PURIFICATION AND CHARACTERIZATION OF NATIVE AND UV IRRADIATED DNA SPECIFIC DEOXYRIBONUCLEASE FROM YOUNG AND AGING RAT BRAIN

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DOCTOR OF PHILOSOPHY

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

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

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ABBREVATIONS USED

A_{260} & A_{280}	: Absorbance at 260 nm and 280 nm.
AP	: Apurinic or Apyrimidinic
Ci	: Curie
CM	: Carboxy methyl
DNase	: Deoxyribonuclease
DPM	: Disintegrations per minute
g	: grams
xg	: multiples of gravitational force
hr	: hour
J	: Joules
min	: minutes
PCA	: perchloric acid
PMSF	: Phenyl Methyl sulfonyl fluoride
RNase	: Ribonuclease
SDS	: Sodium dodecyl sulfate
TEMED	: N,N,N,N-tetra methyl ethylene diamine
UV	: ultraviolet
v/v	: volume by volume

CONTENTS

A NOTE

Throughout this thesis, expressions like acid and NUV DNase are interchangeably used. The term acid DNase refers to **the** acidic pH optimum of the enyzme while the name NUV DNase denotes **the** substrate specificity of **the** enzyme towards native and UV-irradiated **DNA.**

CHAPTER I

General Introduction

CHAPTER - I

GENERAL INTRODUCTION

Aging is one of the most universal, inevitable social **and** scientific challenges confronting man. All multicellular organisms begin with conception, extend through the phases of development, maturity, senescence and finally end in death. Strehler (1962) defined senescence as the changes which occur generally in the post reproductive period, resulting in a decreased survival capacity on the part of the Individual organism.

Aging is a naturally developing biological process, which limits the adaptive possibilities of an organism, increases the likelihood of death, reduces the life span. Death, the ultimate result of aging is sudden, but the process of aging involves a progressive increase in the probability of dying. Another characteristic feature of aging and senescence is that the processes involved are common to all members of a species and are inescapable consequences of getting older. That is to say aging and senescence are the fundamental intrinsic properties of living organism.

Aging is heterochronlc, heterotopic, heteroklnetic and heterocateftenlc. It is heterochronic, because individual tissues, organs and systems age at different times. It is heterotopic, because it is expressed differently in various organs as well as structures of the same organ. It is heterokinetic, because age changes occur at different rates. Aging is

heterocateftenic because age changes occur in different directions, being connected with the suppression of vital process and the activation of others in the aging organism.

Several physiological, morphological, biophysical and biochemical parameters are known to change during aging. Even with the astounding scientific knowledge that has accumulated as on today, the precise molecular mechanisms through which a given organism undergoes the above changes are far from understood.

The process of aging is multlfactorlal. Depending on the experimental findings several theories have been proposed to explain the process of aging. However, there is no single theory which can explain all the biochemical changes, observed during aging or make universal statements about the principles, mechanisms and evolution of aging (Rattan & Clark, 1988, Gensler & Bernstein, 1981).

The Plethora of aging theories that are known today can be largely divided into two categories. (1) Programmed (2) Stochastic (Hayflick, 1975; Medvedev, 1976; Cutler, 1976). According to one view, the programmed aging "fundamentalist's view, the process of aging is genetically fixed. This, concept is supported by theories like Weisman's germ plasm, aging clock theory, codon restriction theory, chromatin reorganization theory, gene regulation theory and DNA damage and repair theory (Shock, 1981; Rattan & Clark, 1988). On the other hand theories that are termed as "epiphenomenalist's view, assume that primary aging is the

result of stochastic (i.e. it is not genetically determined, but is governed by chance) events and express itself by accumulation of errors in DNA, RNA or protein or in combination of all the three. This situation eventually destroys the organism's ability to maintain homeostasis. Theories that lend support to this thinking are the somatic mutation theory, error catastrophe theory, post translatlonal modification theory and free radical theory (Hayflick, 1975; Medvedev, 1976; Cutler, 1976). For the sake of convenience the theories classified as 'Stochastic' are discussed below first followed by those grouped under 'programmed' theories.

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 reports that the symptoms of aging and death of irradiated rodents (Sacher, 1956; Failla, 1960). and humans, (Warren 1956)were similar to those of normal individuals, except that there was a higher incidence of neoplasia in the former, so it was believed that irradiation causes acceleration of the aging process. Szilard (1959) proposed the somatic mutation theory of aging according to which mutations that occur randomly and spontaneously destroy genes and chromosomes in post-mitotic cells during the life span of an organism and gradually increase its mutation load. This increase in mutation and loss of functional genes decrease the production of functional proteins. Cell death

occurs when mutation load in a cell increases beyond a critical level. So the number of post mitotic cells decreases and the overall functional ability of the organism decreases. Other supporters of this were Harman (1962) and Medvedev (1964). Basic difficulties of this theory are discussed by Sacher (1968) and Maynard Smith (1962). At present this theory appears to be unsupportable because although life shortening effects of ionizing radiation have been observed in mice (Llndop & Rotblal, 1961) humans (Seltsen & Sartwell, 1965) and Drospohlla (Gartner, 1973) the symptoms developed in irradiated individuals whose longevity is shortened are different from those seen during 'natural' aging or unlrradlated controls. Hence, ionizing radiation does not accelerate the normal aging process, but may cause early death by increasing the frequency of cancer or through other causes. Thus, 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. Rattan (1985) and Holllday (1986[a,b]) also feel that aging cannot be due to "somatic mutations" because of lack of any evidence for mutations which inactivated whole chromosomes. For example cells treated with various mutagens do not have reduced life span in culture, tetraploid cells have no increased life span and mutations *in vivo* accumulate exponentially but not linearly.

ERROR CATASTROPHE THEORY

Orgel (1963) advanced the error theory of aging according to which errors occurring during Information transfer steps like

transcription and translation may cause accumulation of defective proteins which cause aging. Such errors include incorporation of wrong nucleotldes into mRNAs during transcription that may change the triplet codons or incorporation of wrong amino acids into proteins during translation that may make them partially or totally inactive. Particularly errors in proteins that are involved in protein synthesis like the enzymes required for transcription and translation would amplify such errors in cells. These errors may self propagate and cause exponential increase in defective enzymes and proteins. This may lead to 'error catastrophe' and result in senescence and death of cells.

Incorporation of errors into enzymes responsible for metabolism at the time of transcription of their mRNAs or their translation may not be deleterious. Since the enzymes have short half life and are soon degraded. Thereby the error is erased. If, however an error is introduced into RNA polymerases or aminoacyl tRNA synthetase, it may cause introduction of errors or wrong amino acids into all types of proteins that the cell synthesizes. Hence the level of error containing proteins would increase exponentially. Thus the theory is based on the assumption that the information transfer machinery is prone to error. In other words the fidelity or accuracy of the machinery is not absolute, and once errors arise in enzymes for protein synthesis, they get amplified.

Later Orgel (1970) modified his theory and argued that even though the accuracy of protein synthesizing machinery is not

absolute and permits introduction of errors, such errors may not always accumulate as the successive generations of protein synthetic apparatus are discrete. Orgel himself pointed out, why do errors not accumulate in germ cells? If errors accumulate in the germ line, the species would be wiped out. He has postulated that 'Quality control' processes may operate during oogensls and early development and lead to rejection of ova or embryos having high levels of errors. The question is why and how does such a quality control mechanism, if there is one, fall to operate after the development period? Further, if occurrence of errors is the cause of aging, cessation of cell division and death, it should follow that transformed or tumor cells should have no errors. It is unlikely that transformed or tumor cells should have no errors. It is also difficult to accept that DNA replication and protein synthesis have absolute fidelity in transformed cells.

Kirkwood (1977) has suggested that somatic cells have less ability to regulate errors. He argues that errors are inherent in all processes of macro molecular information transfer. His suggestion "to sustain the prospect of further evolutionary change and so improve its chance of survival, an organism must make occasional copying errors" appears to imply that the organism knows it must evolve and, therefore make errors. Does the organism know where and when to make the errors that would make it evolve? Though it is likely that had the fidelity of the information transfer system been absolute, there would have been no evolution. Hoffman (1974) suggested that translation machinery is such that errors are not possible and hence error catastrophe

cannot occur. On the other hand, Kirkwood & Holliday (1975) point out the unlikelihood of such accuracy and suggest that each protein synthesizing machinery may have some activity even if it has errors, and this determines whether or not stability is attained.

Hopfield (1974) has suggested that errors can be avoided in replication machinery by expenditure of energy, either by proof reading or by destroying erroneous products. Scavenging enzymes may remove error containing products. Accuracy in germ line is vital for gene survival, but may not be so essential for somatic cells. So Kirkwood (1977) has proposed that aging may be due to switching off of the mechanisms responsible for high accuracy in the translation apparatus at or around the time of differentiation of somatic cells from germ line to save energy. So, the somatic cells are left in an unstable state that causes a gradual increase in errors and after a period commits them to error catastrophe and senescence.

Several experimental findings contradict the occurrence of errors in proteins in amounts that may cause aging or error catastrophe. There are sufficient data to show the decrease (30-70%) in specific activities in old age of certain enzymes like aldolase A (Gershon & Gershon, 1973[a]), aldolase B (Gershon & Gershon, 1973) and super oxide dismutase (Relss & Gershon, 1976 ') in rats, isocitrate lyase (Gershon & Gershon, 1970; Relss & Rothsteln, 1975) aldolase (Zeelon *et al.*, 1973) enolase (Sharma *et al.*, 1976)ln *Turbatrix aceti* (free living nematode).

Differences in thermolability and antigenicity have been observed for these enzymes. A few enzymes, however do not differ in their specific activities viz., triose-p- isomerase (Gupta & Rothstein, 1976[a]) of *Turbatrix* and ornithine decarboxylase of rat liver (Obenarader & Prouty, 1977[a,b]).

Measurements of some of parameters of the above enzymes like Km, Ki, electrophoretic mobility and mol.wt do not show any significant differences between the young and old enzymes. What then are reasons for the difference in their catalytic activity? Use of powerful technique like isoelectric focussing also did not show any differences in super oxide dismutase (Reiss & Gershon 1976 ') of young and old rats.

Thus a large bank of available data show that errors like substitution of amino acids in proteins did not occur in any significant amount with increasing age either *in vivo* or *in vitro.* Furthermore errors are random processes, and if they occurred, one would except to find proteins having a spectrum of amino acid substitutions. No such proteins have yet been identified. Moreover, the more or less fixed life span of species and a gradual decline in physiological functions with increasing age cannot be explained on the basis of random errors, because they have to occur at definite rate in order to account for these phenomena. This would mean regulation of the occurrence of errors by genes or other factors and such a proposition would contradict the basic concept of error theory. Hence, increase in errors in functional macro molecules with increasing age is, unlikely to be

the cause of aging.

