MECHANISMS OF AGING: A STUDY ON THE REGULATION OF PROTEIN SYNTHESIS INITIATION AND DNA POLYMERASES IN RAT BRAIN

Thesis submitted for the Degree of

DOCTOR OF PHILOSOPHY

By

DHODDA VINAY KUMAR

Department of Biochemistry
School of Life Sciences
UNIVERSITY OF HYDERABAD
Hyderabad 500 046
INDIA
2000

Enrollment No: 94 LB PH 03

to

my

family members

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Department of Biochemistry
School of Life Sciences
UNIVERSITY OF HYDERABAD
Hyderabad 500 046
INDIA

CERTIFICATE

This is to certify that I, Mr. DHODDA VINAY KUMAR have carried out the research embodied in the present thesis entitled, "MECHANISMS OF AGING: A STUDY ON THE REGULATION OF PROTEIN SYNTHESIS INITIATION AND DNA POLYMERASES IN RAT BRAIN" for the full period prescribed under Ph.D. ordinances of the University.

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 University.

Date: 19/4/2000

D Vinay Kumar
Name: Dhodda Vinay Kumar
Enrollment No: 94 LB PH 03

K. Subba Rao 19/4/2000
Prof. K. Subba Rao
Supervisor
Department of Biochemistry

Ramaiah 19/4/2000
Dr. K.V.A. Ramaiah
Co-Supervisor
Department of Biochemistry

K. Subba Rao 19/4/2000
Prof. K. Subba Rao
Head
Department of Biochemistry

Prof. R.P. Sharma
Dean
School of Life Sciences

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ABBREVIATIONS

AP	: Alkaline phosphatase
Arg	: Arginine
ATP	: Adenosine 5' triphosphate
BCIP	: 5-bromo-4-chloro-3-indolyl phosphate
Bis-acrylamide	: N, N'-methylene-bis-acrylamide
BSA	: Bovine serum albumin
BuAdATP	: {N^2 -(p-n-butyl anilino)-2'-deoxyadenosine-5'-triphosphate}
BuPdGTP	: {N^2 -(p-n-butyl phenyl)-2-deoxyguanosine-5'-triphosphate}
cDNA	: Complementary DNA
CHO	: Chinese hamster ovary cells
Ci	: Curie
CNS	: Central nervous system
cpm	: Counts per minute
dATP	: Deoxyadenosine triphosphate
dCTP	: Deoxy cytosine triphosphate
ddTTP	: 2'3'- dideoxythymidine 5'-triphosphate
DEAE	: Diethyl aminoethyl
dGTP	: Deoxyguanosine triphosphate
DMSO	: Dimethyl sulphoxide
DNA	: Deoxy ribonucleic acid
DPM	: Disintegrations per minute
DSB	: Double strand breaks
dsRNA	: Double stranded RNA
DTT	: Dithiothreitol
E.coli	: Escherichia coli
EDTA	: Ethylene diamine tetra acetic acid
EF	: Elongation factor

EGF	: Epidermal growth factor
eIF2	: Eukaryotic initiation factor 2
eIF2α	: Alpha subunit of eukaryotic initiation factor 2
eIF2(αP)	: Phosphorylated alpha subunit in eIF2
eIF2B/GEF/RF	: Guanine nucleotide exchange factor of eIF2 or reversing factor
eIFs	: Eukaryotic initiation factors
FGF	: Fibroblast growth factor
Fig.	: Figure
g	: Gravitational force
GCN	: General control non-derepressible
GDP	: Guanosine 5' diphosphate
GTP	: Guanosine 5' triphosphate
h	: Hours
Hb	: Haemoglobin
HEPES	: N-[2-hydroxyethyl] piperizine-N'-[2-ethane-sulfonic acid]
HRI	: Heme regulated inhibitor
I.U.	: International units
Ig	: Immunoglobulin
KCl	: Potassium chloride
kDa	: Kilo daltons
LDH	: Lactate dehydrogenase
LTP	: Long term potentiation
Lys	: Lysine
µl	: Microlitre
µM	: Micromolar
M	: Molar; Molecular weight markers
Met.tRNA$_i$	: Initiator transfer RNA
mg	: Milligram(s)
min	: Minutes
ml	: Millilitre

mM	: Millimolar
mRNA	: Messenger RNA
NBT	: Nitro blue tetrazolium
NGF	: Nerve growth factor
nm	: Nanometer
PAGE	: Polyacrylamide gel electrophoresis
PBS	: Phosphate buffered saline
PC12	: Pheochromocytoma cells
PCNA	: Proliferation cell nuclear antigen
PDGF	: Platelet derived growth factor
PEG	: Polyethylene glycol
PKC	: Protein kinase C
PKR	: Protein Kinase Regulated by Double Stranded RNA
PMSF	: Phenyl methyl sulfonyl fluoride
Poly (IC)	: Synthetic polymer of Inosine and Cytosine
POPOP	: 1,4 – bis (5-phenyl-2-oxazolyl) benzene
PPO	: 2,5 – Diphenyl oxazole
PTK	: Protein tyrosine kinase
PVP	: Polyvinyl pyrrolidine
RBCs	: Red blood cells
RNA	: Ribonucleic acid
rpm	: Rotations per minute
RT	: Room temperature
S	: Svedberg
SAM	: S-adenosyl-L-methionine
SDS	: Sodium dodecyl sulphate
Ser	: Serine
SOD	: Superoxide dismutase
SSB	: Single strand breaks
SSC	: Sodium saline citrate
TBS	: Tris buffered saline

TBST	: TBS with Tween-20
TCA	: Trichloro acetic acid
TEMED	: N', N, N, N'- tetramethyl ethyl-ethylene diamine
TNF- α	: Tumor necrosis factor - α
Tris	: Tris (hydroxymethyl) amino methane
tRNA	: Transfer RNA
TTP	: Thymidine triphosphate
UDS	: Unscheduled DNA synthesis
UV	: Ultra violet
V	: Volts

CHAPTER 1

INTRODUCTION

1.0) INTRODUCTION

Aging is an universal and inevitable phenomenon of all higher forms of life. The life span of multicellular organisms can be broadly classified into 3 phases: **development (growth), reproductive phase and senescence.** In every individual after cellular development and maturity, a slow but progressive change in structure and a decline in function appears, even in the absence of any disease. The structural and functional changes occur at all levels of organisation- molecular, cellular, tissue and organismal, in an organism during its life span. Such changes that occur after the attainment of reproductive maturity comprise the phenomenon of **aging or senescence.**

The scientific research in the fields of medicine and biology in the last three decades has only enabled the control of infectious diseases which accounted for most of deaths and resulted in the progressive increase in percentage of elderly population in developed countries. However, the research in these fields has failed to substantiate the basic molecular mechanism of aging. Gerontologists around the globe are confronted with the challenge of identifying the basic fundamental cause of the aging process. Several theories have been proposed to explain the process of aging. However, not a single theory can explain the various physiological and biochemical changes that occur during the process of aging (Rattan and Clark, 1988; Gensler and Bernstein, 1981). The plethora of aging theories that are known today can be broadly classified into two categories:

1) Programmed aging: According to this, the process of aging is predestined and genetically fixed. Therefore all the developmental changes which take place in an organism are a result of the predetermined genetic programme. The manifestations of senescence are brought about by the playing out this programme which contains specified information or due to the presence of "gerontogenes" or "aging genes" which code for these changes.

Weismann's theory of germ plasm (1889), Aging clocks, Codon restriction theory, Chromatin reorganization theory, Gerontogenes (Rattan and Clark, 1988), DNA damage and repair theory (Hart and Setlow, 1974) and Gene regulation theory (Kanungo, 1980) support this view of programmed aging.

2) Stochastic aging: This concept assumes that aging occurs from contingencies of living rather than programmed development. It assumes that the genetic apparatus does not programme for senescent changes per se but that these changes result from an accumulation of random events, which damage the essential information containing molecules leading to a loss of accurate information and an accumulation of mis-specified proteins.

Theories like Cross linkage (Bjorksten, 1974), DNA denaturation (Von Hahn, 1970), Somatic mutation (Szilard, 1959), Error catastrophe (Orgel, 1963), Free radical (Harman, 1956) and Post-translational modification support the view of stochastic aging.

1.1) THEORIES OF AGING

Some of the theories, which have relevance to the present work, are reviewed below in some details.

1.1.1) SOMATIC MUTATION THEORY

Ross and Scott, (1939) first reported that rats exposed to whole body irradiation that was too low to produce any acute syndrome died earlier than unirradiated controls. This was followed by a report that symptoms of aging and death of irradiated rodents and humans was similar to that of normal individuals, except that the former had high incidence of neoplasia. Based on these observations, Szilard, (1959) proposed the somatic mutation theory, according to which, accumulation of DNA damage leading to mutations in somatic cells was the basic mechanism underlying the aging process. Mutations that occur randomly and spontaneously destroy genes and chromosomes in

post-mitotic cells during the life span of an organism and gradually increase the mutation load. This results in loss of functional genes and decreased production of functional proteins. When the mutation load increases beyond a critical level, cell death occurs which when continued, results in decreased functional ability of the organism.

Studies concerning the effects of ionizing radiation on the life span of the cells both ***in vivo*** (Clark and Rubin, 1961) and ***in vitro*** (Hoehn et al., 1975; Thompson and Holliday, 1978) have shown that life shortening due to ionizing radiation may be a non-specific effect and may occur due to "radiation syndrome" which is unrelated to natural aging.

One difficulty in evaluating the validity of somatic mutation theory is that there is no objective means of measuring the range of somatic mutations in the post-mitotic cells. The only way it has been measured is evaluating the mortality rate which may be due to several factors. It cannot explain why germ cells of long lived species - humans are more sensitive to ionizing radiation than those of mice and drosophila (Sinex, 1974). The higher longevity of drosophila (Strehler, 1964) and mice (Walburg et al., 1966) after exposure to ionizing radiation may be due to secondary effects, although at higher doses, the effects of mortality are evident.

Price et al., (1971) studied ***in vitro*** incorporation of [^{3}H] thymidine into DNA of brain, heart and liver of mice and Chetsange et al., (1975) checked the sensitivity of the DNA to S1 nuclease at different ages and reported that DNA damage increases with age. Hart and Setlow, (1974) observed that the extent of unscheduled DNA synthesis is directly related to DNA repair. Thus aging is not caused by somatic mutation resulting from ionizing radiation or external factors.

1.1.2) ERROR CATASTROPHE THEORY OF AGING

Orgel (1963), proposed the error catastrophe theory of aging, according to which the progressive decrease in the accuracy of protein synthesis might be one of the factors contributing to the age-related deterioration of cells. Errors occurring during information transfer steps like transcription and translation may cause accumulation of defective proteins and cause aging. Particularly, errors in enzymes required for transcription and translation would amplify errors in cells. These errors may self propagate and cause exponential increase in defective enzymes and proteins culminating in a cascade of errors. This may lead to the so-called "error catastrophe" and result in senescence and death of cells. This theory was later modified by Orgel (1970), who proposed that even though the accuracy of the protein synthesizing machinery is not absolute and allows introduction of errors, such errors may not accumulate since the successive generations of protein synthesis apparatus are discrete.

Orgel (1973) postulated that the errors do not accumulate in germ cells because if this occurs the species would be wiped out. He postulated that some "quality control" processes might operate during oogenesis and development and lead to rejection of ova or embryos having high level of errors. However this does not hold true because the immortal transformed or tumor cells do make errors.

Several experimental findings contradict the occurrence of errors in proteins at levels that may cause aging. Studies from Kanungo's laboratory on different enzymes (Kanungo and Gandhi, 1972; Patnaik and Kanungo, 1976; Srivastava, 1971) show that no changes in the primary structure of enzymes occur as a function of age. Certain enzymes like aldolase (Gershon and Gershon, 1973 a, b), isocitrate lyase (Reiss and Rothstein, 1975), 3-phosphoglycerate kinase (Gupta and Rothstein, 1976) and acid DNase (Indrapal Singh and Rao, 1984) show lowered specific activity (decrease is about 30 %-70 %) and higher temperature sensitivity in old age. The activity of protease is decreased resulting in increased half life of proteins, thus providing a

greater chance for undergoing modifications. Therefore, these changes have been attributed to post-translational modifications or to conformational changes.

RNA polymerase, according to Orgel, is one of the principal culprits in generating errors. It is shown that this enzyme exhibits no differences in thermolability and specific activity from early and late passage cells of W1-38 fibroblasts (Evans, 1976). Furthermore, the fixed life span of species and the gradual decline in function with increasing age cannot be explained on the basis of errors by genes or other factors which contradicts the concept of error theory. Suggestions for aging, like errors and post-translational modifications are only circular and do not explain the basic cause of aging.

1.1.3) CODON RESTRICTION THEORY

This theory (Strehler et al., 1971) is based on the hypothesis that the fidelity or accuracy of translation in a cell depends on its ability to decode the triplet codons in messenger RNAs. Two factors are responsible for accurate reading of the codons: 1) transfer RNAs 2) aminoacyl-tRNA synthetases. The decoding molecules of the genetic code, the tRNAs, are degenerate. Qualitative changes in the iso-acceptor tRNAs of amino acids may alter the rate of decoding of the message and thus affect translation. Moreover, tRNAs undergo post-translational modifications that may alter their aminoacylation and hence the fidelity in decoding.

Aminoacyl-tRNA synthetases are also crucial for the fidelity of translation as they identify appropriate tRNAs and charge them with specific amino acids. Any deviation in their activity results in the synthesis of wrong proteins. Old rats have a rapidly degradable fraction of tRNA which has a lower ability to be aminocylated and also the number of isoaccepter tRNAs are reduced (Yang, 1971). The profiles of $tRNA^{ser}$, $tRNA^{arg}$ and $tRNA^{lys}$ are altered in the rooster liver after oestrogen treatment (Bernfield and Maenpac, 1971). Since the levels of hormones are known to change with age, concomitant alterations in tRNA profiles may follow.

This theory, however, does not explain the factors responsible for altering the gene expression for tRNAs and synthetases and those responsible for post-translational modifications of tRNAs. These changes are secondary in nature and hence the basic cause of aging remains unexplained.

1.1.4) FREE RADICAL THEORY OF AGING

Free radicals are extremely reactive chemical species produced in metabolic reactions as well as from those reactions which are spontaneous in nature. They play an important role in accelerating the aging process by damaging important biomacromolecules, DNA, proteins and cell membranes (Harman, 1956). Free radicals induce peroxidation of unsaturated fatty acids and lead to the formation of the aging pigment called "Lipofuscin". Membrane damage affects transport processes across cell and organelle membranes. In case of lysosomes, they become leaky and release hydrolytic enzymes that might cause damage to other cytoplasmic and nuclear components. The key enzymes involved in the process of deactivation of dangerous superoxide radical is superoxide dismutase (SOD) and catalase. Reiss and Gershon, (1976) found that the activity of these enzymes decreases in various organs of rat during aging by 60 % which suggests the susceptibility of older animals to the disastrous effects of the free radicals. Superoxide radical at the molecular level causes oxidation of –SH groups to –S-S- and also ferric ion to ferrous ion, which results in conformational changes of many key enzymes and proteins containing sulfhydryl groups. It also facilitates the production of $(O_2)^{--}$ and $(OH)^{-}$radicals. Antioxidants like vitamin E, ascorbic acid and glutathione scavenge/neutralise free radicals and stabilise cellular membranes and there seems to exist a link between free radical associated damage, aging and carcinogenesis. However, the exact molecular mechanism remains unresolved.

1.1.5) GENE REGULATION THEORY OF AGING

Gene regulation theory of aging states that the expression of various genes occurs sequentially till reproductive maturity is attained. Differentiation and growth occur by sequential activation and repression of certain genes unique to these phases. The products and byproducts of these genes after reaching critical levels and at specific time stimulate certain unique genes responsible for reproductive phase. Some of the products of these genes may trigger the expression of these genes whose products like hormones and other factors confer reproductive ability to the organism. Continued reproduction causes alteration in the levels of these products crucial for keeping certain genes expressed or repressed. This variation may trigger some undesirable genes whose products may cause diseases like autoimmune disease. This may lead to the destabilisation and impairment of the homeostatic control of reproductive phase genes. Failure to maintain a balance of factors and modulators results in the loss of reproductive ability and other functions. Thus, decline in physiological functions may begin and lead to senescence (Kanungo, 1980).

Human genetic diseases like Progeria and Werner's syndrome that occur due to mutation of autosomal genes have shortened life spans due to abbreviated developmental and reproductive phases respectively (Setlow, 1978). Thus, alteration in genes at a specific time of a specific phase resulting in change in the duration of life span renders support to the basic tenet of this model.

1.1.6) DNA DAMAGE AND REPAIR THEORY OF AGING

According to this theory, the ability of an organism to repair alterations in the genetic material, DNA, may be directly related to the longevity of that organism. DNA damage can occur either spontaneously or induced by various factors. With time, DNA damage occurs, which if not properly repaired results in accumulation of DNA damage beyond a threshold level which results in the death of the cell and ultimately

affects the survival of the organism. A major stimulus for the DNA damage and repair theory of aging was provided by the observation of Hart and Setlow (1974) that a direct relationship exists between maximum acheivable life span of a species and its capacity for UV-induced unscheduled DNA synthesis (as a measure of DNA repair capacity) in fibroblasts from seven species. Similar observations were made using fibroblasts from primates (Hart and Daniel, 1980) and between two mouse species with a difference in life span of 2.5 fold (Hart et al., 1979), in skin cells of humans (Sutherland et al., 1980) and in lens epithelial cells from rat, rabbit, dog, cow and horse (Treton and Courtois, 1982). However, Francis et al., (1981) and Collier et al., (1982) studying a variety of mammalian species obtained data with a considerable scatter and could not find similar correlations.

DNA, the genetic material of all living organisms is the repository of all the biological information. Hence, any impairment in the structure and function of the genetic material may result in changes in the structure and function of the organism. There are two striking and universal characteristics of all multicellular organisms which appear to be inherited and genetically programmed.

1) All organisms undergo a gradual decline in their adaptibility to the environment after attaining reproductive maturity,

2) All members of a species have more or less a fixed life span.

These are the two theories of aging which have genetic basis and also explain the influence of environmental factors on the process of aging and therefore have attracted the attention of many gerontologists.

1.2) AN OVER-VIEW OF PROTEIN SYNTHESIS:

Protein synthesis is a fundamental process in cell growth and maturation. It is an integral part of gene expression. The flow of information in biological system occurs from DNA to RNA and from RNA to protein. The copying of information present in a DNA template to a DNA daughter molecule is called replication. The synthesis of

RNA from a DNA template is called transcription. Translation or protein synthesis refers to the decoding of information present in mRNA molecule to the corresponding amino acid sequence in proteins. The processes of replication, transcription and translation require specific cellular machineries. The protein synthesis or translation machinery is composed of mRNA, ribosomes, amino acyl tRNA synthetases, transfer RNAs that bring various amino acids, enzymes and several initiation, elongation and termination factors. For convenience, eukaryotic translation is divided into 3 phases- initiation, elongation and termination (Ochoa, 1983; Hershey, 1991; Watson et al., 1987).

Translational control is a broad term, consisting of several kinds of regulation at the level of protein synthesis. In general, translational control is defined as a change in the efficiency of mRNA translation, that is, in the number of amino acids polymerized per unit time per mRNA molecule. This control may effect a quantitative change in the overall amounts of proteins synthesized, or a qualitative change in the species of proteins produced. There are several examples in the literature where the overall rate of protein synthesis is regulated for example in cells starved for serum or amino acids. In virus infected or stress cells, one observes differential translation of one class of mRNAs (cellular) compared with another (viral, stress proteins) (Hershko et al., 1971; Pain et al., 1980).

Regulation of translation can occur at various steps of protein synthesis or by the interaction among protein factors and / or due to the covalent modifications of these factors (Hershey, 1989; Rhoads, 1993). Regulation also occurs at the formation of a protein-mRNA complex wherein the mRNA is not accessible to the ribosomes. This occurs when the protein to be transcribed is to be stored and used later for example during development (Standart and Jackson, 1994). The structural features in the 5' and 3' sequences of mRNA and/or mRNA binding proteins also selectively regulate translation of certain mRNAs or subsets of mRNAs (Walden, 1993; Hershey, 1991; Jackson, 1991; Merrick, 1992; Redpath and Proud, 1994).

The initiation phase of protein synthesis involves the formation of 80S initiation complex carrying initiator tRNA, Met $tRNA_i$ and the mRNA properly placed at the initiation codon. The initiation factors were identified by purification and reconstitution of the initiation pathway ***in vitro*** (Hershey, 1991). The schematic representation of initiation of protein synthesis is illustrated in **Fig.1.1** (Watson et al., 1987).

The initiation process is further subdivided into following steps:

1.2.1) Formation of eIF2.GTP.Met.$tRNA_i$ ternary complex: The initiator tRNA bringing methionine joins the 40S subunit in the presence of initiation factor 2 (eIF2) and GTP. The interaction between eIF2 and Met.$tRNA_i$ is highly specific and does not recognize any other aminoacylated tRNA. GDP inhibits its interaction with Met.$tRNA_i$. This is because eIF2 has a higher affinity for GDP than for GTP in the presence of physiological concentration of Mg^{2+}. eIF2 is an important regulatory factor in initiation and is purified from multiple sources like pig liver (Harbitz and Hauge, 1979), calf liver (Stringer et al., 1979), rabbit reticulocyte (Meyer et al., 1981), yeast (Baan et al., 1980), rabbit skeletal muscle (Proud and Pain, 1982) and rat brain (Cales et al., 1985). Mammalian eIF2 is a trimeric protein with three nonidentical subunits, α (~38 kDa), β (~50 kDa) and γ (~52 kDa) whereas eIF2 purified from rat brain shows subunits α (~42-43 kDa), β (~59 kDa) and γ (~54 kDa) (Cales et al., 1985). All the three subunits are cloned from yeast and humans. Nucleotide sequence information reveals that several sequences are conserved in the subunits obtained from different species. eIF2 is a phosphoprotein and is shown that both the α and β subunits can be phosphorylated by several cAMP independent kinases. Phosphorylation of eIF2α by eIF2α kinases (see later) inhibits protein synthesis through the inactivation of a rate-limiting guanine nucleotide exchange protein called eIF2B. The physiological significance eIF2β (one of the subunits of eIF2) phosphorylation is not clear. eIF2 is also a GDP-GTP binding protein **(Fig.1.2)**. Recent studies indicate that the γ subunit of eIF2 presumably binds to GDP/GTP (Naranda et al., 1995). Hence the

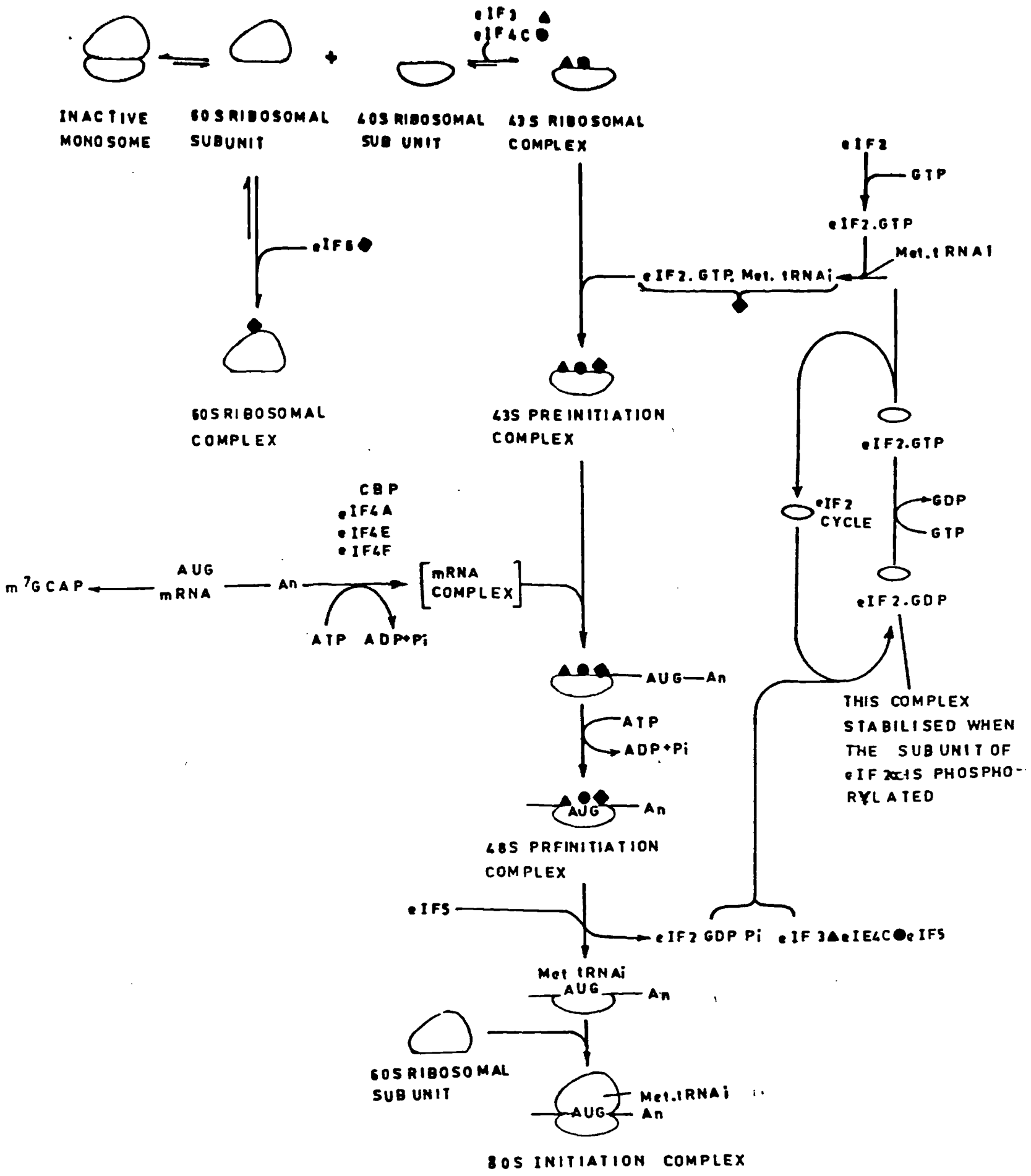


Fig.1.1: Initiation of Protein Synthesis in Eukaryotes (Ref.Watson et al., 1987)

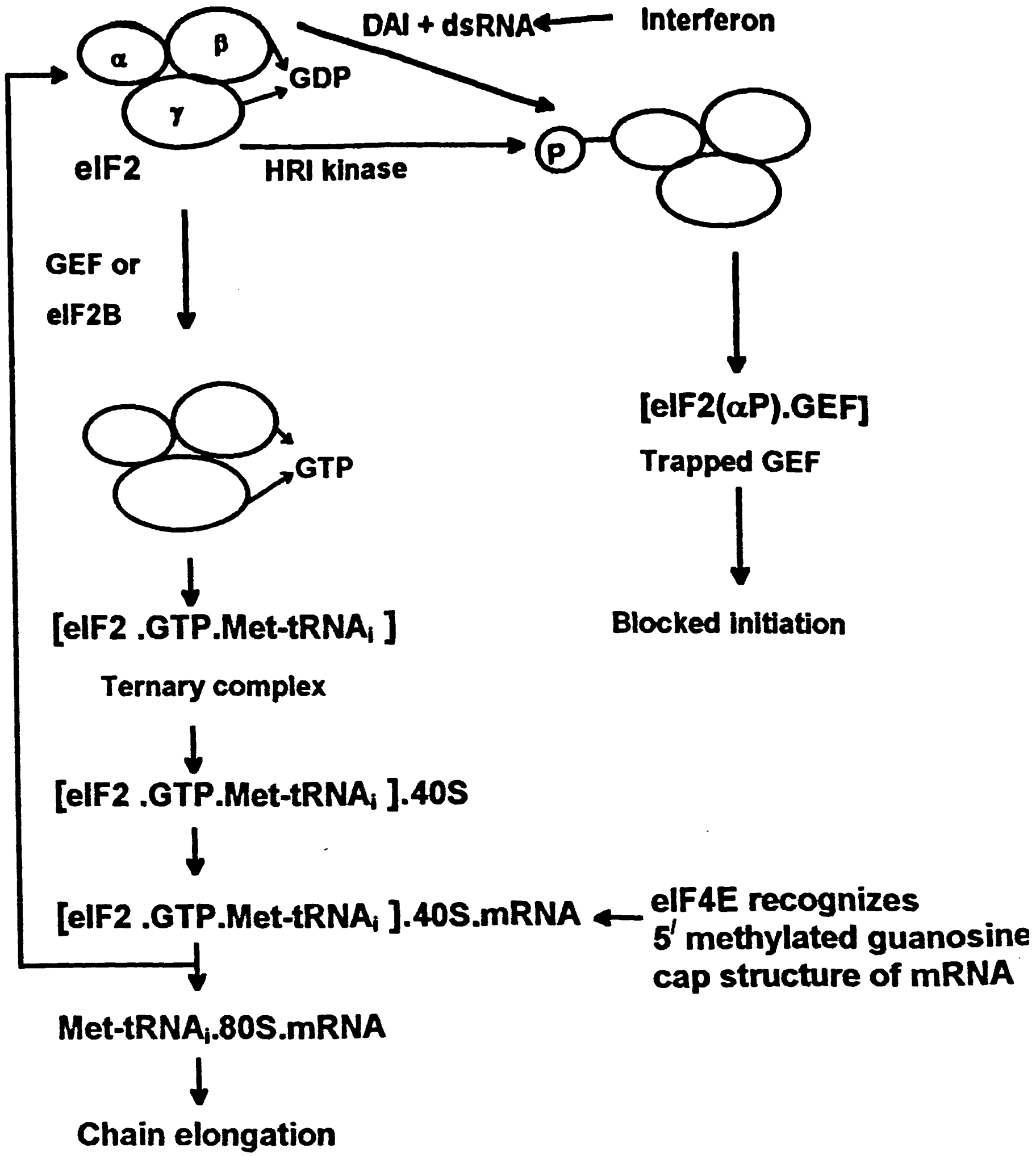


FIG. 1.2: Regulation of polypeptide chain initiation by phosphorylation of eIF2 (Ref. Reichel et al., 1985)

regulation of eIF2 factor activity is dependent not only on eIF2α kinase activation but also is dependent on eIF2α phosphatase activity and the guanine nucleotide exchange activity of eIF2B.

1.2.2) Formation of 48S preinitiation complex: 43S complex consisting of eIF2.GTP.Met-tRNA$_i$ .40S ribosomes, joins the messenger RNA in the presence of eIF4 (consisting of 4A, 4B and 4F initiation factors) and hydrolysis of ATP. Process of mRNA binding to the 43S preinitiation complex involves recognition of cap structure at 5' end of mRNA by eIF4F (reviewed in Hershey, 1991; Merrick, 1992). 4F is a complex of three proteins consisting of:

1) eIF 4E, a 24 kDa cap binding polypeptide (Sonenberg et al ., 1978) previously called as eIF 4α.

2) eIF 4A, a 50 kDa polypeptide which exhibits RNA dependent ATPase and bidirectional RNA winding activities (Ray et al., 1985 ; Rozen et al ., 1990).

3) p220 or eIF 4γ is a 220 kDa polypeptide and the integrity of p^{220} is required for eIF 4F activity in cap dependent translation (Sonenberg, 1987).

eIF 4B is a 80 kDa polypeptide which stimulates the RNA dependent ATPase and helicase activity of eIF 4A (Lawson et al.,1988 ; Rozen et al.,1990).

In addition to eIF4E and eIF4B, eIF2 factor associated with 43S complex may also assist to locate the initiation codon (Dasso et al., 1990).

1.2.3) Joining of 60S subunit to 48S initiation complex to form 80S initiation complex: The formation of 80S initiation complex requires the release of 'anti association' factors like eIF3, eIF4C and eIF6 bound to ribosomal subunits and is stimulated by eIF5 protein which has intrinsic GTPase activity. The GTPase activity facilitates the hydrolysis of GTP bound to eIF2 and the release of eIF2.GDP binary complex from the initiation complex. Several models on eIF2 recycling suggested that eIF2.GDP binary complex is released before joining of 60S subunit to 48S initiation complexes. Some recent studies however indicate that some amount of eIF2.GDP is probably translocated from 48S initiation complex to the 60S subunit of 80S initiation

complex before it is released by yet another rate limiting factor called eIF2B (Ramaiah et al., 1992; Pavitt et al., 1998; Thomas et al., 1985; Gross et al., 1985).

1.2.4) RECYCLING OF eIF2.GDP

The GDP of eIF2.GDP binary complex has to be exchanged for GTP for eIF2 to enter another round of initiation. The heteropentameric eIF2B protein with its guanine nucleotide exchange activity catalyzes this replacement of GDP for GDP (Ramaiah et al., 1992; Pavitt et al., 1998). In addition, eIF2B protein mentioned above helps to release eIF2 GDP from the 80S initiation complexes **(Fig.1.3).**

1.3) ELONGATION OF PROTEIN SYNTHESIS

During the elongation step of protein synthesis, after 80S initiation complex formation, the ribosomes move by three bases towards the 3' end of mRNA in order to read the information present in the template. Depending on the nucleotide sequence information, the respective amino acids brought by the amino acylated tRNAs are properly positioned on mRNA template. The peptide bond formation occurs between adjacent amino acids (amino acid at the 'A' site and growing peptide at the 'P' site of ribosome) probably due to the presence of an enzyme peptidyl transferase (Watson et al., 1987; Spirin, 1986). This process requires EF1 and EF2 (elongation factors 1 and 2). EF1 is equivalent in its function to EF.TU and EF.TS present in prokaryotes.

1.4) TERMINATION OF PROTEIN SYNTHESIS

Termination of nascent polypeptide chain occurs when the 80S complex reaches the termination codons like UAA, UAG or UGA. The termination of protein synthesis requires the releasing factors (RF), which catalyzes termination in the presence of GTP (Caskey, 1977). In prokaryotes there are three RFs whereas in eukaryotes a single releasing factor recognizes all the three stop codons.

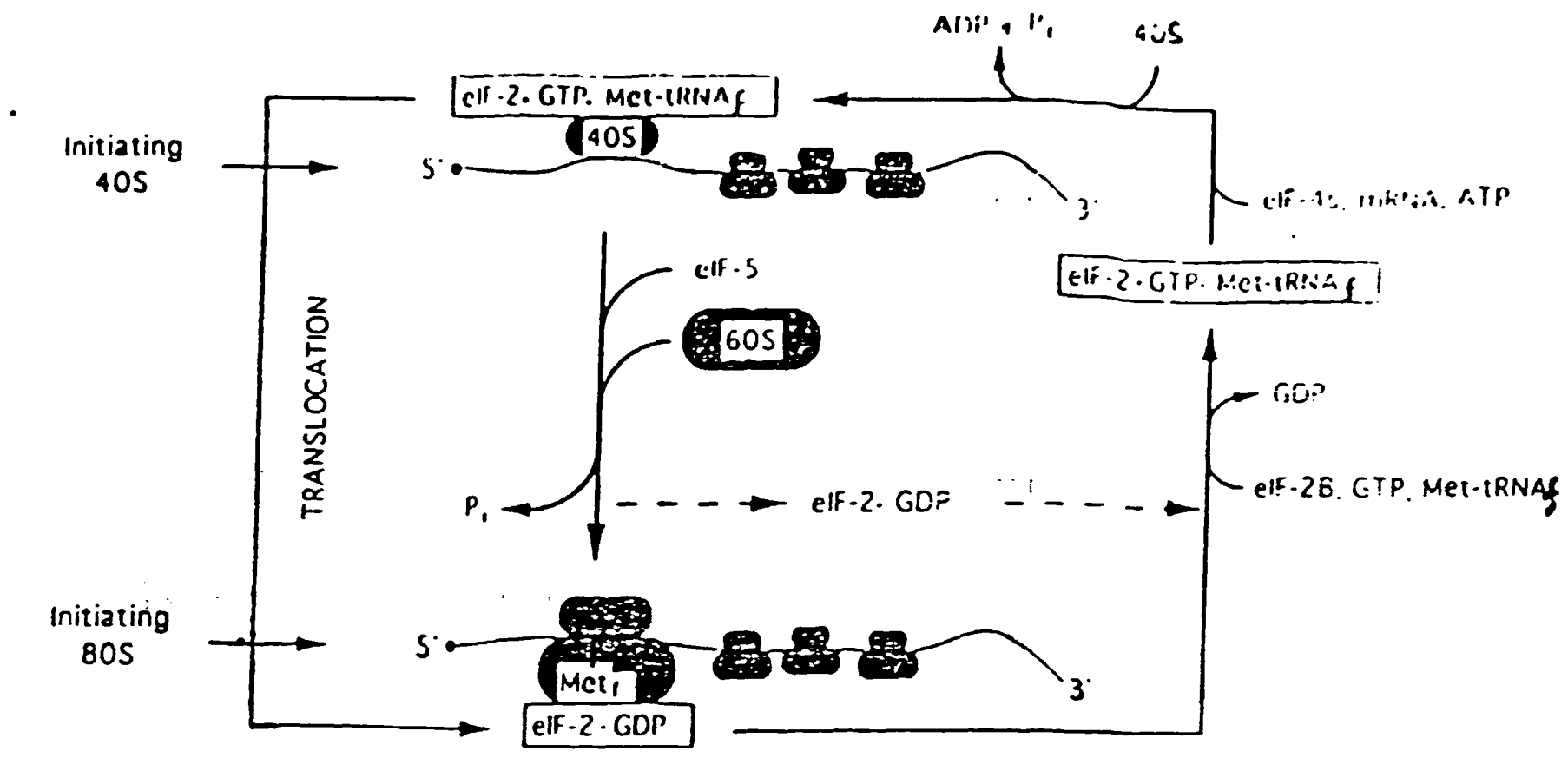


Fig.1.3: Model for the Recycling of eIF2 (Ref. Ramaiah et al., 1992)

1.5) PROTEIN SYNTHESIS IN BRAIN

Protein synthesis is of particular interest in the central nervous system because it has been implicated in memory and information storage processes such as long term potentiation (Gustafsson and Wigstorm, 1988). Protein synthesis in rat brain is altered during aging (Fando et al., 1980; Dwyer et al., 1980; Ekstrom et al., 1980) and development (Johnson, 1976; Vargus and Casteneda, 1981; Dunlop et al., 1977). The alteration in protein synthesis in brain during development depends on the animal's age, nutritional and hormonal status, state of health and response to injury and environmental trauma (Martin et al., 1991). The change in protein synthesis during neural development has been demonstrated ***in vivo***, cortical slices, brain cell suspension, cell free homogenates and microsomal cell free systems. During aging, rate of protein synthesis decreases in rat brain as measured ***in vivo*** (Fando et al., 1980; Goldspink, 1988; Shahbazian et al., 1986), in brain slices ***in vitro*** (Fando et al., 1980; Shahbazian et al., 1986) and in cell free systems from brain (Fando et al., 1980). Various stress conditions effect protein synthesis initiation (Scorsone et al., 1987) and pathological conditons such as ischemia depress translation of mRNA at its initiation step (Cooper et al., 1977; Hu and Weiloch, 1993). Various stress conditions like amino acid imbalance (Aoki and Siegel, 1970; Roberts and Morelos, 1976; Siegel et al., 1971), cerebral ischemia and hypoxia (Cooper et al., 1977; Morimoto et al., 1978; Dienel et al., 1980; Burda et al., 1994; De Gracia et al., 1996), epileptic seizures (Wasterlain, 1977; Wasterlain, 1972), intracranial hypertension (Wasterlain, 1974), hyperthermia (Millan et al., 1979), altered catecholamine metabolism (Weiss et al., 1973; Weiss et al., 1975; Weiss et al., 1971; Weiss et al., 1972; Roel et al., 1974; Moskowitz et al., 1977) and administration of amphetamine (Widelitz et al., 1975; Moskowitz et al., 1975; Nowak and Munro, 1977), ACTH (Schrama et al., 1984; Schotman et al., 1980; Schotman and Allaart, 1981), chlorpromazine (Jakoubek et al., 1980), ethanol (Tewari and Noble, 1971; Lindholm and Khawaja, 1979) affect protein synthesis in the rat brain. Other stresses like serum deprivation and heat shock inhibited protein synthesis in primary neuronal cell cultures (Alcazar et al., 1996; Hu et al., 1993).

