo, R.C. (1975) Science 190, 401-402.
16. Scovassi, A.I., Plevani, P. and Bertazzoni, U. (1980) Trend. in Biochem. Sci. 5, 335-337.
17. Chang, L.M.S. and Bollum, F.J. (1973) J. Biol. Chem. 248, 3398-3404.
18. Loeb, L.A. (1974) in The Enzymes (Boyer, P.D. Ed.), Vol.10, pp 173-209, Academic Press, New York.

19. Hubscher, U., Kuenzle, C.C. and Spadari, S. (1977) Eur. J. Biochem. 81, 249-258.
20. Weissbach, A. (1979) Arch. Biochem. Biophys. 198, 386-396.
21. Krokan, H., Shaffer, P. and Depamphilis, M.L. (1979) Biochem. 18, 4431-4443.
22. Kornberg, A. (1974) in DNA replication, pp 201-229, W.H. Freeman and Company San Francisco.
23. Nagasaka, A. and Yoshida, S. (1984) Endocrinology 115, 1110-1115.
24. Ikegami, S., Taguchi, T., Ohashi, M., Oguro, M., Nagano, H. and Mano, Y. (1978) Nature 275, 458-460.
25. Hesslewood, I.P., Holmes, A.M., Wakeling, W.F. and Johnston, I.R. (1978) Eur. J. Biochem. 84, 123-131.
26. Pedrali-Noy, G. and Weissbach, A. (1977). Biochim. Biophys. Acta. 77, 70-83.
27. Holmes, A.M., Hesslewood, I.P. and Johnston, I.R. (1974) Eur. J. Biochem. 43, 487-499.
28. Subba Rao, K., Martin, G.M. and Loeb, L.A. (1985) J. Neurochem. 45, 1273-1278.