POST TRANSLATIONAL MODIFICATION THEORY

This theory, in a way is a slight modification or extension of error catastrophe theory. While it envisages an 'altered' protein in aging cells, the reasons for such alterations are thought to be conformational changes in the protein, but not any alteration in the primary structure of the proteins. Rothstein 8. co workers studied several enzymes from young and old *Turbatrix aceti* viz., isocitrate lyase (Reiss & Rothsteln, 1975) enolase (Sharma *et al.*, 1976) triose-p-isomerase and phosphoglycerate Kinase (Gupta & Rothstein, 1976 ') and in each case mol.wt,Km, electrophoretlc mobility are similar, but the old enzyme exhibited lowered specific activity. They provided ample evidence to support the idea that 'young' enzyme is converted to an 'old' form without any change in the primary sequences or involvement of intrinsic factors. They suggested that the enzymes from old age are different from those obtained from young the difference being in the conformatlonal aspects of proteins.

Gershon (1979) suggested that the cause of decrease in the specific activity and increase in susceptibility to proteases and heat of enzymes seen in old age was due to subtle post translational modifications like glycosylation, methylation etc., which do not alter the net electric charge of protein. However, half life of altered proteins would increase in old age possibly due to decrease in protease activity. The experimental findings

which support this theory are the reduced turnover of altered proteins with age possibly due to decrease in the concentration or activity of cellular proteases that preferentially degrade altered proteins (Stadman, 1988, 1990).

A some what similar hypothesis was advanced by McKarrow (1979) according to which post translatlonal modifications of various proteins, primarily through deamidation of asparglne and glutamine and racemization of aspartic acid, may be the cause of defective function of these proteins and therefore resulting in aging of the cell.

FREE RADICAL THEORY

This concept was originally proposed by Harman in 1956. He has reviewed this topic once again in 1988, and according to this theory "Sum of deleterious free radical reactions going on continuously throughout the cells and tissues constitutes aging process or is a major contributor to it". In mammalian systems the free radical reactions are largely those involving oxygen. The ubiquitous free radical reactions are initiated continuously throughout cells and tissues from both enzymatic and non enzymatic reactions. Examples include enzymatic reactions involved in the respiratory chain (Harman, 1972; Nohl *et al.,* 1978[a,b]; 1979) phagocytosis (Klebanoff, *et al.,* 1980) and in the cytochrome P-450 system (Sato *et al.,* 1978). Enzymatic reactions of oxygen (Scott, 1965; Mead, 1976) with organic compounds and also similar reactions initiated by ionizing radiation also produce free

radicals (Altman et *al.,* 1970). Since free radicals are highly reactive, it would be expected that all components of the body would be constantly subject to some degree of chemical change in a more or less random manner. The expected changes include (1) adducts or cross links in biologically important molecules such as DNA, proteins and membranes (Harman, 1967; Tas et *al.,* 1980) accumulation of oxldatlve alterations in long lived molecules like collagen (Labella, *et al.,* 1965) elastln (Labella et *al.,* 1966) (3) breakdown of mucopolysacnarides through oxidative degradation (Mat Sumura, et *al.,* 1966) accumulation of metabollcally inert material such as age pigments (lipofuscin) through oxldatlve polymerization reactions involving particularly lipids and proteins (Norkin, 1966).

Lipofuscln (age pigment) accumulates with age (Brizzee, et *al.,* 1974) in various areas of central nervous system (CNS) in parallel with the activities of oxidative enzymes (Friede et *al.,* 1962; Ferrendelli et *al.,* 1971). It is clear, however, that accumulation of age pigment is only a result of aging.

Cells have different defense systems to eliminate free radicals before they can interact with cellular components. Superoxide dismutase (SOD) converts the O radical to H_2O which in turn is converted into water by catalase and glutathione peroxidase. The levels of SOD, catalase and glutathione peroxidase differ in different species (Cutler 1984). Besides enzymatic defense systems, there are several substances generated in the cell or present in the food that have the ability to

scavenge free radicals. Examples of such protectors are selenium, Vit E, Vit C, glutathione and cysteine.

Further, an age related decline in the efficiency of these defense system can accelerate the rate of such free radical dependent damage during aging. An age related decline of anti oxidant systems has been reported (Sohal & Allen, 1985; Benzi et *al.*, 1989). However, there is no report that the level of free radicals is higher in older animals. The question, therefore, is whether free radical reactions could be the sole cause of aging. Although an answer to this question that is satisfactory to all may never be reached, it is apparent that free radicals are secondary products of metabolism and hence are unlikely to explain the primary cause of aging.

Thus, all Stochastic theories explain aging on the basis of changes in various macro molecules and deterioration of different structures of cells like the membranes, lysosomes, mitochondria or changes in homeostasis or defense mechanisms. It is obvious that these changes are secondary in nature. Since mutations, errors in transcription and translation and free radicals production occur randomly. These theories fail to explain how the loss of various functions occur gradually and in a time dependent manner and how the life span of individual species is fixed. However, there are some theories of aging which have genetic basis and at the same time take into account of the influence of environmental factors on the process of aging. These are the gene regulation theory of aging of Kanungo (1980) and DNA damage/repair hypothesis of Hart &

Setlow (1974). Gensler and Bernstein (1981) & Mullart *et al.*, 1990, which is also supported by several others including this laboratory.

GENE REGULATION THEORY

Kanungo (1980) proposed the gene regulation theory of aging. This theory explained two important characteristics of aging (a) deterioration of functional ability (b) fixed life span of individuals in a species. According to his theory, senescence may occur by changes in the expression of genes after reproductive maturity is reached. It is postulated that growth and differentiation occurs as a consequence of sequential activation and repression of certain genes unique to these phases. The products or by products of the genes responsible for these phases on reaching critical levels, stimulate certain other specific genes responsible for the reproductive phase. The products and by-products of these genes, like sex steroid hormones are responsible for conferring reproductive ability to the organism. Some of the gene products of the reproductive phase in turn repress some of the genes required for differentiation and growth and thereby stop further growth of the organism.

The reproductive ability of an organism is highest soon after it attains reproductive maturity. However, as a result of continued reproduction, certain factors may be depleted which may not be replenished as fast as they disappear. Such factors may be of crucial importance for keeping certain genes expressed or

repressed. Likewise, certain factors may accumulate as a result of reproduction and these may not only affect the expression of certain genes but may also cause expression of certain undesirable genes, which should normally remain repressed. This may lead to general destabilization of and the onset of senescence. Therefore, changes in the levels of sex hormones and other modulators which influence genome function directly or indirectly may cause a gradual decline in reproduction and lead to senescence. The effects due to alterations in the levels of modulators may be synerglstlc rather than additive which may be the reason why there occurs an exponential decline in functional abilities of the organism after adulthood.

DNA DAMAGE/REPAIR HYPOTHESIS

Fundamental to aging theories is the importance of genomic integrity. Various physical, chemical, environmental changes and cellular metabolites have been shown to damage cellular DNA. Studies on microorganisms, mammalian and plant cells have shown that DNA damage results in deviation in physiological processes like growth, division, transcription, cell death, mutation and induction of transformation (Hart & Trosko 1976). Various DNA repair pathways exist in the cell to cope with these damages. However, since DNA repair is not always perfect, it is not inconceivable that certain forms of DNA damage accumulate with age; age related decreases in DNA-repair efficiency would enhance such an effect. Inter individual differences in the extent of

exposure to DNA-damaging agents but also in the activity of DNA repair systems can lead to large inter-individual differences in the rate of damage accumulation. This could explain the variation observed in the aging rate among individuals.

DNA DAMAGE

DNA is under continues threat from various chemical and biological factors that can bring about alteration in its structure and impairment or modification of its function. Lesions in DNA can be introduced either by extrinsic chemicals and radiation or intrinsic processes. The more relevant of DNA damage with respect to aging would seem to be those induced by endogenous biochemical and physical reactions (Hart et *al.,* 1979 Ames 1983; Gensler *et al.*, 1987.

The most ubiquitous natural cause of DNA damage is body heat. Due to the thermodynamic instability of DNA, certain alterations in its structure can already occur at the normal human body temperature of 37°C. For instance, the N-glycosylic bond between purlne or pyrimidine base and the deoxyribose is relatively labile and can easily be broken at elevated temperature (37 C) resulting in apurnlc or apyrimidic sites (AP sites). It is estimated that a mammalian cell looses about 10,000 purlnes and 500 pyrlmidlnes from its DNA during a period of 20 hrs. by spontaneous hydrolysis at 37°C (Lindhal & Nyberg, 1972). In a long lived non replicating mammalian cell such as as the human nerve cell, it is estimated that about 10^8 purine bases are

released from DNA of single cell during the life time of an individual *i.e. 3%* of the total number of purines in DNA. AP sites in DNA, if unrepaired, decay to single strand breaks (SSB) in about 100 hrs. (Lindhal & Andersson, 1972). The oxidative deamination of DNA-cytosine to uracil is thought to occur spontaneously at an appreciable rate (Lindhal & Nyberg, 1974).

The other endogenous DNA damaging agents are free radicals. The free radicals generated during several metabolic processes in the cell and also by ionizating radiation react with many cellular components including DNA. Free radicals give rise to variety of oxidative damages, including single strand breaks (SSB) double strand breaks (DSB), AP sites and cross links, but also to several modified bases such as thymine glycol and 5-hydroxymethyl uracil, two forms of oxidized thymine (Cadet and Berger, 1985; Teoule, 1987). Loeb (1989) estimated that in human cells about 10,000 free radical damages are induced per cell per day.

Other reactive species besides oxygen can be the cause of spontaneous DNA damage as well. Bucala *et al.,* (1984, 1985) indicate that non enzymatic reaction of glucose and other reducing sugars with the amino groups of DNA bases can lead to the formation of DNA adducts. Lee *et al.,* (1987) reported that glucose 6-phosphate interaction with the amino group of lysine leads to the formation of DNA protein cross links, the occurrence of which has been observed in various cells during aging (Bojanovic *et al.,* 1970).

Another spontaneously occurring reaction is the alkylation of DNA by S-adenosyl-L-methionine (SAM) the normal intra cellular methyl group donor (Barrows *et al.,* 1982; Rydberg *et al.,* 1982). This reaction can lead to formation of N methyl guanlne, ^{3} N -methyl adenlne, ^{3} N -methyl thymine and small amounts of 0 -methyl guanlne the methylation of guanlne and adenlne bases causes a further destablllzatlon of the N-glycosyllc bond, resulting in an increased spontaneous cleavage and the formation of AP sites (Llndhal & Nyberg, 1972).

The major exogenous source of DNA damage is sun light. The ultraviolet light forms a cyclobutane dimer type pyrimidine dimers (Setlow, 1982; Nlggli *et al.,* 1988). Theoretically there can be twelve isomeric forms of pyrimidine dimers. However, only four of them cis-syn, cis-anti, trans-syn and trans-anti are formed biologically in significant amounts. Formation of pyrimidine dimer is influenced by the nucleotide composition of the DNA (Setlow & Carrier 1966). Other photo products formed in DNA by UV irradiation are non cyclo butane type pyrimldine adducts, pyrimidino-pyrimidine 6-4 lesions, pyrimldlne hydrates and thymine glycols (Roberts, 1978). UV irradiation also induces DNA protein cross links and single strand breaks (Peak *et al.,* 1985 Lai et *al.,* 1987). Ionizing radiation (X-ray, r-ray and fast neutrons) cause single and double strand breaks and cross links (Scholes, 1983; Hutchinson, 1985).