The rate of protein synthesis increases in rat pheochromocytoma cells (PC12 cells are precursors for neurons) upon treatment with nerve growth factor (NGF) and epidermal growth factor (EGF) (Munoz et al., 1998; Kleijn et al., 1998). The nerve growth factor is involved in neuronal differentiation and regeneration (Barde, 1989). NGF promotes neurite regeneration by translation of relatively short-lived mRNA species (Twiss and Shooter, 1995).

However, the mechanism involved in the age-dependent decrease in protein synthesis in brain and activation of protein synthesis by specific stimuli is not yet understood. During mammalian hibernation global physiological functions are virtually arrested and supply of glucose and oxygen are minimal. In brain during hibernation the mechanism of protein synthesis regulation involves inhibition of protein initiation and elongation (Frerichs et al., 1998).

1.6) REGULATION OF TRANSLATIONAL INITIATION

Protein synthesis involves complex sequence of reactions and regulation of protein synthesis occurs at various stages. In such a complex sequence of reactions it is natural for the cells to exert control at the first step of the reaction, i.e., at initiation. Translation regulation of individual proteins or global protein synthesis can occur through reversible covalent modifications of initiation factors and other components. Protein phosphorylation is one of primary mechanisms utilized by eukaryotic cells for post-traslation regulation of protein function. Recent studies have shown that protein phosphorylation is involved in the regulation of neuronal function (Browning et al., 1985). The neuronal proteins like RNA polymerase (Hook et al., 1981), Histones (Langan, 1969; Gurley et al., 1981), non histone nuclear proteins (Johnson, 1982), ribosomal protein S6 (Roberts, 1982) and other ribosomal proteins (Roberts, 1982; Thomas, 1982) involved in the regulation of transcription and translation are phosphorylated. In adult CNS, growth factors are involved in neuronal functions like neurotransmission (O'Dell et al., 1991) or neuronal survival (Shapiro et al., 1991) and

these growth factors activate protein synthesis (Montine and Henshaw., 1989; Morley and Thomas,1991). When viewed from the perspective of translational control, certain features of the initiation process are highlighted. Regulation of eIF2 and formation of Met-tRNA$_i$.40S complex may effect protein synthesis globally, since these steps are common to all mRNAs. Several physiological conditions such as heme-deficiency, amino acid starvation, double-stranded viral infection, heat shock, heavy metal stress, mobilisation of calcium, oxidant stress, serum deprivation and several other conditions are known to stimulate the phosphorylation of eIF2α subunit (London et al., 1987) and inhibit protein synthesis globally or selectively (Hinnebausch, 1993). Several of these stresses are known to stimulate the activation of eIF2α kinases such as heme-regulated inhibitor (HRI), double-stranded RNA induced inhibitor kinase (DSI or PKR), GCN-2 kinase in amino acid starvation, endoplasmic-resident kinase called PERK and a pancreatic eIF2α kinase, PEK (Chen and London, 1995; Samuel, 1993; de Haro et al., 1996; Wek, 1994; Clemens and Elia, 1997; Santoyo et al., 1997; Harding et al., 1999; Shi et al., 1998,99) and thereby facilitate the phosphorylation of serine 51 residue in the α-subunit of eIF2.

Phosphorylation of a limited portion eIF2α inhibits the guanine nucleotide exchange activity of rate limiting eIF2B protein and thereby impairs the recycling of eIF2. GDP and protein synthesis (Clemens et al., 1982; Matts and London, 1984; Naresh and Ramaiah, 1996; Ramaiah et al., 1994,97; Pavitt et al., 1998). In addition to eIF2α phosphorylation, eIF2B activity is influenced by its own covalent modifications such as oxidation – reduction, phosphorylation and dephosphorylation (Clemens, 1996; Singh et al., 1995). Abrogation of eIF2α phosphorylation is shown to cause malignant transformation of NIH 3T3 cells (Donze et al., 1995). Activation of eIF2α kinase like PKR that induces eIF2α phosphorylation can also induce an apoptotic cell death (Der et al., 1997; Lee et al., 1997; Srivastava et al., 1998). Differential regulation of specific mRNAs is expected to occur during the interaction of mRNA with eIF4E. In contrast to eIF2α phosphorylation which was shown to inhibit protein synthesis of several mRNAs, a large body of evidence indicates that phosphorylation of initiation

factor eIF4E stimulates the overall translation rates and specifically enhances synthesis of growth related proteins (Rhoads, 1991). The activity of eIF 4E is regulated in atleast three ways --- by its own phosphorylation state (Pain, 1996; Joshi-Barve et al., 1990; Lamphear and Panniers, 1990; Minich et al ., 1994), by binding to eIF 4G (Haghighat and Sonenberg, 1997) and by association with the eIF 4E binding proteins PHAS-1 or eIF 4E-BP1 (Lin et al.,1994; Pause et al., 1994).

eIF 4E is a phosphoprotein and was initially thought to be phosphorylated on the ser-53 residue (Rychlik et al., 1987). Later on it has been shown that the major site of phosphorylation in eIF 4E is ser 209 and not ser 53. (Bhavesh et al., 1995; Flynn and Proud, 1995). However, mutation studies have shown that ser-53 is important for the physiological activity of the factor. Agents which induce eIF4E phosphorylation include various purified peptide growth factors such as EGF, PDGF, TNF- α and a neuronal differentiation- promoting factor, NGF. These factors exert their effects on cell growth and differentiation through binding and activation of specific cell surface receptors phosphotyrosine kinases (PTKs). Tyrosine-specific protein kinases were first identified as the protein products of the oncogenes of transforming retroviruses (Hunter and Cooper, 1985). It is also shown that tyrosine kinase activity is also associated with various growth factor receptors like epidermal growth factor, the insulin receptor, the platelet-derived growth factor and the insulin-like growth factor (Cohen et al., 1980; Kasuga et al., 1983; Heldin et al., 1983; Jacobs et al., 1983). Enhanced phosphorylation and concomitant activation of downstream serine/threionine/tyrosine protein kinases are thought to further transmit both mitogenic and differentiation-inducing signals to the relevant effector molecules. Thus, oncogenic variants of components of these signalling pathways would also be predicted to maintain elevated rates of eIF4E phosphorylation in the absence of serum or growth factors. There are several studies, which indicate that eIF4E phosphorylation is an important downstream outcome of tyrosine kinase activation. In addition phorbol esters, which bind to and activate various PKC subtypes, induce eIF4E phosphorylation in several cell lines. In addition to changes in the phosphorylation levels of eIF2α and 4E that regulate protein synthesis, quantitative

changes in eIF4E and eIF2α are also known to regulate cell growth. Further, increased expression of both these factors was seen in response to growth induction by c-myc (Rosenwald et al., 1993).

1.7) PROTEINS ALTERED IN THEIR SYNTHESIS DURING AGING

1.7.1) Collagen: Collagen is a structural protein and is the most abundant protein in the body. Collagen is of particular interest to gerontologists firstly because there appears to exist an inverse relationship between mammalian longevity and the aging rate of collagen. Hence several workers have studied molecular properties of collagen as a function of age. Secondly, the progressive increase in stiffness of collagen-rich tissues like arteries, lungs, joints and the extra cellular matrix has been associated with age-related diseases such as hypertension, emphysema, decreased joint mobility and ability to fight infections. The extracellular matrix undergoes progressive changes during aging. This is characterized by decreased solubility, decreased proteolytic digestibility, increased heat denaturation time and accumulation of yellow and fluorescent material, which affect particularly collagen rich tissues and result in the formation of age-related crosslinks.

David and Vincent, (1989) have shown the involvement of pentose-mediated protein cross-linking pentosidine in human extra cellular matrix. Pentosidine is present in almost all tissues of the body including the plasma proteins and red blood cells. Its structure comprises of lysine, arginine residues cross linked to pentose sugars such as ribose, arabinose, xylose and lyxose. Quantitation of pentosidine revealed age-related accumulation in human duramater and skin. It was also detected in human heart, lungs, cartilage, bone, liver, renal cortex and medulla. Possible precursor of pentosidine could be ribose or its metabolites and from ADP-ribosylation reactions which play a crucial role in many cellular functions, including DNA repair mechanisms which are thought to play a role in celluar aging (Ueda and Hayaisha, 1985). This acid resistant, fluorescent molecule may serve as a molecular marker of aging process.

The amount of collagen extracted decreases with increasing age (Heikkinen and Kulonen, 1964). The food restriction retards the aging of collagen (Chvapil and Hruza, 1959). The structural changes in collagen that occur as a function of age may be due to changes in the levels of several enzymes and hence are secondary causes of aging.

1.7.2) Chromatin Associated Proteins: In eukaryotes, the higher level of compact organization of genomic DNA involves chromatin association with both histones and non-histone proteins. The histones are small molecular weight basic proteins rich in lysine and/or arginine. The non-histone proteins are highly heterogenous, rich in acidic amino acids and are tissue and species specific. The association of chromatin with histones results in the repeating units called nucleosomes, which consist of a core of 140 base pairs (bp) of DNA wrapped around an octomer of histones that in turn is made up of two each of the histones H2A, H2B, H3 and H4 (Kornberg, 1977). Variable amounts of DNA ranging from 20 to 60 bp are referred to as linker DNA, which is loosely associated with another histone, H1. The main functions of chromatin are replication and transcription. It is natural that either damage or repair of DNA would depend on the overall chromatin structure. The histone protein content does not alter with age (Carter and Chae, 1975) whereas the nonhistone proteins decrease as a function of age (Kurtz and Sinex, 1967; Zhelabov and Berdyshev, 1972). Both histone and non-histone proteins undergo several post-synthetic covalent modifications like phosphorylation, acetylation, ADP-ribosylation and methylation (Kanungo and Thakur, 1977; Thakur et al., 1978; Kanungo and Thakur, 1979 a,c). The neuronal chromatin undergoes structural changes with age (Berkowitz et al., 1983). The transcriptional activity of chromatin decreases as a function of age (Venugopal and Rao, 1991). In a post-mitotic tissue like brain, structural changes in chromatin causes decline in various functions of the organism and leads to senescence.

1.7.3) CHANGES IN ENZYMES (PROTEINS) DURING AGING

Enzymes are proteins that catalyze various biological reactions in the body and are made up of polypeptide chains. The polypeptide chains are composed of amino acids in a specific sequence linked by peptide bond. These enzymes or isoenzymes are coded by one or two genes. Hence the study on the mechanism of aging at the level of the gene is possible by studying various aspects of enzymes like their levels, isoenzyme pattern, inducibility and molecular properties. The synthesis of some of the proteins is altered in post-mitotic tissues like heart, skeletal muscle and the brain. Indeed the expression and activity of some of the proteins are altered in aging tissues and examples include DNA polymerase α, DNA polymerase β, dehydrogenases, synthetases and ribonucleotide reductase (Dice, 1993; Rao et al., 1994). The activity of certain rat brain proteins like lactic dehydrogenase, pyruvate kinase, glutamate dehydrogenase, choline acetyl transferase and acetylcholine esterase are decreased during aging (Schmuckler and Barrows, 1967; Chainy and Kanungo, 1976; Kaur and Kanungo, 1970a; James and Kanungo, 1978). The activity of rat liver proteins like glucose-6-phosphate dehydrogenases and malate dehydrogenases are increased (Wilson, 1972; Kanungo and Gandhi, 1972) during aging. The activity of rat liver proteins like succinic dehydrogenase remains unaltered during aging (Barrows and Roeder, 1961). The levels or synthesis of human brain acid phosphatase increases during aging, whereas, the levels or synthesis of hexosaminidase and β-glucouronidase in human brain remain unaltered during aging (Brun and Hultberg, 1975).

1.7.4) ISOENZYMES

The isoenzymes are different molecular forms of the same enzyme. Different molecular forms of the isoenzyme appear during development of the organism and the pattern of appearance changes as a function of age. Examples of isoenzymes include haemoglobin (Zuckerkandl, 1965), lactate dehydrogenase (Wieland and Pfleiderer,

1957), pyruvate kinase (Oesterman et al., 1973), creatine kinase (Turner and Eppenberger, 1973), adenylate kinase (Filler and Criss, 1971) and aldolase (Koida et al., 1969). Lactate dehydrogenase is a tetrameric protein with five isoenzymes, H_4, H_3M_1, H_2M_2, H_1M_3 and M_4. The levels of these isoforms vary as a function of age. The level of M_4-LDH decreases with age whereas H_4-LDH increases with age in skeletal muscle and brain. The alterations in the isoenzyme pattern cause significant changes in the metabolic pathways and results in functional changes in the organism during aging. The increase in the level of H_4-LDH in brain, heart and skeletal muscle in the old age may make these tissues more aerobic.

1.7.5) SEMIPURIFIED ENZYMES

The molecular properties of some semipurified enzymes were studied as a function of age. They are acetyl choline esterase (Moudgil and Kanungo, 1973) and pyruvate kinase (Chainy and Kanungo, 1978a) of the brain, myosin ATPase (Kaldor and Min, 1975; Srivastava, 1977) and aldolase (Gershon and Gershon, 1973a) of the skeletal muscle and superoxide dismutase (Reiss and Gershon, 1976) and aldolase (Gershon and Gershon, 1973b) of the liver of mammals. Studies on kinetic and molecular properties of these proteins show that several properties like k_m, k_i, molecular weight and electrophoretic mobility of young animals are similar to those of the old. However, the specific activity decreases while thermal sensitivity increases for several enzymes during aging. This may be due to post-translational modifiation (Kanungo, 1980). Thus the synthesis, expression and activity of various proteins or enzymes are altered during aging. The reasons behind these alterations of these enzymes or proteins are not yet well understood.

1.8) DNA DAMAGE

All the organisms, both unicellular and multicellular are constantly exposed to endogenous or internal (spontaneous) and exogenous or external (environmental) damaging factors like heat, free radicals, glucose, microorganisms, various kinds of

radiation that are dangerous for the maintenance of the structural as well as functional integrity of the genomic apparatus in the cell. Genomic integrity is very vital for the survival of the organism and genomic damage can be defined as any alteration or modification in the chemical structure, including the sequence changes of macromolecular components of the genome and their proper interaction. DNA is subject to damage by both endogenous and exogenous events resulting in the modification or loss of bases, the production of mismatched base pairs, strand breaks, DNA-DNA cross-links and cross-links between DNA and other cellular constituents (Friedberg, 1990; Bernstein and Bernstein, 1991). Many of these DNA modifications have been shown to be mutagenic ***in vitro*** and in ***vivo*** (Loeb, 1989). There are six types DNA damages or alterations – AP sites, altered bases, thymidine dimers, cross-links, single strand breaks (SSB) and double strand breaks (DSB).

1.8.1) ENDOGENOUS SOURCE OF DNA DAMAGE

The cause of DNA damage with respect to aging seems to be induced by endogenous biochemical and physical reactions (Hart et al., 1979; Ames, 1983; Gensler et al., 1987). The endogenous sources include the normal metabolism, body heat, free radicals generated during metabolism, glucose, reducing sugars, alkylation and so forth. The effects of these factors on DNA are found to be mismatched base pairs, alteration in the structure of bases like tautomeric shifts and deamination, loss of bases (depurination and depyrimidination) resulting in apurinic/ apyrimidinic sites (AP sites), single strand and double strand breaks (SSB and DSB).

Perhaps the most ubiquitous natural source of DNA damage is heat. Heat induced DNA alterations measured in double stranded DNA, incubated at different temperatures suggest that about 10,000 depurinations are induced per mammalian cell/ day at 37 ^{o}C. Such apurinic sites (AP sites) are spontaneously converted into single strand breaks (SSB) in about 100 hours (Lindahl and Nyberg, 1972; Lindahl and Anderson, 1972). The other forms of heat induced DNA damage include deamination of cytosine to uracil and to lesser extent of adenine and guanine to hypoxanthine and

xanthine which can be removed enzymatically by specific DNA glycosylases (Lindahl and Nyberg, 1974).

Free radicals generated during cellular metabolism and ionizing radiation reacts with DNA and causes oxidative damages including single strand breaks (SSB), double strand breaks (DSB), AP sites, cross-links and modified bases like thymine glycol and 5-hydroxy-methyl uracil, two forms of oxidised thymine (Cadet and Berger, 1985). Enzymes such as xanthine oxidase, cytochrome P_{450}, aldehyde oxidase and peroxisomal oxidases generate free radicals as by-products of their normal function while superoxide radicals are also liberated during active phagocytosis by polymorphonuclear leukocytes or granulocytes (Freeman and Crapo, 1982; Naqui et al., 1986). Lipid radicals such as lipid-peroxy radicals, fatty acid hydroperoxides and cholesterol epoxides cause loss of cellular membrane functions.

Loeb (1989) estimated that about 10,000 radical damages are induced per day in human cells while according to Fraga et al., (1990) about 90,000 lesions/cell per day are induced in rat. Lee and Ceramini, (1987) reported that glucose-6-phosphate interaction with the amino group of lysine leads to the formatin of DNA-protein cross links, the occurrence which has been observed in various cells during aging (Bojanovic et al., 1970). S-adenosyl-L-methionine (SAM) the intracellular methyl group donor causes DNA damage by alkylation (Barrows and Magee, 1982; Rydberg and Lindahl, 1982) and results in the formation of N^7 methyl guanine, N^3 methyl adenine, N^3 methyl thymine and small amounts of O^6 methyl guanine.

1.8.2) EXOGENOUS SOURCES OF DNA DAMAGE

The exogenous sources of DNA damage are dietary mutagenic compounds, UV and ionizing radiations like X-rays, γ-rays, heavy metals (Hartwig and Beyersmann, 1989) and ultrasound (Miller et al., 1989). The extent of exposure of humans to DNA damaging agents is dependent on the life style, occupation and place of residence. The ultraviolet radiation is major source of exogenous DNA damage which results in the

formation of cyclobutane pyrimidine dimers (Setlow, 1982; Niggli and Rothlisherher, 1988) and formation of pyrimidine dimers is influenced by the nucleotide composition of the DNA (Setlow and Carrier, 1966). UV irradiation also induces DNA-protein cross links and single strand breaks (Peak et al., 1985; Lai et al., 1987) whereas ionizing radiation (X-ray, γ-rays and fast neutrons) causes cross links, single strand breaks (SSB) and double strand breaks (DSB) (Scholes, 1983; Hutchinson, 1985).

The human diet contains natural mutagens and carcinogens such as polycyclic aromatic hydrocarbons (PAH), aflatoxin B1 and nitrosamines (Ames, 1983) which react with DNA and induce several types of damages including single strand breaks, double strand breaks and bulky adducts. In the lymphocytes of coke-oven workers, roofers and foundry workers and cigarette smokers, high levels benzo-α-pyrene (BAP) DNA adducts were detected (Everson et al., 1986; Randerath et al., 1986; Mullaart et al., 1990). DNA adducts were detected in bronchial cells of heavy smokers (Bann et al., 1988).

1.8.3) AP (APURINIC/APYRIMIDINIC SITES)

Mere body temperature is enough to cause the breakage of the N-glycosidic bond between the purine and pyrimidine base and the deoxyribose resulting in AP or baseless sites. The frequency of this breakage is higher at elevated temperatures and at the normal body temperature about 10,000 depurinations and 500 depyrimidinations occur/mammalian cell/day (Lindahl and Nyberg, 1972; Lindahl, 1977). Usually all these baseless sites are converted into SSB if not repaired. Saul and Ames, (1985) calculated that about 20,000-40,000 such breaks take place/cell/day. Unrepaired sites in DNA have been shown to be mutagenic by causing GC-TA and AT-TA mutations (Schaaper and Loeb, 1981; Loeb and Preston, 1986). In a non-proliferating cell like neuron of a human brain, about 10^8 purines are lost because of spontaneous depurination during a life span which amounts to about 3 % of the total number of purines in the cell (Lindahl and Nyberg , 1972).

Thus the genetic material (DNA) is constantly exposed to exogenous and endogenous assaults which poses a threat to its maintenance and integrity.

1.9) DNA REPAIR

Organisms have developed a number of mechanisms to counteract various deleterious alterations of the genetic material (Hanawalt et al., 1979; Linn, 1982; Friedberg, 1985; Sedgwick, 1986; Collins et al., 1987; Sancar and Sancar, 1988; Friedberg, 1990;1991). There are several systems to eliminate DNA damaging agents before they react with DNA. The activity of the free radicals is antagonized by the enzymes like superoxide dismutase, catalase, glutathione peroxidase. Besides these enzymatic defence systems, there are several substances generated in the cell or present in the food that have ability to scavenge free radicals such as selenium, vitamin E (α-tocopherol), vitamin C, glutathione and cysteine.

In addition to this 'first line' of defence systems, cells possess a multitude of potential DNA repair pathways. The DNA repair events can be divided into three classes- **Reversal of damage, Recombinational repair and Excision repair.**

Reversal of damage: The DNA damage is simply reversed without involving the breakage of phosphodiester bond, e.g., monomerization of pyridine dimers by an enzymatic reaction dependent on light of wavelength more than 300nm, removal of methyl groups and simple rejoining of the strand breaks. The enzymes implicated in reversal of damage are DNA photolyase, O^6 methyl guanine-methyl transferase and DNA ligase.

Recombinational repair: The mechanism of this type is least understood and is of no significance to a non-dividing cell like neuron since the recombination repair is essentially a post-replicational event to take care of the possible repairs introduced during the active DNA synthesis.

Excision repair: There are two types of excision repairs like -- **Nucleotide excision repair** and **Base excision repair.** Except for the initial differences, these two processes actually constitute a common pathway and are diagrammatically represented in **Fig: 1.4.**

Nucleotide excision repair: The first step in nucleotide excision repair pathway is the recognition and incision of the damaged site by an incision endonuclease. From the plethora of endonucleases found in different organisms, it appears that these enzymes have rather broad and varied specificities (Linn, 1982; Lambert et al., 1988). Some recent information suggests that the recognition of the site by endonucleases is facilitated by the binding of the damaged specific proteins. For example, a protein that has specificity for UV damaged DNA has been isolated from HeLa cells (Chao et al., 1991). Similarly Satoh and Lindahl, (1992) have shown that the enzyme poly(ADP-ribose) polymerase has the capacity to recognize and attach to damaged site in the DNA molecule. This attachment activates the synthesis of poly ADP-ribose resulting in the auto poly ADP-ribosylation of the enzyme. This automodified enzyme has a reduced affinity for DNA and is therefore released, making the damaged site accessible to DNA repair enzymes. On the same lines specific T:G mismatch binding proteins in mammalian cells are reported (Heywood and Burke, 1990).

The second step is the excision of the damaged region, which may include some adjacent nucleotides as well by an exonuclease. However, in the case of UV induced damage the incision seems to be precisely at 6 bases 3' to the damage and 22 bases 5' to the damage, thus releasing a 29 nucleotide fragment (Tanaka and Wood, 1994).

B) Base excision repair: In the case of base excision repair, first the baseless site formed either spontaneously or by the action of DNA-glycosylase would be recognised by an apurinic/ apyrimidinic endonuclease and the site incised. Henceforth, the subsequent steps are similar to that of the nucleotide excision repair.

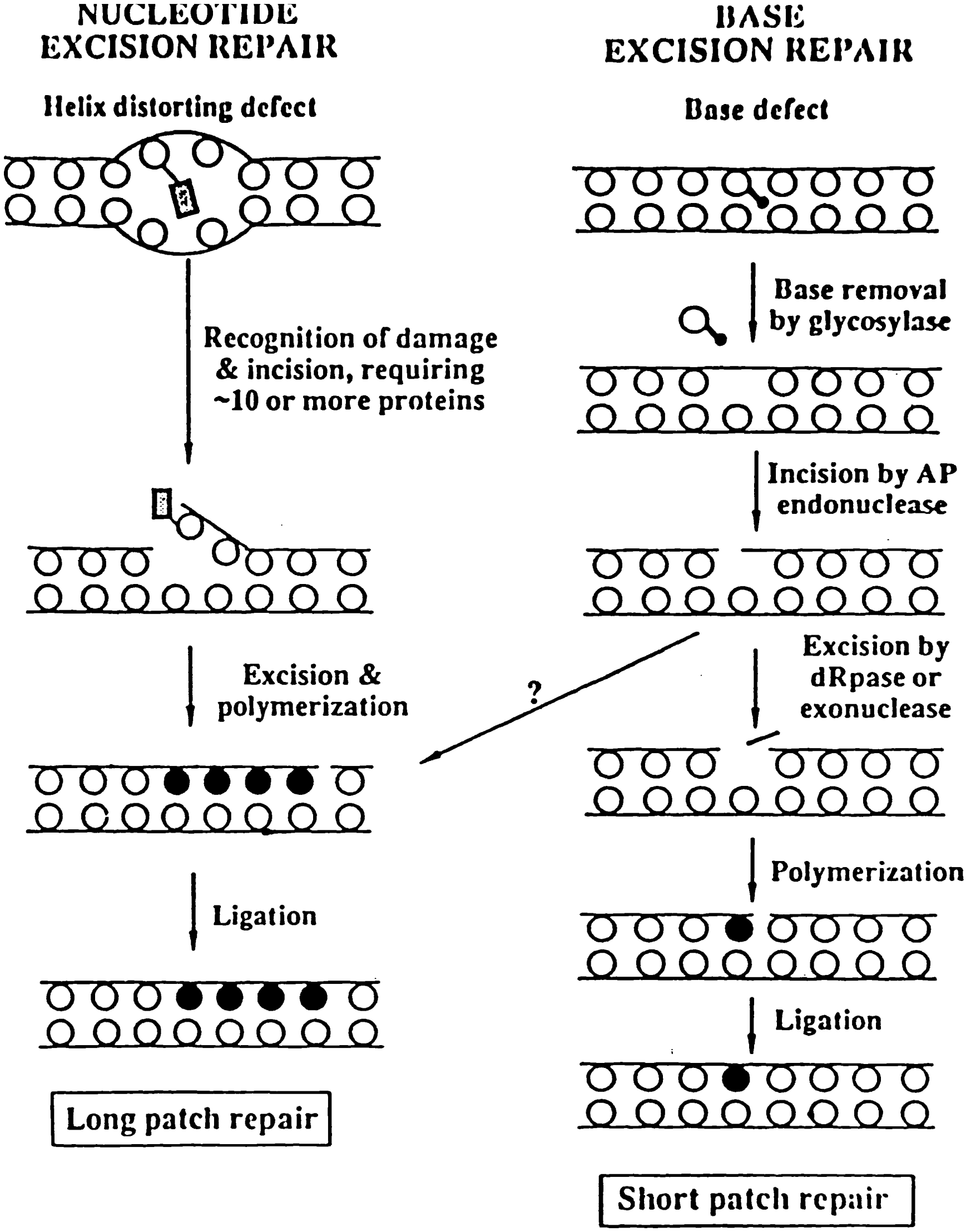


Fig.1.4: DNA repair process – The four steps involved in Excision pathway are: a) Recognition of the damage, b) Excision of the damaged portion, c) Resynthesis of the excised portion by DNA polymerase, d) Ligation of the last nucleotide gap by DNA ligase. Nucleotide excision repair and Base excision repair are shown. This diagram is kindly provided by Larry H. Thompson of Lawrence Livermore National Laboratory, Livermore, CA

It is generally believed that the nucleotide excision repair is characterized by a long patch repair based on the number of nucleotides incorporated per each repair segment, whereas in the base excision repair the patch is a short one comprising only three to four nucleotides. Much of the information about the enzymology of DNA repair has emanated from prokaryotic systems. Information regarding enzymes involved in the eukaryotic DNA repair system is scanty. Comparable information from brain is even more scanty (Linn, 1982; Friedberg, 1985; 1990; Rao, 1990). DNA repair system in eukaryotes appears to be more complex than in prokaryotes. If one considers the initial step of damage recognition, the number of genes tends to increase from prokaryotes to humans. Thus in T4 infected E.Coli only a single T4 coded gene is required for the incision. However in the uninfected E.coli four separately encoded proteins (UVR A, B, C and D) are required for the same process. In mammalian cells (including humans) there are indications to suggest that atleast 15 separate gene products are required in the incision process (Fischer et al., 1985; Hoy et al., 1985; Cleaver and Karentz, 1986).

Hanawalt (1987) have shown that there exists a genomic heterogenity in DNA repair process, which means that damages in certain regions of the genome may be repaired preferentially. Although the overall dimer removal is poor in Chinese Hamster Ovary cells (CHO), the repair of dimers in the restriction fragment of active and essential dihydrofolate reductase gene is very efficient (Mellon et al., 1987). Studies further demonstrated that in mouse cells, the expressed c-abl proto-oncogene is preferentially repaired, whereas the unexpressed c-mos protooncogene is poorly repaired (Madhani et al., 1986). Some types of damage such as interstrand cross links are repaired more efficiently than other lesions like psoralen photomonoadducts (Vos and Hanawalt, 1987).

The enzymes involved in DNA repair and present in brain tissues are endonuclease of lamb brain (Healy et al., 1963), acid and alkaline DNases of rat (Sung, 1968), acid DNase (UV DNase) and alkaline DNase (AP DNase) from chick and rat brain (Rao, 1990; Suvarchala and Rao, 1994), AP-endodeoxy nuclease from rat neocortex

chromatin (Ivanov et al., 1988), exonuclease (DNase B III) from rat brain neuronal nuclei (Ivanov et al., 1983), uracil-N-glycosylase of human foetal brain (Krokan et al., 1983), DNA polymerase β of rat and mouse brain (Hubscher et al., 1979), DNA ligase of neuronal and glial cells of guinea pig brain and rat cerebellum (Inoue and Kato, 1980), poly (adenosine diphosphate ribose) synthase of bovine brain (Bilen et al., 1981), o^6 alkyl-guanine-DNA-alkyl transferase of rat and human brain (Wiestler et al., 1984) and photolyase of marsupial brain (Rupert, 1975). However, a systematic examination of the activities of these DNA repair enzymes in brain as a function of age has been carried out only in a couple of them (Rao, 1993).

1.9.1) DNA REPAIR, BRAIN AND AGING

Many studies were conducted to study the changes in DNA repair potential in brain as a function of age. Alexander (1967), for the first time reported that the DNA repair system is at a low key once cells are differentiated into a post mitotic state. A number of subsequent observations confirmed this postulation with a special reference to brain cells (Bernstein and Bernstein, 1991). Studies on DNA repair suggests that repair is not completely shut off, but lower in adult brain by autoradiography. Korr and Shultz, (1989) have demonstrated low but significant levels of DNA repair in various types of cells of adult mouse brain ***in vivo***. Waser et al., (1979) and Rao and Rao, (1984) reported significant levels of DNA polymerase β, a repair enzyme, in adult and aging brain. The DNA polymerase β levels are significant in adult and old mouse brain and no change occurs in the fidelity of this enzyme between young, adult and old ages (Rao et al., 1985). A nonspecific alkaline DNase of rat brain, a putative DNA repair enzyme was found to exhibit high activity during adult and old ages (Rao and Rao, 1982; Rao, 1990). Jensen and Linn, (1988) found that when neuroblastoma cells differentiate in culture to a postmitotic state, the levels of DNA polymerase β and uracil DNA glycosylase remain unchanged, whereas AP endonuclease activity shotup three fold. The DNA repair capacity measured by the ***in vitro*** incorporation [^{3}H] thymidine into DNA of isolated neuronal cells, as a function of age shows that the DNA repair potential decreased markedly from young (10 days) to adult (6 months)

and no further decrease occurred between adult and old age (540 days). These results suggests that the adult and aging brain possess the necessary machinery to take care of atleast some forms of DNA damage (Subrahmanyam and Rao, 1991).

Wheeler and Lett, 1974 working with beagle dog cerebellar internal granular layer of neurons did not find any deterioration in joining the single strand breaks induced by gamma radiation. However, they have reported an age-dependent decline in the size of the DNA extracted from these cells. Thus the result can be interpreted as a decline in some form of DNA repair in these cerebellar neurons with age.

De Souza et al., (1986) have studied UV-induced unscheduled DNA synthesis (UDS) in neuronal cultures of mouse dorsal root ganglia. They have reported a significant decrease in the unscheduled DNA synthesis (UDS) in old age as compared to the adult in the both strains of mice. However, in the same study no age related decrease in unscheduled DNA synthesis (UDS) was observed in lymph node cells. Subrahmanyam and Rao, (1991) working with the isolated neurons as a model system in rat brain demonstrated that neurons isolated from the adult and old animals offer a good model system to measure the UV-induced UDS without any interference of DNA-replicative synthesis. They also reported that the response of the aging neurons to mutagenic challenge like UV-irradiation was limited and it is this lack of responsive DNA-repair against a given damage that may lead to metabolic deterioration and senescence. There is no preponderance of evidence to support a decline of DNA repair in brain with increasing age. The inconsistencies are largely due to comparison among different experimental systems. In studies with mice, Ono and Okada, (1978) found no age associated increase in γ-ray induced strand breaks, while De Souza et al., (1986) reported decreased DNA repair by UV-induced damage in dorsal root ganglia. The hamster studies of Gensler and Bernstein, (1981) were carried out with whole brain rather than with a specific neuronal population. Comparison of data should perhaps be made by measuring the repair of the particular type of DNA damage in specific cell types of the brain and in an identical manner, especially in view of the predicted complexities of DNA repair pathways in a highly

evolved organ like brain. All these studies are tempting to uphold the DNA damage and repair theory of aging. Evidence to support this contention comes from the work of Niedermuller, (1985) and Washington et al., (1989). Niedermuller has studied the repair of four types of DNA damage: UDS after treatment with the DNA damaging agents like N-nitrosomethyl urea and methylmethane sulphonate, single strand breaks (measured through nucleoid sedimentation) and double strand breaks as measured by neutral elution and removal of endonuclease sensitive sites (measured by velocity sedimentation in alkaline sucrose gradient). The first type of repair declined with age in all the tissues including brain. Single strand repair was significantly reduced only in testis and brain in old rat. No reduction was found in the double strand break repair. The ability to repair endonuclease sensitive sites was significantly lost in the brain during aging. The activities of 3-methyladenine DNA glycosylase and O^6-methylguanine DNA methyltransferase were measured in the mouse brain by Washington et al., (1989). While no change was found in the case of methyltransferase, a significant lowering of the 3-methyladenine glycosylase activity was observed with age.

Besides inter-species and inter-individual differences in DNA repair activities, there are some hereditary disorders associated with a defect in DNA repair activities. A study was made on these genetic disorders showing premature aging to know whether there is any effect on the DNA damage and repair capacities in these disorders characterized by symptoms of premature aging. Martin (1978) listed 162 genetic syndromes in humans with some or many signs of premature aging. About 21 features are considered as markers for accelerated aging. Those aspects along with possible accumulation of the diverse forms of DNA damage and decline in DNA-repair capacity in some of these syndromes have been discussed in detail by Friedberg (1985); Warner and Price, (1989); Bohr et al., (1989); Bernstein and Bernstein, (1991). Most of them show elevated levels of DNA damage and a large number of them suffer from neurodegeneration. The genetic syndromes characterized by nervous debility, premature aging and DNA damage/decreased repair capacity are Down's syndrome, Ataxia telangiectasia (Delabar et al., 1987).

Down's Syndrome: Down's syndrome has several features of premature aging and the genetic defect is trisomy of the distal part of the long arm of chromosome 21. The critical segment of chromosome 21 is shown to have three genes coding for copper and zinc-dependent superoxide dismutase, oncogene ets-2 and cystathione β-synthase (Delabar et al., 1987). Since elevated levels of superoxide dismutase are found in various tissues of these individuals, it is postulated that the accelerated aging of these patients may be caused by overproduction of superoxide dismutase, which is responsible for the production of H_2O_2 while scavenging the oxygen-free radicals. The brains of Down's syndrome individuals are particularly vulnerable to oxidative DNA damage because the high levels of superoxide dismutase found in this tissue are not accompanied by an elevation in the glutathione peroxidase and catalase (Balazs and Brookshank, 1985) that would have normally helped in removing the overproduced H_2O_2. Several lines of evidence suggest that aluminum may be involved in the aetiology of Alzheimer's disease and Down's syndrome. Recent study suggests that the gastrointestinal absorption of aluminum is increased in Down's syndrome patients (Moore et al., 1997).