The human diet contains a great variety of natural mutagens and carcinogens, such as polycycllc aromatic hydrocarbons (PAH),

aflatoxin B , and nitrosamines (Ames,1983). These agents can react with DNA, inducing several types of damages including SSB, DSB and bulky adducts. Cigarette smoke also contains various carcinogenic compounds including benzo (a) pyrene (BAP). (Everson et *al.*, 1986; Randerath, et *al.*, 1986) DNA adducts were detected in bronchial cells of heavy smokers (Bann et *al.*, 1988).

DNA REPAIR PATHWAYS

The immediate cellular response to DNA damage is to repair its damaged DNA. Cells are equipped with a battery of repair systems to remove such damages. Eukaryotic DNA repair is a complex process involving multistep reactions catalyzed by different enzymes and has been extensively reviewed (Hart & Trosko 1976; Lehmann & Karran, 1981; Friedberg, 1985; Bohr & Wasserman, 1988). The repair systems are generally divided into 3 categories i.e. direct repair, excision repair and post replicational repair.

a) DIRECT REVERSAL OF THE DAMAGE

Enzymatic photo reactivation (EPR) is a highly specific and simple mechanism for the direct reversal of pyrimidine dimer to monomers. DNA photolyase catalyzes the monomerization of cys-syn cyclobutane type of pyrimidine dimers utilizing the energy source from light generally between 300 and 500 nm. An enzyme substrate complex forms before the absorption of photo reactivating light. The precise wavelength utilized by the enzyme varies with the source of enzyme and also the chromophore composition. This

enzyme has been purified and characterized from a number of species including placental mammals (Rupert, 1975; Sancar & Sancar, 1988). Another mode of simple reversal of damage is the repair of 0 -alkylguanine. This lesion has considerable biological importance in mammalian systems since it is implicated in mutagenesis (Loveless, 1969; Newbold et *al.*, 1980). An enzyme activity called alkylguanlne methyl transferase, has been found in various mammalian species including human, which transfers alkyl group of the DNA to cystelne residue of enzyme molecule itself (llndhal, 1982).

b. DNA EXCISION REPAIR

This is brought about by a complex multi enzyme system. The concept of excision repair was first put forward by setlow and carrier (1964) which envisaged a four step process for the removal of damaged sections of DNA. According to this, after making an incision at the damaged site by an endonuclease, the damaged section was removed by an exonuclease, the resulting gap was filled by DNA polymerase with the opposite intact strand as template and finally the nick was llgated by DNA ligase.

Depending upon the nature of incision event the excision repair has been further classified into two types viz., base excision repair and nucleotide excision repair. In the former type, the damage is recognized by a class of narrow specific enzymes called DNA glycosylases (Llndhal, 1979). These enzymes remove the damaged base, leaving 'AP' sites in the DNA. Whether

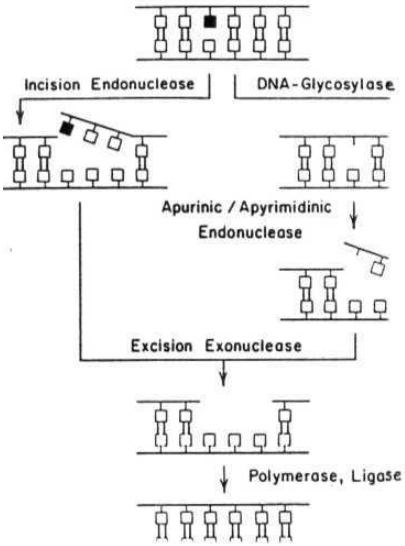
these arise by the action of DNA glycosylases or through spontaneous hydrolysis of N-glycosydic bonds, their removal orexclslon requires the action of one or more nucleases (like endodeoxyrlbo- nucleases & exodeoxyrlboneculeases) (See Fig.1). Enzymes that specifically recognize sites of base loss (like purines or pyrimidines) in DNA are called AP endonucleases. The sequential action of 5' acting and 3' acting AP endonucleases (or vice versa) can result in the excision of AP moieties, leaving a gap of Just one nucleotide. Alternatively the action of Just single AP endonuclease (either 5' or 3' acting) will generate an incision (nick or strand break) that provides substrate sites for enzymes called exonucleases which degrade DNA in either 5'_____> 3' or 3'_____> 5' directions at free ends created by incisions.

Several AP endonucleases from *B subtilis* (Inoue *et al.,* 1978), S *cerevisiae* (Futcher *et al.*,1979) calfhymus (Lijungquist *et al.,* 1975) calf liver (Kuebler, 1977) Mouse epidermis, (Ludwlg 1979). human lymphocytes (Brent, 1979) human skin fibroblasts and human placenta (Llnsely *et al.,* 1977) were purified and shown that these AP endonucleases are involved in excision of 'AP' sites from DNA. Loeb (1986) reported that unrepaired AP sites lead to GC _____> AT and AT_____> TA mutations, and these mutations could lead to carclnogensls.

In the nucleotide excision repair, a damage specific endonuclease makes a nick at the damaged site. Examples of such enzymes or gene products involved in nucleotide excision repair are, UV specific endonuclease and UVr ABC gene products in

Figure 1. Repair of damaged DNA. Excision-repair pathways. The sequence of reactions on the left represent nucleotide excision repair pathway while on the right base excision repair pathway. Figure taken from T inn .

FIG-1


Incision Endonuclease
DNA-Glycosylase
Apurinic / Apyrimidinic
Endonuclease
Excision Exonuclease
Polymerase, Ligase

prokaryotes. A repair specific endonuclease was first isolated from M. luteus (Takagi et *al.,* 1966). This enzyme was shown to make nicks in single strand DNA containing cyclobutane dimer and in UV irradiated double stranded DNA but does not act upon unirradiated or denatured DNA Haseline *et* aJ.,(1980), had shown that the UV specific endonuclease from M. luteus removes dlmer in a two step mechanism thus making the distinction between the two repair processes less clear. Several endonucleases which preferentially attack UV-irradiated relative to unirradlated DNA have been purified and characterized (Freidberg, 1977). Van Lancker & Tomura (1974) purified an enzyme from rat liver and showed that it catalyze the formation of single strand breaks in duplex UV irradiated or acetyl aminoflourence-treated DNA. Waldstein *et al.,* (1979) reported a 50 fold purification of a UV DNA incising activity from calf thymus that apparently recognizes pyrimidine dimers specifically.

Enzymes and other gene products involved in ER in prokaryotes have been identified, purified and characterized eg. UV specific endonuclease from *Micrococus luteus* , DNA glycosylases, AP endonucleases, UVr ABC gene products. In contrast the enzymology of ER in eukaryotes is less understood, even though some damage specific nucleases, AP endonucleases, DNA glycosylases, DNA polymerases, DNA llgase II, poly (ADPR) polymerase and five RAD gene products have been found to be involved in the repair of DNA damage in vitro (Llndhal, 1979; Lehman & Karran, 1981; Freidberg, 1987; Sancar & Sancar, 1988). Some repair endonucleases which recognize damaged regions in DNA

induced by chemicals, UV or r-irradiation have been purified from a variety of mammalian sources (van Lancker & Tomura, 1974; Bachetti & Benne, 1975; Brent, 1983; Waldstein et *al.*, 1979; Helland *et al.*, 1985, Doetsch, *et al.*, 1986, Sanderson, *et al.*, Ivanov, et *al.*, 1988).

In mammalian cells including human there are indications to suggest that at least 15 gene products are involved in incision repair process. This number could be predicted making use of a series of human diseases characterized by DNA repair deficiencies *(eg. Xeroderma pigmentosum)* and also a variety of mouse and hamster cell mutants selected *in vitro* (Collins et *al.*, 1987). Efforts are underway in several laboratories to identify and isolate genes responsible for DNA repair in normal human cell lines as well as from rodent cell lines. The possible overlap of mutants in humans and rodent cell lines is also being tested. Already a couple of genes in human cells involved in UV repair have been cloned and when these genes are introduced into hamster mutant cells, they seem to function well by correcting the defect in UV repair of the mutant cells. Similarly genes cloned from rodent cells could be tested for correcting DNA repair deficiency in human cells. Work is continuing in these existing lines (Hoejmakes, 1987).

On the basis of the number of substituted nucleotides per repair patch, this DNA repair has been classified into short patch (2-10 nucleotides) and long patch (25-100 nucleotides) repair. Damage induced by UV, as well as that induced by 'UV' like

chemicals (such as polycycllc aromatic hydrocarbons) is removed via long patch repair, while damage induced by r-rays and r-like chemicals (such as alkylatlng agents) is repaired via short-patch repair (Regan & Setlow, 1974).

c. POST REPLICATIONAL REPAIR

This mode of DNA repair was first proposed by Rupp and Howard-Flanders in prokaryotes (1968). According to this model DNA damage like pyrimidine dimers interrupt DNA chain elongation during synthesis, which then resumes beyond the damaged site, leaving a gap opposite to the damage that can be filled by recombination or de *novo* synthesis. In prokaryotes both recombinational repair and transleslon synthesis are very well characterized. Two possible recombinational events can occur, one in which the dimer remains in the parental strand and the other in which the dimer is transferred to daughter strand (Cox & Lehman, 1987). It is hypothesized that in transleslon syntheses, the DNA polymerase I 'idles' at the lesion, adding and removing the nucleotldes with the proof reading function of 3'——> 5' exonuclease activity. The DNA damage induces 'SOS' response which somehow suppress the 3'——> 5' exonuclease activity, thus allowing the synthesis continuously. The recomblnatlonal repair and transleslon synthesis have been described in mammalian cells also. (Park & Cleaver, 1979; Meneghini, *et al.*, 1981).

Proper and immediate repair of the damaged regions in the DNA is necessary for the maintenance of DNA structural integrity and thereby the cell function. Delay or reduced rate of DNA repair may lead to accumulation of damages and loss of function. Alexander (1967) was the first to propose that the DNA damage is the primary cause of aging. Later Painter 8. Cleaver (1969) after comparing the DNA repair capacities of several kinds of mammalian cells declared 'DNA repair' as the longevity assurance system. Hart & Setlow (1974) observed strong log-linear correlation between UV induced UDS and maximum achievable life span in skin fibroblasts of different mammalian species. According to Gensler and Bernstein (1981) DNA damage is the primary cause of aging and it is determined by the balance between the rate at which DNA damage occurs and the rate of DNA repair. If the DNA repair rate is less than the rate at which DNA gets damaged, lesions in the DNA accumulate, when the cell no longer cope up with damaged DNA, it dies, which will be followed by tissue dysfunction and death of the animal. Several reviews appeared asserting the importance of maintenance of genomic integrity and DNA damage/repair in relation to aging process (Hart & Trosko, 1976; Tice, 1978; Hart et al., 1979; Gensler & Bernstein, 1981; Lehman, 1985; Vijig & Knook, 1987; Rattan, 1989; Mullart, *et al.,* 1990).

Thus the existing literature shows some relation between DNA damage repair, transcriptional and translational activity and aging process.