Ataxia Telangiectasia (Louis-Bar syndrome): This is a growth deficiency occuring during infancy with progressive cerebellar ataxia and chronic dilation of small blood vessels, mental retardation and impaired cellular immunity. The basic defect appears to be in the recognition and repair of γ-induced damage suggesting an under activity of endonucleases specific for this damage (Patterson et al., 1976).

The syndromes having characteristics common to aging include Werner's chromosome instability syndrome, Hutchinson-Gilford syndrome (Progeria), Cockayne's syndrome. Mullenders et al., (1988) have reported that cells from patients with Cockayne's syndrome have a diminished capacity to repair actively transcribed genes. Alzheimer's disease is also characterized by a defect in DNA repair mechanism (Robbins, 1983; Mullaart et al., 1990a).

Thus in a postmitotic tissue like brain the activity, efficiency and reliability of DNA repair system plays a major role in gene expression and maintenance of structural, functional integrity of DNA. Brain transcribes a greater portion of its DNA than other non-cerebral tissues (Hahn and Laird, 1971). The cerebral cortex with high metabolic rate generates DNA damaging free radicals and hence efficient DNA repair system helps to overcome the genetic instability.

The large number of DNA lesions produced by exogenous and endogenous factors leads to altered gene expression and results in the alteration of protein translational machinery and protein synthesis during aging. This could be the underlying cause for age-related changes in the protein synthesis and dysfunctioning of organs and cells. However, the 'KEY DNA' lesion responsible for age-related changes in protein synthesis and dysfunctioning of organs and cells still remains to be identified and more pointed work is needed to gain definitive answers in this context.

2.0) SCOPE OF THE THESIS

Accumulation of the DNA damage and decrease in the DNA repair capacity is one of the causes for aging and age-related disorders (Gensler and Bernstein, 1981; Hart and Setlow, 1974). Genomic integrity is very essential for the survival of any organism, as any damage to it will ultimately lead to the death of the organism.

For the past few years, our laboratory has been studying DNA damage and repair mechanism as a function of age in the rat brain. Extensive studies were conducted on the DNA repair enzymes, such as DNases, DNA polymerases, RNA polymerase activity, single strand breaks and double strand breaks in aging rat brain cells. The number of single and double strand breaks increased with increasing age and accumulation of DNA damage in old neurons is not due to apoptosis but is due to a natural phenomenon like aging (Bhaskar and Rao, 1994; 1996). The transcriptional ability decreases with increasing age (Venugopal and Rao, 1991) in rat brain cells and decline in the transcriptional ability may be due to a conformational change in the

DNA. The decline in transcriptional ability may lead to the alterations in the protein translation or protein synthesis. Recent studies have shown a general decline in protein synthesis during aging. The reduction in protein synthesis may be due to reduced mRNA levels or mRNA may be present but reduction may be due to a defect in protein translation. Hence, in this study we have examined the changes in protein synthesis and DNA repair enzymes as a function of age and also looked at the mechanism behind these changes at DNA replication, translation and post translation levels.

The main objectives of the present study are:

1. To study protein synthesis, the levels of initiation factors – eIF2, eIF4E and tyrosine phosphorylation in rat brain neuronal cells as a function of age.

2. To assess the synthesis and levels, including the accessory factors if any, of some DNA polymerases, in particular DNA polymerase β in rat brain.

Firstly, to study the protein synthesis, the [^{35}S] methionine incorporated into acid precipitable proteins was studied as a function of age. The levels of eukaryotic initiation factors - eIF2α and eIF4E were estimated in neuronal extracts during aging. Using a polyclonal antiphosphotyrosine antibody the levels of phosphorylated tyrosine residues in the rat brain neuronal extracts was determined during aging. The effect of genistein, a potent inhibitor of tyrosine kinase and phosphorylation of eIF2α by PKR like kinase or double stranded RNA dependent kinase were determined in the young and old extracts by ***in vitro*** phosphorylation. The results on the study of protein synthesis in rat brain cells as a function of age are discussed in detail in chapter III.

There are 5 different polymerases in mammalian system. They are DNA polymerases α, β,γ, δ and ε. DNA polymerase β is considered as a repair enzyme in some forms of damage (Miller and Chinault, 1982). Recent studies have shown that polymerase β from mouse neurons carries out its function throughout life with same fidelity (Rao et

al., 1985) and significant amounts of polymerase β are present in both adult and old rat neuronal cells (Subrahmanyam and Rao, 1988). The presence of these putative DNA repair enzymes indicate the occurrence of active DNA repair process even in aging rat brain. In the present study, the active levels of DNA polymerase β were estimated by general enzyme assay, activity staining, western blotting, northern blotting and immunotitration. The regulation of polymerase β by ***in vitro*** phosphorylation and degradation of polymerase β through ubiquitination were also examined. The results on DNA polymerase β in aging rat brain neuronal cells are discussed in detail in chapter IV.

DNA polymerase δ is involved in DNA replication whereas DNA polymerase ε is required for DNA repair. Recent studies have shown that DNA polymerase δ and ε are present in the young and developing rat brain (Prapurna and Rao, 1997). In the present study we have partially purified and characterized the DNA polymerase δ and ε from rat brain and the results are illustrated in detail in chapter V.

Proliferation cell nuclear antigen (PCNA) an auxillary protein from DNA polymerase δ is known to stimulate processivity of DNA polymerase δ by several fold. In the present study we have purified PCNA from rat brain and characterized the protein by SDS-PAGE, western blotting and by enzyme activity. We have also assessed the levels of PCNA in young, adult and old rat brain. The results of these experiments are shown in detail in chapter V.

CHAPTER 2

MATERIALS AND METHODS

2.0) MATERIALS:

2.01) Antibodies and chemicals for western analysis: Antibodies against eIF2α (anti-human) and eIF4E (anti-mouse) were received as kind gifts from Dr.Jane Jane Chen, MIT, Cambridge, USA and Prof.Nahum Sonenberg, McGill university, Canada respectively. Anti-phosphotyrosine antibody was purchased from Upstate Biotechnology Inc., New York. Monoclonal anti proliferation cell nuclear antigen (anti-PCNA) antibody was received as kind gift from Dr.M.Y.W.T.Lee, University of Florida, USA. Pure DNA polymerase β protein from rat liver and polyclonal anti DNA polymerase β were a kind gift from Dr.A.Matsukage, Aichi Cancer Research Centre, Japan. Monoclonal α antibody SJK-132-20, against human DNA polymerase α was obtained from PL-Biochemicals. Alkaline phosphatase conjugates anti-mouse IgG and anti-rabbit IgG, NBT and BCIP detection systems were purchased from Promega Inc., USA. Horse radish peroxidase conjugate anti-rabbit IgG was purchased from Sigma,USA.

2.02) Radioactive Isotopes: (^{35}S) methionine (Sp. act.1000 Ci/mmole), (γ^{32}-P) ATP (Sp. Act.3000 Ci/mmole), (α^{32}-P) dCTP (Sp. act.3000 Ci/mmole) and (α^{32}-P) TTP (Sp. act. 3000Ci/mmole) were purchased from Bhabha atomic research centre, Trombay, Mumbai. (^{3}H) TTP (Sp. act. 40 Ci/mmole) was purchased from Amersham,UK.

2.03) Chemicals: Chemicals for protein estimation, protein molecular weight markers and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Biorad, USA. Guanidium isothiocyanate was purchased from Fluka, USA. DTT and poly(IC) were obtained from Roche diagnostics, Germany and Calbiochem, USA respectively. Acetyl phenyl hydrazine, ATP, sarcosyl, ficoll,

polyethylene glycol, formamide, poly vinyl pyrrolidine, dATP, dGTP, dCTP and dTTP nucleotides, highly polymerised 'activated' calf thymus DNA, DEAE-sephacel, hydroxyl apatite, phenyl sepharose, dimethyl sulphoxide (DMSO), pepstatin, leupeptin, diaminobenzidine, magnesium acetate, DNA, bromo phenol blue, coomassie R250, BSA, ponceau S stain, PMSF and GF/C filters were purchased from Sigma,St.Louis,USA. BuPdGTP [N^2- (p-n-butyl phenyl)-2'-deoxy guanosine – 5'-triphosphate] and BuAdATP [N^2- (p-n-butyl anilino)-2'-deoxy adenosine – 5'-tri phosphate], the nucleotide analogs, were kindly gifted by Dr.George Wright,USA. Gene screen plus nylon membranes were purchased from Dupont, USA. Synthetic substrates poly (dA.dT) and poly (dA).oligo $(dT)_{12-18}$ were purchased from Midland Certified Reagent Co., Midland,Texas,USA. DEAE Cellulose (DE-52), phosphocellulose, filter papers and nitrocellulose membranes were purchased from Whatmann, UK. Tris, glycine, PPO and POPOP were purchased from Spectrochem,India. Potassium chloride, sodium chloride, hydrogen peroxide, triton x-100, sodium hydroxide, ammonium sulphate, glucose, calcium chloride, magnesium chloride, EDTA, hydrochloric acid, acetone, toulene, glycerol, trichloroacetic acid, potassium dihydrogen phosphate, disodium hydrogen phosphate, sodium fluoride, acetic acid, silver nitrate, methanol and ammonium per sulphate were purchased from Qualigens, India while TEMED, β-mercaptoethanol were purchased from Loba-chemie, India. Non-fat dry milk powder and X-ray films, developer, fixer were purchased from Nestle, India and Indu, India respectively. Heparin and New Zealand white rabbits were procured from Biological Evans Ltd., India. Goat serum was prepared in our laboratory. Rats of Wistrar strain were obtained from our university animal house. They were fed ad libitum standard rat feed of Hindustan Lever Ltd., India. Rats of three age groups were used in the present study and referred to as young (4 days), adult (180 days), old (>730 days)—all postnatal.

2.1) METHODS:

2.1) CELL FREE TRANSLATION SYSTEM:

2.1.1) Heme-Deficient rabbit Reticulocyte lysate preparation:

New Zealand white male rabbits (2-3 months old) were made anemic by injecting them sub-cutaneously with 1 % acetyl phenyl hydrazine in water for four consecutive days (Hunt et al., 1972; Ernst et al., 1978). Five days later, blood was collected into precooled heparinised 30 ml corex tubes (300 IU for 30-50 ml blood). The blood was centrifuged in a Remi high speed centrifuge at 3000 rpm for 5 minutes at 4 ^{0}C and supernatant was discarded. The Cell pellet was washed thrice (at 3000 rpm for 5 minutes) in the presence of isotonic buffered saline (7.5 mM $MgCl_2$, 5 mM KCl, 130 mM NaCl, 5 mM glucose and 10 mM HEPES pH 7.2). The supernatant and white buffy coat present over the cell pellet was carefully removed. The packed volume of the cell pellet (RBCs) was noted and an equal volume of ice cold deionised water was added to lyse the RBCs. The lysed red blood cells suspension was centrifuged at 10,000 rpm for 20 minutes at 4 ^{0}C. The supernatant lysate was collected, aliquoted and stored under liquid nitrogen for protein synthesis assays.

2.2) PREPARATION OF NEURONAL CELL EXTRACTS FROM YOUNG AND AGING RAT BRAIN:

2.2.1) Scheme for isolation of neurons:

Young (4 days), adult (180 days) and old (>730 days) rat brains

↓

Remove the white matter and blood vessels

↓

Slice the gray matter

↓

Incubate young in isolation medium, adult and old in 0.1 % trypsin in isolation medium for one hour at 37 ^{0}C

↓

Remove the supernatant and incubate adult and old rat brains in 0.1 % trypsin inhibitor in isolation medium for 5 minutes at 4 ^{0}C

↓

Pass all the age group brains, three times each through 103 μM, 80 μM and 48 μM nylon meshes

↓

Centrifuge at 760 x g for 15 minutes at 4 ^{0}C

↓

Cell rich pellets suspended in 7 % ficoll

↓

Centrifuge at 270 x g for 10 minutes at 4 ^{0}C

↓

Pellet (Neuron-enriched)

↓

Neuronal pellet suspended in 5ml of isolation medium, layered onto discontinuous ficoll gradient containing 10 %, 22 %, 28 % ficoll and centrifuged at 7800 x g for 15 min. at 4^{O}C.

↓

Neurons are obtained as a pellet in 28 % ficoll gradient.

2.2.2) Procedure for Isolation of Neurons:

Neurons were isolated from young and aging rat brains according to the procedure of Usha Rani et al., (1983). The rats were decapitated and the entire cerebral hemispheres were removed. Gray matter and white matter were separated physically. The Gray matter from cerebral hemispheres was taken, sliced into pieces and incubated at 37 ^{0} C in isolation medium having 8 % (w/v) glucose, 5 % (w/v) fructose, 2 % (w/v) ficoll in 10 mM potassium dihydrogen phosphate buffer, pH 6.0 and 0.1 % trypsin for 60 minutes. After the incubation trypsin containing medium was removed and equal amount of isolation medium containing 0.1 % soya bean trypsin inhibitor was added and chilled on ice for 5 minutes. The remaining procedure was carried out at 0-4 ^{0} C. The trypsin inhibitor medium was discarded. The tissue was washed with ice cold isolation medium and then passed three times each through nylon meshes of pore sizes 103 μM, 80 μM and 48 μM successively using porcelain hirsch funnel and glass rod. The resulting crude cell suspension was centrifuged at 760 x g for 15 minutes. The supernatant was discarded and the cell enriched pellet which consisted of both neurons and astrocytes was suspended in 20 ml of 7 % ficoll in isolation medium. The cell suspension thus obtained was centrifuged at 270 x g for 10 minutes to obtain a pellet composed mostly of neurons. The pellet was suspended in 5ml of isolation medium and the suspension was loaded onto discontinuous ficoll gradients for further purification. Gradients were prepared in the polycarbonate gradient centrifuge tubes from the bottom up, of 5 ml each of 28 %, 22 % and 10 % ficoll (w/v) in isolation medium. The cell suspension was loaded onto 10% ficoll and centrifuged at 7800 x g for 15 minutes. Neurons were collected as a pellet in 28 % ficoll gradient. Neurons were collected from the gradients, washed once with isolation medium and twice with 1x phosphate buffered saline.

2.2.3) Preparation of cell extracts:

The neuronal brain cells thus obtained were lysed in a lysis buffer containing 20 mM Tris-HCl pH 7.6, 80 mM KCl, 2 mM magnesium acetate, 1 mM DTT and 1 mM PMSF and sonicated at 10 amplitude for 10 seconds. The suspension was centrifuged at 10000 rpm for 15 minutes at 4 ^{0}C. The supernatant was used as the enzyme source to study the protein synthesis.

2.2.4) Protein estimation in the brain cell extracts:

Protein was estimated in the neuronal brain cell extracts by Bradford's Biorad reagent (Bradford, 1976) as per the instructions of the manufacturer.

2.3) Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE):

Proteins were separated on a modified Laemmli method (1970). The 10 % separation gel mix, 30 ml contained 7.5 ml of 1.5 M Tris-HCl pH 8.8, 10 ml of 30 : 0.8 acrylamide: bisacrylamide mixture, 0.3 ml of 10 % SDS, 0.2 ml of 10 % ammonium per sulphate, 75 µl of TEMED and 12.0 ml of double distilled water. The 4.5 % stacking gel mix in a total volume of 6 ml contained 0.9 ml of 30 : 0.8 acrylamide : bisacrylamide, 1.5 ml of 0.5 M Tris-HCl pH 6.8, 0.1 ml of 10 % SDS, 0.06 ml of 10 % ammonium per sulphate, 6 µl of TEMED and 3.6 ml of double distilled water. Protein samples were prepared in a sample buffer containing Tris-HCl pH 6.8, glycerol, SDS, β-mercaptoethanol and bromophenol blue. Vertical slab gel

electrophoresis was carried out at 120 volts with Tris-SDS-Glycine buffer until the dye front ran into the lower buffer tank. The gel was stained by coomassie R250 stain and destained with 7 % acetic acid and 50 % methanol solution.

2.4) WESTERN BLOTTING:

The proteins were electrophoretically separated on a 10 % SDS-PAGE gel and transferred onto a nitrocellulose membrane in a Towbin transfer buffer containing 25 mM Tris, 195 mM glycine and 20 % methanol for 3 hours at 70 volts (Towbin et al., 1979). After the transfer, the membrane was stained with ponceau S red stain to check that the transfer had occurred and also to mark the molecular weight marker proteins. The stain was removed by thoroughly rinsing the membrane with water. The membrane was blocked with 5 % non-fat milk powder in TBS containing 10 mM Tris-HCl pH 8.0, 150 mM NaCl for one hour. Thereafter the membrane was placed in primary antibody solution with the required dilution in TBST (TBS with 0.05% Tween-20) for overnight. The membrane was later washed thrice with TBST for 10 minutes each time to remove the unbound antibody. Later, the membrane was incubated in TBST containing the appropriate secondary antibody anti-IgG-AP conjugate for 2 hours. The membrane was once again washed thrice with TBST for 10 minutes each time. Then the membrane was developed with a colour development solution with NBT (66 µl) and BCIP (33 µl) as substrates in 10 ml of AP buffer containing 100 mM Tris-HCl pH 9.5, 100 mM NaCl and 5 mM magnesium chloride. The colour development was arrested by washing the membrane with double distilled water. The membrane was air dried and stored in between filter papers and protected from the light.

2.5) *IN VITRO* PHOSPHORYLATION:

In vitro phosphorylation assays were carried out by incubating rat brain neuronal cell extracts with (γ^{32}-P) ATP (3000 Ci/mmole) for 7 minutes in a 20 µl cocktail containing 20 mM Tris-HCl pH 7.8, 2 mM Mg^{2+}, 80 mM KCl and 30 µM ATP. The protein kinase assays were terminated by addition of 2x SDS sample buffer (Tris-HCl pH 6.8, SDS, glycerol, β-mercaptoethanol and bromophenol blue). The samples were heated for 3 minutes in boiling water and electrophoretically separated on 10 % polyacrylamide–0.1 % sodium dodecyl gel. The gel was dried and analyzed by autoradiography.

2.6) DETERMINATION OF TOTAL PROTEIN SYNTHESIS IN BRAIN CELLS:

2.6.1) Preparation of Crude Brain Cells:

The crude brain cells were prepared from young, adult and old rats. The rats were decapitated, white matter and blood vessels were removed physically. The Gray matter was sliced in artificial cerebro spinal fluid containing 124 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1.2 mM KH_2PO_4, 30 mM glycyl-glycine, 10 mM glucose, pencillin and streptomycin (pH 7.4). The cell suspension was passed through 330 µM nylon mesh four times. The resulting suspension was centrifuged at 1000 rpm for 5 minutes and the cell rich pellet was suspended in artificial cerebro spinal fluid.

2.6.2) [^{35}S] Methinone Uptake and Incorporation:

To the crude brain cell suspension 20 μCi of [^{35}S] methionine (1000 Ci/mmole) was added and incubated at 37 ^{0}C for 60 minutes. Aliquots were taken out at 60 minutes, centrifuged and pellet was washed once with artificial cerebro spinal fluid. The final pellet was suspended in artificial cerebro spinal fluid. The aliquots were spotted on two sets of GF/C filters. One set of filters were dried and counted directly in a liquid scintillation counter to determine uptake. Another set of filters was kept in 10 % cold TCA for 5 minutes. Afterwards, the filters were washed for 5 minutes with 5 % cold TCA and boiling TCA. Thereafter filters were washed with ethanol, dried and radioactivity was counted in a liquid scintillation counter to determine incorporation of labelled amino acid [^{35}S] methionine into trichloroacetic acid precipitable proteins. The percent incorporation of labelled amino acid into trichloroacetic acid precipitable proteins was determined from the total label taken up by these cells (incorporation / uptake x100).

2.7) DETERMINATION OF LEVELS OF DNA POLYMERASE β IN YOUNG AND AGING RAT BRAIN EXTRACTS:

2.7.1) Preparation of Brain extracts from young and aging rat Brain:

The young, adult and old rats were decapitated and 10 % homogenates of the brain tissues were prepared by homogenizing sliced brains in DNA polymerase extraction buffer containing 20 mM Tris-HCl pH 7.5, 0.1 mM β – mercaptoethanol, 1 mM $MgCl_2$, 0.1 mM EDTA, 5 % glycerol, 0.1 % triton x-100, 0.5 M KCl and protease

inhibitors like pepstatin, leupeptin (both 1 μg/ml) and 0.5 mM phenylmethyl sulfonyl fluoride (PMSF). The homogenate was kept at 0 - 4 ^{o}C for one hour and then centrifuged at 100000 x g for one hour in a Beckman ultracentrifuge. The supernatant was used as a source of DNA polymerase β.

2.7.2) Activity gel assay of DNA Polymerase β:

Procedure followed here is essentially same as previously described (Suzuki et al., 1991; Hirose et al., 1989). Brain extracts of young, adult and old animals containing 25 and 40 μg of protein were run on 10 % polyacrylamide gel containing 150 μg/ml of 'activated' calf thymus DNA. After the electrophoretic run the gel was incubated in the renaturation buffer containing 50 mM Tris-HCl pH 7.5, for 90 minutes, with two changes. Then the gel was left at 4 ^{o}C for 17.5 h in 50 mM Tris-HCl pH 7.5 / 1 mM EDTA followed by 6.5 h in the same buffer with 1 mM DTT. The gel was immersed in the incubation buffer, 50 mM Tris-HCl pH 7.5, 7 mM $MgCl_2$, 1 mM DTT, 12 μm each of dATP, dGTP and dTTP and 60 μCi (α-^{32}P) dCTP (3000 Ci/mmole) for 17 h at 37 ^{o}C. The gel was then thoroughly washed with cold 5 % TCA / 1 % sodium pyrophosphate for 40 h with 3 - 4 changes. The gel was dried and autoradiographed.

2.7.3) Levels of DNA Polymerase β of young, adult and old rat brain by Immunoblotting:

About 30 μg of protein from these tissues were electrophoretically separated on 10 % polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was blocked for 1 h with 3 % goat serum in 10 mM Tris-HCl pH 7.8, 0.15 M NaCl (TBS) and incubated overnight with polyclonal primary antibody (1:3000) to rat liver β-

polymerase (Hirose et al., 1989) in TBS buffer containing 0.3 % goat serum. Then blots were washed four times with TBS containing 0.1 % triton x-100 and then incubated with secondary anti rabbit IgG antibody conjugated to horse radish peroxidase for 2 h. The immunoblot was washed again four times with TBS buffer containing 0.1 % triton x-100 and developed with diaminobenzidine and hydrogen peroxide.

2.7.4) RNA preparation and Northern dot blotting:

Three pooled brains from young, adult and old rats were dissected and immediately frozen on liquid nitrogen. Tissues were powdered and RNA was extracted as per the procedure of (Chomczynski and Sacchi, 1987) which is as follows. The powdered tissue was homogenized with 4 M guanidium isothiocyanate containing 25 mM sodium citrate buffer, pH 4.0, 0.1 M β-mercaptoethanol, 0.5 % sarcosyl, 10 ml of water saturated phenol, 2 ml of chloroform: isoamyl alcohol mix (49:1) and thoroughly mixed. The final suspension was cooled on ice for 15 minutes and centrifuged at 10000 x g for 20 minutes at 4 ^{O}C. The aqueous phase was mixed with isopropanol and RNA precipitated overnight at –20 ^{O}C. The samples were centrifuged at 10000 x g for 20 minutes at 4 ^{O}C, resulting pellet suspended in homogenization medium and RNA precipitated with ice cold isopropanol at –20 ^{O}C for 1 hour. Then the samples were centrifuged at 10000 x g for 10 minutes at 4 ^{O}C, pellet suspended in 75 % ethanol and resulting RNA pellet was suspended in formamide for further use. Northern dot blotting was performed as described by (Hirose et al., 1989). 15-30 μg of RNA was dot blotted on to Genescreen plus membrane (Dupont, USA) and prehybridized for 6 h at 42 ^{O}C in 50 % formamide, 5X sodium saline citrate (SSC), 65 mM KH_2PO_4, 0.1 % Polyvinylpyrrolidine (PVP), 0.1 % ficoll, 1 % BSA, 5 mM EDTA and 100 μg/ml of calf thymus DNA. Hybridization was performed at 42 ^{O}C for 24 h in

50 % formamide, 5X SSC, 0.2 % PVP, 0.02 % ficoll, 0.2 % BSA, 10 % PEG, 5 mM EDTA and 50 ng (about 10^7 CPM) of β-polymerase cDNA probe 10SL (573 nucleotides) (Hirose et al., 1989). The membrane was washed with 2X SSC containing 1 % SDS at 60 ^{o}C for one hour and then with 0.1X SSC with 0.1 % SDS at room temperature for 30 min. The blot was then autoradiographed.

2.7.5) Biochemical assay for 50 % Inhibition of DNA Polymerase β Activity (Immunotitration):

Polymerase assay was performed with 'activated' calf thymus DNA as template primer. The incubation mixture contained in a total volume of 50 µl, 40 mM Tris-HCl pH 7.5, 8 mM $MgCl_2$, 1 mM β–mercaptoethanol, 4 mM ATP, 100 µM each of dATP, dGTP, dCTP, 25 µM TTP, 5 µM of 'activated' DNA, 1 µCi [^{3}H] TTP (Sp.act. 40 Ci/mM Amersham , England) and 20 µl of the crude extract contained 90 µg of protein. Reaction mixture also contained different concentrations of β-polymerase antibody at 1:100 dilution. The reaction mixture was incubated at 37 ^{o}C for 20 min and 200 µg of DNA and BSA were added and the reaction was stopped with 1 ml of cold 10 % trichloro acetic acid containing 10 mM sodium pyrophosphate. The samples were kept on ice for 10 min and filtered through GF/C filters. Precipitate was washed twice with 5 % TCA and once with cold 95 % ethanol. The fillers were counted for radioactivity in a Beckman LS-1800 counter equipped with automatic quench correction.

2.8) PARTIAL PURIFICATION OF DNA POLYMERASE δ and ε FROM RAT BRAIN:

2.8.1) Scheme for Purification of DNA Polymerase δ and ε:

Brain homogenate in Buffer A

↓

Centrifuged at 15000 rpm for 10 minutes at 4 ^{O}C

↓

Supernatant fractionated by ammonium sulphate, 30 % - 50 %

↓

Passed through DEAE-Sephacel column

↓

Eluted with linear gradient of 0 – 600 mM NaCl

↓	↓
125 mM NaCl (DNA Polymerase δ)	**200 mM NaCl (DNA Polymerase ε)**
↓	↓
Passed through phosphocellulose	**Passed through phosphocellulose**
↓	↓
Proteins eluted with 0-600 mM NaCl	**Proteins eluted with 0-600 mM NaCl**
↓	↓
Peak activity around 350 mM NaCl	**Peak activity around 300 mM NaCl**
↓	↓
Passed through hydroxyl apatite	**Passed through hydroxyl apatite**
↓	↓
Peak activity at 200 mM phosphate in PGDT and Protein concentrated (δ)	**Peak activity at 300 mM phosphate in PDGT and Protein concentrated (ε)**

2.8.2) Partial Purification of DNA Polymerase ε:

We followed basically the protocol as described by Syvaoja et al., 1990. About 140 adult brains were homogenized in 20 mM Tris-HCl pH 7.5, 1 mM magnesium chloride, 0.1 mM EDTA. 0.1 mM β-mercaptoethanol, 5 % glycerol, 1 % triton x-100, 0.5 M KCl, protease inhibitors like pepstatin, leupeptin (both 1 μg/ml) and 0.5 mM phenyl methyl sulphonyl fluoride (PMSF) **(Buffer A)** and centrifuged at 15000rpm. The supernatant was precipitated with ammonium sulphate upto 30 % saturation. The solution was centrifuged at 20000 x g in Kubota centrifuge for 20 mins. The supernatant was raised to 50 % ammonium sulphate saturation. The precipitate was obtained by centrifugation as above and dissolved in TDEG buffer and dialysed in the same buffer against two liters. The dialysate was centrifuged to remove any precipitate that is formed and loaded directly onto a DEAE-sephacel (12 cm x 2.5 cm) column equilibrated in the buffer containing 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 20 % glycerol in 20 mM Tris-HCl pH 7.5 (TDEG). The column was washed thoroughly and eluted with a linear gradient of 0-600 mM NaCl in TDEG buffer. Sample eluting around 200 mM NaCl ionic strength (based on activity) was pooled and diluted to 1:1 and loaded on to a phosphocellulose column (8 cm x 2.5 cm) equilibrated with TDEGT buffer (20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM DTT, 20 % glycerol and 0.05 % triton x-100) containing 100 mM NaCl. The column was washed with the same buffer and then eluted with 100 ml of 0-600 mM NaCl in TDEGT buffer. Activity eluting around 300 mM ionic strength was pooled, concentrated and dialyzed with 100 mM potassium phosphate pH 7.5, 1 mM DTT, 20 % glycerol, 0.05 % triton x-100. The dialysate was chromatographed on hydroxyl apatite (5 cm x 1.5 cm) column equilibrated with 100 mM potassium phosphate pH 7.5, 1 mM DTT, 20 % glycerol and 0.05 % triton x-100 (PDGT) buffer. The column was washed with the

equilibration buffer and eluted with 0.1 M, 0.2 M, 0.3 M, 0.4M and 0.5 M potassium phosphate in PDGT buffer. Fraction showing highest activity around 0.3M phosphate in PDGT was dialyzed and used for subsequent experiments. Activity of these samples was tested with different substrates and activators.

2.8.3) Partial Purification of DNA Polymerase δ:

Protocol was essentially same as Syvaoja et al., 1990. The sample eluting at 125 mM ionic strength from DEAE-sephacel column was pooled and applied to phosphocellulose column equilibrated with TDEGT buffer containing 100 mM NaCl. The column was eluted with a linear gradient 0 to 600 mM NaCl in TDEGT. The fractions were tested for activity of DNA polymerase δ. The fraction having highest activity around 350 mM NaCl concentration was pooled, concentrated and dialyzed with 100 mM potassium phosphate pH 7.5, 1 mM DTT, 20 % glycerol, 0.05 % triton x-100. The dialysate was chromatographed on hydroxyl apatite column equilibrated with 100 mM potassium phosphate pH 7.5, 1 mM DTT, 20 % glycerol and 0.05 % triton x-100 (PDGT). The column was washed with equilibration buffer and eluted with 0.1 M, 0.2 M, 0.3 M, 0.4 M and 0.5 M potassium phosphate in PDGT buffer. Fraction showing highest activity around 0.2 M phosphate in PDGT was dialyzed and used for subsequent experiments. Activity of these samples was tested with different substrates and activators.

2.8.4) Biochemical Assay For DNA Polymerases δ and ε:

The partially purified DNA polymerases δ and ε were characterized using the following assay system in the presence of various activators and inhibitors. Poly (dA.dT) was used as a substrate for DNA polymerase δ and poly(dA).oligo$(dT)_{12\text{-}18}$ as

a substrate for DNA polymerase ε. With poly(dA.dT) or poly(dA).oligo$(dT)_{12-18}$ as template primer, the incubation mixture contained in a total volume of 100 µl, 40 mM Tris-HCl pH 7.5, 0.8 mM magnesium chloride, 5 µg of BSA, 2 % glycerol, 2 mM DTT, 25 µM TTP, 1 µCi (^{3}H) TTP (Sp. act. 40 Ci/mmole), 0.025 (A260) units of template primer and appropriate amount of partially purified extracts as source of the DNA polymerase. In either case, after a 20 minutes incubation at 37 ^{o}C, 200 µg of each of DNA and BSA were added and the reaction was terminated with 1ml of 10 % TCA containing 10 mM sodium pyrophosphate. The samples were kept on ice for 10 minutes and filtered through GF/C filters. Precipitate on the GF/C filters was washed twice with 5 % TCA and once with cold 95 % ethanol. The filters were dried and counted for radioactivity in a Beckman LS-1800 counter equipped with automatic quench correction in toulene based liquid scintillation fluid.

Using the above assay system, based on their differential sensitivities towards various inhibitors/activators the properties of partially purified DNA polymerase δ and ε in rat brain were determined. In the incubation protocols, two concentration levels of inhibitors were used. For BuPdGTP the concentrations were 1 and 200 µM, for BuAdATP the concentrations were 1 and 100 µM and for ddTTP the concentrations were 50 µM and 1 mM. We have arrived at these figures, after careful consideration of the sensitivities exhibited to these inhibitors by DNA polymerases δ, ε and the $I_{0.5}$ (the amount of analog that gave 50 % of inhibition of the polymerase activity) for DNA polymerses δ and ε with respect to a given template. Recent data about the sensitivity of DNA polymerase δ towards ddTTP were also taken into account (Prapurna and Rao, 1997; Lee et al., 1985; Wahl et al., 1986 and Dressler and Kimbro, 1987)

2.9) PURIFICATION OF PROLIFERATION CELL NUCLEAR ANTIGEN (PCNA) FROM RAT BRAIN:

2.9.1) Scheme for purification of PCNA:

Brain homogenate in buffer A

↓

Centrifuged at 15000 rpm for 10 minutes at 4 ^{O}C

↓

Supernatant fractionated by ammonium sulphate, 30 % - 50 %

↓

Passed through DEAE-52 column

↓

Eluted with linear gradient of 0 – 600 mM NaCl

↓

Peak activity of PCNA around 300 mM NaCl ionic strength

↓

Passed through hydroxyl apatite

↓

Eluted with 75 mM, 100 mM, 125 mM, 150 mM, 175 mM, 200 mM phosphate in PDGT

↓

Peak activity of PCNA at 150 mM phosphate in PDGT

↓

Fractionated by ammonium sulphate, 20 %

↓

Passed through phenyl sepharose

↓

Eluted with 10, 20, 30, 40, 50 % ethylene glycol in TDEG

↓

Peak activity of PCNA at 50 % ethylene glycol in TDEG

↓

Fractionated by ammonium sulphate, 0 - 80 %

↓

Pellet obtained after centrifugation, dissolved in 200ul of TDEG

↓

Dialyzed against TDEG

↓

Aliquoted and stored in –70 ^{O}C

2.9.2) Purification of PCNA from Rat brain:

Proliferation cell nuclear antigen was purified from adult rat brain following the protocol as described by Anne Nicholas and Aziz Sancar, 1992; Syvaoja et al., 1990 with modifications and protocol was carried out at 0–4 ^{0}C. About 70 adult brains were homogenized in a buffer containing 20 mM Tris-HCl pH 7.5, 1 mM magnesium chloride, 0.1 mM EDTA, 0.1 mM β-mercaptoethanol, 5 % glycerol, 1 % triton x-100, 0.5 M KCl, protease inhibitor like pepstatin, leupeptin (both 1 µg/ml) and 0.5 mM phenyl methyl sulphonyl fluoride (PMSF) (**Buffer A**). The homogenate was centrifuged at 15000 rpm for 10 mins. The supernatant was precipitated with ammonium sulphate upto 30 % saturation and the solution was centrifuged at 15000 rpm for 20 mins. The supernatant was raised to 50 % ammonium sulphate saturation and centrifuged at 15000 rpm for 20 mins. The pellet was dissolved in TDEG buffer containing 20 mM Tris-HCl pH 7.5, 1 mM DTT, 1 mM EDTA, 20 % glycerol, 100 mM NaCl and dialyzed against 3 litres of TDEG buffer with 100 mM NaCl. The dialysate was centrifuged and loaded on DEAE cellulose (DE52) column (10 cm x 2.5 cm) equilibrated with TDEG buffer containing 100 mM NaCl. The column was washed thoroughly and eluted with a linear gradient of 0-600 mM NaCl in TDEG buffer. The activity in the fractions was assayed and the active fractions around 300 mM NaCl ionic strength were pooled. The active pooled fractions were concentrated and dialyzed against buffer containing 75 mM potassium phosphate pH 7.5, 1 mM DTT, 10% glycerol and 0.05 % triton x-100. The dialysate was loaded onto hydroxylapatite column (5 cm x 1.5 cm), equilibrated with 75 mM potassium phosphate pH 7.5, 1 mM DTT, 10% glycerol and 0.05 % triton x-100 buffer. The column was washed with equilibration buffer and eluted in steps with 75 mM, 100 mM, 125 mM, 150 mM, 175 mM, 200 mM potassium phosphate in PDGT buffer. The

activity in the fractions was assayed and the activity eluted at 150 mM potassium phosphate ionic strength. The peak fraction was concentrated and dialyzed against TDE buffer. The dialysate was raised to 20 % ammonium sulphate prior to loading onto phenyl sepharose which was pre-equilibrated with TDE buffer containing 20 % ammonium sulphate. The column was washed thoroughly with TDE buffer containing 20 % ammonium sulphate and the proteins were eluted in steps with 10, 20, 30, 40 and 50 % ethylene glycol in TDE buffer. The activity in the fractions was assayed and the activity eluted at 50 % ethylene glycol. The peak fraction was raised to 80 % ammonium sulphate. The pellet obtained after centrifugation was suspended in 200 µl of TDEG buffer, dialyzed against TDEG buffer, aliquoted and stored in –80 ^{0}C.

2.9.3) Biochemical assay for stimulation of DNA polymerase δ activity by PCNA:

DNA polymerase δ activity in the presence of PCNA was performed with poly(dA.dT) as template primer. The incubation mixture contained in a total volume of 100 µl, 40 mM Tris-HCl pH 7.5, 0.8 mM magnesium chloride, 5 µg of BSA, 2 % glycerol, 2 mM DTT, 25 µM TTP, 1 µCi (α^{32}-P) TTP (Sp. act. 3000 Ci/mmole), 0.025 (A_{260}) units of template primer, appropriate amounts of DNA polymerase δ and purified proliferation cell nuclear antigen from adult rat brain. After 20 minutes of incubation at 37 ^{O}C, 200 µg of each of DNA and BSA were added. The reaction was terminated with 1ml of 10 % TCA containing 10 mM sodium pyrophosphate. The samples were kept on ice for 10 minutes and filtered through GF/C filters. Precipitate on the GF/C filters was washed twice with 5 % TCA and once with cold 95 % ethanol. The filters were dried and counted for radioactivity in a Beckman LS-1800 counter equipped with automatic quench correction in toulene based liquid scintillation fluid.

CHAPTER 3

PROTEIN SYNTHESIS IN AGING BRAIN: LEVELS OF INITIATION FACTORS eIF2, eIF4E AND TYROSINE PHOSPHORYLATION IN RAT BRAIN NEURONAL CELLS AS A FUNCTION OF AGE

INTRODUCTION

Protein synthesis is one of the crucial steps in gene-expression. Brain is a master organ of the body that controls all other functions directly or indirectly. It is composed of two major types of cells - the neurons and the glial cells. Neurons once differentiated are nondividing (Korr, 1980), non-erythroid and post-mitotic cells. Neurons are well differentiated very early in organism's life span (Dobbing, 1971) and neuron's life span is almost equal to that of the whole animal. They have high metabolic integrity for a long time in order to keep up the fidelity of the cellular processes (Rao, 1993). Neurons are characterized by tremendous physiological activity, which includes high level of gene expression. In neurons extracellular signals like neurotransmitters, hormones and growth factors produce diverse physiological responses and the molecular mechanisms involved in these responses are not yet fully understood (Shapiro et al., 1991). The brain cells are known to transcribe atleast 30% of genomic DNA sequences which is much higher than in other tissues and presumably neurons contain a much larger number of proteins compared to other cells. Various studies suggest that mammalian brain contains 15,000 novel brain specific proteins (Bantle and Hahn, 1976; Chikaraishi, 1979; Milner and Sutcliffe, 1983). The transcriptional activity is two to three times more in neurons as compared to cells in other organs (Tobin and Khrestchatisky, 1989). In brain, complexity of mRNA species is very high and thousands of unique and brain specific mRNA species, both Poly A^+ and Poly A^- are reported (Chaudhari and Hahn, 1983). In brain, most of the rare complex class mRNA species are present at less than one copy per cell (Chikaraishi, 1979). Eberwine et al., (1992) using a novel RNA amplification technique in a single neuron, have shown that each single neuron can be distinguished at the molecular level by its mRNA complexity.

During aging protein synthesis undergoes decremental changes in many organs and tissues and certain enzymes were shown to be altered (Dice, 1993; Rao et al., 1994). The protein synthetic activity in rat brain is high during fetal life and declines during post-natal development as the brain matures (Lerner and Johnson, 1970; Gilbert and Johnson, 1974; Fando et al., 1981). The mechanism involved in the regulation of

protein synthesis in post-natal brain is not well understood but it is suggested that regulation is exerted at the initiation step of the protein translation (Kisilevski, 1972; Dwyer and Wasterlain, 1980). Protein phosphorylation is one of the most important mechanisms in the regulation of cellular functions. In brain, the protein phosphorylation regulates neuronal function (Browning et al., 1985). Tyrosine phosphorylation plays a role in virtually every step in the development and function of a neuron, including survival and differentiation, the extension of axons to their targets and synapse formation and function. A number of synaptic molecules, such as neurotransmitter receptors, voltage-gated ion channels, enzymes and proteins involved in neurotransmitter release are tyrosine phosphorylated. The involvement of protein tyrosine kinases in long term potentiation (LTP) and/or learning and memory has been demonstrated (Grant et al., 1992). In addition, protein tyrosine kinases implicated in cell transformation and proliferation are abundant in brain (Hunter and Cooper, 1985; Hirano et al., 1988; Hunter, 1989; Sugrue et al., 1990). The activity levels and regional distribution of protein tyrosine kinases varies in the central nervous system. High protein tyrosine kinase activity is found in the cerebellum, hippocampus, olfactory bulb and pyriform cortex. The regional distribution of $pp60^{csrc}$ a well characterized protein tyrosine kinase is high in hippocampus, pyriform cortex, neocortex and followed by cerebellum and brain stem (Walaas et al., 1988). Various compounds inhibit protein tyrosine kinase activity. The isoflavone compound genistein isolated from Pseudomonas sp. (Ogawara et al., 1986) is a specific inhibitor of tyrosine-specific protein kinase. Genistein specifically inhibits tyrosine kinase activity associated with EGF receptor kinase, $pp60^{v\text{-}src}$ and $pp110^{gag\text{-}fes}$ kinases but scarcely inhibits protein kinase C activity (Akiyama et al., 1987). Genistein inhibits protein synthesis in neurons in a dose-dependent manner (Hu et al., 1993). Genistein blocks long term potentiation in hippocampal slices (O'Dell et al., 1991). The protein tyrosine kinase activity is altered in Alzheimer's disease, a neurodegenerative disorder (Shapiro et al., 1991). In Alzheimer's disease the protein phosphorylation is highly abnormal (Saitoh and Iimato, 1989). In brain tissues afflicted with Alzheimer's disease, several protein kinases are altered. The levels of protein tyrosine kinase activity in the brain tissue of Alzheimer's disease is reduced in the frontal cortex

(Shapiro et al., 1991). In Alzheimer's disease patients there is an increased production of free radicals. Iron plays a central role in free radical mechanisms. In the hippocampus of Alzheimer's disease patient's brain iron is associated with cells containing neurofibrillary tangles (NFT) and glial cells. The association of non-haem iron with glial cells in Alzheimer's patients may be a secondary response of glial cells, in particular microglia, to neuronal damage (Morris et al., 1994). It is well estabilished that apoptosis is a normal feature in developing brain and may play a role in some neurodegenerative diseases and aging as well (Sastry and Rao, 2000). Translation initiation is a complex phenomenon leading to the formation of 80S initiation complex and involves the participation of 12 eukaryotic initiation factors. Recent studies implicated that initiation factor 2 and 4E (eIF2 and eIF4E) phosphorylation may be involved in the regulation of cell growth and development (Kimball et al., 1992). Hu et al., 1993 proposed that protein synthesis initiation is modulated by protein tyrosine kinase, presumably linked to growth factor receptors, that, directly or indirectly through a protein kinase cascade, modulates ternary complex formation. Recent studies demonstrated the presence of eIF2 in the nucleus, its interaction with DNA-dependent protein kinase (DNA-PK) and the phosphorylation of β-subunit of eIF2 by DNA-PK (Ting et al., 1998; Goldstein et al., 1999). These findings suggest that probably the role of eIF2 is not limited to translational initiation but it may well be associated with other functions such as DNA repair. In addition to regulating protein synthesis initiation, phosphorylation of eIF2α plays an important role in growth and development (Donze et al., 1995) and apoptosis (Srivastava et al., 1998). Consistent with this idea that eIF2α phosphorylation contributes to the phenomenon of apoptosis, caspases whose activation is central to apoptosis, are found to cleave eIF2α protein on the C-terminus (Satoh et al., 1999).

Keeping in view of these recent developments on gene expression at the translational level, we have studied the age-dependent changes in protein synthesis, in protein profiles; in eIF2α and eIF4E levels; in tyrosine phosphorylation and in eIF2α kinase activity.

RESULTS

1) Age - Dependent Decrease in Protein Synthesis of Brain Cells:

Protein synthesis in brain cells was estimated from the incorporation of [^{35}S] methionine into trichloroacetic acid precipitable proteins and from the total labelled methionine taken up by these cells. The results were presented in Table 1 and Table 2. Although the uptake of [^{35}S] methionine is not significantly affected between young and adult cells, the percent incorporation of the labelled amino acid into protein (incorporation / uptake x 100) decreased significantly in adult brain cells. The protein synthetic activity was much higher in cells obtained from young rat brain and it was substantially decreased in the cells obtained from adult and old rat brains. However, the difference in protein synthetic activity between adult and old rat brains is not significantly affected. The age-dependent decline in protein synthesis observed here in brain cells is consistent with earlier observations wherein it was shown that aging caused a general reduction in protein synthesis in rat brain ***in vivo*** (Fando et al., 1980; Goldspink, 1988; Shahbazian et al., 1986), in brain slices ***in vitro*** (Fando et al., 1980; Shahbazian et al., 1986) and in cell-free systems prepared from brain (Fando et al., 1980).

2) Age-Dependent Changes in Proteins of Neuronal Cells:

Neuronal cell extracts were prepared from young, adult and old rat brains and the proteins were separated on 10 % SDS-PAGE (Fig.3.1 and Table 3) to determine the changes in protein profiles. Several significant changes were observed in various proteins with increase in aging. These include:

A) A general decline in the high molecular weight proteins and other proteins like the 25 kDa and 20 kDa in the extracts prepared from adult and old

Protein Synthesis in Rat Brain Cells As A Function of Age: Incorporation of [^{35}S] Methionine Into Proteins:

The crude brain cell suspensions from young and aging rat brains were prepared. To one milligram of crude cell suspension in 500 µl, 20 µci of [^{35}S] methionine (Sp.act. 1000 µi/mmole) was added and incubated at 37 ^{O} C for 60 minutes. The uptake and incorporation of labelled amino acid [^{35}S] methionine into trichloroacetic acid precipitable proteins was determined as described in materials and methods. The % incorporation of the labelled amino acid into trichloroacetic acid precipitable proteins was determined by incorporation / uptake x 100. Table 1 shows the uptake, incorporation and % incorporation of labelled amino acid into trichloroacetic acid precipitable proteins in the young and aging rat brain cells for 60 minutes. Table 2 shows the incorporation into proteins as relative percentages. The pattern of the results was consistent in four different experiments.

PROTEIN SYNTHESIS IN RAT BRAIN CELLS AS A FUNCTION OF AGE: INCORPORATION OF [S^{35}] METHIONINE INTO PROTEINS

TABLE – 1

Experimental Conditions	Uptake of [^{35}S] Methionine	Incorporation into Proteins	% Incorporation
Young	354279	42927	12.11
Adult	347016	24441	7.04
Old	317861	20890	6.50

TABLE - 2

Experimental Conditions	Uptake of [^{35}S] Methionine(%)	Incorporation into Proteins(%)	% Incorporation(%)
Young	100	100	100
Adult	97.9	56.9	58
Old	89.7	48.6	53.6

brains (lanes 2 and 3 respectively vs lane 1 that contains the proteins of extracts prepared from young rat brain).

B) Some of the proteins with a molecular masses of approximately 76 kDa, 31.1 kDa and 21kDa remained unchanged in the three lanes, where as,

C) The expression of some proteins like 14.6 kDa and 44 kDa increased in the neuronal cells prepared from adult and old rat brains when compared with the young neuronal cells (lanes 2 and 3 vs lane 1).

D) A new protein of approximately 27 kDa appears to be made ***de novo*** with aging.

E) The protein with a molecular mass of 22 kDa present in the young extracts disappeared altogether in the adult and old extracts.

Although the nature and function of these proteins is not clear at this time, very significant differences were observed in the protein profiles of these extracts prepared from young, adult and old rat brain neuronal cells as mentioned above.

3) Age-Dependent Reduction in eIF2α and eIF4E levels in Neuronal cells:

Previous studies suggested that the decrease in protein synthesis was due to a decrease in the rate of initiation of protein synthesis as there was a reduction in the large size class polysomes and increased ribosomal sub units and monosomes. The translational efficiency could be regulated through alterations in the activity of peptide chain initiation factors like eIF2 and eIF4E which mediate the formation of 43S preinitiation complex (eIF2.GTP.Met.tRNAi 40S ribosomes) and 48S preinitiation complex (43S Complex~mRNA) respectively. Hence we have evaluated the relative levels of some important peptide chain initiation factors like eIF2 and eIF4E. Using equal amounts of protein extract, the levels of eIF2α and eIF4E initiation factors in neuronal cell extracts are estimated with the help of human monoclonal anti-eIF2α antibody and a mouse monoclonal anti-eIF4E antibody by western blotting (Fig.3.2 and 3.3).

ıre 3.1 : Protein profile of young and aging rat brain neuronal extracts:

Extracts of neuronal cells were prepared from young, adult and old rat brains. Based on protein estimation, equal amount of protein was taken for all samples and the proteins were separated on 10 % SDS-PAGE. This is a comassie gel.

Lane M : Molecular Weight Markers

Lane 1 : Young neuronal extract

Lane 2 : Adult neuronal extract

Lane 3 : Old neuronal extract

Five different bands of the profile were quantitated and values are shown in Table 3.

FIGURE 3.1

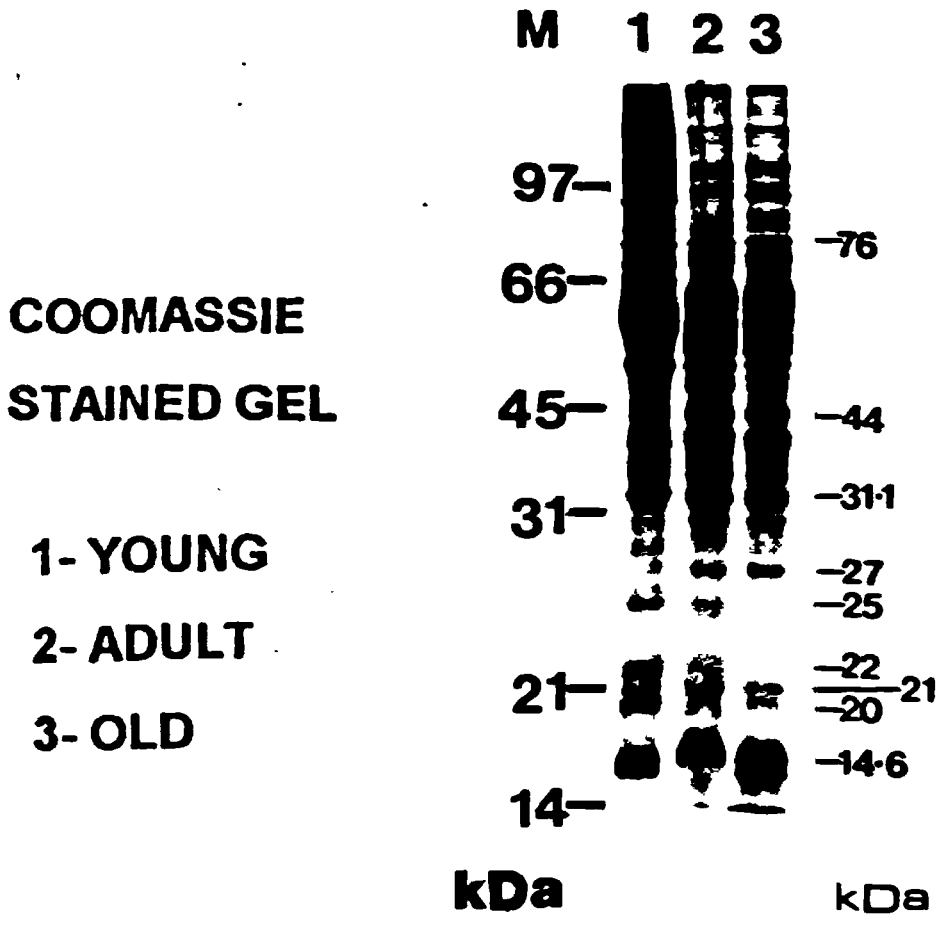


TABLE 3

S.No.	Molecular Weight (kDa)	Young (Area)	Adult (Area)	Old (Area)
1	76.0	1040.0	977.8	971.9
2	44.0	1140.0	2186.2	1891.4
3	31.1	4437.5	4927.8	4768.7
4	24.7	2719.8	2328.4	1945.6
5	14.6	5032.6	8005.3	9108.8

The monoclonal human anti-eIF2α antibody cross reacted with the 38 kDa eIF2α sub-unit in the control reticulocyte lysate (Fig.3.2, lane 1) and a 42 kDa protein in neuronal cell extracts. This finding suggests that the alpha sub-unit of the trimeric eIF2 complex of neuronal cells has a higher molecular mass compared to reticulocyte and human eIF2α and can be recognised by the human eIF2α antibody. The molecular mass of eIF2α in different systems varies anywhere from 36-42 kDa. Reticulocyte eIF2α has an estimated molecular mass of 38 kDa and wheat germ eIF2α has a molecular mass of 42 kDa (Janaki et al., 1995; Krishna et al., 1997). The neuronal eIF2α has a molecular mass of 42 kDa as has been estimated here and is consistent with the earlier observations (Cales et al., 1985). Further, the eIF2α level in neuronal extracts decreased with increase in aging (Panel B, Fig.3.2) and quantitative data was shown in Panel C, Fig.3.2. The intensity of eIF2α signal was higher in the young extracts and it decreased by 20% in extracts prepared from adult rat brain cells and by 30 % in the extracts prepared from old rat brain cells (Table 5).

The level of eIF4E initiation factor which recognizes the 5' methylated guanosine structure of eukaryotic mRNAs was also decreased in aged neuronal extracts (Fig.3.3, Panel B lane 2 vs Lanes 3 and 4). The decrease in eIF4E signal has been quantified (Panel C, Fig.3.3). The intensity of eIF4E signal was higher in the young extracts and it decreased by 11 % in the extracts prepared from adult rat brain cells and by 52 % in the extracts prepared from old rat brain cells (Table 5). The molecular mass of eIF4E however appears to be the same in reticulocyte lysates (lane 1) and in the rat neuronal cell extracts (lanes 2-4) as judged from the western blot.

The decrease in eIF2 and eIF4E levels is consistent with the reduction in protein synthesis in aged neuronal cells as has been observed here and supports the earlier observations of others that initiation step in protein synthesis may be affected in aging.

Figure 3.2 : eIF2α Protein expression in young and aging rat brain neuronal extracts:

Panel A: Neuronal cells obtained from young, adult and old rat brains were lysed as described in materials and methods. Based on protein estimation, equal amount of neuronal extracts (300 μg) was resolved on a 10 % SDS-PAGE. The figure is a comassie stained gel. The various lanes are as follows:

Lane M : Molecular Weight Markers

Lane 1 : Young neuronal extract

Lane 2 : Adult neuronal extract

Lane 3 : Old neuronal extract

Panel B: An immunoblot of rat brain neuronal extracts shown in *Panel A* showing cross-reactivity with human eIF2α monoclonal antibody. Neuronal extracts containing equal amounts of proteins were resolved on a 10 % SDS-PAGE gel, transferred onto a nitrocellulose membrane and probed with an eIF2α monoclonal antibody.

Lane M : Molecular Weight Markers

Lane 1 : Rabbit reticulocyte lysate (20μl)

Lane 2 : Young neuronal extract

Lane 3 : Adult neuronal extract

Lane 4 : Old neuronal extract

Panel C : Quantification of eIF2α band by UVP gel documentation. The bar diagram represents the area of the band.

FIGURE 3.2

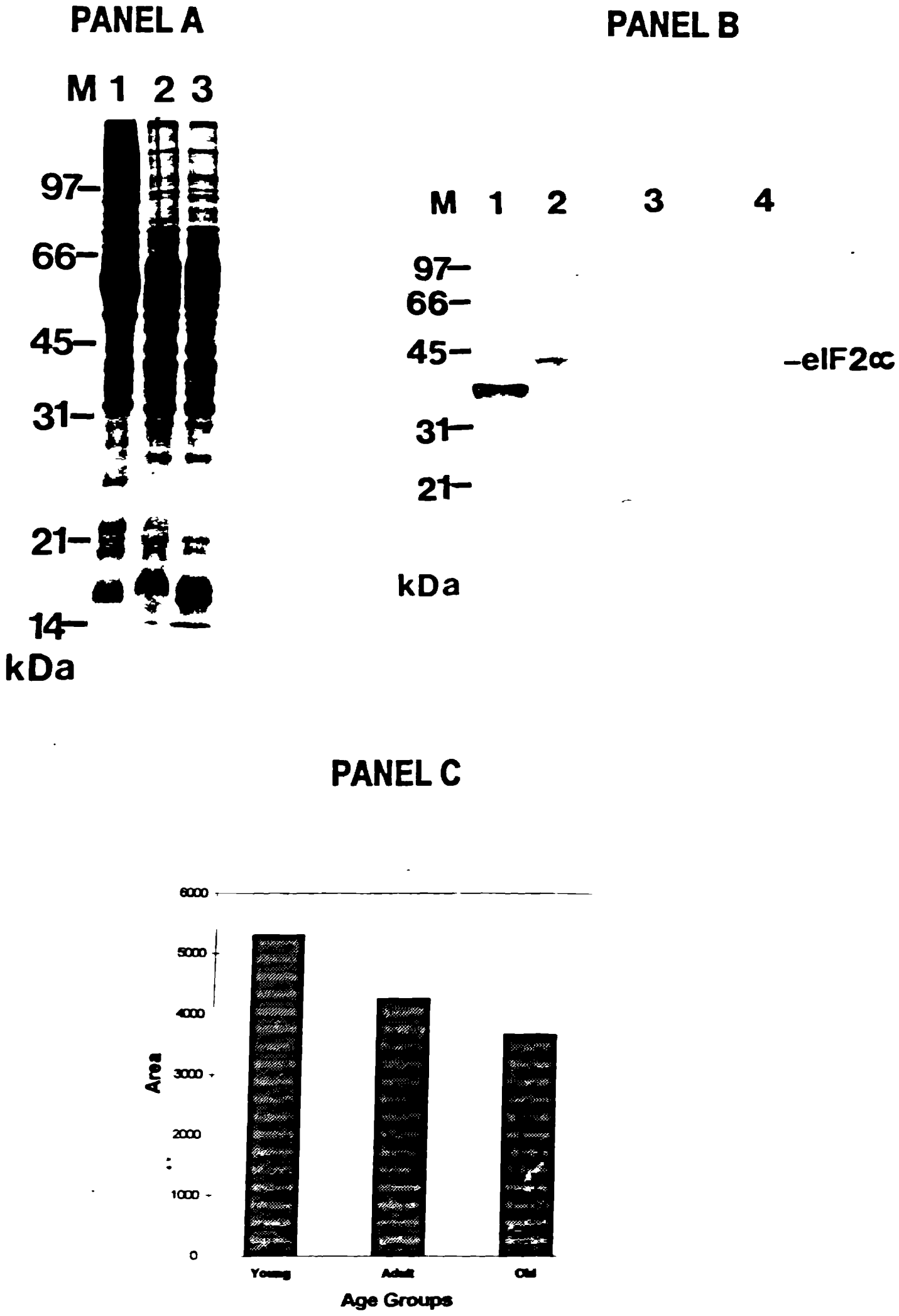

PANEL A
M 1 2 3
97–
66–
45–
31–
21–
14–
kDa
PANEL B
M 1 2 3 4
97–
66–
45–
31–
21–
kDa
–eIF2α
PANEL C
6000
5000
4000
3000
2000
1000
0
Area
Young
Adult
Old
Age Groups

Figure 3.3 : eIF4E Protein expression in young and aging rat brain neuronal extracts:

Panel A: Neuronal cells obtained from young, adult and old rat brains were lysed as described in materials and methods. Based on protein estimation, equal amount of neuronal extracts (300 μg) was resolved on a 10 % SDS-PAGE. The figure is a comassie stained gel. The various lanes are as follows:

Lane M : Molecular Weight Markers

Lane 1 : Young neuronal extract

Lane 2 : Adult neuronal extract

Lane 3 : Old neuronal extract

Panel B: An immunoblot of rat brain neuronal extracts shown in *Panel A* showing cross-reactivity with mouse eIF4E monoclonal antibody. Neuronal extracts containing equal amounts of proteins were resolved on a 10 % SDS-PAGE gel, transferred onto a nitrocellulose membrane and probed with an eIF4E monoclonal antibody.

Lane M : Molecular Weight Markers

Lane 1 : Rabbit reticulocyte lysate (20 μl)

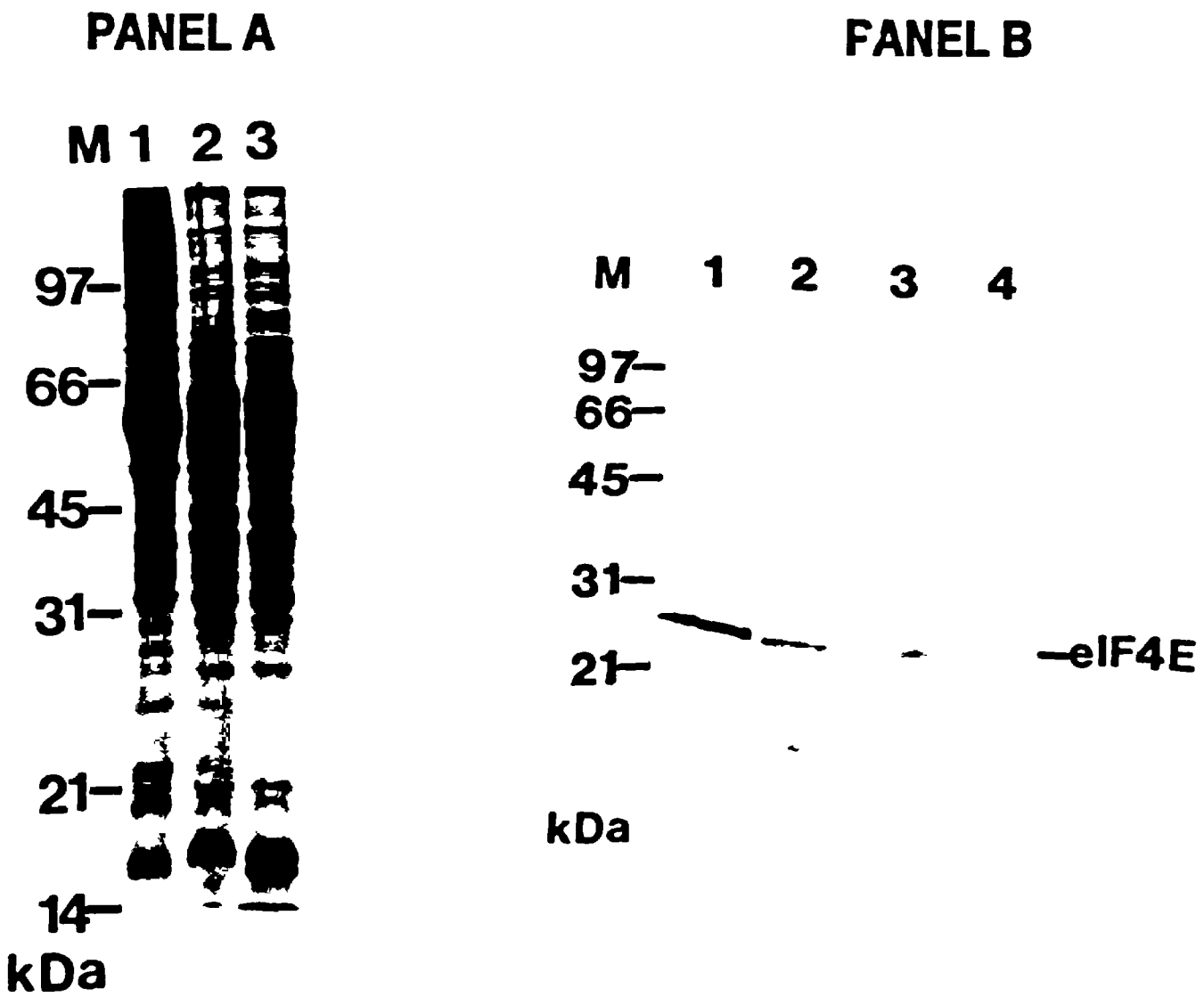
Lane 2 : Young neuronal extract

Lane 3 : Adult neuronal extract

Lane 4 : Old neuronal extract

Panel C: Quantification of eIF4E band by UVP gel documentation. The bar diagram represents the area of the band.

FIGURE 3.3


PANEL A
M 1 2 3
97–
66–
45–
31–
21–
14–
kDa
PANEL B
M 1 2 3 4
97–
66–
45–
31–
21–
–eIF4E
kDa
PANEL C
6000
5000
4000
3000
2000
1000
0
Area
Young
Adult
Old
Age Groups

4) Age-Dependent Decrease in Tyrosine Phosphorylation:

Recent studies by Hu et al., (1993) reported that protein tyrosine kinase inhibitors can depress neuronal protein synthesis thereby suggesting that phosphotyrosine kinases may also play an important role in the regulation of protein synthesis. Hence, we have studied the level of tyrosine phosphorylation in the extracts. The extracts were incubated with [γ^{32}P] ATP and with or without genistein, an inhibitor of tyrosine phosphorylation (Fig.3.4). Since genistein is prepared in 1% DMSO, the phosphorylation of the extracts was also carried out in the presence of DMSO alone as control (Fig.3.4, Panel B, lanes 3 and 6). The findings of this experiment are as follows:

a) High phosphorylation of various proteins was observed in old neuronal extracts than in young extracts in the presence of DMSO (lane 6 vs lane 3 respectively) or in the absence of the DMSO (lane 4 vs lane 1 respectively).
b) DMSO appears to reduce marginally the phosphorylation of various proteins.
c) Addition of genistein reduces the phosphorylation of several proteins significantly (lanes 2 and 5 vs lanes 3 and 6) suggesting that a number of polypeptides may be phosphorylated on their tyrosine residues.
d) The effect of genistein on phosphorylation of proteins in the rat brain neuronal extracts of young and old has been quantified (Panel C, Fig. 3.4). In case of a high molecular weight protein genistein inhibited phosphorylation by 27 % in young extracts and by 39 % in old extracts. In case of a low molecular weight protein genistein inhibited phosphorylation by 31 % in young extracts and by 46 % in old extracts (Table 4).

The phosphorylation of proteins in young extracts however are relatively less susceptible to inhibition by genistein than proteins in the old extracts (Fig.3.4 lanes 2 vs 5; Table 4).

Further the level of tyrosine phosphorylation was determined using a polyclonal anti-phosphotyrosine antibody. Our results (Fig.3.5) indicate that the antibody recognizes about four polypeptides in the young extracts (Fig.3.5, Panel B, lane 2) in the region around 60 - 64 kDa and 43 - 45 kDa. The 43-45 kDa polypeptides are strongly recognised by the antibody than the 60-64 kDa polypeptides. The antibody cross reacted with the 43 - 45 kDa polypeptides only in the adult and old extracts (lanes 3 and 4) and the signal intensity in these extracts was found to be weak compared to the signal intensity observed in the young extracts and has been quantified (Panel C). The intensity of 43 - 45 kDa polypeptide is relatively higher in the young extracts and is decreased by 29 % in the extracts prepared from adult rat brain cells and by 61 % in the extracts prepared from old rat brain cells (Table 5). These findings suggest that the adult and old neuronal cells have relatively low level of tyrosine phosphorylation compared to the extracts obtained from the cells of young rat brain. These findings are consistent with idea that a decline in the tyrosine phosphorylation is related to a decrease in the protein synthesis in neuronal cells (Hu et al., 1993).

5. Age-Dependent Enhancement in eIF2α Phosphorylation:

Phosphorylation of eIF2α is one of the best characterized mechanisms which is known to regulate the initiation of protein synthesis. A small amount (20 – 30 %) of phosphorylated eIF2α inhibits protein synthesis completely in many mammalian systems and yeast studied till today. This is because eIF2 (αP) inhibits another important rate limiting initiation factor called eIF2B. The latter exchanges GTP for GDP in eIF2.GDP binary complex and converts inactive eIF2.GDP to active eIF2.GTP which can then join the Met-tRNAi (initiator tRNA) and 40S

Figure 3.4: ***In vitro*** **phosphorylation of young and old rat brain neuronal extracts in presence of genistein, a tyrosine kinase inhibitor:**

Young and old neuronal extracts (50 μg) were phosphorylated *in vitro* with [γ-^{32}P] ATP without and with genistein (20 μg) as described in materials and methods.

Panel A : Comassie stained gel

Panel B : Autoradiogram of comassie stained gel

Lane M : Molecular weight marker

Lane 1 : Young neuronal extract without genistein

Lane 2 : Young neuronal extract with genistein

Lane 3 : Young neuronal extract with DMSO control

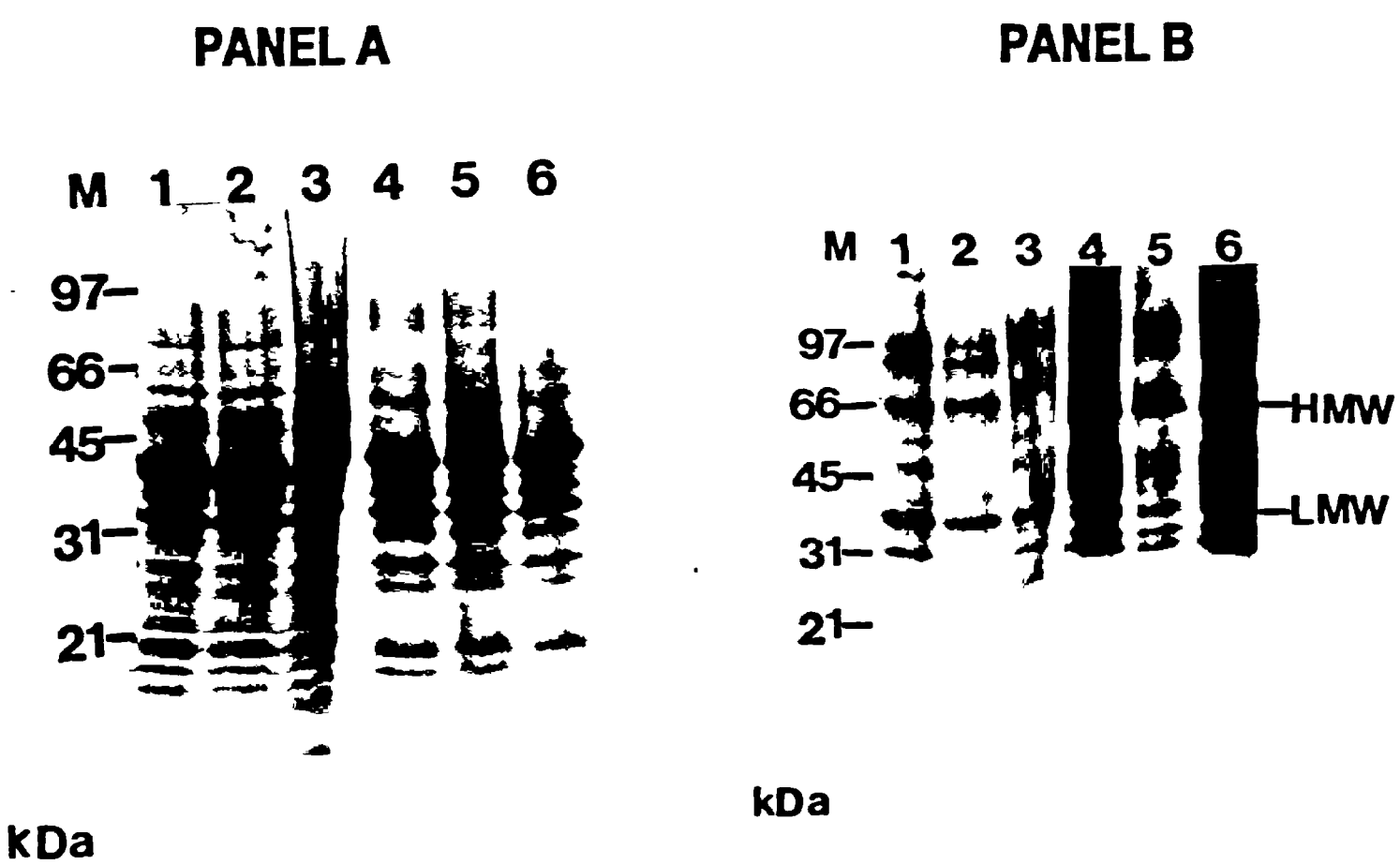
Lane 4 : Old neuronal extract without genistein

Lane 5 : Old neuronal extract with genistein

Lane 6 : Old neuronal extract with DMSO control

Panel C : **Quantified data of the phosphorylated high molecular weight (HMW) and low molecular weight (LMW) proteins by UVP gel documentation. The bar diagram represents the area of the band.**

FIGURE 3.4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M 1 2 3 4 5 6
97–
66–
45–
31–
21–
kDa
PANEL B
M 1 2 3 4 5 6
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66–
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31–
21–
HMW
LMW
kDa

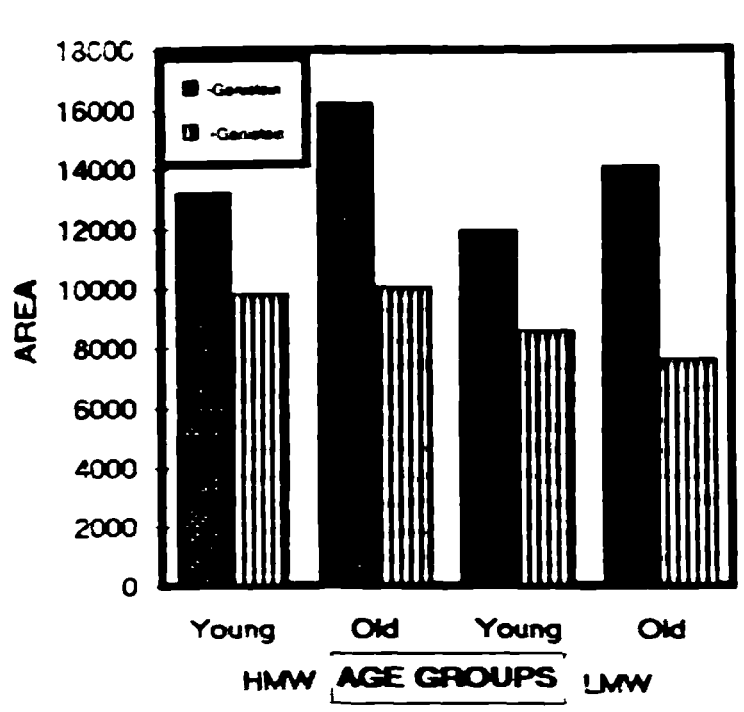

PANEL C
16000
14000
12000
10000
8000
6000
4000
2000
0
AREA
Young
Old
Young
Old
HMW
AGE GROUPS
LMW

TABLE - 4

***IN VITRO* PHOSPHORYLATION OF RAT BRAIN NEURONAL EXTRACTS IN ABSENCE AND PRESENCE OF GENISTEIN, A TYROSINE KINASE INHIBITOR.**

	Molecular weight	Young	Old
- Gen	High molecular weight	100	100
+ Gen		73	61
- Gen	Low molecular weight	100	100
+ Gen		69	54

Young and old neuronal extracts were incubated with [γ^{32}-P] ATP in absence or presence of genistein, a tyrosine kinase inhibitor. The areas of a high molecular weight (HMW) band and a low molecular weight (LMW) band on the autoradiogram were determined in the UVP gel documentation system. The percentage of the areas was calculated by considering (-) genistein condition of young and old extracts as 100 % and (+) genistein condition of young and old extracts were expressed as relative percentages.