TABLE - 1

DNA REPAIR ENZYMES IN BRAIN

Endonuclease of lamb brain	Attacks single strand DNA releasing oligonucleotides with 5' phosphate (Healy, *et al.*, 1963).
Acid and Alkaline DNases of rat brain	Acid DNase attacks native DNA with pH optimum of 5.0 releasing oligo-nucleotides with 3'-phosphate alka-line DNase, pH optimum between 7.4 to 8.9 degrades denatured single stranded DNA releasing 5'-phosphate oligonu- cleotides (Sung, 1968).
Acid DNase (UV DNase) and alkaline DNase (AP DNase from chick and rat brain	Acid DNase (UV DNase) attacks native alkaline DNase (AP DNase) acts on either denatured DNA or depurinated DNA (Subba Rao, 1990).
AP-endodeoxynuclease from rat neocortex chromatin	Incises near AP sites of a super-coiled $DNA_{1,2}$ Optimal pH 7.8 requires Mg or Mn (Ivanov, *et al.*, 1988).
Exonuclease (DNase B III) from rat brain neuronal nuclei	Excises single strand DNA of single strand termini in a duplex DNA in 5'___> 3' direction. Nucleoside-5'-monophosphate is released (Ivanov, *et al.*, 1983).
Uracil-N-Glycosylase of human foetal brain	Action similar to that found in other mammalian tissues (Krokan, et *al.*, 1983).
DNA polymerase β of rat and mouse neurons	Action same as in other tissues and (Hubschur, *et al.*, 1979).
DNA ligase of neuronal & glial cells of guinea pig brain and rat cerebellum	Action same as In E.Coli but requires ATP (Inoue & Kato, 1980)
Poly (ADPR) synthase of bovine brain	Action same as in other mammalian tissues (Bilen, *et al.*, 1981).
O^6-alkyl-Guanine-DNA alkyl transferase of rat and human brain	Action same as in other mammalian tissues (Wiestler, 1984).
Photolyase of Marsupial brain	Monomerization of pyrimidine dimers (Rupert. 1975).

The specific information is taken from a different sources and the references are cited.

BRAIN, DNA REPAIR AND AGING

Although aging may be a generalized phenomenon throughout the mammalian body, attention has frequently been focused on such controlling and integrative organs like brain and endocrines in which age dependent changes may have widespread consequences. Many researchers interconnected the species life span and the brain's regulatory abilities. Sacher (1973) identified a significant correlation between brain weight and life span and he proposed that brain may play an important 'pacemaker' role in aging or it may be an organ of 'longevity' due to its unique role in adaptation to the external environment and in homeostatlc integration of all organs of the body.

The high metabolic rate of cerebral tissue probably results in higher rate of production of DNA damaging free radicals from electron transport and other normal metabolic reactions (Slater, 1984). Thus, it is possible to expect that these cells should possess good DNA repair machinery to repair and maintain the integrity of the genome. The study of DNA repair in brain is important because neurons do not posses mitotic ability. A search has begun for DNA repair capacity of brain and indeed some DNA repair enzymes have been detected in brain and the list is shown in Table 1. However, details regarding the actual mechanisms of DNA-repair in brain are still awaited.

SCOPE OF THE PRESENT INVESTIGATION

The phenomenon of DNA damage and its repair is of special significance to a post mitotic tissue like brain, because it brings homeostatic integration of all the bodily organs. Although there is extensive literature regarding the enzymology and pathways of DNA repair in prokaryotes these aspects are not yet clearly studied in mammalian cells, and much less in brain cells. (Kuenzle, 1985).

This laboratory has been involved in studies on brain aging phenomenon during the past several years. These studies have been essentially related to changes in nucleic acids, protein, structural changes in DNA and levels of some DNA metabolizing enzymes like DNases and DNA polymerases (Subba Rao, 1986). During these studies, two major DNase activities were identified both in chick and rat brain. From the profiles of changes of these activities through the life span of rat, it appeared that while one of the DNases with an acidic pH optimum, shows highest activity during early embryonic stages and decreased during postnatal age. These results revealed that the metabolic role of acid DNase is quite likely linked to DNA repair process connected to DNA replication i.e. post replicatlonal repair. However, this enzyme shows acidic pH optimum which warranted a comparison of its main properties with that of DNase II, studied extensively by Coordinnier & Bernardi, (1968). Subsequent studies of Subrahmanyam (1989) on acid DNase in brain cells showed the

presence of this enzyme, in nuclei of neurons, astrocytes and oligodendrocytes in substantial amounts. These results raised several pertinent questions as follows:

1. What is the precise physiological role of acid DNase in brain?
2. What is the mechanism of action of this enzyme?
3. How brain DNase differs from that of classical DNase II?
4. Why the specific activity of acid DNase decreases with age?
5. How this enzyme activity is regulated in the brain?
6. Is the nuclear DNase similar to that of cytoplasmic DNase?

The present investigation is undertaken to gain some answers to the above questions.

As a first step the Putative DNA repair enzyme, has been purified to nearly 240, fold by using ammonium sulfate ppt, Gel filtration and affinity chromatography. The properties of purified enzyme were studied in order to establish the mechanism of action of the enzyme as well as its substrate specificity. The purified enzyme was found to be an endonuclease and acting on both native and native UV irradiated DNA. The enzyme does not show activity towards apurnic DNA and denatured DNA. The properties of brain DNase were compared with the classical spleen acid DNase (DNase II). It appears that the brain enzyme is different from the DNase II. The results are presented and discussed in Chapter III.

The specific activities of brain acid DNase decreases with age. In order to understand the reasons for this decrease, the enzyme was also purified by 'old' brain and the properties were compared with 'young' enzyme. The results suggest accumulation of inactive enzyme molecules in aging brain. These results are presented and discussed in Chapter IV.

If the acid DNase has a role in DNA replication/repair one would expect to find this enzyme in nuclear fraction of brain cells. Indeed this enzyme is found in nuclear fraction of the cells and the enzyme was purified from nuclear fraction as well. The nuclear enzyme was similar to the soluble one. These results are shown and discussed in Chapter V.

In an attempt to know how the brain acid DNase activity is regulated, a specific inhibitor, protein in nature was found in the brain and this inhibitor was also purified and its properties were studied. The results are presented and discussed in Chapter VI.

CHAPTER II

Materials and Methods

CHAPTER - II

GENERAL MATERIALS AND METHODS

ANIMALS

Rats of wistar strain and of specific age (both sexes) were obtained from this university animal house. They were fed *ad libitum* standard rat feed of Hindustan Lever, India. Animals were divided into two categories on the basis of age. Young (7 days: the day of birth was considered as day zero) and old (> 2 years). Rabbits (2-2.5 Kg) used for the production of antisera were purchased from Indian Immunologicals animal house, Hyderabad.

MATERIALS

Highly polymerized calf thymus DNA, RNase (Bovine pancreas) protease (*B. subtilus*), Bovine serum albumin, Acrylamide, Bis-acrylamide. Ammonium per sulfate, deoxyguanosine and trizma base were purchased from Sigma Chemical Company, St.Louis, MO, USA. H thymidine (18 ci/mmole) was purchased from Bhabha Atomic Research Centre, Bombay, India. All other chemicals used were of analytical grade.

ASSAY OF ACID DNase

The assay medium consisted of 100 mM sodium acetate buffer

pH 5.0, 200 µg of native or native DNA exposed to UV light (UV DNA) and the enzyme fraction. The final assay volume was 600 µl. The incubation was carried out at 37°C in Dubnoff metabolic shaker water bath for 2 hrs. After the incubation the tubes were transferred to ice and 400 µl of 1.4 N perchloric acid (PCA) was added. After keeping in ice for 10 min., the tubes were centrifuged at 10,000 rpm for 10 mln. and the supernatants were transferred to another set of tubes and the optical density was measured at 260 nm in a Hitachi 150-20 spectrophotometer. The enzyme activity was expressed as µg of DNA phosphorus liberated from native or UV DNA as acid soluble products/2 hrs at 37°C.

ASSAY OF ALKALINE DNase

The reaction mixture volume, method of assay and expression of activity and other details are the same as in the case of acid DNase, except that the reaction mixture consisted of either heat denatured or depurinated calf thymus DNA as the substrate and 50 mM Tris HC1 buffer pH 8.0.

ESTIMATION OF PROTEIN

Protein content was determined according to the method of Lowry (1951). An aliquot of the sample or protein standard (10-100 µg BSA) was taken into test tubes and the volume was made upto 1 ml with distilled water. To this 5 ml of solution A was added (solution A: To 100 ml 2% sodium carbonate in 0.1 N NaOH. 1

ml of 2'/. sodium potassium tartarate and 1 ml of 1% copper sulfate were added and used immediately. After 20 min. 0.5 ml of 1 N Folin's reagent was added to the tubes, while stirring. After keeping the tubes at room temperature for 30 mln. the developed blue colour was read at 670 nm against a proper blank and standard.

PREPARATION OF ^{3}H-LABELLED DNA FROM *E.Coli*

DNA of *E. Coli* A 19 cells was labelled with ^{3}H-thymidine according to the procedure of Inga Mahler (1967) 250 ml flask containing 50 ml growth medium (1 g NH Cl, 0.5 g NaCl, 3 g KH PO , 6 g Na HPO , 1 ml of 1 M MgSO 0. 1 ml of 1 M CaCl and 1.5 g casamino acids per liter, after autoclaving, was supplemented with 10 ml 50% sterile glucose) and inoculated with *E.Coli* A 19 and kept on a shaker overnight at 37 C. The overnight grown culture was added as inoculum to 500 ml of growth medium in a 2 liter flask and the incubation was continued for 2 hrs. Then 100 mg of deoxyguanosine and 1 mci (methyl H)-thymidlne were added to the medium. After further incubation for 1 hr, the cells were chilled with crushed ice and harvested, ^{3}H labelled DNA from cell pellet was isolated by the method of Marmur (1961).

2 to 3 g wet packed cells were washed with 50 ml of saline-EDTA consisting of 0.15 M NaCl and 0.1 M EDTA pH 8.0. The final cell pellet was suspended in 25 ml of saline EDTA and 10 mg of lysozyme was added and incubated at 37 C for 1 hr. 2 ml of 25%

SDS were added and kept at 60°C for 10 min and then brought to room temperature. 5 M solution of sodium perchlorate was added to bring the final concentration to 1 M. The whole mixture was shaken with equal volume of chloroform : isoamyl alcohol (24:1) for 30 min, and then centrifuged for 10 min, at 5000 rpm. The upper aq. phase containing the nucleic acids was taken out. To this 2 vols of ethylalcohol (95%) was added gently and the nucleic acids dissolved in 10 to 15 ml of saline citrate (0.015 M NaCl and 0.0015 M trisodium citrate pH 7.0). Then an equal volume of chloroform : isoamyl alcohol (24:1) was added and shaken for 15 min. and then centrlfuged for 5 min, at 5,000 rpm. Two volumes of ethylalochol was added to the aqueous phase containing nucleic acids and the precipitate was dissolved in saline citrate. Then ribonuclease, at a conc. of 50 jig/ml was added and incubated at 37 C for 30 min. The whole digestion mixture was extracted with chloroform:isoamyl alcohol (24:1) and the DNA in the aqueous phase was precipitated once again with ethyl alcohol. The DNA was dissolved in 9 ml of saline citrate and 1 ml of acetate-EDTA consisting of 3 M sodium acetate and 1 mM EDTA pH 7.0 was added. To this solution 0.54 volumes of isopropylalcohol was added dropwise while stirring rapidly. The DNA was spooled and made free of acetate-EDTA by successive washing with increasing per centage of ethylalcohol (70% to 90%). The final DNA precipitate was dissolved in 10 mM Tris HCl pH 8.0 containing 1 mM EDTA, 2 drops of chloroform were added and stored at 4 C. The specific activity of the isolated DNA was around 7 x 10 DPM/mg of DNA.