Figure 3.5 : Expression of phosphorylated tyrosine residues in young and aging rat brain neuronal extracts:

Panel A: Neuronal cells obtained from young, adult and old rat brains were lysed as described in materials and methods. Based on protein estimation, equal amount of neuronal extracts (300 μg) was resolved on a 10 % SDS-PAGE. The figure is a comassie stained gel. The various lanes are as follows:

Lane M: Molecular Weight Markers

Lane 1: Young neuronal extract

Lane 2: Adult neuronal extract

Lane 3: Old neuronal extract

Panel B: An immunoblot of rat brain neuronal extracts shown in *Panel A* showing cross-reactivity with phosphotyrosine polyclonal antibody. Neuronal extracts containing equal amounts of proteins were resolved on a 10 % SDS-PAGE gel, transferred onto a nitrocellulose membrane and probed with a phosphotyrosine polyclonal antibody.

Lane M: Molecular Weight Markers

Lane 1 : EGF stimulated positive control protein for the antibody (10 μl).

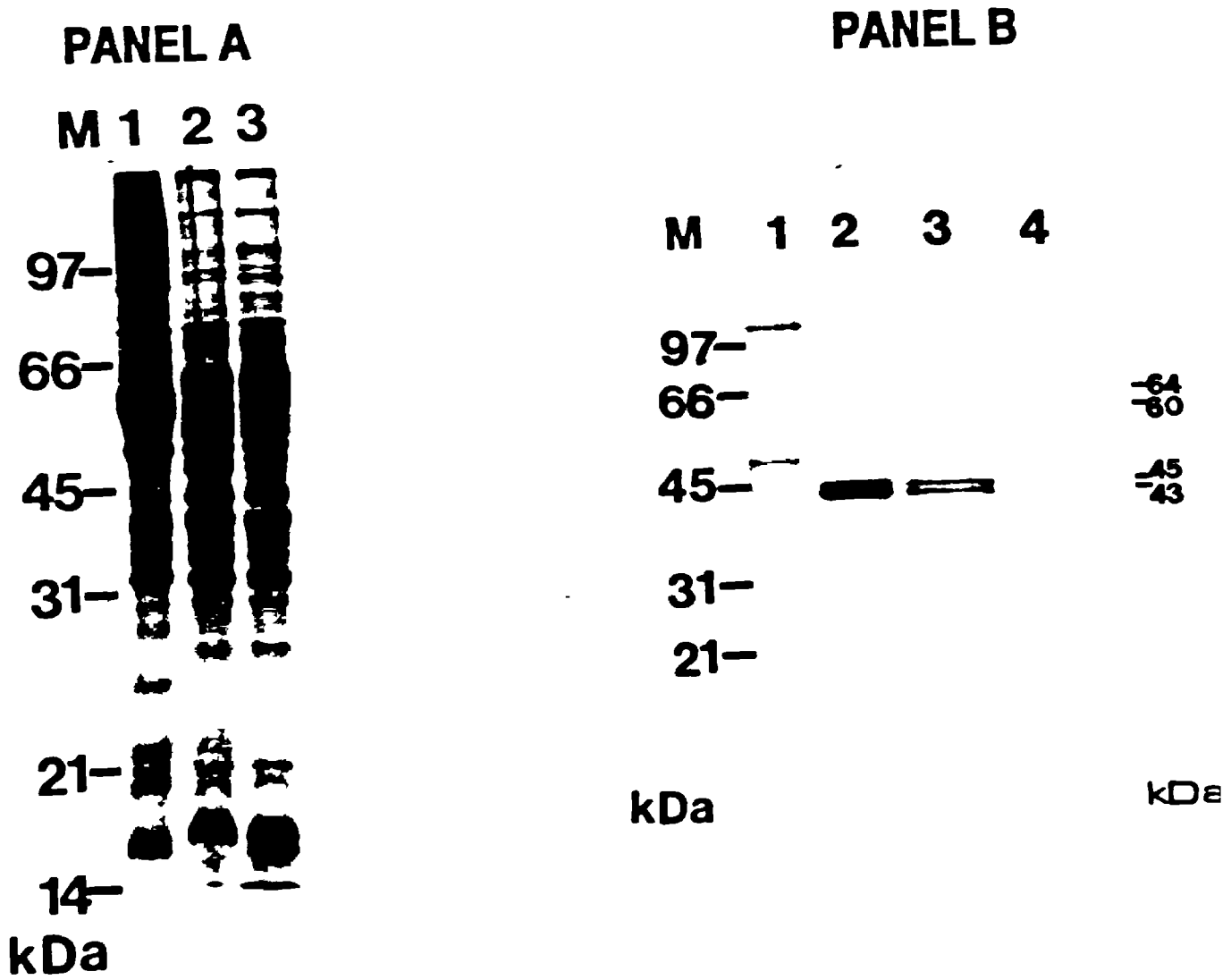
Lane 2 : Young neuronal extract

Lane 3 : Adult neuronal extract

Lane 4 : Old neuronal extract

Panel C: Quantification of phosphotyrosine band by UVP gel documentation. The bar diagram represents the area of the band.

FIGURE 3.5



PANEL C

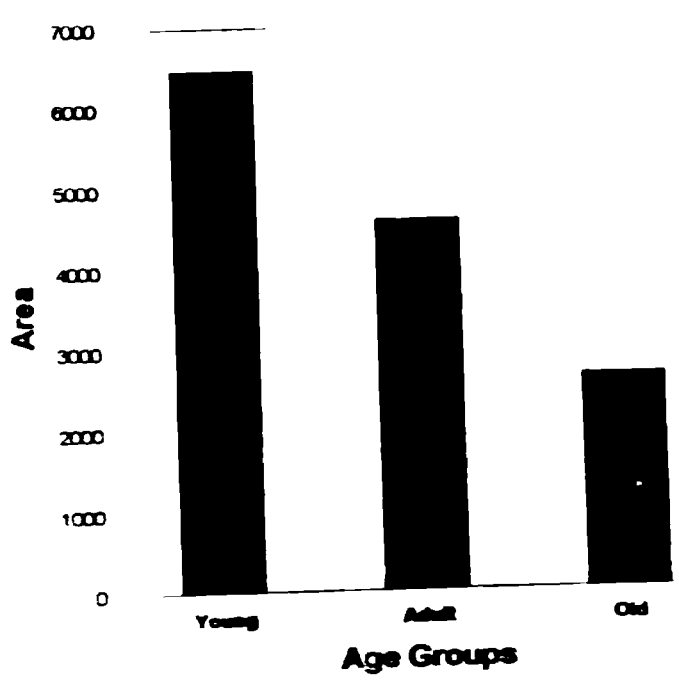


TABLE - 5

AREA (%) OF THE IMMUNOBLOT BANDS OF eIF2α, eIF4E AND PHOSPHORYLATED TYROSINE RESIDUES IN THE EXTRACTS OF RAT BRAIN NEURONAL CELLS.

Antibody	Molecular Weight (kDa)	Young (Area %)	Adult (Area %)	Old (Area %)
eIF 2α	42	100	80.2	69.2
eIF4E	24	100	89.2	47.8
Phosphorylated tyrosine	43-45	100	71	38.7

The area of eIF 2α, eIF4E and phosphorylated tyrosine residue bands of the respective immunoblots in young and aging rat brain neuronal extracts was determined in the UVP gel documentation system. The percentage of areas was calculated by considering young age group area as 100 %.

ribosomes and enter the initiation cycle. Several eIF2α kinases are known to phosphorylate eIF2α under different conditions such as heme-deficiency, viral infection, in the presence of double stranded RNA, amino acid starvation, mobilisation of calcium and other stresses that affect the functioning of endoplasmic reticulum. It is not known however if there is any eIF2α kinase like activity present in neuronal cells and is altered due to aging. PKR, a double stranded RNA-dependent kinase, is ubiquitously expressed in most of the cells. Hence we have studied here the phosphorylation of purified reticulocyte eIF2 in neuronal cells extracts prepared from young and old rat brains. The phosphorylation of eIF2 was studied in the presence and absence of poly (IC), a synthetic double stranded RNA which can activate the double stranded RNA dependent eIF2α kinase called PKR. The idea is to determine if PKR like activity is enhanced with aging. Our observations indicate:

a) That the net phosphorylation of various proteins in aged extracts is relatively high compared to the phosphorylation of various proteins in the young extracts ***in vitro*** (Fig.3.6, Panel B, lanes 1 and 2 vs lanes 3 and 4).
b) The phosphorylation of various proteins increased with the addition of poly (IC) [300 ng / ml] (lanes 1 vs 2 or lanes 3 vs 4.)
c) Phosphorylation of purified eIF2α was higher in poly (IC) treated lysates [lane 1 {- poly (IC)} vs lane 2 {+ poly (IC)}] and (lane 3 vs lane 4). The old extracts phosphorylated eIF2α more efficiently relatively (lanes 3 and 4) than the young extracts (lanes 1 and 2) in the absence of poly (IC) (lanes 1 and 3) and also in the presence of poly (IC) (lanes 2 and 4).
d) A control lane containing phosphorylated purified reticulocyte eIF2α was loaded in lane 5 to assess the phosphorylation of neuronal eIF2α by purified reticulocyte heme-regulated eIF2α kinase.

e) The effect of poly (IC) on the phosphorylation of the eIF2α in the rat brain neuronal extracts of young and old has been quantified. Poly (IC) enhanced the phosphorylation of eIF2α in the old extracts more significantly than in the young extracts (Panel C, Fig.3.6).

These findings suggest that poly (IC) or double stranded RNA dependent eIF2α kinase activity may be relatively high in the old neuronal extracts than in the young extracts and is consistent with the age-dependent decrease in protein synthesis observed here (Table 1 and Table 2).

Figure 3.6: *In vitro* phosphorylation of young and old rat brain neuronal extracts:

Young and old neuronal extracts (50 μg) were phosphorylated *in vitro* with γ-^{32}P- ATP and reticulocyte eIF2, without and with poly (IC) (300 ng/ml) as described in materials and methods.

Panel A: Comassie stained gel

Panel B: Autoradiogram of comassie stained gel

In *Panels A & B* Lane M is the Molecular weight marker

Lane 1 : Young neuronal extract without poly (IC)

Lane 2 : Young neuronal extract with poly (IC)

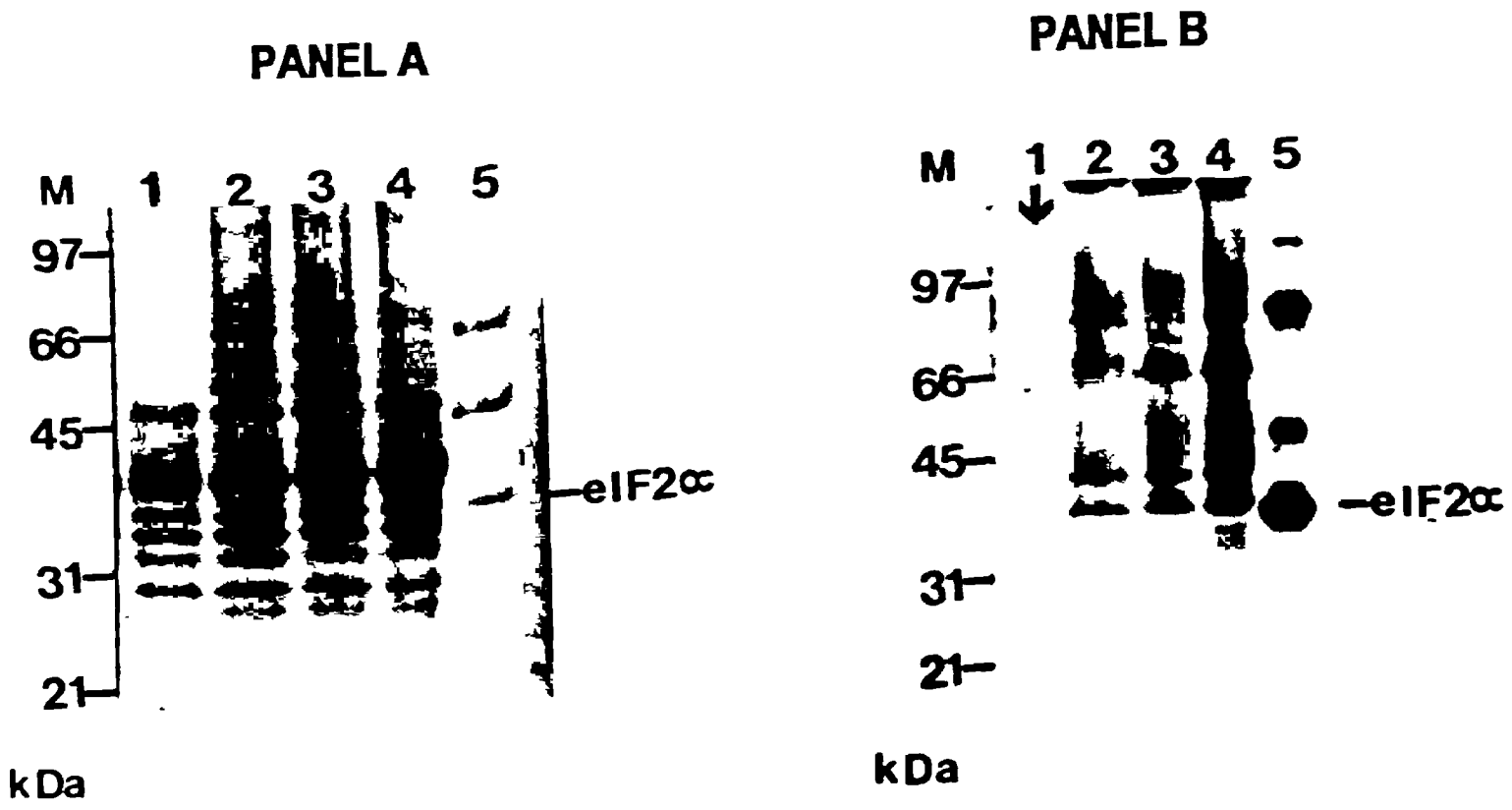
Lane 3 : Old neuronal extract without poly (IC)

Lane 4 : Old neuronal extract with poly (IC)

Lane 5 : Purified reticulocyte eIF2 (1 μl)

Panel C : Quantified data of the phosphorylated eIF2 by UVP gel documentation. The bar diagram represents the area of the band.

FIGURE 3.6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1
2
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97
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45
31
21
eIF2α
kDa
PANEL B
M
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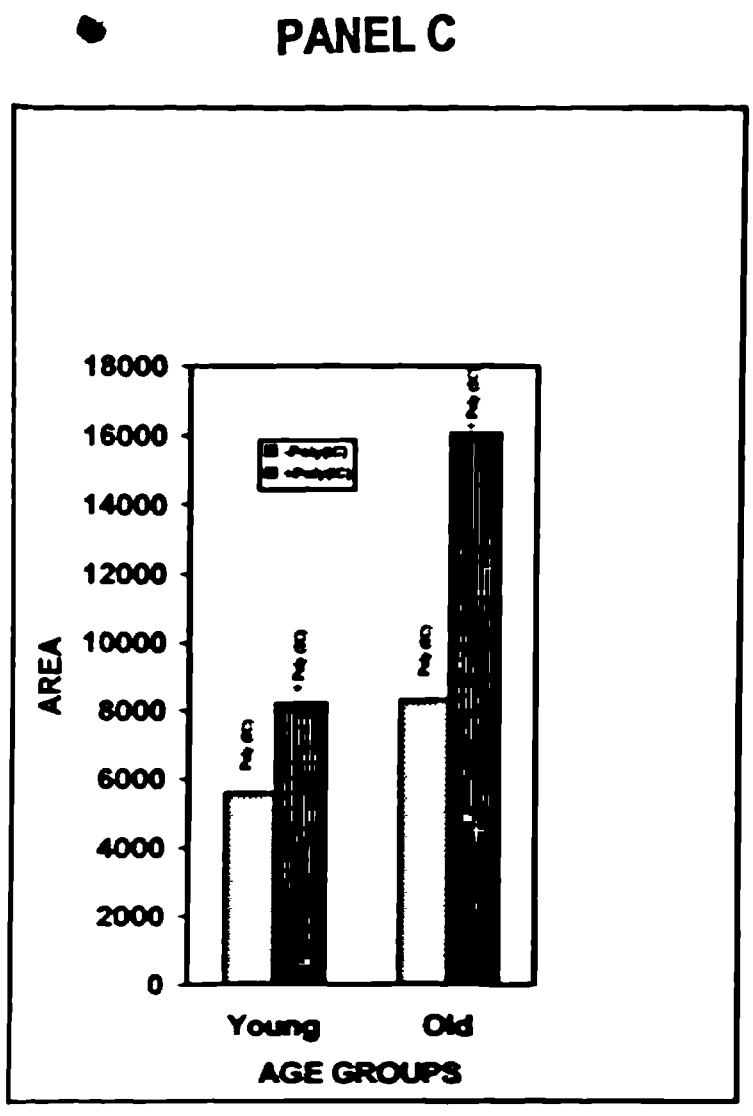
45
31
21
eIF2α
kDa


PANEL C
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
AREA
Young
Old
AGE GROUPS

DISCUSSION

Protein synthesis is a fundamental process in cell growth and maturation and is of particular interest in the central nervous system as it has been implicated in memory and information storage processes such as long term potentiation (Gustafsson and Wigstorm, 1988). Various physical, physiological, chemical and pathological conditions are known to affect gene expression at the translational level in many tissues (reviewed by Hershey, 1991). The rate of protein synthesis can be regulated by quantitative changes of the components involved in the translational machinery or through alterations in the activity of some of these components. Previous studies indicated that the rate of protein synthesis undergoes decremental changes in many organs and tissues during aging. Protein synthesis in rat brain was shown to decrease with age, as measured ***in vivo*** (Fando et al., 1980; Goldspink, 1988; Shahbazian et al., 1986), in brain slices ***in vitro*** (Fando et al., 1980; Shahbazian et al., 1986) and cell free systems prepared from brain (Fando et al., 1980). Aminoacyl tRNA synthetases which are involved in charging tRNAs with proper aminoacids are crucial for the fidelity of translation. Earlier studies showed that old rats have a rapidly degradable fraction of tRNA which has a lower ability to be aminoacylated (Yang, 1971). Recent studies in yeast indicate that accumulation of uncharged tRNAs during aminoacid starvation is thought to stimulate GCN4, a transcriptional activator which in turn stimulates the synthesis of several enzymes which are involved in the biosynthesis of aminoacids (Lanker et al., 1992). GCN4 induction is found to be an ***in vivo*** barometer of initiation factor activity in yeast (Hinnebusch, 1994). Aminoacid starvation in yeast results in the activation of an eIF2α kinase called GCN2 which regulates protein synthesis in a gene-specific manner (Wek, 1994). While phosphorylation of eIF2α decreases protein synthesis of several genes, it is also found to upregulate GCN4 and other mRNAs that code for proteins that are required for the biosynthesis of aminoacids. It is proposed that the histidyl-tRNA synthetase related domain in GCN2 protein functions to monitor the concentration of uncharged tRNAs. GCN2 like kinase or its homologue is found in drosophila (Santyo et al., 1997) and also in mammalians (Berlanga et al., 1999). The recent

studies suggest that a decrease in eIF2 level may be one of the reasons for the diminished protein synthesis in aging brain (Kimball et al., 1992). Our observations here on age–dependent decline in protein synthesis (Table 1 and 2) and eIF2 levels (Fig.3.2; Table 5) in neuronal cells are consistent with the above observations obtained from whole brain tissue. When analyzed the protein profiles of neuronal extracts (Fig.3.1; Table 3) prepared from young, adult and old rat brains, we found that there are several changes in the proteins. Some proteins are not altered, whereas, many others are either decreased or enhanced. In addition, there are some totally new proteins that were found in aged extracts (adult and old) which were not found in young extracts. It is difficult to assume that these changes in proteins can be solely explained by changes in translation alone or to changes in eIF2 levels. The present study suggests that the diminished rate of protein synthesis in brain during aging may have a multifactorial etiology. Therefore we have evaluated other activities such as the eIF2α kinase, eIF4E levels and tyrosine phosphorylation level which are known to affect the rates of protein synthesis (Kimball et al., 1992).

Our observations for the first time provide evidence that the levels of eIF4E, which is implicated in recognizing 5'-capped mRNAs, is also decreased like eIF2 in neuronal cells during aging (Fig.3.3; Table 5). In addition, we observed for the first time that aged cell extracts can phosphorylate purified eIF2α more efficiently than the young extracts suggesting that the eIF2α kinase activity may be higher in aged extracts. Indeed, addition of poly (IC) (a synthetic double stranded RNA) that stimulates PKR (double stranded RNA dependent protein kinase) has enhanced purified eIF2α phosphorylation in old extracts than in young extracts there by suggesting that PKR activity may be higher in old extracts than in young extracts (Fig.3.6). Decreased eIF4E levels and enhanced eIF2α kinase activity in old extracts are consistent with the general decline in the protein synthesis in these extracts compared to the neuronal extracts prepared from young rat brains.

Our results suggest that the neuronal eIF2α has a higher molecular mass (approximately 42-43 kDa) than reticulocyte or human eIF2α wnich is a 38 kDa protein (Fig.3.2). This observation is consistent with the earlier observations obtained from the purified eIF2 of rat brain (Cales et al., 1985).

We have also studied the levels of tyrosine phosphorylation in young, adult and old neuronal extracts. This is because protein tyrosine kinases, generally involved in cell transformation and proliferation (Hunter and Cooper, 1985) are abundant in the central nervous system (Hirano et al., 1988; Hunter, 1989; Sugrue et al., 1990). In the adult central nervous system, neurons are non-proliferating and the growth factors may be more involved in neuronal functions such as neurotransmission (O` Dell et al., 1991) or neuronal survival (Shapiro et al., 1991). Growth factors also activate protein synthesis (Montine and Henshaw, 1989; Morley and Thomas, 1991). Recent studies suggest that tyrosine kinase inhibitors cause depression of neuronal protein synthesis (Hu et al., 1993). In addition, some of the unpublished observations of Ramaiah and Chen, 1997 (abstracted in meeting on Growth, Development and Differentiation in Mahabaleshwar) suggest that purified tyrosine kinases inhibit reticulocyte heme-regulated eIF2α kinase (HRI) and thereby stimulate protein synthesis in heme-deficient lysates in which HRI is active. Our findings indicate that the adult and old neuronal extracts contain low levels of phosphorylated tyrosine containing proteins compared to the young extracts. In contrast, when these extracts were phosphorylated ***in vitro*** in the presence and absence of genistein, a tyrosine kinase inhibitor, it was observed that the various proteins of aged extracts are phosphorylated more efficiently than the young extracts and genistein caused more severe inhibition in the phosphorylation of the aged extracts than in the young extracts (Fig.3.4; Table 4). These findings are different from the ***in vivo*** observations obtained by using antiphosphotyrosine antibody (Fig.3.5; Table 5). This may be because many of these proteins in young extracts were well phosphorylated and are not accessible for any further phosphorylation ***in vitro*** whereas the proteins of old extracts were not well phosphorylated ***in vivo*** and were phosphorylated ***in vitro*** when incubated with [γ^{32}P] ATP and magnesium.

Recent studies suggest that the family of eIF2α kinases like HRI (Chen and London, 1995), GCN2 (Wek,1994) and PKR (Clemens and Elia, 1997) which are found to phosphorylate hitherto only the serine 51 residue in eIF2α, are also found to phosphorylate threonine and tyrosine residues in the place of serine 51 residue in eIF2α (Lu et al., 1999). These findings raise the possibility that there are alternate substrates for these kinases and they are probably able to phosphorylate not only serine and threonine residues but also tyrosine residues in proteins. Thus eIF2α kinases may be involved in cellular signalling and growth control pathways.

Thus from this study we have shown for first time that in neuronal cells, the levels of eIF4E decreases like eIF2 levels during aging. We have also shown for first time that neuronal extracts contain a double-stranded RNA-dependent eIF2α kinase activity which increases during aging. We have also observed an age-dependent decline in the levels of phosphorylated tyrosine residues in the neuronal cell extracts and ***in vitro*** phosphorylation suggests that the phosphate group on tyrosine residue of protein is not turned over as efficiently in the old neuronal cells as in young neuronal cells. All these findings are consistent with a general decline in protein synthesis during aging (Kimball et al., 1992; Hu et al., 1993; Fando et al., 1980; Shahbazian et al., 1986).

CHAPTER 4

LEVELS OF DNA POLYMERASE β IN RAT BRAIN DURING AGING

INTRODUCTION

Brain is a heterogeneous, intricately designed organ of diversified organization with regional compartmentalization of certain functions. The different regions of the brain are cerebral cortex, corpus striatum, hippocampus, hypothalamus, pituitary, cerebellum and brain stem. The DNA repair enzymes are widely distributed in these regions of the brain. The presence of five different DNA polymerases in mammalian cells has been shown. They are DNA polymerase α, β, γ, δ and ε (Fry and Loeb, 1986; Burgers, 1989; Burgers et al., 1990). Polymerase α has been classically regarded as an enzyme involved in DNA replication although some reports suggest its role in DNA repair as well. Polymerase β, a small and highly conserved molecule, is essentially considered as a repair enzyme in some forms of DNA damage whereas polymerase γ is required for the replication of mitochondrial DNA. Polymerase δ is involved in replication and is tightly associated with intrinsic 3'-5' exonuclease activity. The significance of the 3'-5' exonuclease activity is that it has the potential to provide a proof reading or editing function. Kunkel et al., (1987) have reported that DNA polymerase δ is more accurate than DNA polymerase α during ***in vitro*** DNA synthesis. DNA polymerase ε is a repair enzyme required at least for the initiation of repair synthesis, after which DNA polymerase α and β could participate in the process (Nishida et al., 1988; Burgers, 1998). The most striking difference between DNA polymerase δ and ε is that the activity DNA polymerase δ is dependent on proliferating cell nuclear antigen (PCNA), a co-factor, while DNA polymerase ε activity is independent of PCNA (Fry and Loeb, 1986; So and Downey, 1988). The properties of different DNA polymerases are catalogued in the **Table-6** (Wang, 1991). While the various DNA polymerases are referred to as 'replication' and 'repair' enzymes, the possibility that in an actual ***in vivo*** situation these functions could be overlapping does exist. In this laboratory we investigated the activities of putative DNA repair enzymes, including DNA polymerases in order to understand the DNA repair capacity of rat brain at different ages (Rao and Rao, 1984; Subrahmanyam and Rao, 1988). These studies have revealed that the unscheduled DNA synthesis (UDS)

TABLE - 6: EUKARYOTIC DNA POLYMERASES – STRUCTURE, LOCATION AND PROPERTIES (Ref. Wang, 1991)

Property	α	β	γ	δ	ε
STRUCTURE					
Catalytic Polypeptide (kDa)	165	38	180	125	200
Associated subunits (kDa)	70,58,48	—	—	48	55
LOCATION	nuclear	nuclear	mitochondria	nuclear	nuclear
ENZYME ACTIVITY					
3'-5' endonulclease activity	—	—	yes	yes	yes
Primase	yes	—	—	—	—
Template	activated DNA	activated DNA	Poly(rA). Oligo(dT)	Poly(dA.dT)	Poly(dA). $Oligo(dT)_{12-18}$
PCNA	—	—	—	high	—
Fidelity	high	low	high	high	high
INHIBITORS					
Aphidicolin	sensitive	sensitive	sensitive	—	—
BuPdGTP				relatively	relatively
BuAdATP	sensitive	—	—	resistant	resistant
ddTTP	relatively resistant	highly sensitive	—	sensitive	sensitive
DMSO	—	—	—	activates	inhibits
FUNCTION	replication	repair	replication	replication	repair

potential of aging neurons is quite low (Subrahmanyam and Rao, 1988). DNA polymerase β, a 38 kDA protein is essentially considered as a repair enzyme at least with respect to short patch repair of some forms of DNA damage (Wang, 1991). Suzuki et al., (1991) reported transcriptional enhancement of DNA polymerase β gene in CHO cells treated with DNA damaging agents. cDNAs for rat and human DNA polymerase β have been cloned (Zmudzka et al., 1986; Sengupta et al., 1986) and comparison of their nucleotide sequences suggested a 96 % homology (Sengupta et al., 1986). DNA polymerase β is the predominant polymerase in rat and mouse neurons (Waser et al., 1979; Rao et al., 1985). DNA polymerase β therefore has a vital role in the DNA repair process of neurons. Thus it is important to assess the levels of DNA polymerase β in rat brain of different ages.

REGULATION OF DNA REPAIR ENZYMES

Protein phosphorylation has been implicated in cell growth control and cell cycle regulation. It provides a mechanism for the modulation of proteins in eukaryotic cells and has been shown to be important in the regulation of biological activities of many proteins involved in DNA and RNA synthesis. A key regulatory protein controlling the cell cycle progression from interphase to mitosis is a 33-34 kDa serine/ threonine Kinase (Murray and Kirschnes, 1989; Draetta, 1990; Maller, 1990). In eukaryotic cellular DNA polymerases, phosphorylation DNA polymerase α is the best studied. The DNA polymerase α is a principal replicative enzyme. The catalytic polypeptide (180 kDa) and 70 kDa subunit of human DNA polymerase α are phosphorylated in a cell cycle-dependent manner. The catalytic polypeptide (180 kDa) of DNA polymerase α is phosphorylated throughout the cell cycle and is hyperphosphorylated in G2/M phase whereas 70 kDa sub unit is phosphorylated only in G2/M phase (Nasheuer et al., 1991). Wong et al., 1986 have shown that the catalytic polypeptide 180 kDa and 70 kDa subunit of human DNA Polymerase α are phosphoproteins and are phosphorylated by protein kinase C. It has been demonstrated that the activity of human DNA polymerase α is stimulated upon phosphorylation by protein kinase C *in*

vitro (Krauss et al., 1986). In the rat (Donaldson and Gerner, 1987) and human (Vishwanatha, 1989) DNA polymerase α dephosphorylation has been shown to reduce enzyme activity significantly. The DNA polymerase β, a repair enzyme is phosphorylated by protein Kinase C. However the ***in vitro*** phosphorylation by protein kinase C inactivates the DNA polymerase β (Tokui et al., 1991). The DNA polymerase β inactivated by phosphorylation retained the DNA binding potential.

Phosphorylation has also a stimulatory effect on the activities of topoisomerases I (Durban et al., 1985) and II (Ackerman et al., 1985), RNA polymerases I (Duceman and Jacob, 1980) and II (Kranias et al., 1977) and poly (A) polymerase (Rose and Jacob, 1979). In view of the importance of phosphorylation as a mechanism of regulation of DNA polymerases, we have studied ***in vitro*** phosphorylation of DNA polymerase β in the rat brain as a function of age.

UBIQUITINATION

Ubiquitination is novel mechanism for selective protein degradation (Hershko and Tomkins, 1971; Hershko and Ciechanover, 1982). Ciechanover et al., (1980) demonstrated that several molecules of ubiquitin were conjugated to the proteins to be degraded in an ATP-dependent manner by isopeptide linkages to the ε-amino lysine of the protein substrate. The C-terminus of ubiquitin is linked by an isopeptide bond to specific internal lysine residues of target proteins in a multi step process (Hershko et al., 1980).

Several types of cell stress have been shown to increase the expression of polyubiquitin C gene. These include toxins (Nenoi, 1992), heavy metals (Mulier-Taubenberger et al., 1988), starvation (Finley et al., 1987), water stress in plants (Borkird et al., 1991), irradiation (Nenoi, 1992), heat (Bond and Schlesinger, 1985), disease progression (Heggie et al., 1989) and viral infection (Latchman et al., 1987). Kenward et al., (1994) have reported that the expression of mouse polyubiquitin C gene increased several fold in the brains of mice infected with strains of scrapie.

Various short lived proteins like cyclins, Myc, Fos and P^{53} are all ubiquitinated. Various growth factors like epidermal growth factor (EGF), platelet derived growth factor (PDGF), Fibroblast growth factor (FGF) and various repair enzymes are polyubiquitinated (Seijiro et al., 1995). Ubiquitin protein conjugates are present in chronic neurodegenerative diseases (Lowe et al., 1988) and Alzheimer's disease (Lowe et al., 1993). In our work for the first time we have looked at ubiquitinated protein conjugates of DNA polymerase β in the rat brain as a function of age.

RESULTS

1. Age-Dependent Decrease in mRNA Levels of DNA Polymerase β in Young and Aging Rat Brain Extracts:

The DNA polymerase β is a single polypeptide protein. The cDNAs for rat and human DNA polymerase β were cloned (Zmudzka et al., 1986; Sengupta et al., 1986) and comparison of their nucleotide sequences suggested that their respective aminoacid sequences have 96 % homology. The testis of mouse had the highest content of DNA polymerase β mRNA followed by brain, spleen and thymus (Hirose et al., 1989). The DNA polymerase β mRNA levels in young and aging rat brain were quantified by Northern dot blotting using rat β-polymerase cDNA probe 10SL (573 nucleotides). The results of this experiment elucidate the following:

A) The expression of mRNA of DNA polymerase β was dependent on total RNA concentration and increased with an increasing concentration of total RNA (Fig. 4.1, Panel A, Lanes 1,2 and 3).

B) The mRNA levels of DNA polymerase β was higher in young rat brain and it substantially decreased in the adult and old rat brains (Fig. 4.1, Panel A, Lane Y vs Lanes A and O). However, the decrease in mRNA levels of DNA polymerase β between adult and old rat brains is not very significant (Fig. 4.1, Panel A, Lane A vs O). The decrease in the mRNA levels of DNA Polymerase β has been quantified for 15 μg (Fig. 4.1, Panel B), 20 μg (Fig. 4.1, Panel C) and 25 μg (Fig. 4.1, Panel D) of total RNA.

C) The mRNA levels of DNA polymerase β decreased in adult and old rat brain by 21 % and 29 % respectively with respect to young rat brain (Table 7).

The results were consistent in three separate experiments.

Figure 4.1: mRNA Dot Blotting of DNA polymerase β in young and aging rat brain:

Total RNA was prepared, blotted onto a nylon membrane and dot blotting was performed as described in materials and methods.

Panel A: Corresponds to the autoradiogram.

Lane 1: 15 μg of total RNA

Lane 2: 20 μg of total RNA

Lane 3: 25 μg of total RNA

Y, A and O refers to Young, Adult and Old rat brain total RNA.

Panel B, C and *D* shows the quantified data of expression of mRNA in the UVP gel documentation system. The bar diagram represents the area of the RNA dot on the autoradiogram.

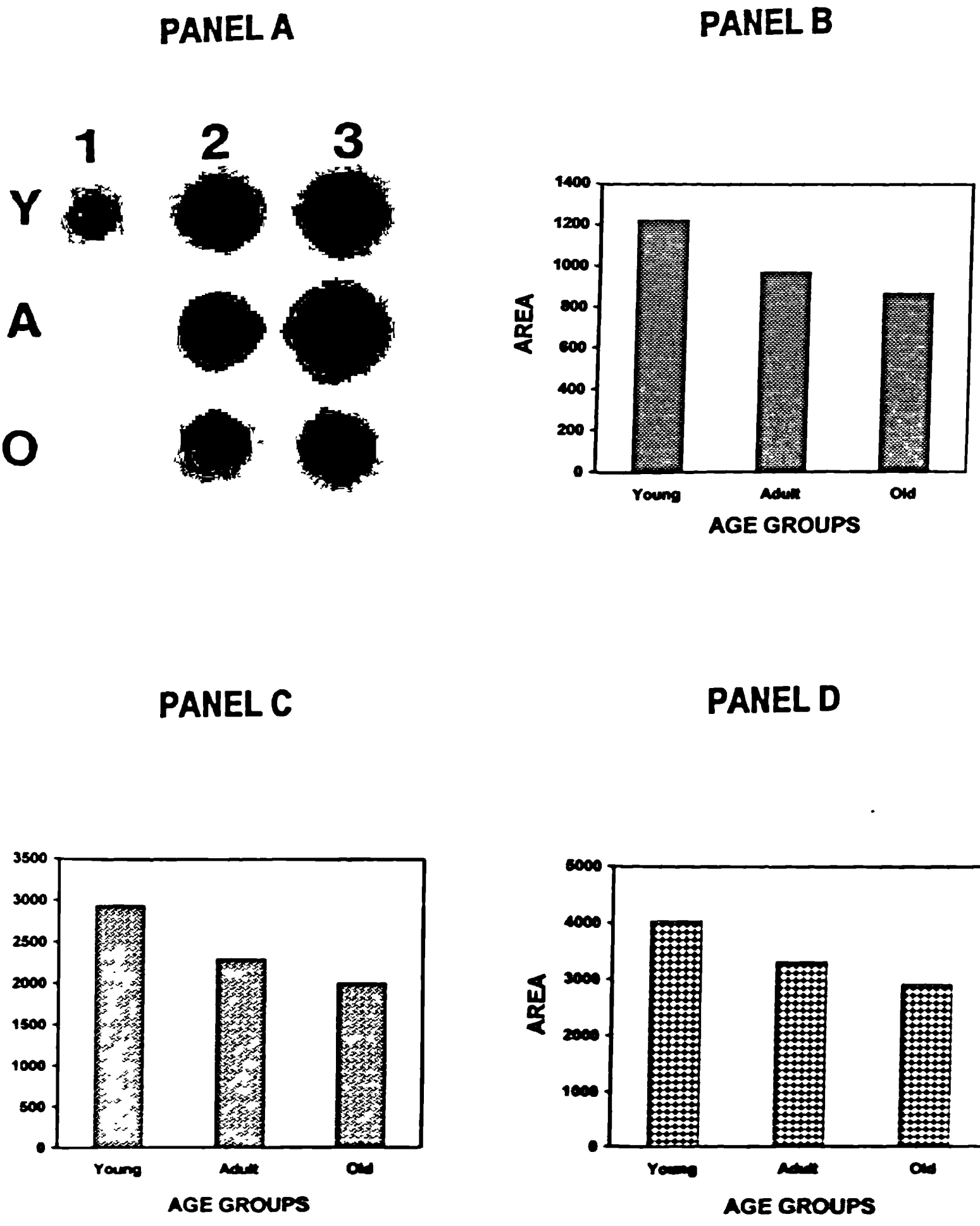
Panel B represents 15 μg of total RNA dot on the autoradiogram

Panel C represents 20 μg of total RNA dot on the autoradiogram

Panel D represents 25 μg of total RNA dot on the autoradiogram

The results were consistent in three separate experiments.