CHAPTER III

Purification and Characterization of Native and UV irradiated DNA Specific Deoxyribonuclease (acid DNase) from Young Rat Brain

CHAPTER - III

PURIFICATION AND CHARACTERIZATION OF NATIVE AND UV IRRADIATED DNA SPECIFIC DEOXYRIBONUCLEASE (ACID DNase) FROM YOUNG RAT BRAIN

INTRODUCTION:

Deoxyribonucleases are ubiquitous in nature, apparently occurring in all bacterial, plant and animal cells. Although the function of deoxyribonucleases include degradation of DNA (Bernard!, 1971) a possible role in DNA synthesis (Lehman, 1967; Walker & Edmunds, 1969) and the excision of damaged portions from the DNA molecule (Sneider, 1971; Kirtikar *et ml.,* 1976).

Several Deoxyribonucleases are identified in prokaryotes and eukaryotes, which are suspected to be participating in DNA synthesis or repair (Lehman, 1967; Bernardi, 1968; Slor & Lev, 1971; Hanawalt *et* aJ., 1979; Lindhal, 1982; Linn, 1982). Repair specific endonucleases involved in excision repair pathway, which act on UV-induced thymidine dimers and AP sites are reported In some mammalian sources and the specificities of these enzymes have been studied *in vitro* (Van Lancker & Tomura, 1974; Bacchetti & Benne, 1975; Waldstein *et al.*, 1979;Nes, 1980. Kane and Linn, 1981; Shaper & Grossman, 1980, Brent, 1983). Much of the information about the enzymology of DNA repair has emanated from either prokaryotes or from some tissues of eukaryotes, but Information regarding DNA-repair enzymes in nervous tissue Is scanty.

A search has begun for the DNA repair capacity of brain and

indeed some DNA repair enzymes have been detected in brain (Table 1). In addition enzymes like DNA glycosylases and DNA polymerase β two DNases have been reported in brain tissue. This was first reported by Sung (1968) who described them as acid and alkaline DNases based on their pH optima.

Earlier studies from this laboratory on human, chick and rat brains also indicated the existence of two major DNases in brain, one of them with an acidic pH optimum and the other with alkaline pH optimum. The results of these studies have been reviewed (Subba Rao, 1986). The acid DNase showed highest activity during very early stages of brain development, at a time where DNA-synthesis would also be expected to be proceeding rapidly. The activity, however, showed a marked decline in adult and old brains, once again in parallel with the DNA-synthetic potential of the brain. This curious relationship was also noticed by Chanda *et al.*, (1975) in rat cerebellum.

These results were taken to indicate a positive relationship between acid DNase and DNA replication or a DNA repair process that is closely linked to DNA replication. Subsequent investigations by Subrahmanyam (1989) of this laboratory revealed the presence of acid DNase in nuclear fraction of brain cells thereby adding strength to its possible role in nuclear DNA metabolism.

A pure sample of an enzyme is a prerequisite to understand the mechanism of action and physiological role of that enzyme. In order to understand more about the possible function of this so

called acid DNase, in DNA-replication/repair, a project to purify this enzyme to homogeneity is undertaken. The present chapter deals with the steps that lead to the isolation of apparently homogeneous preparation of acid DNase and study of some of its properties.

MATERIALS AND METHODS:

CHEMICALS:

Sephadex G-100, protein molecular weight standards, p-hydroxy mercuric benzoate (PHMB) β-mercapto ethanol, actinomycin D, mitomycin C, Iodoacetic acid, Iodoacetamide, diisopropylflouro-phosphate, poly dA, poly A, Poly I, Poly U, were purchased from Sigma Chemical Company, USA. Poly dA-dT, poly (dA-dT).(dA-dT), poly dG-dC, poly (dG-dC).(dG-dC), poly (dA).oligo (dT) 12-18 were purchased from Pharmacia, Uppsala, Sweden. Freund's complete and incomplete adjuvants were purchased from DIFCO Laboratories, Detroit, Michigan, U.S.A. Ecteola cellulose (ET II) was purchased from Whatman Company, England. Phenyl isothiocyanate(PITC) 4-N-N Dimethylamino benzene 4'isothiocyanate (DABITIC) were obtained from Pierce Chemical Company, Rockford, Illinois, USA. Trifluoro acetic acid (TFA) was purchased from British Drug House. Micropolyamide thin layer chromatography plastic sheets were obtained from Carl Schleicher and Schuell (Basel, West Germany). Agarose and sodium dodecyl sulphate were obtained from Sisco research Laboratories (Pvt) Ltd., Bombay, India. All other chemicals and reagents used were of analytical grade.

PREPARATION OF THE SUBSTRATES:

UV IRRADIATED DNA (UV-DNA):

UV irradiated DNA was prepared by irradiation of highly polymerized calf thymus DNA (2 mg/ml of water). The DNA solution was taken into a petrldlsh as a thin layer, kept on ice and exposed to a UV (254 nm) dose of 2 x 10^4 J/m^2 using Phillips TUV 8 15 W germicidal lamp. When needed, labelled DNA isolated from E.coll (^{3}H-DNA) was also treated similarly.

DEPURINATED DNA:

Depurlnatlon of the DNA was done essentially as described by Sharper & Grossman (1980). Native calf thymus DNA (2 mg/ml) was mixed with equal volume of depurination buffer (40 mM sodium citrate, 40 mM NaCl, 40 mM potassium phosphate pH 5.0 and incubated at 70°C for 15 min.

DENATURED DNA:

Calf thymus DNA dissolved in distilled water at 2 mg/ml concentration was kept in a boiling water bath for 10 min and then rapidly cooled in ice.

PURIFICATION OF ACID DNase (NUV-DNase):

Throughout the purification the activity was assayed using native DNA as well as native DNA exposed to UV light as

substrates. Since preliminary results showed that with these two substrates, the activity was highest.

PREPARATION OF BRAIN EXTRACT (STEP-1):

Brains of albino rats of 10 days postnatal age were taken for purification. Rats were sacrificed by decapitation. Brains were dissected out and washed in ice cold double distilled water. The cerebral hemispheres were homogenized with potter elvehjem homogenizer with teflon pestle in ice cold double distilled water, containing 10 mM Phenyl methyl sulfonyl fluoride (PMSF) to make 10% homogenate. The brain homogenate was centrifuged at 30,000 g for 45 min in a Beckman J2-21 high speed refrigerated centrifuge. Most of the activity was present in the supernatant. The supernatant was taken as enzyme source for further purification. All operations were carried out at 0-4 C.

AMMONIUM SULFATE FRACTIONATION (STEP-2):

The supernatant was brought to 60-95% saturation by means of solid ammonium sulfate and centrifuged at 10,000 xg for 20 mln. The pellet contained >90% of the activity was taken for further purification. The pellet was dialysed against cold glass distilled water until the ammonium ions were completely removed. The acid DNase preparation was then dialysed against 200 volumes of 0.01 M sodium acetate buffer pH 5.0 for 12-15 hrs and clarified by centrifugation.

GEL FILTRATION ON SEPHADEX G-100 (STEP-3):

The partially purified acid DNase preparation as obtained at step-2 was loaded on 1.5 cm x 84 cm. Column of Sephadex G-100, which was preequilibriated with 0.01 M sodium acetate buffer pH 5.0. The column was developed with the same buffer at a flow rate of 15 ml/hour. Fractions of 2 ml were collected. Fig. 2 shows the elution profile of acid DNase from young rat brain. The enzyme activity was found in the first protein peak. Fractions 15 to 25, consisting of the major peak of DNase activity were pooled and dialysed against 0.01 M Na acetate buffer pH 5.0.

AFFINITY CHROMATOGRAPHY USING A DNA-ECTEOLA CELLULOSE COLUMN **(STEP-4):**

PREPARATION OF DNA-ECTEOLA-CELLULOSE:

20g Ecteola-cellulose was suspended in 250 ml of 0.2 N NaOH for 30 min. The alkali was removed by repeated washings with distilled water. Until the pH of the washings was approximately 6.0. The exchange was finally suspended in 0.01 M sodium acetate buffer pH 5.0.

The washed Ecteola cellulose was kneaded with 20 ml of calf thymus DNA (2 mg/ml). The slurry was spread on petridish and exposed to 450 W Hanovla UV light lamp from a distance of 15 cm for 20 min. The material was washed with 0.01 M sodium acetate buffer pH 5.0 to remove the unbound DNA. The amount of bound DNA was estimated by measuring the OD of washings at 260 nm. About

90% of the DNA was bound to Ecteola cellulose. These preparations are stable at 4 C for longer periods.

The pooled active fractions from step 3 was loaded on 2 cm x 7 cm column of DNA bound Ecteola cellulose column. The column was preequlllbrlated with 0.01 M sodium acetate buffer pH 5.0. After washing the column with the same buffer the bound proteins were eluted by step wise increase in the concentration of NaCl (0.05 M, 0.1 M, 0.2 M and 0.3 M) in the buffer. The flow rate of the column was 5 ml/hr. The enzyme activity was eluted with 0.2 M NaCl. Fig.3 shows the elution pattern of acid DNase from the affinity column. The fractions containing acid DNase activity were pooled and dialysed against 0.01 M sodium acetate buffer pH 5.0 to eliminate excess of NaCl. In this step 240 fold purification of the enzyme was achieved. (Table 2).

NON DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS:

The purified enzyme was electrophoresed according to the method of Davis (1964) in 7'/. gels in alkaline conditions using Tris-glycine buffer pH 8.0 (separating gel pH 8.9). These gels were silver stained for proteins as follows:

Silver staining of gels was carried out as described by Sammons & Adams (1981). Gels were fixed overnight in SOX methanol, 10% acetic acid mixture. Then the gels were washed for 30 min with three changes in 50% ethanol. The gels were rinsed with double distilled water and kept in $Na_2S_2O_3$ (200 mg/lit of distilled water) for 2 min and then washed thoroughly with 2-3

changes of distilled water. Thereafter the gels were stained with $AgNO_3$ (2 g/lit of distilled water) formaldehyde (150 µl /200 ml) solution for 20 min. The AgNO solution was then drained off and the gels were developed in solution containing Na CO (60 g/ lit) $Na_2S_2O_3$ (4 g/lit) and 0.5 ml formaldehyde, till the bands appeared. The solution was discarded and the gels were washed with 50% methanol, 7% acetic acid and stored at 0-4°C.

ACTIVITY STAINING:

Gel Electrophoresls was carried out in the presence of calf thymus DNA at a concentration of 100 µg/ml which was incorporated into the separating gel, containing 7% acrylamide and 0.5% bis acrylamide. Electrophoresis was carried out in Tris-glycine buffer pH 8.0 after which the gels were washed for three 20 min periods with 200 ml of 0.01 M Na acetate buffer pH 5.0. The gels were incubated at 37°C overnight in 0.1 M Na acetate buffer pH 5.0. Ethidium bromide was added to a concentration 10 µg/ml. Gels were photographed under UV light. Nuclease activity appeared as a white band against fluorescent background of DNA-bound ethidlum bromide.

GEL ELECTROPHORESIS (DENATURING CONDITIONS (SDS-PAGE):

Gel electrophoresls under denaturing conditions was carried out according to the method of Laemmli (1970) in 10% gels in the presence of 0.1% SDS using Tris-glycine buffer pH 8.0. After the electrophoresls, the gels were fixed and stained with commassie brilliant blue R and destained as described by Weber and Osborn (1962).

DETERMINATION OF MOLECULAR WEIGHT:

SDS-PAGE:

The molecular weight of the purified protein was determined by SDS-PAGE, which was carried out according to the procedure of Weber and Osborn (1969). The standard, proteins of known molecular weights, BSA (66 KD) egg albumin (45 KD) chymotrypsin (25 KD) and the purified enzyme protein were run on separate lanes. After electrophoresis the gels were fixed and stained with commassie Brilliant Blue R and destained with methanol:acetic acid and water. The relative mobility of respective bands were determined by dividing its migration distance by the migration distance of tracking dye (bromophenol blue) from the top of the gel. The Rf values (Abscissa) obtained for standard proteins were plotted against their molecular weight (ordlnate) on semi log graph paper. The molecular weight of brain acid DNase was calculated from the above calibration curve.

GEL FILTRATION:

The mol.wt of acid DNase from young brain was also determined by gel filtration using Sephadex G-100 column (1.5 cm x 84 cm) equilibriated with 0.01 M sodium acetate buffer pH 5.0. The standard proteins of known molecular weights (given in parenthesis) BSA (66 KD) egg albumin (45 KD) chymotrypsin (25 KD)

and cyto C (12.5 KD) were used to calibrate the column. The enzyme activity and absorbance at 280 nm was measured in each fraction (2 ml). The mol.wt. of the acid DNase was determined by plotting a graph with elutlon volume against the molecular weights of respective marker proteins on semi log graph paper.

HPLC PROTEIN PAK 300 COLUMN:

Waters HPLC fitted with protein pak 300 SW column size of 7.5 mm (ID) x 30 cm (800 13) was used both for checking the purity of the final sample as well as to determine the molecular weight. The column was preequilibriated and developed with 0.01 M sodium acetate buffer pH 5.0. Fractions of 1 ml were collected and the protein values were monitored by 0D at 280 nm. The molecular weight of purified enzyme was determined on the basis of retention time (compared to that of BSA taken as standard protein). The retention time for BSA was 6.65 min, while the enzyme protein was eluted at a retention time of 6.766 mln.

AGAROSE GEL ELECTROPHORESIS:

Agarose gel electrophoresls of the products of the reactions was carried out according to the method described by Maniatis (1982) using \'A agarose gels with electrode buffer containing 89 mM Tris, 89 mM Borate and 2 mM EDTA pH 8.0.

AMINOACID ANALYSIS:

2 µg of pure enzyme was taken into sample tube and placed in

a reaction vial. The sample was evaporated under vaccum, after completion of drying, 200 µl of HCl/phenol solution was added into the bottom of reaction vial and it was placed in oven and hydrolyzed the sample. The sample was redrled by addition of 10 µl of redrying solution consists of a 2:2:1 mixture (by volume) of ethanol:water:triethylamine. Then sample was derlvatlzed with 200 µl derivatizing reagent consist of 7:1:1:1 solution (by volume) of ethanol:trl ethylamine:water:phenyl isothiocyanate (PITC) and redrled. The sample was dissolved in sample diluent solution and aminoacids were analyzed by reverse phase HPLC column.

MANUAL PARTIAL SEQUENCING OF PURIFIED ENZYME:

The partial sequencing of enzyme protein was carried out according to Jui-Yoa Chang (1983). The protein (4 nmol) was subjected to the first coupling by adding 150 µl water and 300 µl DABITIC solution (10 n mol/µl in pyridine freshly prepared). The tube is flushed with N gas, closed and kept at 54°C for 50 min with stirring. PITC (30 µl) is added and the second coupling is allowed to continue at 54°C for 20 mln. The excess reagent is removed by gentle centrifugation 20 µl of Trlfluoro acetic acid (TFA) is added and the cleavage reaction is performed at 54 C for 10 min. The TFA is evaporated under vaccum and the cleaved derivative is extracted with one portion of 250 µl of methanol* after gentle centrifugation. The lower aqueous phase was then subjected to the next cycle. The methanol extract wich contains the thiazoline is dried and transferred to 0.5 ml eppendorf tubes and evaporated to dryness over P_2O and KOH. The pellet is dissolved in 5 µl of 80% ethanol.

IDENTIFICATION OF DABTHS:

Micro TLC analysis was performed 0.5 - 1 µl of DABTH sample is applied to the origin (about 6 mm from the edges of two adjacent sites) of a 2.5 x 2.5 cm polyamide sheet. Internal standard DABTC-dlethylamine (20-30 pmol) is always cochromatographed with unknown samples. The sheet is run two dimensionally in one direction with acetic acid :water (1:2 v/v) and second dimension with toluene n-hexane and acetic acid (2:1:1 v/v/v) by ascending manner. The sheet is dried after the second dimension and exposed to HCl vapors when the yellow spots turn red for identification.

PREPARATION OF **BRAIN ACID DNase** ANTI SERUM:

Pure DNase (500 µg/ml) was dlalysed against water and wasemulsified with an equal volume of Freund's complete adjuvant and injected into rabbit intradermally at multiple sites. The rabbit was given a booster dose after one month of initial injection by emulsifying 250 µg enzyme protein with Freund's incomplete adjuvant. One week after the booster dose, the animal was bled from the marginal ear vein and the anti serum was obtained by allowing the blood to clot overnight at 4 C. The sample was centrlfuged at 10,000 xg for 15 min and the sera were stored in sodium azide (0.002%) at -20°C.

OCHTERLONY'S DOUBLE IMMUNODIFFUSION:

Agar double diffusion analysis was performed by using the

technique described by Ochterlony (1958) Agar (1%) plates were made in 10 mM sodium phosphate buffer pH 7.6 containing 150 mM NaCl and 0.02'/. (w/v) sodium azide. The agar solution was poured on to the petrldlsh to a height of 3 mm and allowed to cool at room temperature. The wells were punched in the gel and the anti serum (25 µl) was placed in central well, while encircling peripheral wells were filled with pure DNase (5 µg) or crude extract or partially purified fractions (10-15 µg). The diffusion was allowed to take place for 24 hours at 0-4 C. The precipitin lines were visualized and photographs were taken against a dark field illumination.

COUNTER IMMUNOELECTROPHORESIS:

Agar solution was prepared by dissolving 0.5 g of agarose in 0.25 M sodium barbital buffer containing 0.01% sodium azlde. The solution poured on a horizontally levelLed glass slide and allowed to cool at room temperature. The wells were punched in the gel. The pure enzyme and antiserum were placed in the wells electrophoresis was done at 200 volts for 1 hour in cold room. The agar slide and electrode buffer tanks were connected by Whatman paper (5 mm thickness). At the end of electrophoresis, the plates were washed with several changes of 0.15 M NaCl. The precipitin lines were visualized after 24 hrs incubation at room temperature.

RESULTS

Table 2 shows typical purification schedule of acid/NUV

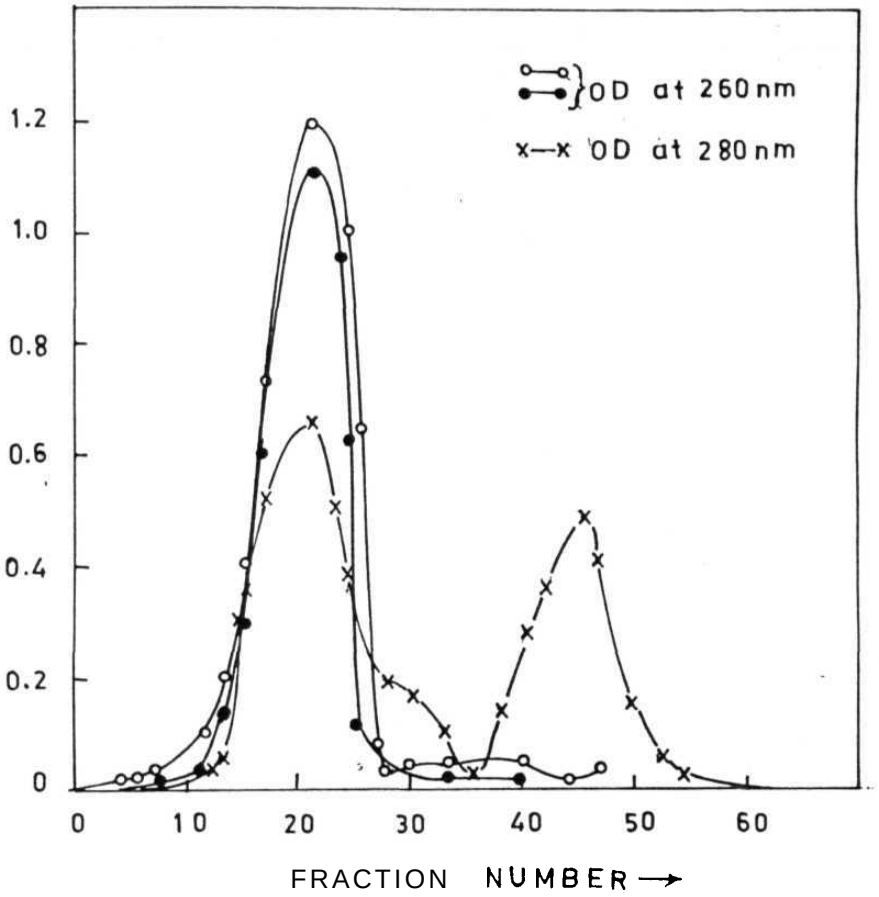


Fig. 2 Elution profile of acid/NUV DNase activity from Sephadex-G-100: The 95V. ammonium sulphate precipitate containing the activity was **dialysed** and loaded on **1.5** Cm x 84 **Cm** column of sephadex **G-100.** The column was preequlllbrlated with 10 **mM** sodium acetate buffer pH 5.0 and developed **with** same buffer at a flow rate of 15 ml per hour. 2 ml fractions were collected. Fractions 15 to 25 were pooled for further **purification.** Elution profile was monitored by taking OD at 280nm of each fraction (x-x) An aliquot of each fraction was taken for the enzyme assay.(0-0) **Activity with native DNA as** substrate **(●–●) Activity with UV DNA as substrate.**