FIGURE 4.1


PANEL A
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AGE GROUPS
PANEL C
AREA
3500
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500
0
Young
Adult
Old
AGE GROUPS
PANEL D
AREA
5000
4000
3000
2000
1000
0
Young
Adult
Old
AGE GROUPS

C) The activity of DNA polymerase β decreased by 45 % in adult brain extract and by 53 % in old brain extract when compared to young brain extract (Table 7).

The results were consistent in four different experiments.

3) Age-Dependent Decrease in the Immmunological Levels of DNA Polymerase β in Young and Aging Rat Brain Extracts:

Using equal amounts of protein (30 μg) the immunological levels of DNA polymerase β was determined in the young, adult and old rat brain extracts with the help of rat polyclonal anti-polymerase β antibody in the western blotting (Fig. 4.3). The polyclonal rat anti-polymerase β antibody cross-reacted with the 38 kDa pure polymerase β (Fig. 4.3, Panel A, Lane 1) and polymerase β in the brain extracts (Fig. 4.3, Panel A, Lanes 2,3 and 4). The findings of this experiment are as follows:

A) The immunological levels of DNA polymerase β decreased in the adult and old brain extracts when compared to young brain extracts (Fig. 4.3, Panel A, Lane 2 vs Lanes 3 and 4). However the levels of DNA polymerase β increased marginally in the old brain extract when compared to adult brain extract (Fig. 4.3, Panel A, Lane 3 vs Lane 4).

B) The decrease in the levels of DNA polymerase β in the rat brain extracts has been quantified (Fig. 4.3, Panel B)

C) The immunological expression of DNA polymerase β decreased by 30 % in adult brain extract and by 20 % in the old brain extract with respect to young brain extract (Table 7).

The results were consistent in three different experiments.

Figure 4.2: Activity gel assay of DNA polymerase β in young and aging rat brain extracts:

Crude extracts containing 25 μg (lanes 1, 2 and 3) and 40 μg (lanes 4, 5 and 6) of protein from young, adult and old brain homogenates were separated by 10% SDS-PAGE gel and the activity of DNA polymerase β in different lanes was determined as described in materials and methods. Pure polymerase β (50ng) was loaded in the lane P as control.

Panel A: Corresponds to autoradiogram.

Lane M: Molecular weight markers

Lane P: Pure polymerase β

Lanes 1and 4: Young brain extracts

Lanes 2 and 5: Adult brain extracts

Lanes 3 and 6: Old brain extracts

Panel B and *C*: shows the quantified data of polymerase β band on the autoradiogram in UVP gel documentation system. The bar diagram represents the area of the band.

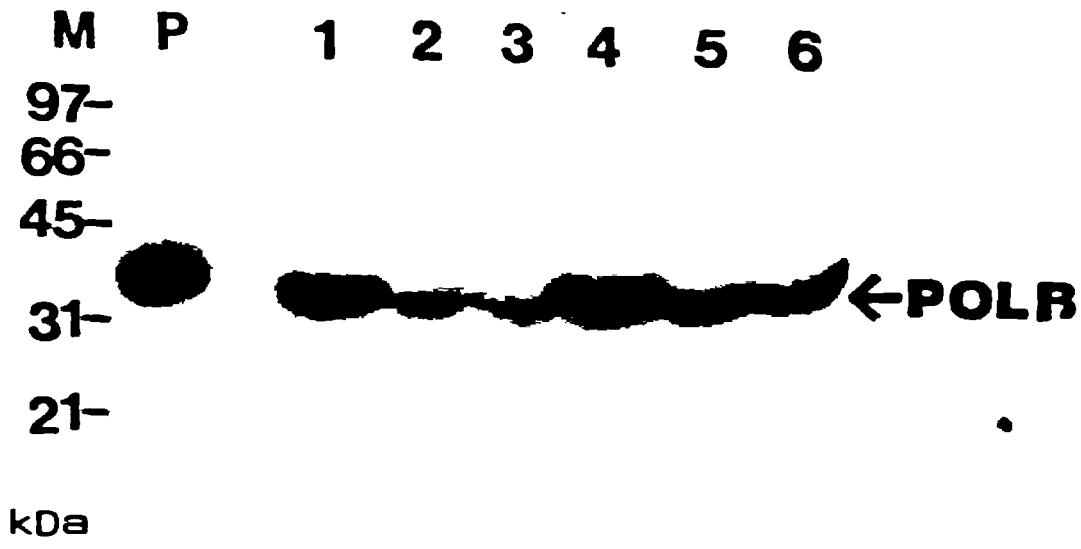
Panel B: 25 μg of brain protein extract.

Panel C: 40 μg of brain protein extract

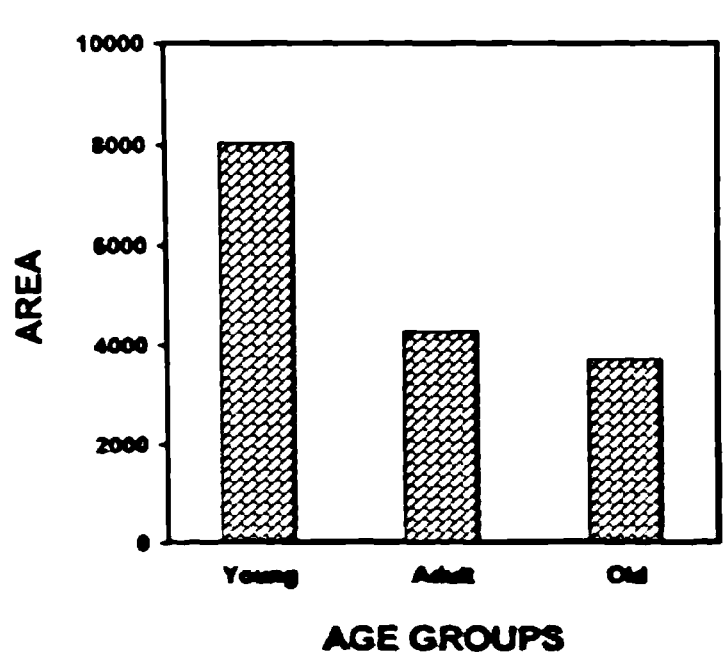
The results were consistent in four different experiments.

FIGURE 4.2

PANEL A



PANEL B



PANEL C

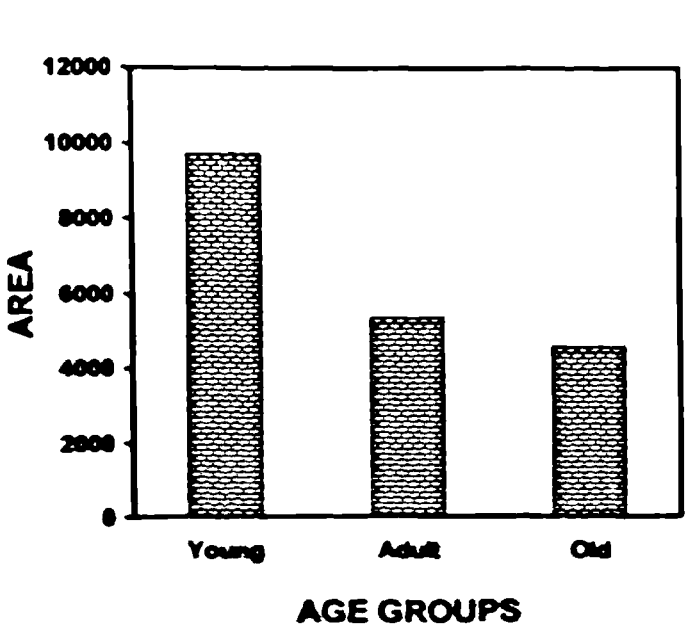


Figure 4.3: Immunoblotting of DNA polymerase β in young and aging rat brain extracts:

30 μg of protein from crude extracts of young, adult and old brain homogenates was separated by 10 % SDS-PAGE gel, transferred onto nitrocellulose membrane and immunoblotted with polyclonal polymerase β antibody as described in materials and methods.

Panel A: Corresponds to an immunoblot.

Lane M: Molecular weight markers

Lane 1: Pure polymerase β enzyme (50 ng)

Lane 2: Young brain extract

Lane 3: Adult brain extract

Lane 4: Old brain extract

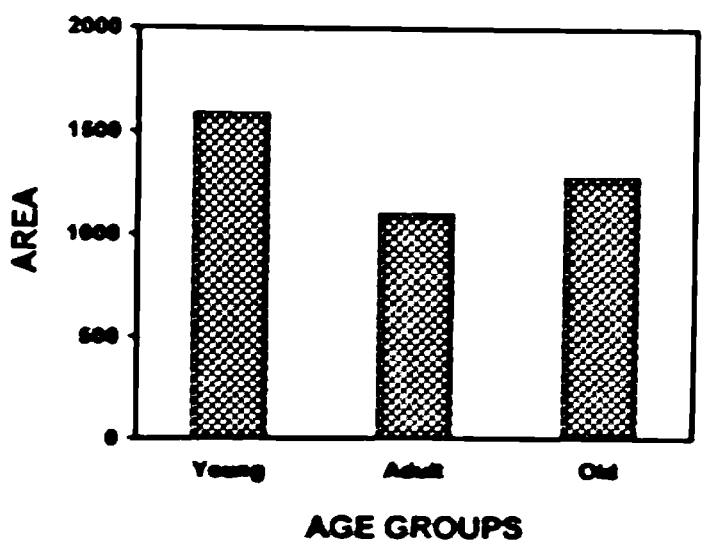
Panel B: shows the quantified data of polymerase β band on the immunoblot in the UVP gel documentation. The bar diagram represents area of the band.

The results were consistent in three separate experiments.

FIGURE 4.3

PANEL A

PANEL B



4) Immunotitration of DNA Polymerase β Activity in the Young, Adult and Old Rat Brain Extracts:

The amount of antiserum required for 50 % inhibition of DNA polymerase β activity were presented in this figure 4.4. DNA polymerase β protein from young, adult and old brain extracts whose activity was equivalent to incorporation of 60 picomoles of [^{3}H] TMP into DNA were taken and assayed in the presence of different amounts of the antibody. The following results were obtained.

A) To inhibit 50 % of DNA polymerase β activity, the young and adult extracts required about 12 and 22 µl of antibody respectively whereas old brain extracts required about 34 µl (Fig. 4.4, Table 7).

B) The total polymerase activity was inhibited by 70 % which suggests that the DNA Polymerase β is the predominant type of polymerase in the total brain homogenates (Fig. 4.4).

The results were consistent in three sets of experiments.

5) Age-Dependent Increase in the Phosphorylation Status of DNA Polymerase β in the Young and Aging Rat Brain Extracts:

As mentioned earlier phosphorylation provides a mechanism for the modulation of protein function in an eukaryotic cell. The biological activities of many of the eukaryotic DNA replication proteins are modulated by protein phosphorylation. Nasheuer et al., (1991) have reported that DNA polymerase α is phosphorylated throughout cell cycle in leukemia cell lines. Mammalian DNA polymerase β is linked to DNA synthesis associated with repair and recombination. Tokui et al., (1991) have shown that DNA polymerase β purified from recombinant rat was inactivated upon *in vitro* phosphorylation and activity recovered upon dephosphorylation with alkaline phosphatase. Using equal amounts of protein from young, adult and old rat brain extracts the level of *in vitro* phosphorylation of DNA

Figure 4.4: Biochemical assay for 50 % inhibition of DNA polymerase β activity in young and aging rat brain (Immunotitration):

DNA polymerase β activities were assayed in presence of different amounts of antibody for young, adult and old brain homogenates as described in materials and methods. Y, A and O denotes Young, Adult and Old respectively. Activity of DNA polymerase β equivalent to incorporation of 60 picomoles of [^{3}H] TMP into DNA was taken in all cases before addition of antibody. Three sets of experiments were performed and averages plotted. In this graph X-axis represents volume of antibody added and Y-axis represents % inhibition of DNA polymerase β activity.

FIGURE 4.4

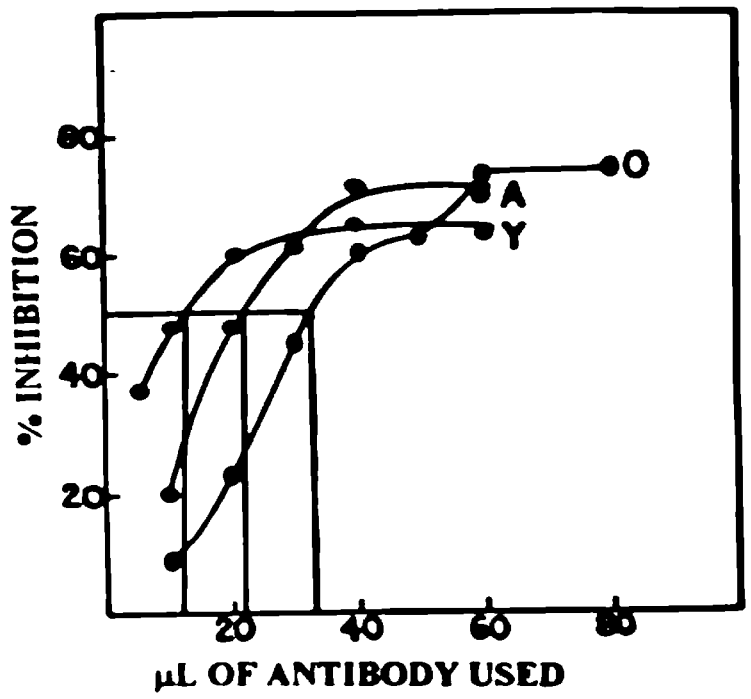


TABLE - 7

DNA POLYMERASE β LEVELS IN YOUNG AND AGING RAT BRAIN ASSESSED THROUGH DIFFERENT STRATEGIES

AGE	mRNA	WESTERN BLOT	ACTIVITY ON GEL	IMMUNOTITRATION
		PERCENTAGES		Anti serum required for 50 % inhibition (µl)
Young	100	100	100	12
Adult	79.4	69.4	55	22
Old	70.9	80.6	47	34

The levels of DNA polymerase β in young and aging rat were assessed by RNA dot blotting, activity gel assay, Western blotting and immunotitration. The area of the band corresponding to DNA polymerase β in young, adult and old brains in the respective autoradiograms and blot were estimated in a UVP gel documentation system. The densitometric scanning values of young were taken as 100 % and the values of adult and old were presented as relative percentages. The table also depicts the volume of antiserum required for 50 % inhibition of DNA polymerase β activity.

polymerase β was estimated by incubating the brain extracts with [γ^{32}P] ATP. The phosphorylation status of rat brain DNA polymerase β increased with increasing age (Fig. 4.5, Panel A). The amount of phosphorylation was more in old brain extracts when compared to young brain extracts (Fig. 4.5, Panel A, Lane 3 vs Lane 1). The increase in the phosphorylation status of DNA polymerase β upon aging has been quantified (Fig. 4.5, Panel B). The phosphorylation status of DNA polymerase β was less by 49 % and by 21 % in young and adult extracts respectively when compared to old extracts (Table 8).

The above results suggest that the decrease in the activity of DNA polymerase β upon aging may probably be due to protein phosphorylation, a post-translational modification.

6) Age-Dependent Decrease in Ubiquitinated Levels of DNA Polymerase β in Young and Aging Rat Brain Extracts:

Ubiquitination is a novel mechanism for selective protein degradation. During ubiquitination, ubiquitin molecules are conjugated to a protein via an isopeptide bond between the C-terminal glycine of the ubiquitin and ε-NH_2 of an internal lysine of the protein under consideration. This isopeptide bond is not destroyed by SDS or β-mercaptoethanol. Thus ubiquitinated proteins are seen in SDS gels as a ladder of higher molecular weight species as ubiquitin a 8.5 kDa molecule is conjugated to protein during ubiquitination. Using the above principle, we have examined the phenomenon of ubiquitination of DNA polymerase β in rat brain as a function of age. Equal amounts of protein (100 μg) from young, adult and old rat brain extracts were first immunoprecipitated with polyclonal ubiquitin antibody (1:100 dil). The immunoprecipitate blots were probed with polyclonal anti polymerase β antibody. The level of ubiquitination was observed as a ladder of high molecular protein-ubiquitin conjugates. The polyclonal anti polymerase β antibody cross reacted with 38 kDa pure DNA polymerase β protein (Fig. 4.6,

Figure 4.5: *In Vitro* phosphorylation of DNA polymerase β in young and aging rat brain extracts:

The crude extracts containing 50 µg of protein from young, adult and old brain homogenates were phosphorylated *in vitro* with [γ-^{32} P] ATP as described in materials and methods and were separated on a 10 % SDS-PAGE gel.

Panel A: Corresponds to autoradiogram.

Lane M: Molecular weight markers

Lane 1: Young brain extract

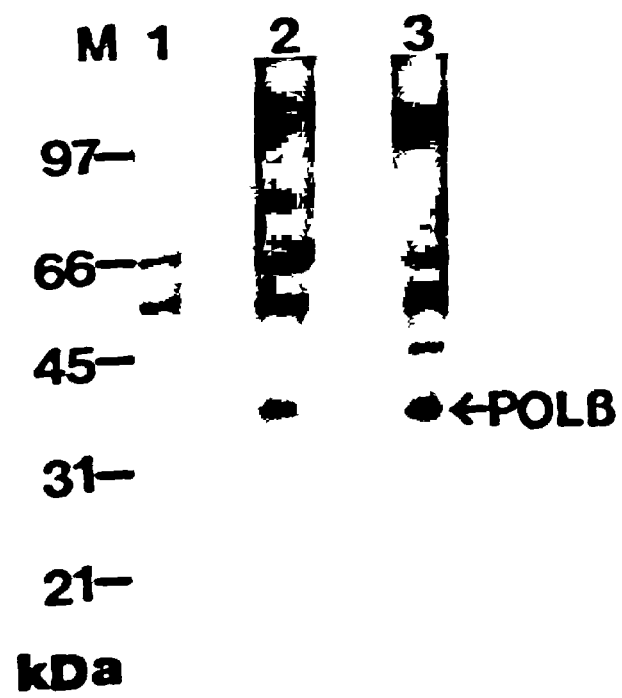
Lane 2: Adult brain extract

Lane 3: Old brain extract

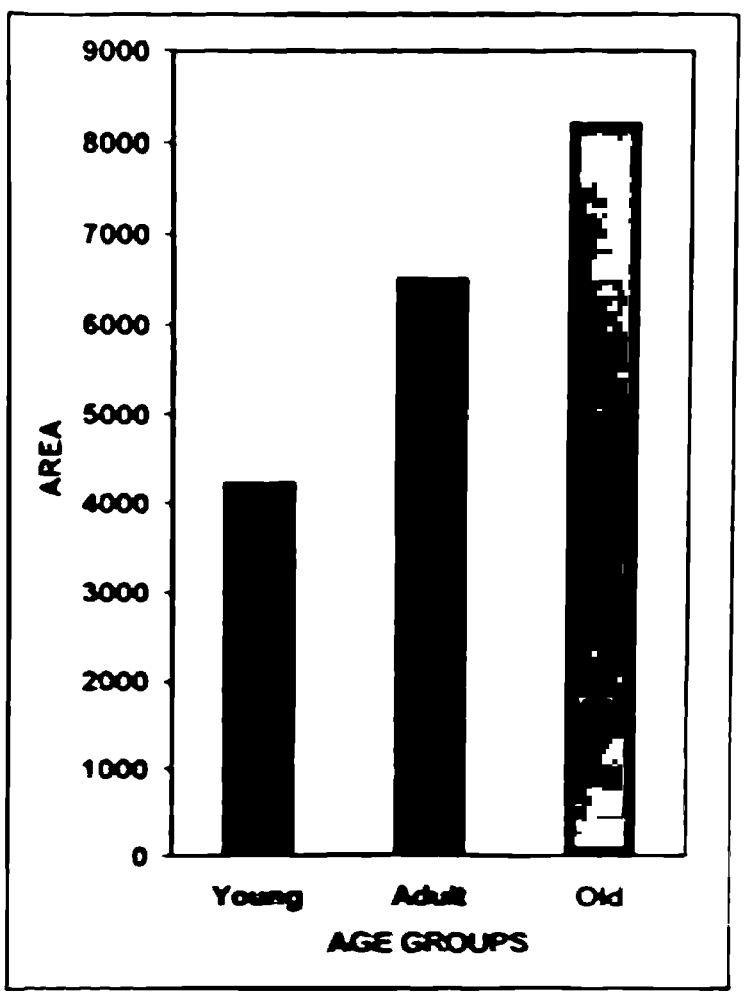
Panel B: shows the quantified data of the phosphorylated polymerase β band in the UVP gel documentation. The bar diagram represents to the area of the band on the autoradiogram.

FIGURE 4.5

PANEL A



PANEL B



Panel A, Lane 1) and 46.5 kDa DNA polymerase β protein in young, adult and old rat brain extracts (Fig. 4.6, Panel A, Lanes 2, 3 and 4). The levels of ubiquitinated DNA polymerase β was higher in young extracts and decreased in adult and old extracts (Fig. 4.6, Panel A, Lane 2 vs Lanes 3 and 4). The levels of ubiquitinated DNA polymerase β in young, adult and old rat brain extracts have been quantified (Fig. 4.6, Panel B). The ubiquitinated DNA polymerase β decreased by 22 % in adult extracts and by 29 % in old extracts when compared to young extracts (Table 9).

Figure 4.6: Expression of ubiquitinated DNA polymerase β in young and aging rat brain extracts:

100 μg of protein from young, adult and old brain homogenates were immunoprecipitated with polyclonal antibody (1:100 dilution). The immunoprecipitates were separated on a 10 % SDS-PAGE gel, transferred onto nitrocellulose membrane and probed with polymerase β polyclonal antibody as described in materials and methods.

Panel A: Corresponds to immunoblot

Lane M: Molecular weight markers

Lane 1: Pure polymerase β enzyme

Lane 2: Young brain extract

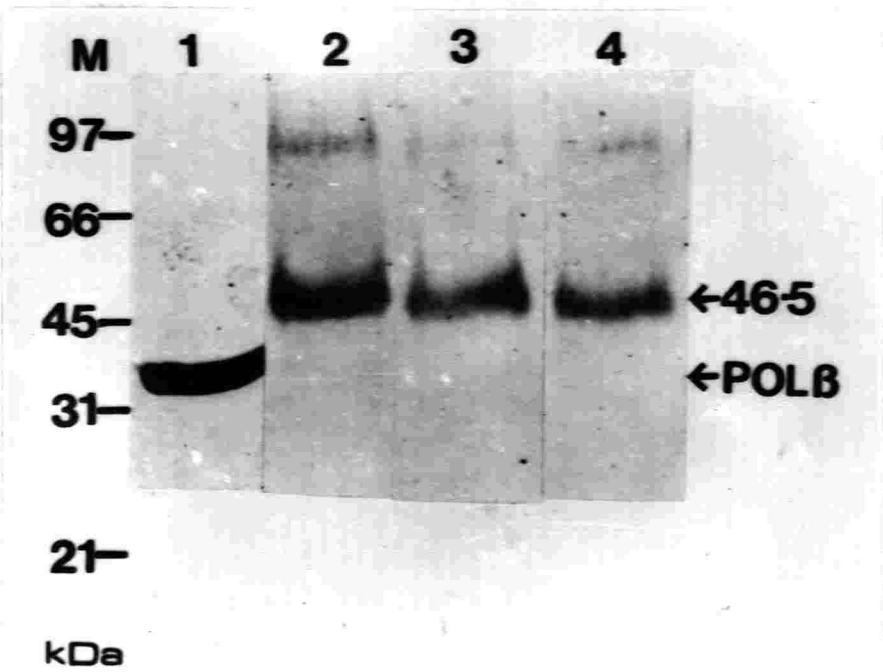
Lane 3: Adult brain extract

Lane 4: Old brain extract

Panel B: shows the quantified data of the ubiquitinated polymerase β band in the UVP gel documentation. The bar diagram represents the area of the band on the immunoblot.

FIGURE 4.6

PANEL A



PANEL B

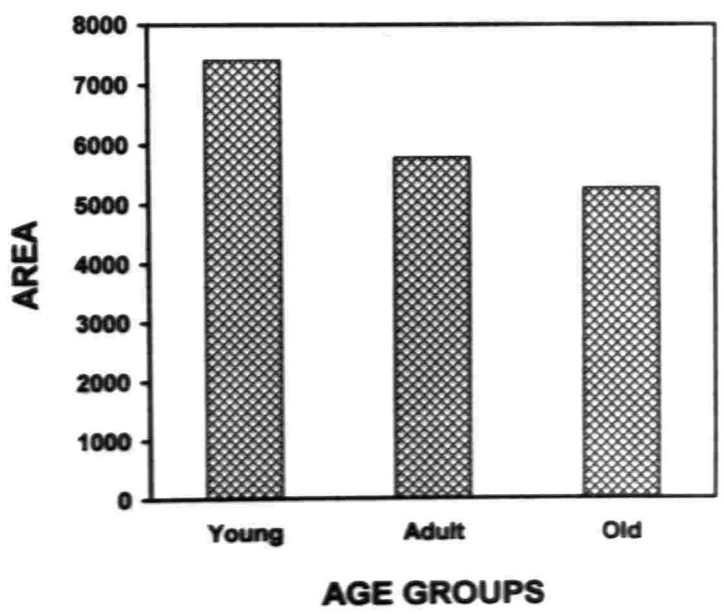


TABLE 8: DNA polymerase β from young, adult and old rat brain extracts was phosphorylated with [γ^{32}P] ATP. The area of the band corresponding to DNA polymerase β was determined in UVP gel documentation system. The percentage of the areas was calculated by considering old age group area as 100 %, and, young and adult age groups were presented as relative percentages.

TABLE 9: The area of the band corresponding to the ubiquitinated DNA polymerase β in young, adult and old rat brain extracts was determined in UVP gel documentation system. The percentage of the areas was calculated by considering young age group area as 100 %, and, adult and old age groups were presented as relative percentages.

TABLE 8

***IN VITRO* PHOSPHORYLATION OF DNA POLYMERASE β IN YOUNG, ADULT AND OLD RAT BRAIN EXTRACTS.**

AGE GROUP	PHOSPHORYLATION (PERCENTAGES)
YOUNG	51
ADULT	79
OLD	100

TABLE 9

LEVELS OF UBIQUITINATED DNA POLYMERASE β IN YOUNG, ADULT AND OLD RAT BRAIN EXTRACTS.

AGE GROUP	UBIQUITINATION (PERCENTAGES)
YOUNG	100
ADULT	78
OLD	71

DISCUSSION

Neurons are characterized by tremendous physiological activity and transcribe 30 % of genomic DNA. The transcriptional activity is two to three times more in the brain cells compared to the cells in other organs (Tobin and Khrestchatisky, 1989). A postmitotic tissue like brain, where the cells have lost their potential to divide but are endowed with high metabolic activity, is one of the most appropriate systems to examine DNA-repair as a function of age. Studies from different laboratories support an association between accumulation of DNA damage, DNA-repair mechanisms and the aging process. DNA polymerase is a DNA repair enzyme in the brain and the existence of five different polymerases in mammalian cells has been shown. They are DNA polymerase α, β, γ, δ and ε (Fry and Loeb, 1986; Burgers, 1989; Burgers et al., 1990). DNA polymerase β, a DNA repair enzyme is involved in short patch repair mechanism. DNA polymerase β is present in both proliferating and non proliferation cells (Matsukage et al., 1983; Chang and Bollum 1972; Bertazzoni et al., 1976) and through cell cycle (Spadari and Weissbach, 1974; Chang and Bollum, 1973). In one of the studies (Subrahmanyam and Rao, 1991), it was found that the unscheduled DNA synthesis (UDS) potential of aging neurons is quite low. Our observations here show significant differences between the activity and the expression levels of DNA polymerase β in the extracts of young and old brain homogenates (Fig. 4.1, 4.2, 4.3, 4.4, Table 7). The mRNA levels of DNA polymerase β decreased by 30 % in old rat brain extracts when compared to the young rat brain extracts (Fig. 4.1, Table 7) whereas the immunoreactive levels of DNA polymerase β decreased by 20 % in the old extracts when compared to the young rat brain extracts (Fig. 4.3, Table 7). However, the activity levels of DNA polymerase β decreased by 51 % in the old brain extracts with respect to the young rat brain extracts (Fig. 4.2, Table 7). We observed from the immunotitration experiment that the DNA polymerase β from old rat brain extracts required more of the antibody for 50 % inhibition of the activity than that from the young rat brain

extracts (Fig.4.4, Table 7). Immunotitration has revealed that DNA polymerase β is the predominant type in the total brain homogenate.

These results suggest an accumulation of catalytically inactive molecules of DNA polymerase β in rat brain as a function of age. These results also indicate that DNA polymerase β expression levels and activity levels do not correlate and atleast 25 % reduction in the activity could be due to structural changes in DNA polymerase β of old rat brain. Such structural changes in DNA polymerase β from old brain could be due to many reasons. Several changes in the proteins were observed by us when the proteins profiles prepared from neuronal extracts of young, adult and old rat brain were analysed (Fig 3.1, Table 3). Indeed several 'altered' enzymes have been found in aging organs and some of the examples include DNA polymerase α, dehydrogenases, synthetases, ribonucleotide reductase etc., (Dice, 1993). The present study conclusively adds DNA polymerase β to this list. The present study shows that the decreased efficiency of DNA-excision repair in neurons from aging rat brain observed earlier (Subrahmanyam and Rao, 1991) could be due to the lowered levels of catalytically active DNA polymerase β molecules.

Further, we have also studied the post-translational modifications like phosphorylation and ubiquitination of DNA polymerase β in the extracts prepared from young, adult and old rat brains (Fig 4.5 and Fig. 4.6). It is known that protein phosphorylation is one of the primary mechanisms utilized by eukaryotic cells for post-translational regulation of protein function (Browning et al., 1985). It is also becoming increasingly apparent that covalent post-translational modifications of proteins renders them susceptible for degradation and such molecules accumulate in aging tissues (Dice, 1993). The protein kinases are abundant in central nervous system and play a major role in signal transduction process. Recent studies have shown that the phosphorylation of DNA polymerase β by protein kinase C resulted in the inactivation of DNA polymerase β activity (Tokui et al., 1991). The findings here indicate that the ***in vitro*** phosphorylation of DNA polymerase β in rat brain

extracts increases with increasing age. (Fig 4.5, Table 8). TheDNA polymerase β in the old brain extracts is more phosphorylated than the DNA polymerase β in the old extracts. This probably explains the reason for decreased activity of polymerase β upon aging.

Ubiquitination is a novel mechanism for selective protein degradation and is a post-translational modification. The results of this study suggest that the levels of ubiquitinated DNA polymerase β decreased during aging (Fig. 4.6, Table 9). Fewer number of DNA polymerase β molecules are ubiquinated in old brain extracts than in the young brain extracts which probably reflects a lower turnover of this enzyme in old brain extracts compared to young brain extracts. Such low turnover of the DNA polymerase β molecules coupled with higher phosphorylation should be resulting in the accumulation of catalytically inactive molecules. It is possible that efficient ubiquitination may not occur on the already phosphorylated molecules.

CHAPTER 5

PURIFICATION OF DNA POLYMERASE δ, ε AND PROLIFERATION CELL NUCLEAR ANTIGEN (PCNA) FROM ADULT RAT BRAIN

INTRODUCTION

As already mentioned in the previous chapter it is the general consensus now that in adult brain, the most predominant DNA polymerase is DNA polymerase β. However, the presence of small amounts of other DNA polymerases cannot be ruled out. It is possible that while DNA polymerase β might be taking care of most of the DNA repair activity in brain, there may be such damage and lesions that require other polymerases for repair. In a recent study it has been noticed that a small percentage of total DNA polymerase activity in adult and aging brain may be of DNA polymerase δ and ε type (Prapurna and Rao, 1997). These studies were however based on the usage of various inhibitors and the activities observed were relative but not absolute. It would therefore be important to know whether polymerases other than DNA polymerase β are totally absent in the adult brain or they are present in such low amounts that could escape detection.

Attempts were therefore made to purify the DNA polymerase δ and ε from adult brain following the already published procedure for achieving the purification.

Similarly Proliferating Cell Nuclear Antigen (PCNA), a 37 kDa protein, is a replicative factor that functions as a cofactor to the DNA polymerase δ holoenzyme and increases its activity many fold. It was purified first as a cell cycle dependent protein and then as a DNA polymerase δ auxillary factor. The gene encoding PCNA was cloned from many different organisms including human, yeast, drosophila and plants (Kelman and Odonnell, 1995). PCNA Crystal structure was elucidated (Krishna et al., 1994) and PCNA is a ring shaped molecule made up of 3 identical 29 kDa subunits (Burgers and Yoder, 1993; Brand et al., 1994). The central cavity of the ring is sufficiently large to allow passage of a double stranded DNA molecule. The equivalent of PCNA in E.Coli, the β subunit of the DNA polymerase III, was shown to have an identical three dimensional structure (Kong et al., 1992).

Recently, PCNA is also been implicated in nucleotide excision repair (Anne Nichols and Aziz Sancar, 1992). In the DNA replication system, different polymerases and PCNA have a role to play at the replication fork. While the DNA polymerase α with its tightly associated primase activity is essential for the synthesis of Okazaki fragments and lagging strand, DNA polymerase δ in the presence of PCNA synthesizes long stretches of DNA required for the leading strand. It has been shown that in the absence of PCNA, initiation of DNA synthesis and lagging strand synthesis can occur, but there is no leading strand synthesis (Waga and Stillman, 1994) **(Fig. 5.1)**. Recent study suggests that the translesion replication by DNA polymerase δ depends on the activity of PCNA, an accessory protein (Daube et al., 2000). Further more, PCNA was shown to be a requirement even for DNA polymerase β dependent DNA-repair when the patch size is large (Wilson, 1998).

In view of the role of PCNA in nucleotide excision repair and our interest in DNA repair mechanisms in brain, a study to examine the levels and possible role of PCNA in developing and aging brain has also been undertaken.

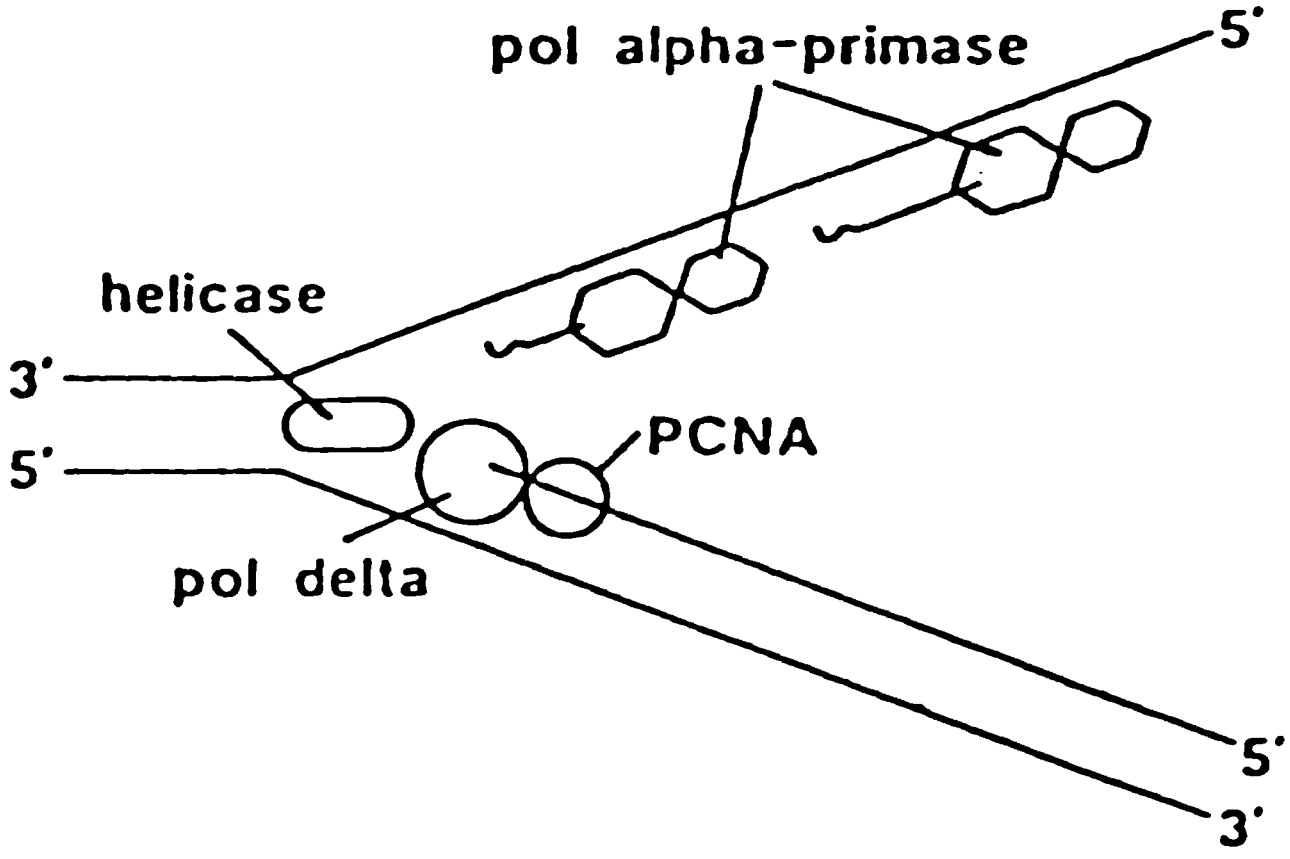


Fig.5.1: Model of a eukaryotic replication fork showing the proposed roles of DNA polymerase α (lagging strand replicase), DNA polymerase δ (leading strand replicase) and Proliferation Cell Nuclear Antigen (PCNA – an auxillary protein for DNA polymerase δ) (Ref. Downey et al., 1990)

RESULTS

1) PARTIAL PURIFICATION OF DNA POLYMERASE δ AND ε FROM ADULT RAT BRAIN:

In this laboratory, the activities of DNA polymerases were assessed earlier in order to understand the DNA repair capacity of rat brain at different ages (Rao and Rao, 1984, Subrahmanyam and Rao, 1988). DNA polymerase δ and ε are considered to be involved in DNA replication and repair respectively. These enzymes play a critical role in DNA-repair mechanisms in the brain especially in aging, as aging is clearly associated with genetic instability (Crute et al., 1986; Dresler and Kimbro, 1987). DNA polymerase δ is tightly associated with intrinsic 3'-5' exonuclease activity. DNA polymerase δ along with its auxillary protein, proliferation cell nuclear antigen (PCNA), is the prime candidate for leading strand synthesis of the replication fork (Waga and Stillman, 1994). DNA polymerase ε is independent of PCNA for its activity. Recent studies have shown the presence of DNA polymerase δ and ε in young and aging rat brain extracts (Prapurna and Rao, 1997). To further study the specific properties of DNA polymerase δ and ε, and their role, if any, in brain DNA-repair, we have made attempts to purify these two DNA polymerases from adult rat brain.