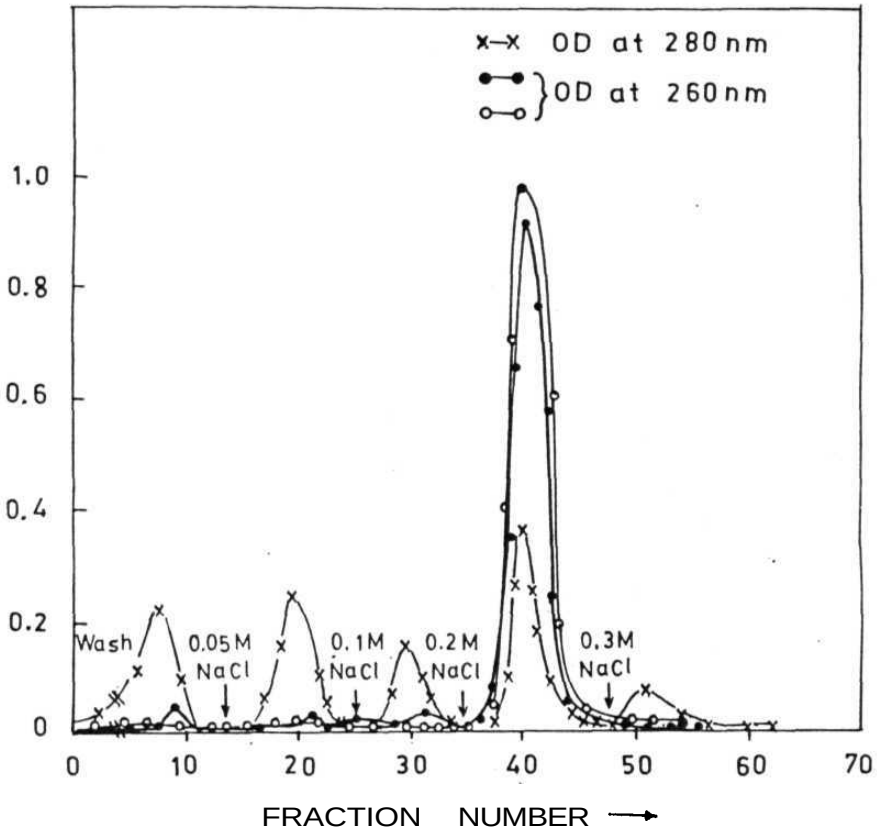


Fig 3 Elution profile of Acid/NUV DNase from DNA bound Ecteola cellulose affinity column: The active fractions from the sephadex G-100 column were loaded on 2 Cm x 7 Cm affinity column. The column was preequilibriated and developed with 10 mM sodium acetate buffer pH 5.0. The unbound proteins were washed out. The bound proteins were eluted by NaCl step wise gradient in the buffer. The concentrations of NaCl used were 0.05,0.1, 0.2 and 0.3M. Other details were shown in Fig.2.

Table - 2

Purification of **Acid/NUV DNase** from Young Rat Brain

Purification step	Total protein (mg)	Total activity (units)	Sp.act	Yield '/.
Brain Extract				
Native DNA	1792	39728	**22.1**	100
UV - DNA		42000	23.4	
$(NH_4)_2SO_4$ PPt				
Native DNA	65	6372	98	16.0
UV - DNA		5922	92	
Sephadex G-100				
Native DNA	**18.4**	2700	150	6.8
UV - DNA		2689	146	
Affinity Chromatography				
Native DNA	0.2	968	4840	**2.4**
UV - DNA		982	**4910**	

Specific activity is expressed as **µg** of acid soluble DNA-P **liberated/2 hrs/mg** protein. Total activity is obtained by **multiplying** the specific activity with the protein content of the tissue in mg. For the assay of NUV DNase the reaction mixture consisted of 200 **µg** of native or UV DNA, 0.1 M sodium acetate buffer pH 5.0, and **enzyme** in a total volume of 0.6 **ml.At** the end of 2 hr incubation at 37°C the reaction was stopped by adding 0.4 ml of 1.4 N PCA and **immediate** chilling. The whole reaction mixture was centifuged at 4,000 **rpm** for 10 **min** and the OD of supernatant was read at 260 **nm** against appropriate blank.

DNase from rat brain. As can be seen about 240 fold purification was achieved with about 2% yield. The final preparation showed almost equal specific activity towards double stranded native DNA and UV-irradiated DNA. To our knowledge this is the first time that a DNase activity from brain showing an activity towards UV exposed DNA has been observed.

The purity of enzyme was checked by PAGE (Fig. 5) and SDS-PAGE (Fig.6). A single band was noticed in either case. The band was also identified as DNase by "activity staining". (Fig.4).

The optimal conditions for the activity of acid/NUV DNase were found by using both native as well as native DNA exposed to UV light as substrates. The optimum pH for brain DNase was determined by assaying the activity in different buffers (100 mM) containing 200 μg/ml native or UV DNA as substrates. The optimum pH for enzyme activity was found to be pH 5.0 (Fig. 7). The neutral DNase activity was determined by assaying the DNase activity in 10 mM potassium phosphate buffer pH 7.0 as well as with 10 mM sodium acetate buffer pH 5.0 containing various concentrations of NaCl. Under the conditions of low ionic strength (0.02 M NaCl) the enzyme showed considerable activity at physiological pH *i.e.* pH 7.0 (Fig. 8). The acid/NUV DNase assay was conducted with different amounts of enzyme (1 to 20μg) to study the effect of enzyme concentration on the rate of reaction. The activity of enzyme was linear up to enzyme concentration of 10 μg/ml (Fig.9) and two hours time (Fig.10).

The optimal of substrate concentration was determined by

Fig.4 Activity staining of pure enzyme.
For details of the technique please see the Materials and Methods.

Fig.5 Polyacrylamide gel electrophresls of purified acid/NUV after step 3 (Sephadex G-100) and step 4 (DNA- Ecteola cellulose column). The gels were silver stained.

Lanes 1 to 3 after the affinity column.
lane 4 after sephadex G-100 fraction.
For details of the technique please see Materials and Methods section of this chapter.

FIG. 4

FIG.5

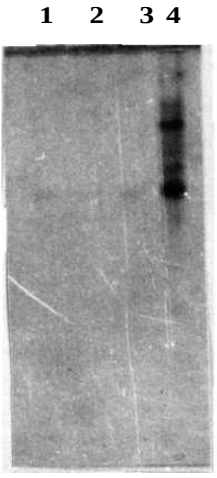


Fig.6 Coomassle blue stained Gel chromatogram of SDS-PAGE of rat brain acid/NUV DNase at different steps of purification.

Lane - 1 Ammonium sulphate fraction (step 2)
Lane - 2 After sephadex G-100 chromatography (Step 3)
Lane - 3 After affinity chromatography (Step 4)

Fig.15 SDS-PAGE of purified DNase along with known mol.wt standard proteins (Lane 1 & 7)Gel Coomassle blue stained.

Lane 2 to 4 : pure enzyme fraction

Lane 5 & 6 : enzyme fraction after G-100 and ammonium sulfate fraction.

FIG. 6

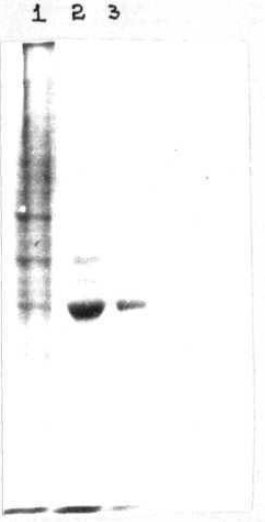
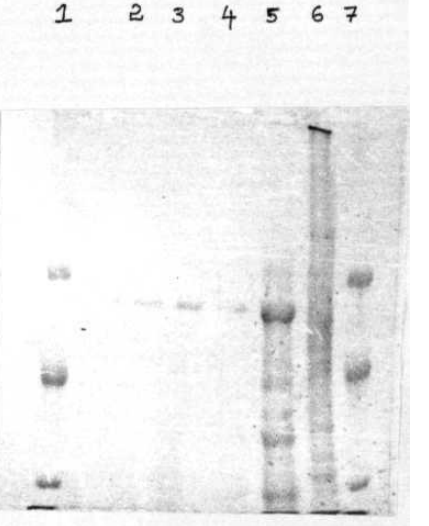


FIG. 15



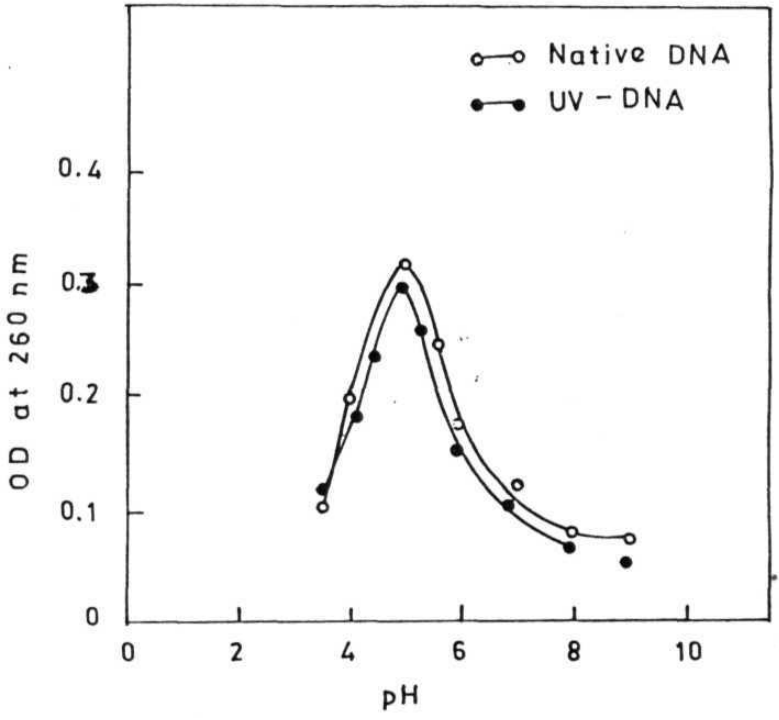
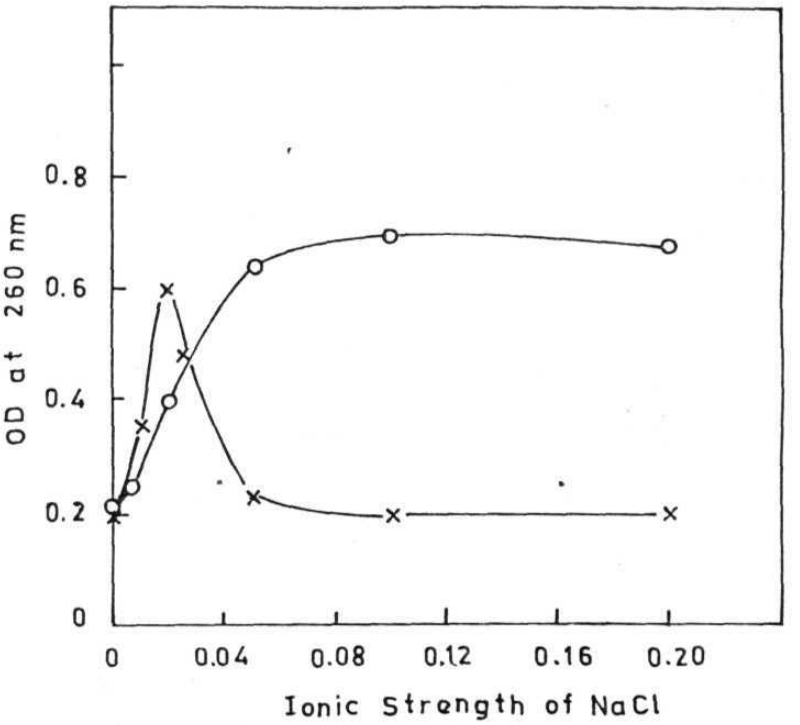


Fig.7 Effect of pH on the activity of purified acid/NUV DNase from rat brain. The buffers used were sodium acetate buffer pH (3-5.5) potassium phosphate buffer (pH 6-7.5) and Tris HC1 buffer (pH 8-9.5). Each point represents average of three experiments.