2) Preparation of Crude Extract from Adult Rat Brain:

The crude extract for partial purification of DNA polymerase δ and ε from adult rat brain was prepared as described in materials and methods and the procedure was essentially as described by Syvaoja et al., 1990. The ammonium sulphate fraction thus obtained was used for partial purification of DNA polymerase δ and ε as shown in the flow sheets (Fig. 5.2 and Fig. 5.3). The specific activity in the dialysate was determined using poly(dA.dT) as substrate. The specific activity at

this stage was observed to be 27.3 picomoles of [^{3}H] TMP incorporated into the acid insoluble portion per milligram of protein per 60 minutes (Tables 10).

3) DEAE-Sephacel Chromatography for Partial Purification DNA polymerase δ from Adult Rat Brain:

DEAE-Sephacel (12 cm x 2.5 cm; 60 ml) column was equilibrated with 120 ml buffer containing 20 mM Tris-HCl pH 7.5, 1 mM DTT, 1 mM EDTA, 20% glycerol (TDEG) and 100 mM NaCl. The ammonium sulphate fraction was loaded onto the column and the column was washed with 120 ml of equilibration buffer. The column was eluted with 120 ml of linear gradient of 0-600 mM NaCl in TDEG. 1.5 ml fractions were collected and the activity was determined in the fractions using poly(dA.dT) as substrate. The peak activity was eluted around 125 mM NaCl concentration. The specific activity of DNA polymerase δ in this fraction was found to be 386 picomoles of [^{3}H] TMP incorporated into the acid insoluble portion per milligram of protein per 60 minutes (Table 10).

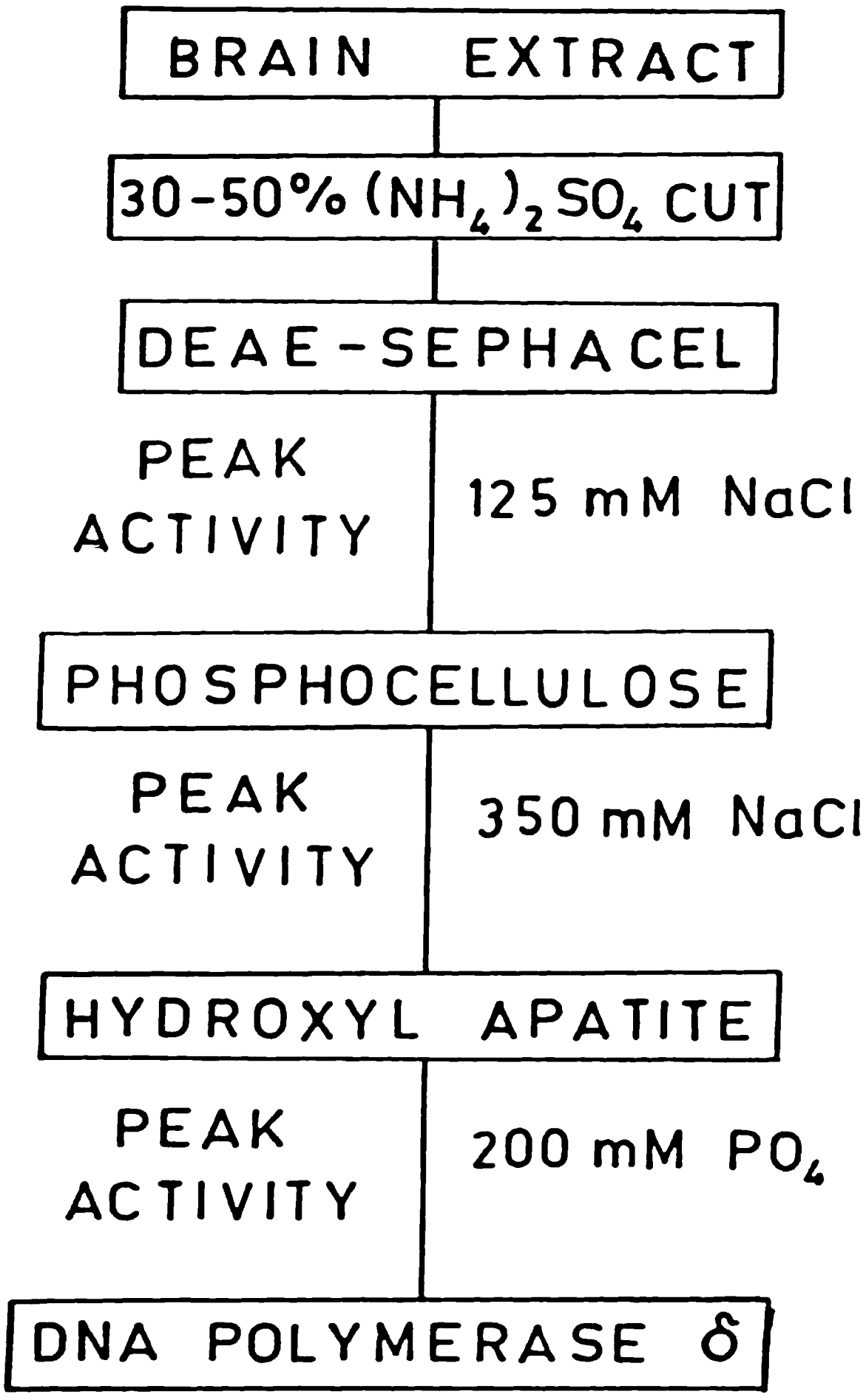
4) Phosphocellulose Chromatography for Partial Purification DNA Polymerase δ from Adult Rat Brain:

Phosphocellulose (8 cm x 2 cm; 40 ml) column was equilibrated with 100 ml of equilibriation buffer containing 20 mM Tris-HCl pH 7.5, 1 mM DTT, 1 mM EDTA, 20 % glycerol, 0.05 % triton x-100 (TDEGT) with 100 mM NaCl. The peak fractions from DEAE-Sephacel column were loaded onto the phosphocellulose column and the column was washed with 100 ml of equilibration buffer. The column was eluted with 100 ml of linear gradient 0-600 mM NaCl in TDEGT buffer. 1.0 ml fractions were collected and activity in the fractions was determined using poly(dA.dT) as substrate. The peak activity was eluted at 350 mM NaCl concentration. The specific activity of DNA polymerase δ in the peak fraction was

Figure 5.2: SCHEME FOR PARTIAL PURIFICATION OF DNA POLYMERASE δ FROM ADULT RAT BRAIN.

FIGURE 5.2

PURIFICATION OF DNA POLYMERASE δ



found to be 2502 picomoles of [^{3}H] TMP incorporated into the acid insoluble portion per milligram of protein per 60 minutes (Table 10).

5) Hydroxyl apatite Chromatography for Partial Purification DNA Polymerase δ from Adult Rat Brain:

Hydroxyl apatite (5 cm x 1.5 cm; 10 ml) column was equilibrated with 20 ml of 100 mM potassium phosphate pH 7.5, 1 mM DTT, 20 % glycerol and 0.05 % triton x-100 buffer. The peak fractions from phosphocellulose column were concentrated and dialyzed against buffer containing 100 mM potassium phosphate pH 7.5, 1 mM DTT, 20 % glycerol, 0.05 % triton x-100 (PDGT). The dialysate was loaded onto the hydroxyl apatite column. The column was washed with 20 ml of equilibration buffer. The column was eluted with 20 ml of 100 mM, 200 mM, 300 mM, 400 mM and 500 mM of potassium phosphate in PDGT buffer in stepwise manner. The stepwise fractions were concentrated and activity in the fractions was determined using poly(dA.dT) as template. The peak activity was eluted around 200 mM potassium phosphate in PDGT buffer. The specific activity of DNA polymerase δ in the peak fraction was found to be 4205 picomoles of [^{3}H] TMP incorporated into the acid insoluble portion per milligram of protein per 60 minutes (Table 10).

With the above described procedure 154 fold purification of DNA polymerase δ was achieved. However, the yield was extremely poor.

6) DEAE-Sephacel Chromatography for Partial Purification DNA polymerase ε from Adult Rat Brain:

DEAE-Sephacel (12 cm x 2.5 cm; 60 ml) column was equilibrated with 120 ml buffer containing 20 mM Tris-HCl pH 7.5, 1 mM DTT, 1 mM EDTA, 20 % glycerol (TDEG) and 100 mM NaCl. The ammonium sulphate fraction was loaded onto the column and the column was washed with 120 ml of equilibration buffer.

TABLE - 10

PURIFICATION OF DNA POLYMERASE δ FROM ADULT RAT BRAIN

FRACTION	SPECIFIC ACTIVITY	FOLD PURIFICATION
30 – 50% Ammonium sulfate cut	27.3	1.0
DEAE Sephacel	386	14.1
Phosphocellulose	2502	91.6
Hydroxylapatite	4205	154.0

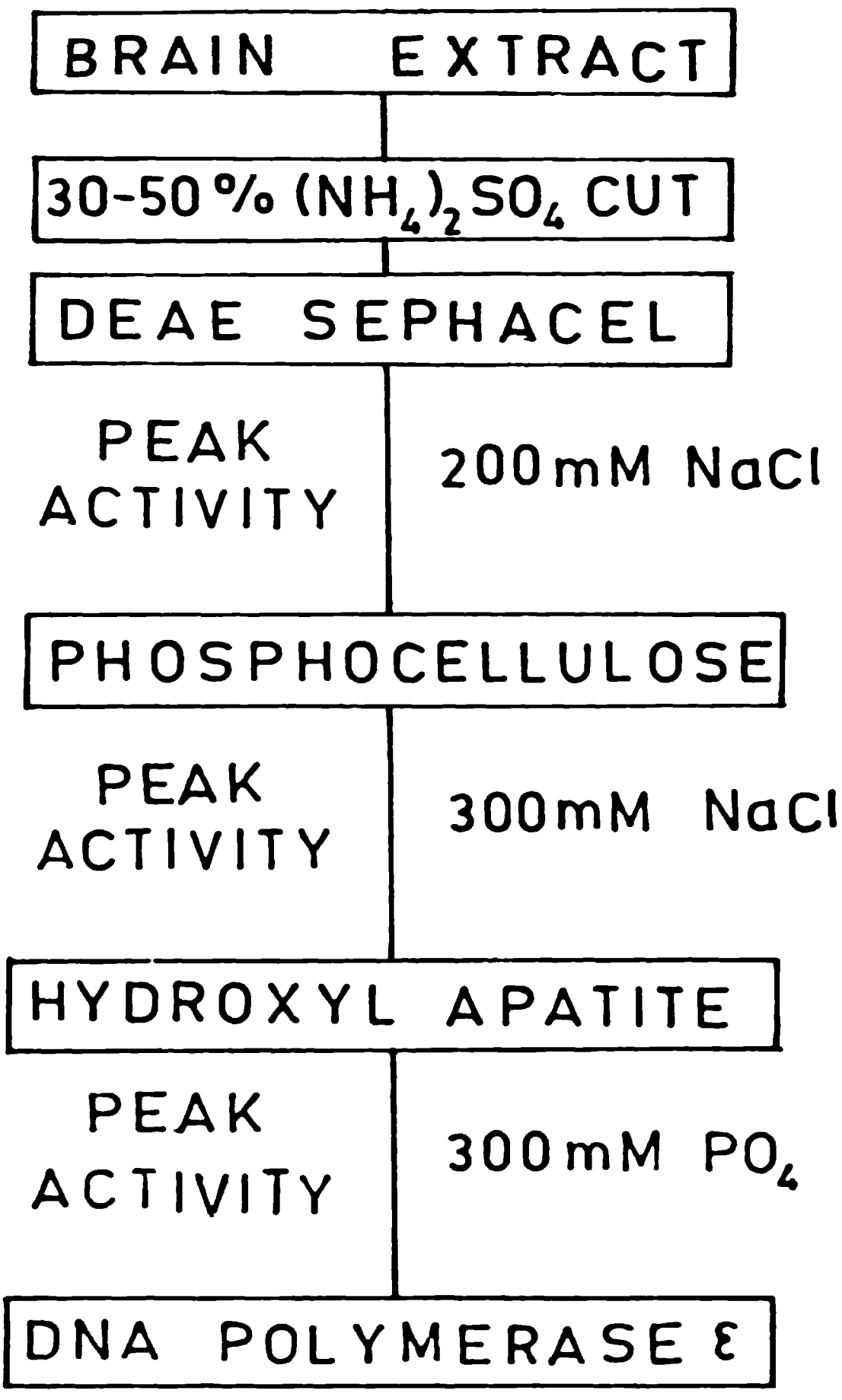
The DNA polymerase δ was purified from adult rat brain as described in materials and methods. The table depicts specific activity and fold purification of DNA polymerase δ through various steps of partial purification. In the assay system poly (dA.dT) was used as template for DNA polymerase δ.

All the values are given as picomoles of [^{3}H] TMP incorporated into the acid insoluble portion per milligram of protein per 60 minutes.

Figure 5.3: SCHEME FOR PARTIAL PURIFICATION OF DNA POLYMERASE ε FROM ADULT RAT BRAIN.

FIGURE 5.3

PURIFICATION OF DNA POLYMERASE ε



The column was eluted with 120 ml of linear gradient of 0-600 mM NaCl in TDEG. 1.5 ml fractions were collected and the activity was determined in the fractions using poly(dA).oligo$(dT)_{12-18}$ as substrate. The peak activity was eluted around 200 mM NaCl concentration. The specific activity of DNA polymerase ε in the peak fraction was found to be 147 picomoles of [^{3}H] TMP incorporated into the acid insoluble portion per milligram of protein per 60 minutes (Table 11).

7) Phosphocellulose Chromatography for Partial Purification DNA Polymerase ε from Adult Rat Brain:

Phosphocellulose (8 cm x 2 cm; 40 ml) column was equilibrated with 100 ml of equilibriation buffer containing 20 mM Tris-HCl pH 7.5, 1 mM DTT, 1 mM EDTA, 20 % glycerol, 0.05 % triton x-100 (TDEGT) with 100 mM NaCl. The peak fractions from DEAE-Sephacel column were diluted 1:1 with TDEGT buffer and loaded onto the phosphocellulose column. The column was washed with 100 ml of equilibration buffer and eluted with 100 ml of linear gradient 0-600 mM NaCl in TDEGT buffer. 1.0 ml fractions were collected and activity in the fractions was determined using poly(dA).oligo$(dT)_{12-18}$ as substrate. The peak activity was eluted at 300 mM NaCl concentration. The specific activity of DNA polymerase ε in the peak fraction was found to be 1137 picomoles of [^{3}H] TMP incorporated into the acid insoluble portion per milligram of protein per 60 minutes (Table 11).

8) Hydroxyl apatite Chromatography for Partial Purification DNA Polymerase ε from Adult Rat Brain:

Hydroxyl apatite (5 cm x 1.5 cm; 10 ml) column was equilibrated with 20 ml of 100 mM potassium phosphate pH 7.5, 1 mM DTT, 20 % glycerol and 0.05 % triton x-100 buffer. The peak fractions from phosphocellulose column were concentrated and dialyzed against buffer containing 100 mM potassium phosphate pH 7.5, 1 mM DTT, 20 % glycerol, 0.05 % triton x-100 (PDGT). The dialysate was loaded onto

the hydroxyl apatite column. The column was washed with 20 ml of equilibration buffer. The column was eluted with 20 ml of 100 mM, 200 mM, 300 mM, 400 mM and 500 mM of potassium phosphate in PDGT buffer in a stepwise manner. The stepwise fractions were concentrated and activity in the fractions was determined using poly(dA).oligo$(dT)_{12-18}$ as template. The peak activity was eluted around 300 mM potassium phosphate in PDGT buffer. The specific activity of DNA polymerase ε in the peak fraction was found to be 3328 picomoles of [^{3}H] TMP incorporated into the acid insoluble portion per milligram of protein per 60 minutes (Table 11).

With the above described procedure a 22.6 fold purification of DNA polymerase ε was achieved. However, the yield was extremely poor.

The above partially purified fractions of DNA polymerase δ and ε were concentrated and dialyzed against 20 mM potassium phosphate pH 7.5, 1 mM DTT, 20 % glycerol and 0.05 % triton x-100. The activity of DNA polymerase δ and ε partially purified from adult rat brain was tested with different substrates and activators. The yield of DNA polymerase δ and ε achieved during partial purification was extremely poor and hence could not be shown on a SDS-PAGE gel.

9) Properties of Partially Purified DNA polymerase δ and ε from Adult Rat Brain:

The activity of DNA polymerase δ and ε partially purified was assayed using poly(dA.dT) and poly(dA).oligo$(dT)_{12-18}$ as templates. The effect of various activators and inhibitors was determined as described in materials and methods. Partially purified DNA polymerase δ and ε tested for the presence of DNA polymerase α with DNA polymerase α antibody SJK 132-20 showed its presence to be 15 % and 28 % respectively (Table 12). Activators like PCNA and DMSO

TABLE - 11

PURIFICATION OF DNA POLYMERASE ε FROM ADULT RAT BRAIN

FRACTION	SPECIFIC ACTIVITY	FOLD PURIFICATION
30 – 50% Ammonium sulfate cut	—	—
DEAE Sephacel	147	1.0
Phosphocellulose	1137	7.7
Hydroxylapatite	3328	22.6

The DNA polymerase ε was purified from adult rat brain as described in materials and methods. The table depicts specific activity and fold purification of DNA polymerase ε through various steps of partial purification. In the assay system poly (dA).oligo$(dT)_{12-18}$ was used as template for DNA polymerase ε.

All the values are given as picomoles of [^{3}H] TMP incorporated into the acid insoluble portion per milligram of protein per 60 minutes.

were used to confirm the presence of DNA polymerase δ and ε in our preparations. 5 % DMSO activated DNA polymerase δ activity but not DNA polymerase ε activity (Table 12). PCNA stimulated DNA polymerase δ activity very significantly but not DNA polymerase ε (Table 12). The DNA polymerase ε was activated by 120 mM KCl but it showed no effect on DNA polymerase δ activity (Table 12). At 1 mM concentration of ddTTP the activity DNA polymerase δ and ε were significantly inhibited. The ddTTP inhibition of polymerase δ activity was 60 % while that of polymerase ε was 23 %. However at 50 μM concentration of ddTTP, the activity was inhibited to a lesser extent. The activity of DNA polymerases δ and ε was inhibited significantly by BuPdGTP at 200 μM. The BuPdGTP inhibited DNA polymerase δ activity by 22 % and DNA polymerase ε activity by 60 %. At 100 μM concentration BuAdATP inhibited DNA polymerases δ and ε activities significantly. The activity of DNA polymerase δ was inhibited by 84 % and that of DNA polymerase ε activity by 56 % (Table 12). Our present study is consistent with our earlier results on DNA polymerase δ and ε (Prapurna and Rao, 1997) and clearly demonstrate the presence of DNA polymerase δ and ε in brain although at a very low level.

TABLE – 12: PROPERTIES OF PARTIALLY PURIFIED DNA POLYMERASES δ AND ε PURIFIED FROM ADULT RAT BRAIN – This table shows the specific properties of partially purified DNA polymerase δ and ε from adult rat brain. In the assay system poly (dA.dT) and poly (dA).oligo(dT)$_{12-18}$ were used as substrates for DNA polymerase δ and ε respectively.

All values are given as picomoles of [^{3}H] TMP incorporated into the acid insoluble portion per milligram of protein per 60 minutes. The values are compared with a control value taken as 100.

TABLE - 12

PROPERTIES OF PARTIALLY PURIFIED DNA POLYMERASES δ AND ε PURIFIED FROM ADULT RAT BRAIN

Activators / Inhibitors		δ	ε
Control		100	100
+ α antibody SJK 132-20		85	72
+ β antibody		100	100
+ 5% DMSO		180	88
+ PCNA		468	100
+ KCl		—	400
+ddTTP	50μM	64	120
	1Mm	40	77
	1μM	109	125
+BUPdGTP			
	200μM	78	40
	1μM	90	116
+BUAdATP			
	100μM	16	44

10) PURIFICATION OF PROLIFERATION CELL NUCLEAR ANTIGEN FROM ADULT RAT BRAIN:

10.1) Preparation of Crude Extract from Adult Rat Brain:

The crude extract for purification of the proliferation cell nuclear antigen (PCNA) from adult rat brain was prepared as described in materials and methods and the procedure was essentially as described by Anne Nichols and Aziz Sancar, 1992 and Syvaoja et al., 1990. The ammonium sulphate fraction was used for the purification of the PCNA as shown in the Fig. 5.4.

11) DEAE-Cellulose Chromatography:

DE-52 (10 cm x 2.5 cm; 40 ml) column was equilibrated with 80 ml of 20 mM Tris-HCl pH 7.5, 1 mM DTT, 1 mM EDTA, 20 % glycerol (TDEG) with 100 mM NaCl. The ammonium sulphate fraction was loaded onto the DE-52 column and the column was washed with 80 ml of equilibration buffer. The column was eluted with 80 ml of linear gradient of 0-600 mM NaCl in TDEG buffer. 1.0 ml fractions were collected and the activity in the fractions was determined by following the activation of DNA polymerase δ with poly (dA.dT) as template. The peak activity was eluted around 300 mM NaCl concentration (Fig 5.5). The activity for PCNA in the peak fraction was found to be 129.8 picomoles of [α^{32}P] TMP incorporated into the acid insoluble portion per milligram of protein per 60 minutes. This is the activity over and above the basal activity observed with DNA polymerase δ alone (Table 13).

Figure 5.4: SCHEME FOR PURIFICATION OF PROLIFERATION CELL NUCLEAR ANTIGEN (PCNA), AN AUXILLARY PROTEIN FOR DNA POLYMERASE δ FROM ADULT RAT BRAIN.

FIGURE 5.4

PURIFICATION OF PCNA

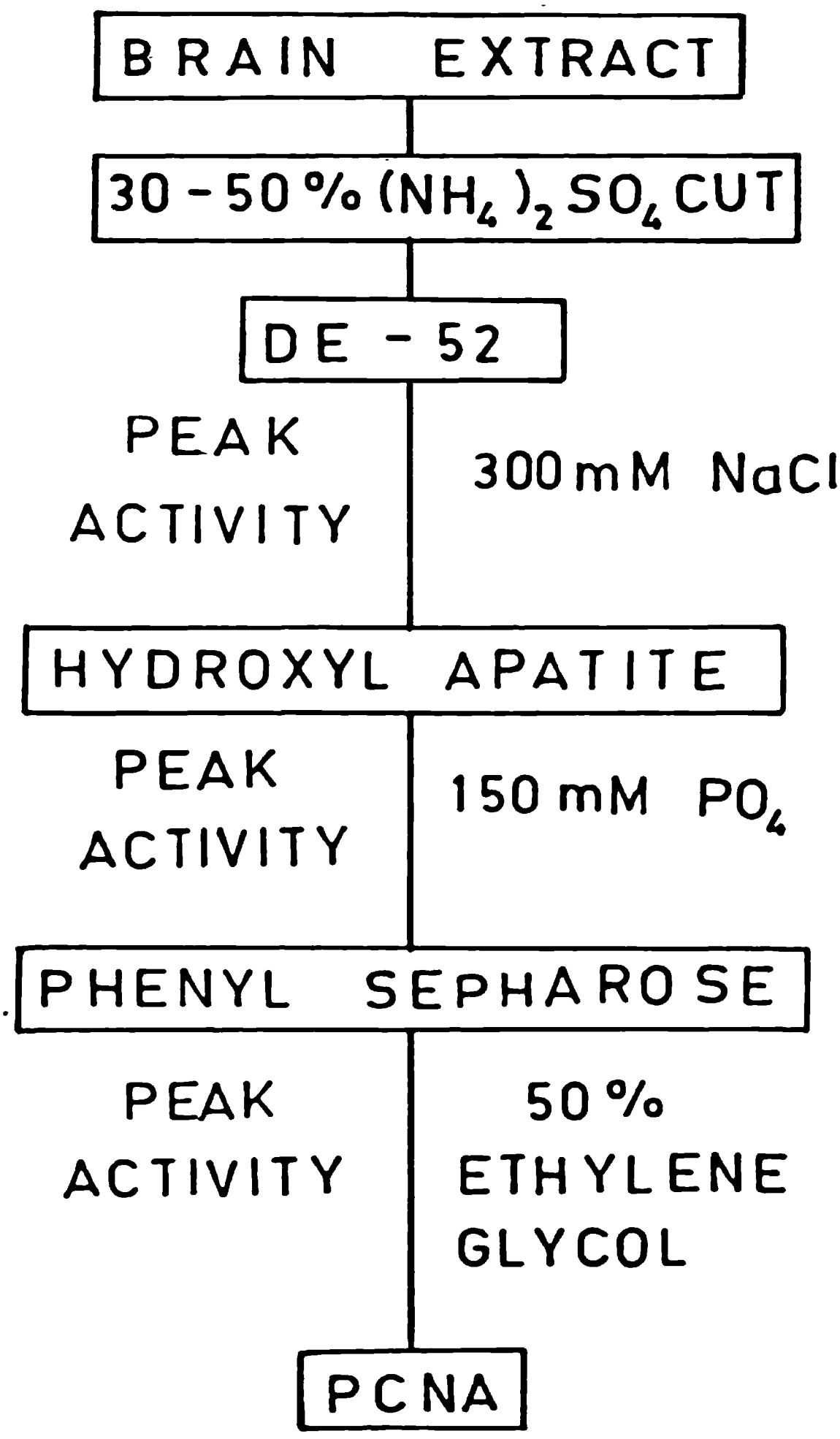
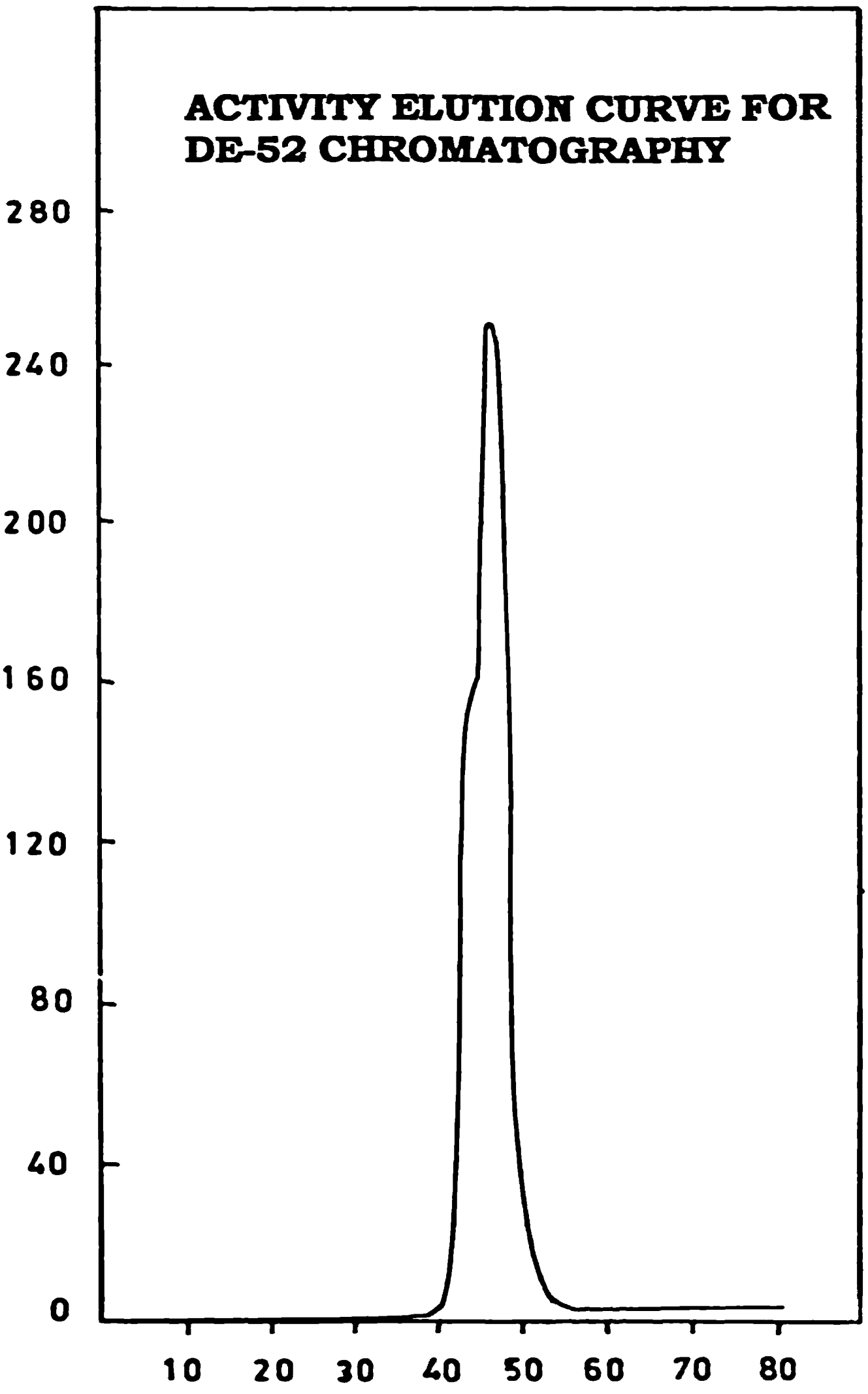


Figure 5.5: Activity elution curve for DE-52 chromatography of PCNA from adult rat brain: DE-52 (10 cm. X 2.5 cm.) was equilibrated with 20 mM Tris-HCl pH 7.5, 1 mM DTT, 1 mM EDTA, 20 % glycerol (TDEG) with 100 mM NaCl. The 30 % - 50 % ammonium sulphate precipitate was dissolved in TDEG with 100 mM NaCl and dialyzed against TDEG with 100 mM NaCl buffer. This was loaded onto DE-52 column and the column was washed with the equilibration buffer. The column was eluted with a linear gradient of 0-600 mM NaCl in TDEG buffer. 1.0 ml fractions were collected. The activity in the fractions was determined using DNA polymerase δ extract and poly(dA.dT) as templates as described in materials and methods. The activity is expressed as picomoles of [^{3}H] TMP incorporated over and above the basal activity shown by DNA polymerase δ. Peak activity eluted around 300 mM NaCl ionic strength from DE-52 column.

FIGURE 5.5


ACTIVITY ELUTION CURVE FOR
DE-52 CHROMATOGRAPHY
ACTIVITY UNITS/ml
280
240
200
160
120
80
40
0
10
20
30
40
50
60
70
80
FRACTION NUMBER

12) Hydroxyl apatite Chromatography:

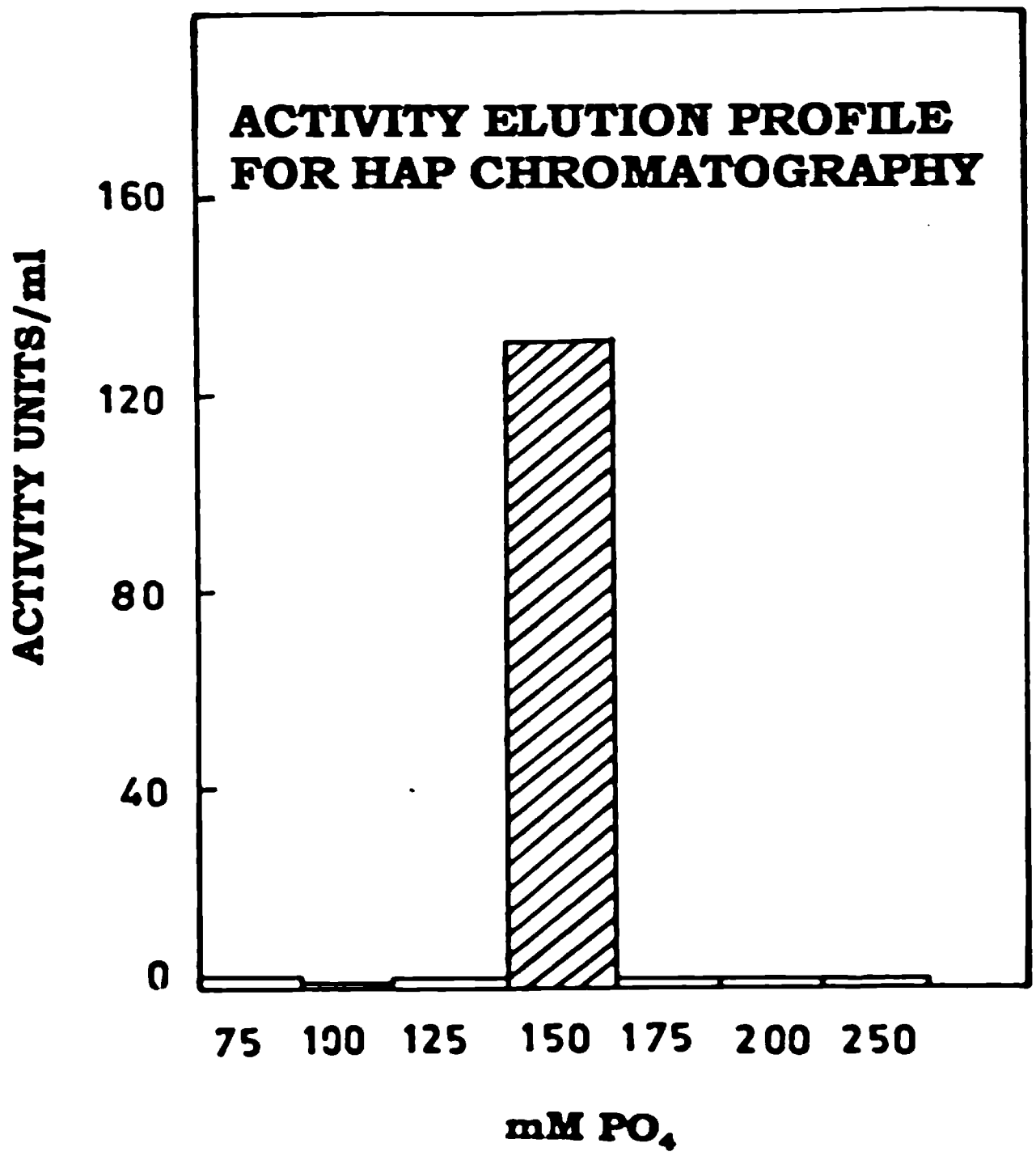
Hydroxyl apatite (5 cm X 1.5 cm; 10ml) co'umn was equilibrated with 20ml of 75 mM potassium phosphate pH 7.5, 1 mM DTT, 10 % glycerol and 0.05 % triton x-100 (PDGT). The peak fractions from DE-52 column were pooled, concentrated, dialyzed against PDGT buffer containing 75 mM potassium phosphate. The dialysate was loaded onto the hydroxyl apatite column. The column was washed with 20 ml of equilibration buffer. The column was eluted with 20 ml of 75 mM, 100 mM, 125 mM, 150 mM, 175 mM and 200 mM potassium phosphate in PDGT buffer in a stepwise manner. The fractions obtained after stepwise elution were concentrated and the activity in these fractions was determined using DNA polymerase δ and poly(dA.dT) as template. The peak activity was eluted at 150 mM potassium phosphate ionic strength (Fig 5.6). The activity of PCNA in the peak fraction was found to be 389.2 picomoles of [α^{32}P] TMP incorporated into the acid insoluble portion per milligram of protein per 60 minutes. This is the activity over and above the basal activity observed with DNA polymerase δ alone (Table 13).

13) Phenyl-Sepharose Chromatography:

Phenyl Sepharose (15 ml) column was equilibrated with 30 ml of 20 mM Tris-HCl pH 7.5, 1 mM DTT, 1 mM EDTA (TDE) buffer containing 20 % ammonium sulphate. The peak pooled fractions from hydroxyl apatite column were concentrated and dialyzed against TDE buffer. The dialysate was raised to 20 % ammonium sulphate and loaded onto phenyl sepharose column. The column was washed with 30 ml of equilibration buffer. The column was eluted with 20 ml of 10, 20, 30, 40 and 50 % ethylene glycol in TDE buffer in a stepwise manner. The stepwise fractions were concentrated and activity was determined using DNA polymerase δ and poly(dA.dT) as template. The peak activity was eluted at 50 % ethylene glycol in TDE buffer (Fig 5.7). The activity for PCNA in the peak fraction

Figure 5.6: Activity elution profile for Hydroxylapatite chromatography of PCNA from adult rat brain: Hydroxylapatite column (5 cm. X 1.5 cm.) was equilibrated with 75 mM potassium phosphate pH 7.5, 1 mM DTT, 10 % glycerol, 0.05 % triton X-100 (PDGT) buffer. The peak fractions from DE-52 column after dialysis were loaded onto hydroxylapatite column. The column was washed with equilibration buffer and eluted with 75, 100, 125, 150, 175, 200 mM potassium phosphate in PDGT buffer. The activity in the fractions was determined using DNA polymerase δ extract and poly(dA.dT) as templates as described in materials and methods. The activity is expressed as picomoles of [^{3}H] TMP incorporated over and above the basal activity shown by DNA polymerase δ. Peak activity eluted at 150 mM NaCl ionic strength from hydroxylapatite column.

FIGURE 5.6


ACTIVITY ELUTION PROFILE
FOR HAP CHROMATOGRAPHY
ACTIVITY UNITS/ml
160
120
80
40
0
75
100
125
150
175
200
250
mM PO4

was found to be 527.0 picomoles of [α^{32}P] TMP incorporated into the acid insoluble portion per milligram of protein per 60 minutes. This is the activity over and above the basal activity observed with DNA polymerase δ alone (Table 13).

The peak fraction after phenyl sepharose column was concentrated by 80 % ammonium sulphate precipitation. The pellet obtained after centrifugation was suspended in 200 µl of 20 mM Tris-HCl pH 7.5, 1 mM DTT, 1 mM EDTA and 20% glycerol (TDEG) and dialyzed against TDEG buffer. The purified PCNA was aliquoted and stored in –80 ^{0}C.

14) SDS-PAGE Gel Analysis of Purified PCNA from Adult Rat Brain:

About 10 µg of phenyl sepharose purified PCNA was electrophoretically separated on 10 % SDS-PAGE gel and the gel was silver stained. The SDS-PAGE analysis shows the presence of a single band of purified PCNA. The mobility of the band suggests that the PCNA of rat brain is a 24 kDa protein (Fig. 5.8, Lane 1).

15) Immunoblotting of Purified PCNA from Adult Rat Brain:

The purified PCNA from adult rat brain has cross reacted with a monoclonal anti-PCNA antibody in the western blot analysis. The immunoblot showed that the antibody cross reacted with a polypeptide band of 24 kDa (Fig 5.9, Lane 1).

The SDS-PAGE analysis and the western blot analysis indicated that the PCNA protein in rat brain is possibly of different molecular weight (24 kDa) (Fig 5.8, Lane 1 and Fig 5.9, Lane 1) and differs from that of HeLa cell PCNA, which was shown to have a molecular weight of 36 kDa (Anne Nichols and Aziz Sancar, 1992).

Figure 5.7: Activity elution profile for Phenyl Sepharose chromatography of PCNA from adult rat brain: Phenyl Sepharose column was equilibrated with 20 mM Tris-HCl pH 7.5, 1 mM DTT, 1 mM EDTA (TDE) buffer containing 20 % ammonium sulphate. The peak fractions from hydroxylapatite column were dialyzed, raised to 20 % ammonium sulphate and loaded onto phenyl sepharose column. The column was washed with equilibration buffer and eluted with 10, 20, 30, 40, 50 % ethylene glycol in TDE buffer. The activity in the fractions was determined using DNA polymerase δ extract and poly(dA.dT) as templates as described in materials and methods. The activity is expressed as picomoles of [^{3}H] TMP incorporated over and above the basal activity shown by DNA polymerase δ. Peak activity eluted at 50 % ethylene glycol from phenyl sepharose column.

FIGURE 5.7

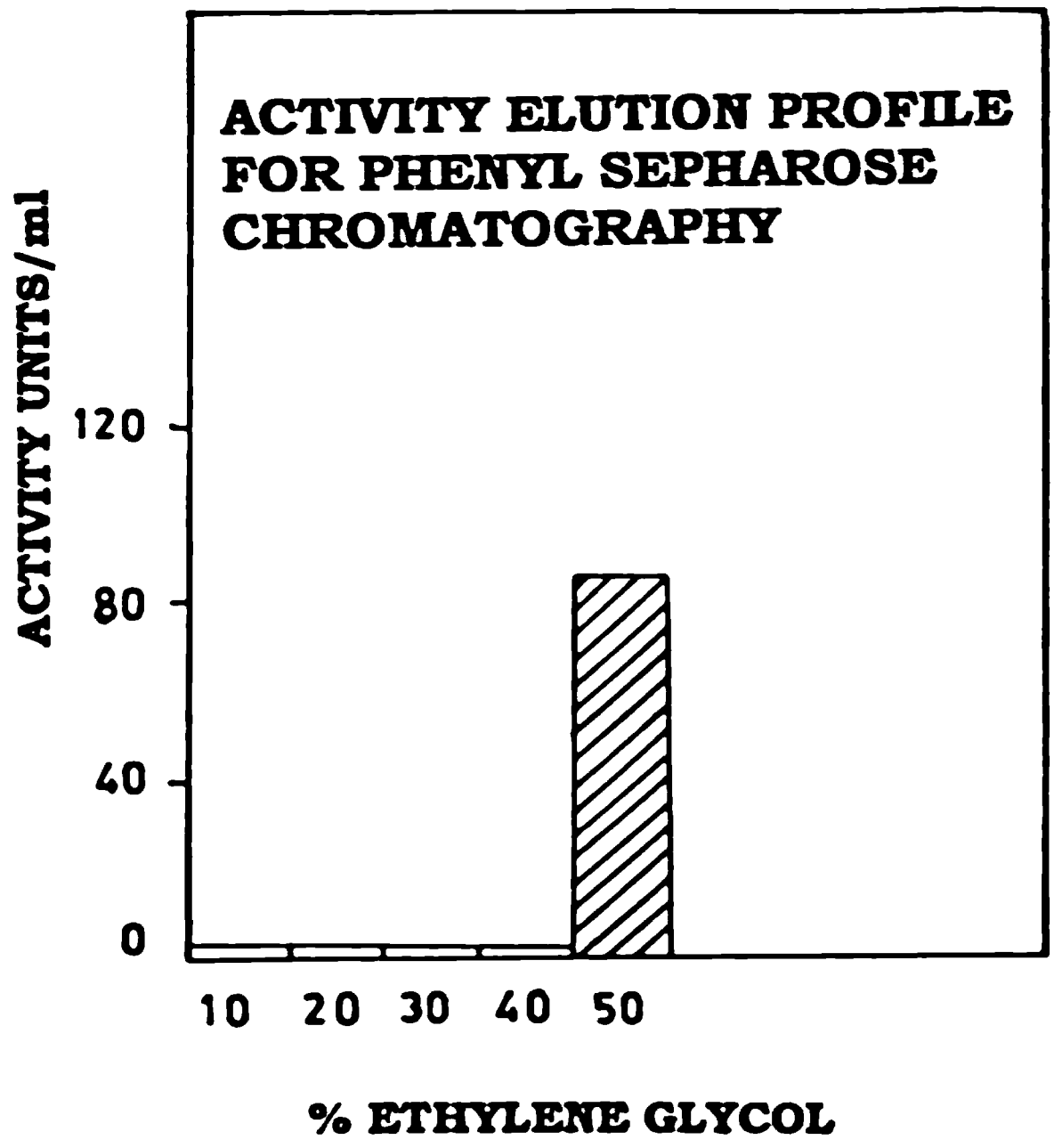

ACTIVITY ELUTION PROFILE FOR PHENYL SEPHAROSE CHROMATOGRAPHY
ACTIVITY UNITS/ml
120
80
40
0
10
20
30
40
50
% ETHYLENE GLYCOL

TABLE – 13

PURIFICATION OF PROLIFERATION CELL NUCLEAR ANTIGEN (PCNA) FROM ADULT RAT BRAIN

Step	Total protein	Total activity	Specific activity	Fold Purification
30-50% ammonium sulfate cut	300mg	—	—	—
DE 52	151mg	19613.00	129.8	1.00
HAP	17mg	6616.00	389.2	2.99
PHE-SEPH	200µg	105.41	527.0	4.06

PCNA was purified from adult rat brain as described in materials and methods. The total protein, total activity, specific activity and fold purification of PCNA obtained through various steps of purification are shown in this table. For determination of PCNA activity, in the assay system poly (dA.dT) is used as the template for DNA polymerase δ.

The PCNA activity is expressed in terms of picomoles of [α^{32}-P] TMP incorporated into the acid insoluble portion per milligram of protein per 60 minutes over and above the basal activity found with DNA polymerase δ alone.

FIGURE 5.8

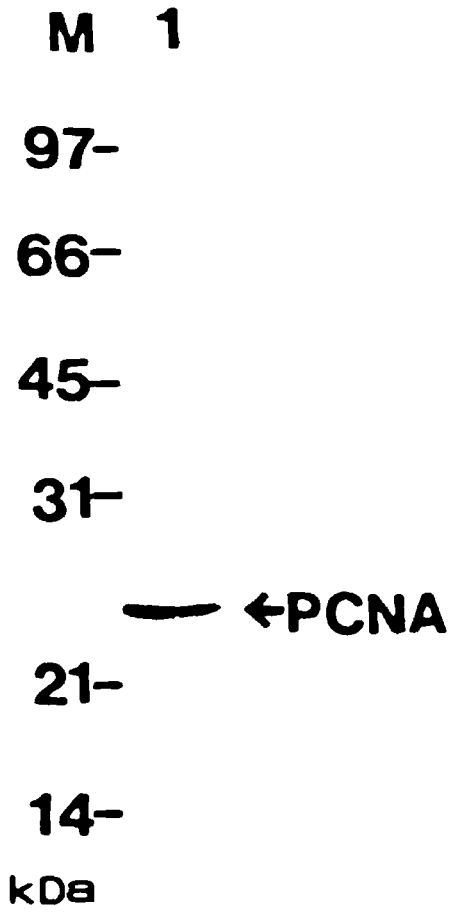


Figure 5.8: Purified Proliferation Cell Nuclear Antigen (PCNA), an auxillary protein of DNA polymerase δ from rat brain: The peak fraction from phenyl sepharose column was raised to 80 % ammonium sulphate and centrifuged. The pellet was suspended in 20 mM Tris-HCl pH 7.5, 1 mM DTT, 1 mM EDTA, 20 % glycerol (TDEG) buffer and dialyzed against TDEG buffer. 10 µg of this sample was separated on 10 % SDS-PAGE gel and silver stained.

Lane M: Molecular weight markers

Lane 1: 10 µg of purified PCNA protein from adult rat brain.

FIGURE 5.9

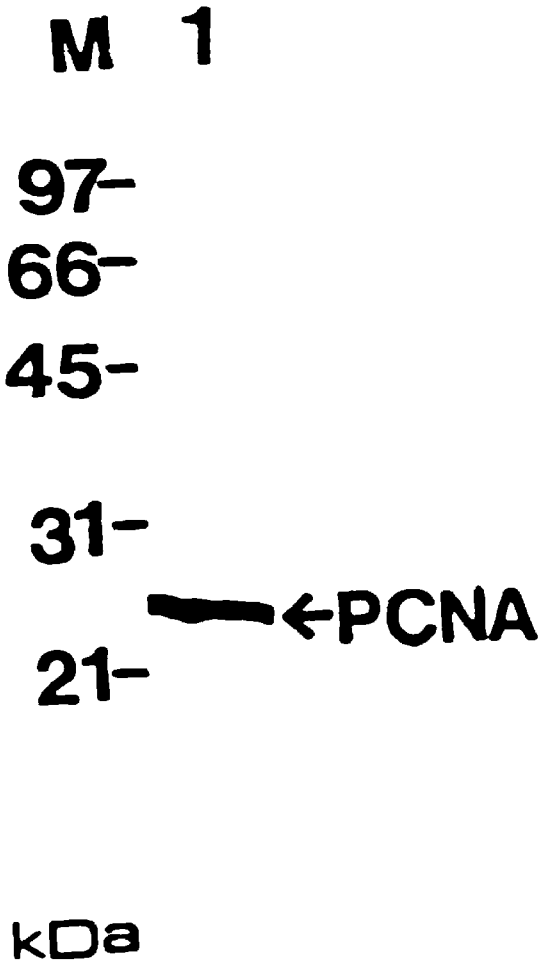


Figure 5.9: Immunoblot of purified Proliferation Cell Nuclear Antigen (PCNA) from adult rat brain: 10 μg of purified PCNA protein from adult rat brain was separated by 10 % SDS-PAGE gel, transferred onto nitrocellulose membrane and immunoblotted with PCNA monoclonal antibody (1:500 dilution) as described in materials and methods.

Lane M: Molecular weight markers

Lane 1: Purified PCNA from rat brain.

16) Stimulation of DNA Polymerase δ Activity by Purified PCNA from Adult Rat Brain:

The activity of purified proliferation cell nuclear antigen (PCNA) from adult rat brain was determined by its ability to stimulate DNA polymerase δ activity. The purified PCNA from adult rat brain has stimulated rat brain DNA polymerase δ activity very significantly by about 5 fold.

17) Expression of PCNA in Young and Aging Rat Brain Extracts:

Using equal amounts of protein the levels of PCNA was estimated in the young, adult and old rat brain extracts with the help of a monoclonal anti-PCNA antibody by western blotting (Fig. 5.10). The following results were obtained:

A) The monoclonal antibody cross reacted with a 24 kDa pure PCNA protein (Fig. 5.10, Panel A, Lane 1) and PCNA from rat brain extracts (Fig. 5.10, Panel A, Lanes 2, 3 and 4).
B) The expression of PCNA decreased in the adult and old rat brain extracts when compared to young rat brain extracts (Fig. 5.10, Panel A, Lane 2 vs Lanes 3 and 4).
C) The decrease in the expression of PCNA as a function of age in the rat brain extracts has been quantified (Fig. 5.10, Panel B).
D) The expression of PCNA decreased by 25 % in adult rat brain extracts and by 53 % in old rat brain extracts as compared to young rat brain extracts.

Figure 5.10: Expression of Proliferation Cell Nuclear Antigen (PCNA) in young and aging rat brain extracts: 100 μg of protein from crude extracts of young, adult and old brain homogenates were separated by 10 % SDS-PAGE gel, transferred onto nitrocellulose membrane and immunoblotted with PCNA monoclonal antibody (1:500 dilution) as described in materials and methods.

Panel A: Corresponds to an immunoblot.

Lane M: Molecular weight markers

Lane 1: Purified PCNA from adult rat brain (10 μg)

Lane 2: Young rat brain extract

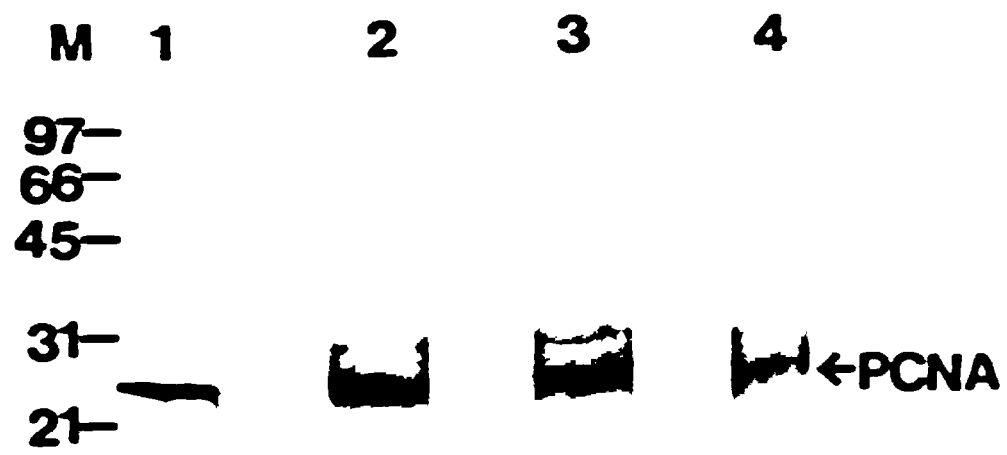
Lane 3: Adult rat brain extract

Lane 4: Old rat brain extract

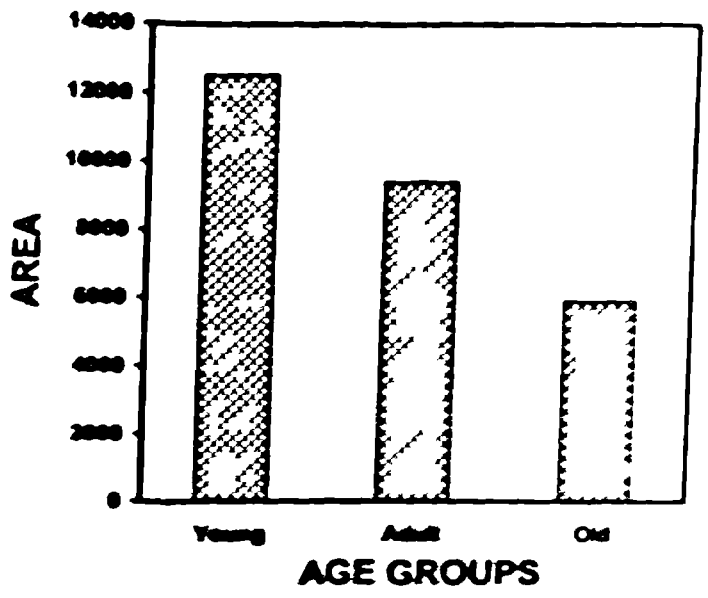
Panel B: shows the quantified data of PCNA band on the immunoblot in the UVP gel documentation. The bar diagram represents area of the band.

FIGURE 5.10

PANEL A



PANEL B



DISCUSSION

DNA polymerase δ is involved in the replication process and DNA polymerase ε is involved in the repair process. Earlier studies revealed the presence of DNA polymerase δ and ε in rat brain throughout the life span and their levels changed significantly from young to old (Prapurna and Rao, 1997). The presence of these enzymes in the brain, throughout the life span emphasizes that these enzymes have a role in the maintenance of genomic integrity. In this study we have partially purified and characterized DNA polymerase δ (Fig 5.2) by chromatography on DEAE Sephacel, phosphocellulose and hydroxyl apatite and DNA polymerase ε (Fig.5.3) by chromatography on DEAE Sephacel, phosphocellulose and hydroxyl apatite from adult rat brain. The specific activity of DNA polymerase δ and ε increased significantly during the purification protocol (Tables 10 and 11). However the yield recovered during the partial purification of DNA polymerase δ and ε was so low that it could not be seen on a polyacrylamide gel. Activation of DNA polymerase δ activity by PCNA and DMSO indicates the presence of DNA polymerase δ in the rat brain (Table 12). Activation by KCl and inhibition by DMSO of DNA polymerase ε activity suggests the presence of DNA polymerase ε in the preparation (Table 12). The activity of DNA polymerase δ and ε were inhibited by high concentration of various inhibitors like ddTTP, BuPdGTP and BuAdATP confirming the presence of DNA polymerase δ and ε in the rat brain (Table 12). Thus this study clearly differentiates between DNA polymerase δ and ε and confirms their presence albeit at a low level in the rat brain.

DNA polymerase δ along with its auxillary protein, proliferation cell nuclear antigen (PCNA), are the prime proteins for leading strand synthesis of the replication fork (Waga and Stillman, 1994). In this study, we have purified and characterized PCNA from the adult rat brain. The PCNA was purified (Fig 5.4) by passing through various chromatographic columns like DEAE-Cellulose (Fig. 5.5), hydroxyl apatite (Fig. 5.6) and phenyl sepharose (Fig. 5.7). The specific activity of the PCNA

increased significantly during purification protocol (Table 13). SDS-PAGE analysis revealed that the PCNA purified from adult rat brain was a pure preparation (Fig. 5.8). The pure PCNA cross reacted with the monoclonal anti-PCNA antibody (Fig. 5.9). The SDS-PAGE analysis and the western analysis revealed that the PCNA obtained from the rat brain is a 24 kDa polypeptide. This shows that PCNA in rat brain is probably of different molecular mass than that reported in HeLa cells which is a 36 kDa protein (Anne Nichols and Aziz Sancar, 1992). The purified PCNA from adult rat brain stimulated DNA polymerase δ activity with poly(dA.dT) as template by about 5 fold. This shows that PCNA functions as an auxillary protein for DNA polymerase δ. The expression levels of PCNA in the extracts prepared from young, adult and old rat brains decreased as a function of age. The levels decreased by 53 % in the old brain extracts when compared to youmg brain extracts (Fig. 5.10). It is known that the human DNA replication complex consists of DNA polymerase δ, PCNA, replication factor A, replication factor C and other proteins. The PCNA is directly involved in human nucleotide excision repair system (Anne Nichols and Aziz Sancar, 1992). An indepth study on the PCNA at the molecular level in the rat brain would be interesting and may be useful in understanding the aging phenomenon.

In summary, the present study consisting the evaluation of different DNA polymerase activities revealed that DNA polymerase β, δ and ε are present in the rat brain, DNA polymerase β being the predominant one. Our data also indicate that rat brain has sufficient levels of DNA polymerases and is well equipped to affect the DNA repair process. This is so, because, the brain cells (Neurons) with high transcriptional activity would maintain high and efficient DNA repair mechanisms in order to keep up the fidelity of vital processes like replication, transcription and translation throughout the life span.

CHAPTER 6

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Aging is an universal and inevitable phenomenon of all higher multicellular organisms. The process of aging is one of the most intriguing problems of the day and gerontologists around the globe are confronted with the challenge of identifying the basic fundamental cause of the aging process. Aging is a basic and general phenomenon, involving a complex interplay of genetic and epigenetic factors resulting in the functional deterioration at all levels of organisation. Several theories have been proposed to explain the process of aging. Among all the theories, the most acceptable theory of aging is the DNA damage and repair theory proposed by Hart and Setlow (1974). The theories of aging proposed by various groups as well as the process of DNA damage, DNA repair and their relation to aging was discussed in Chapter I. Protein synthesis is the most basic and fundamental activity of every living cell and all cellular activities depend on it. It is a complex process comprising of many stages, involving many initiation and regulatory factors. A brief account of this complex process was described in Chapter I. In this thesis some aspects of the role of protein synthesis and its initiation and regulatory factors as well as DNA polymerases in the aging phenomenon were investigated. The investigation led to the following conclusions:

1) Protein synthesis, as measured by labelled [S^{35}] methionine uptake and its incorporation into the trichloro acetic acid precipitable proteins in the crude extracts of brain cells, showed a decrease with aging.

2) SDS-PAGE analysis of proteins in the neuronal extracts indicated several age-related changes in the protein profiles. A general decline in several high molecular weight proteins was observed. The protein of molecular mass 25 kDa decreased in adult and old extracts. Proteins of molecular masses 76 kDa and 31 kDa are not altered whereas synthesis of proteins like 14.6 kDa and 44 kDa are enhanced in adult and old extracts. A protein of molecular mass 27 kDa was found exclusively in the adult and old extracts.

3) The levels of the initiation factors eIF 2α and eIF4E decreased with increasing age. A decline in their levels is consistent with the general decline in protein synthesis. Our studies also revealed that the molecular mass of neuronal eIF2α is higher than reticulocyte eIF2α subunit.

4) ***In vitro*** phosphorylation of the neuronal extracts revealed that the various proteins of aged extracts are phosphorylated more efficiently than the young extracts. Addition of genistein, an inhibitor of tyrosine phosphorylation caused more severe inhibition in the phosphorylation of the aged extracts than in the young extracts. However the ***in vivo*** levels of phosphorylated tyrosine residues in the proteins of the neuronal extracts decreased during aging. This may be because many of these proteins in young extracts were well phosphorylated and are not accessible for any further phosphorylation ***in vitro*** whereas the proteins of old extracts were not well phosphorylated ***in vivo***.

5) The neuronal extracts contain a double stranded RNA dependent eIF2α kinase activity, which increased with aging.

6) The mRNA levels of DNA polymerase β decreased as a function of age. The mRNA levels decreased in adult and old rat brains by 21 % and 29 % respectively when compared to young rat brain.

7) The activity gel assay indicates a decrease in the DNA polymerase β activity with increasing age. The activity decreased by 45 % in the adult rat brain extract and by 53% in old rat brain extract with respect to young rat brain extract.

8) Expression of DNA polymerase β decreased from young to old rat brain extracts as determined immunologically with specific antibody. The levels decreased by 30 % in adult rat brain extract and by 20 % in the old rat brain extract with respect to young rat brain extract.

9) The immunotitration revealed that old extracts require more antibody than young extracts for 50 % inhibition of activity of DNA polymerase β. The old extracts required 34 µl of antibody whereas young and adult extracts required 12 µl and 22 µl of antibody respectively.

10) The study revealed that DNA polymerase β is the predominant type in the brain as 70 % of polymerase activity was inhibited by its antibody.

11) The phosphorylation of DNA polymerase β increased with increasing age leading to inactivation of DNA polymerase β. This shows that the decrease in the activity of DNA polymerase β upon aging may probably be due to protein phosphorylation.

12) The levels of ubiquitinated DNA polymerase β decreased with increasing age. This probably reflects a lower turnover of this enzyme in old brain extracts compared to young brain extracts.

13) The study confirms the presence of DNA polymerases δ and ε in rat brain. DNA polymerases δ and ε were partially purified from adult rat brain. During the procedure a 154 fold purification for DNA polymerase δ and a 22.6 fold purification for DNA polymerase ε was achieved.

14) DNA polymerase δ was activated significantly in the presence of PCNA and DMSO but not DNA polymerase ε.

15) The KCl (120-mM) activated DNA polymerase ε but not DNA polymerase δ.

16) Various inhibitors like ddTTP, BuPdGTP and BuAdATP inhibited DNA polymerases δ and ε activities very significantly.

17) The study showed conclusively the presence of PCNA in rat brain.

18) SDS-PAGE and western blot analysis indicate that PCNA in rat brain is a 24 kDa protein. This study showed that PCNA in rat brain is probably of different molecular mass than that reported in HeLa cells.

19) The purified PCNA from adult rat brain stimulated DNA polymerase δ activity by several fold. This showed that PCNA functions as an auxillary protein for DNA polymerase δ.

20) The levels of PCNA in the rat brain extracts decreased as a function of age

21) The above findings are consistent with general decline in protein synthesis and DNA repair polymerase activity, suggesting that aging may have a multifactorial etiology.

CHAPTER 7

REFERENCES

REFERENCES

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ON THE 'ACTIVE' MOLECULES OF DNA-POLYMERASE β IN AGING RAT BRAIN

Subba Rao, K.,* Vinay Kumar, D., Bhaskar, M. S. and Sripad, G.

Neurochemistry Laboratory,
Department of Biochemistry, School of Life Sciences, University of Hyderabad, HYDERABAD-500134, INDIA

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Summary: DNA polymerase β, a 38kD protein is essentially considered to be a repair enzyme atleast with respect to some forms of DNA damage. Through northern and western blotting, it was found that mRNA and immunologically reactive molecules of DNA-polymerase β are reduced by 30% and 20% respectively in old rat brain as compared to the young. However, activity gel assay and immunotitration revealed a 50% reduction in the activity in old brain. These data are taken to indicate accumulation of catalytically inactive molecules of DNA-polymerase β in rat brain with age.

DNA Polymerase β, a small highly conserved enzyme, is essentially considered as a repair enzyme atleast with respect to short-patch repair of some forms of DNA damage (1). Recently transcriptional enhancement of the DNA polymerase β gene in CHO cells treated with DNA damaging agents was reported (2). Also, cDNAs for rat and human DNA polymerases β have been cloned (3,4) and comparision of their nucleotide sequences suggested a 96% homology (4). DNA polymerase β is the predominant polymerase in rat and mouse neurons (5,6). It may therefore have a vital role in the DNA repair process of neurons.

For the past few years, our laboratory has been examining the DNA-repair capacity of rat brain at different ages and the parameters used for this purpose were the activities of

* corresponding author. Tel. 91-40-289451 Fax. 91-40-253145

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putative DNA-repair enzymes, including DNA polymerases (7-9). In one of the studies (10), we found that the unscheduled DNA-synthesis (UDS) potential of aging neurons is quite low. However the precise locus of the defect is not clear. In the present studies, we have monitored changes in expression and activity of DNA-polymerase β in aging rat brain.

Materials and Methods

Animals: Wistar strain rats of different ages were maintained under optimal conditions for growth in the university animal house. Rats of three different age groups were used in the present study and referred to as young (4 days), adult (180 days) and old (730 days)-all postnatal.

RNA preparation and Northern dot blot: Three pooled brains from young, adult and old rats were dissected and immediately frozen in liquid nitrogen. Tissues were powdered and RNA was extracted as per the procedure of Chomczynski and Sacchi (11) which is as follows. The powdered tissue was homogenized with 4 M Gdn.SCN containing 25 mM sodium citrate buffer, pH 7.0, 0.1 M β-mercaptoethanol and 0.5% Sarcosyl and centrifuged. The supernatant was phenol extracted and precipitated with isopropanol. Northern dot blotting was performed as described by Hiroshi et al.,(12). 15-30 μg of RNA was dot blotted on to Genescreen plus membrane (Dupont, USA) and prehybridized for 6 h at 42°C in 50% formamide, 5X SSC, 65 mM KH_2PO_4, 0.1% Polyvinylpyrrolidine (PVP), 0.1% Ficoll, 1% BSA, 5 mM EDTA and 100 μg/ml of calf thymus DNA. Hybridization was performed at 42°C. for 24 h in 50% formamide, 5X SSC, 0.2% PVP, 0.02% Ficoll, 0.2% BSA, 10% PEG, 5mM EDTA and 50 ng (about 10^7 CPM) of β-polymerase cDNA probe 10SL (573 nucleotides) (12). The membrane was washed with 2X SSC containing 1% SDS at 60°C for 1h and then with 0.1X SSC with 0.1% SDS at room temp. for 30 min. The blot was then autoradiographed.

Preparation of brain extracts: 10% homogenates of the brain tissues were prepared by homogenizing sliced brains in DNA polymerase extraction buffer contaning 20 mM Tris-HCl, pH 7.5, 0.1 mM β-mercaptoethanol, 1 mM $MgCl_2$, 0.1 mM EDTA, 5% glycerol, 0.1% Triton X-100 and 0.5 M KCl and protease inhibitors, pepstatin, leupeptin (both 1 μg/ml) and 0.5 mM phenylmethyl sulfonylfluoride (PMSF). The homogenate was kept at 0-4°C for 1h and then centrifuged at 100,000 xg for 1 h. The supernatant was used as source of DNA-polymerase β.

Immunoblotting of extracts from young, adult and old brain: About 15-30 μg of protein from these tissues were electrophoretically separated on 10% polyacrylamide gel and transferred to nitrocellulose (Schleicher and Schull, W. Germany). The membrane was blocked for 1 h with 3% goat serum in Tris-HCl buffer pH, 7.8; 0.15 M NaCl (TBS) and incubated

overnight with polyclonal primary antibody (1:3000) to rat liver β-polymerase (12) in TBS bufer containing 0.3% goat serum. Then blots were washed four times with TBS containing 0.1% triton X-100 and then incubated with secondary anti-rabbit IgG antibody conjugated to horseradish peroxidase. The immunoblot was washed again four times with TBS buffer containing 0.1% triton X-100 and developed with diaminobenzidine and hydrogen peroxide.

Activity gel assay of β-polymerase: Procedure followed here is essentially same as previously described (2,12). Brain extracts of young, adult and old animals containing 15-30 μg protein were run on 10% polyacrylamide gel containing 150 μg/ml of activated calf thymus DNA as given in the immunoblot procedure. After the electrophoretic run the gel was incubated in the renaturation buffer containing 50 mM Tris-HCl, pH 7.5, for 90 min, with two changes. Then the gel was left at 4°C for 17.5 h in 50 mM tris-HCL, pH 7.5/1 mM EDTA followed by 6.5 hr in the same buffer with 1 mM DTT. The gel was immersed in the incubation buffer, 50 mM tris-HCl, pH 7.5; 7 mM $MgCl_2$, 1 mM DTT, 12 μM each of dATP, dGTP and dTTP and 60 μci [α-^{32}P] dCTP(3000Ci/m.mole) for 17 h at 37°C. The gel was then thoroughly washed with cold 5% TCA/1% Sodium pyrophosphate for 40 h with 3-4 changes. Then the gel was dried and autoradiographed. Scanning of autoradiograms and western blots was done in a UV-P gel documentation unit, Cambridge, UK. Two separate experiments were performed and scanned to check the reliability of results.

Biochemical assay of DNA polymerase β: Polymerase assay was performed with 'activated' calf thymus DNA as template primer. The incubation mixture contained in a total volume of 50μl, 40mM Tris-HCl, pH 7.5, 8 mM $MgCl_2$, 1 mM β-mercaptoethanol, 4 mM ATP, 100 μM each of dATP, dGTP, dCTP, 25 μM TTP, 5μM of activated DNA, 1 μCi ^{3}H TTP (Sp.act. 40Ci/mM, Amersham, England) and 20 μl of the crude extract containing 90 μg of protein. Reaction mixture also contained different concentrations of β-polymerase antibody at 1:100 dilution. The reaction mixture was incubated at 37°C for 20 min and 200 μg of DNA and BSA were added and the reaction was stopped with 1 ml of cold 10% trichloro acetic acid containing 10 mM sodium pyrophosphate. The samples were kept on ice for 10 min and filtered through GF/C filters. Precipitate was washed twice with 5% TCA and once with cold 95% ethanol. The filters were counted for radioactivity in a Beckman LS-1800 counter equipped with automatic quench correction.

Results

Expression levels of β-polymerase mRNA: Reduction of mRNA was seen in adult and old rat brain by 21% to 29% with respect to young, (Fig. 1). The average reduction of mRNA from young to

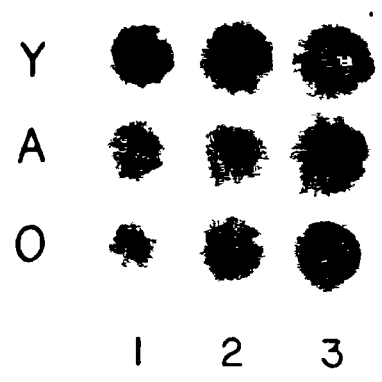

Fig. 1. mRNA levels of β Polymerase in young, adult and old rat brains. Total RNA about 15, 20 and 25 μg was blotted in each slot in lanes 1-3 respectively. Y, A and O refers to young, adult and old brain extracts respectively.The results are consistent with two separate experiments.

Fig. 2. Western blot analysis of β-polymerase: 30 μg of protein from crude extracts were separated by SDS-PAGE on 10% gel and immunoblotted on to nitrocellulose and stained. Lanes 1, 2 & 3 contains young, adult and old samples respectively and in lane 4, pure β-polymerase 50 ng was loaded as control. The amounts in each lane were calculated by scanning. The results are similar in two independent experiments.

old was about 24.9%. The results were consistent from two sets of experiments and at different concentrations of RNA.

Analysis of Immunoblot: The levels of immunologically competent molecules of β polymerase were determined by western blot (Fig. 2). There was a similar marginal decrease in this case also. The exact decrease being 30.6% in adult and 19.4% in old with respect to young value.

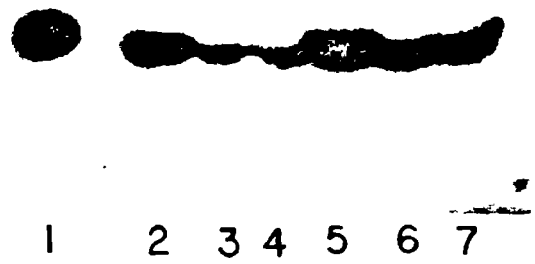

Fig. 3. Quantitation of β-polymerase activity in young, adult and old brain extracts by activity gel method. Crude extracts containing 25 μg (lanes 2, 3 & 4) and 40 μg (lanes 5, 6 & 7) of protein from young, adult and old brain homogenates were seperated by 10% SDS-PAGE and activity staining was performed as described previously (12). Pure β-polymerase (50 ng) was loaded in lane 1 as control. This pattern of results is consistent in four different experiments.

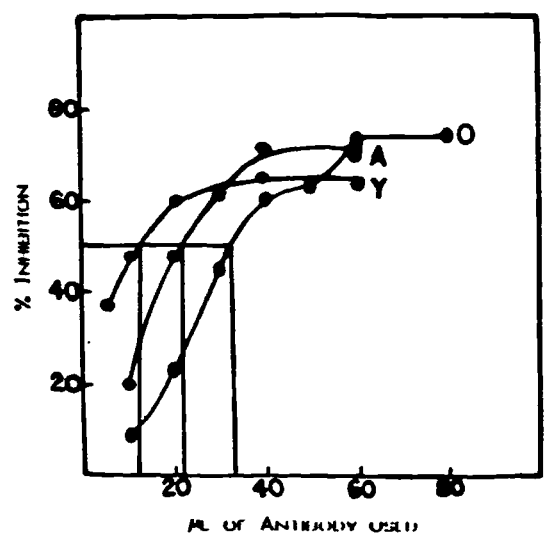

Fig. 4. Immunotitration of β-polymerase activity of young, adult and old brain extracts. β-polymerase activities were titrated in presence of different amounts of antibody for young, adult and old samples.Three sets of experiments were performed and averages plotted. Y, A and O denotes young, adult and old respectively. Activity of β-polymerase equivalent to incorporating 60 picomoles of ^{3}H-TMP into DNA was taken in all the cases before the addition of antiserum.

Activity gel assay of β-polymerase: β-polymerase activity by activity gel method however showed more marked differences between young, adult and old brain samples (Fig. 3). The average reduction of β-polymerase activity was by about 51% for adult and old samples. The exact reductions were 45% for adult and 53% for old.

Immunotitration of β-polymerase activity: To inhibit 50% of β-polymerase activity in young and adult extracts, it required about 12 and 22 μl of antibody respectively, whereas old sample required about 34 μl (Fig. 4.). Total polymerase activity was inhibited by 70% showing that β-polymerase is the predominant type in the total brain homogenate.

Discussion

Significant differences were noticed between the activity and the expression levels of β-polymerase in extracts of young and old brain homogenates. The results of all the experiments are summarized in Table. 1. The percentage of reduction of β polymerase from mRNA analysis and western blot is about 25% whereas from activity gel assay it is about 51% between young, adult and old brain extracts. Also from immunotitration experiment, β polymerase from old rat brain required more of antibody than that from young rat brain. These results thus indicated that β polymerase expression levels and activity did

Table: β-polymerase levels in aging brain assessed through different strategies.

Age	mRNA	Western Blot	Activity on gel	Immunotitration
	Percentages			Antiserum reqd. for 50% inhib.
Y	100	100	100	12 μl
A	79.4	69.4	55	22 μl
O	70.9	80.6	47	34 μl

The densitometric scanning values of 'young' are taken as 100 and other values are presented as relative percentages.

not correlate and atleast 25% of reduction in activity could be due to structural changes in β polymerase of old brain. Such structural changes in β-polymerase from old brain could be due to many reasons. It is becoming increasingly apparent that covalent post-translational modifications of proteins renders them susceptible to degradation and ineffective and such molecules accumulate in aging tissues (13). Indeed several 'altered' enzymes have been found in aging organs and some of the examples include DNA polymerase α, dehydrogenases, synthetases, ribonucleotide reductase etc., (13). The present results add β-polymerase to that list. In this respect it would be interesting to study which regions of β-polymerase are altered and in what manner by appropriate probes or specific reagents that would facilitate structural measurements. Be as it may, the present results pointedly show one of the reasons for decreased efficiency of DNA-excision repair in neurons from aging rat brain observed earlier by us (10) could be the lowered levels of catalytically active DNA polymerase β molecules.

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