FigIG.8: **Acid/NUV** DNase activity at pH 5.0 and 7.0 as a function of ionic strength. The activity (o-o) at pH 5.0 was assayed in **0.01M** sodium acetate buffer pH 5.0 **with** addition of various quantities of **NaCl**. The activity, (x-x) at pH 7.0 was assayed in 10 **mM potassium** phosphate buffer pH 7.0 with various quantities of NaCl. DNase activity expressed as OD at 260 **nm** of acid soluble nucleotldes/hr at 37°C/5 **μg** enzyme.

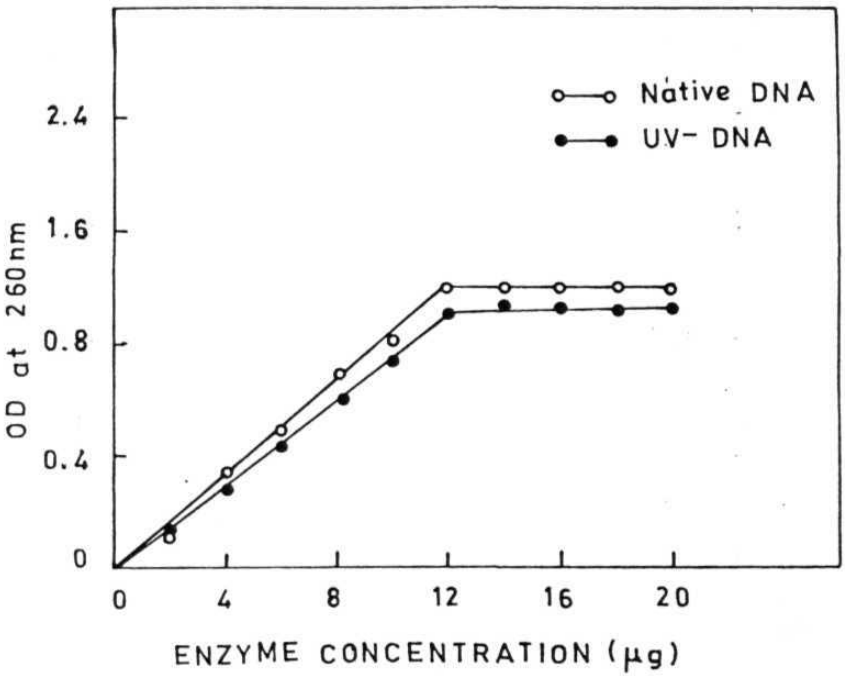


Fig.9 Effect of enzyme concentration on the activity of purified acid/NUV DNase from rat brain. Each point represents average of three experiments.

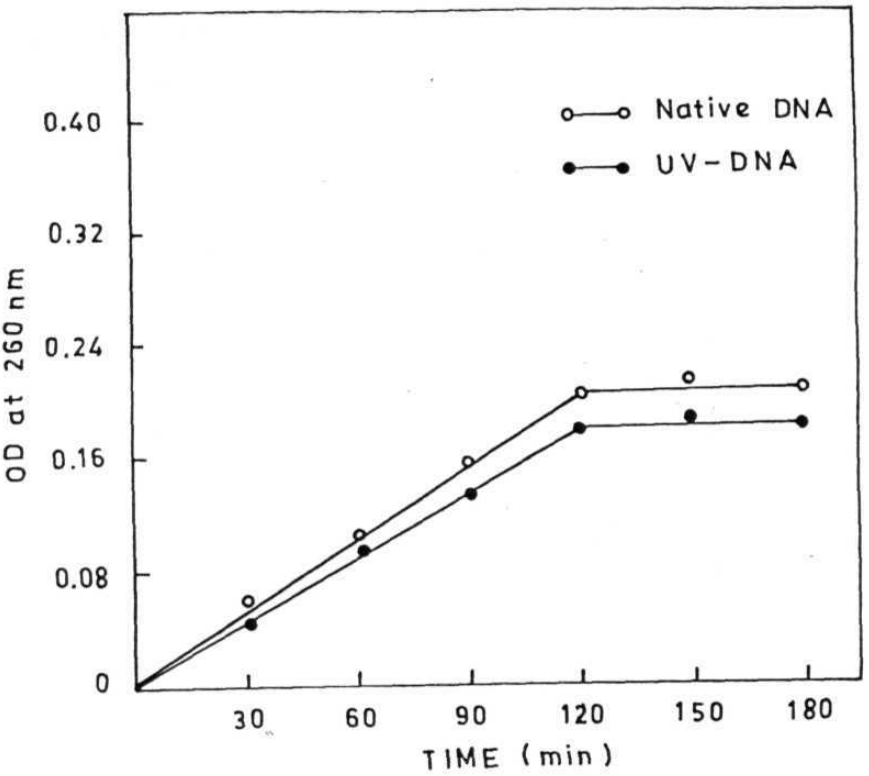


Fig.10 Effect of time on the activity of purified acid/NUV DNase from rat brain. Each point represents average of three experiments.

Table - 3

Effect of inorganic ions on the purified Acid/NUV DNase activity

Cations & anions	*V.* activity					
	2.5 mM	5 mM	10 mM	20 mM	50 mM	100 mM
$MgCl_2$	80	90	100	100	110	13
CaCl	80	80	90	100	**120**	40
$HgCl_2$	12	10	15	10	ND	ND
Na_2SO_4	72	70	60	40	10	0
$MgSO_4$	64	34	21	18	10	0
NaH_2PO_4	ND	56	50	26	15	0

ND-not determined. All the values are average of three individual experiments. Activities are expressed as **µg** of acid soluble DNA-P liberated/2 hrs at 37°C. Activities are expressed as percentages where control value is taken as 100V.

taking different concentrations of native and UV DNA in assay medium and assayed, the enzyme activity at 37°C. The velocity (V) was defined as increase in absorbency at 260 nm. Fig. 11 and 12 shows the effect of increasing amounts of native DNA and UV irradiated double stranded DNA concentration on DNase activity. The optimal substrate concentration were found to 200 µg/ml. From the line Weaver-Burk plots (assuming the molecular weight of calf thymus DNA to be 8.6 x 10 daltons) the Km was calculated to be 4.3 x 10^{-9} (Fig.13) in the case of native DNA and 5.2 x 10^{-9} with respect to UV-irradiated DNA (Fig.14).

DNase was incubated in 100 mM sodium acetate buffer pH 5.0 with different mM concentrations of various cations as well as anions at 37°C for one hour and assayed the enzyme activity, the percentage of enzyme activity without metal ions was taken as 100%. Table 3 shows the effect of various ions on the enzyme activity. There was no significant increase of activity in the presence of Mg or Ca whereas higher concentrations (0.01 M) were found to be inhibitory, the enzyme activity was strongly inhibited by anions like SO ~ , PO ~ and heavy metals like Hg^{+} .

The enzyme was incubated with different concentrations of PHMB, β-mercaptoethanol, urea, NH and EDTA and enzyme activity was measured. Where the control activity was considered as 100X solutions of various compounds, except those of EDTA and PHMB, were made in glass distilled water. In the case of EDTA, care was taken to see that the stock solutions pH is 7.0. Stock solution of PHMB was prepared in 0.1 N NaOH. Table 4 shows the effect of sulfhydryl reagents, EDTA and NH ions on the DNase activity.

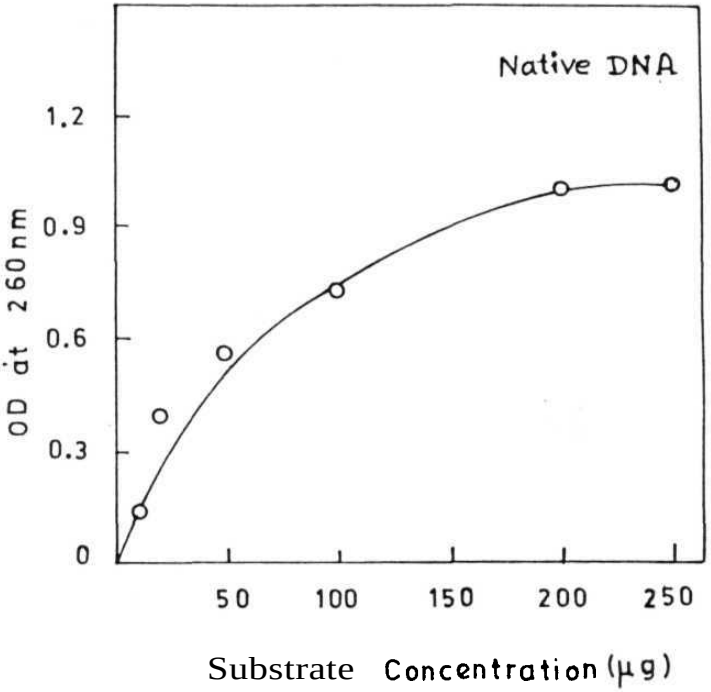


Fig.11 Effect of substrate (native DNA) concentration on the activity of purified acid/NUV DNase. Each point represents the average of three experiments

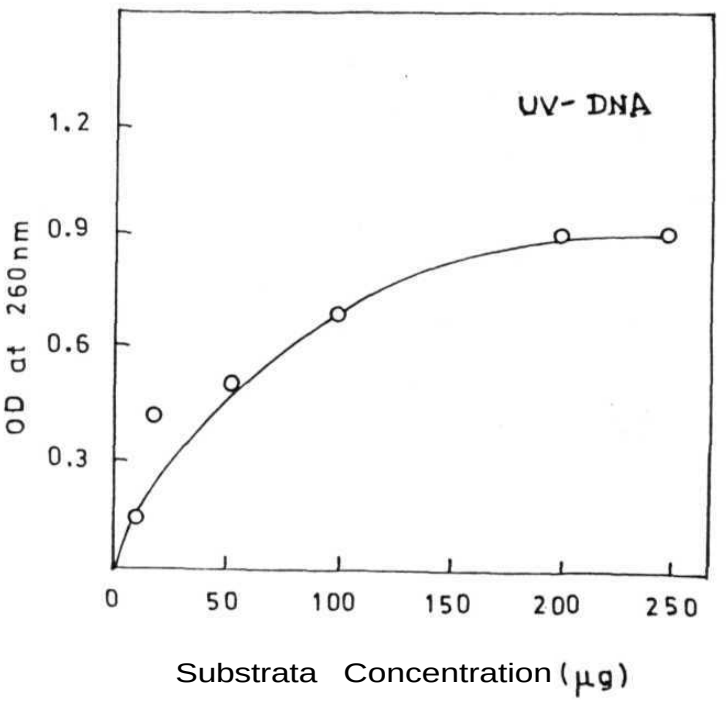


Fig. 12 Effect of substrate (UV-irradiated DNA) concentration on the activity of purified acid/NUV DNase. Each point represents the average of three experiments.

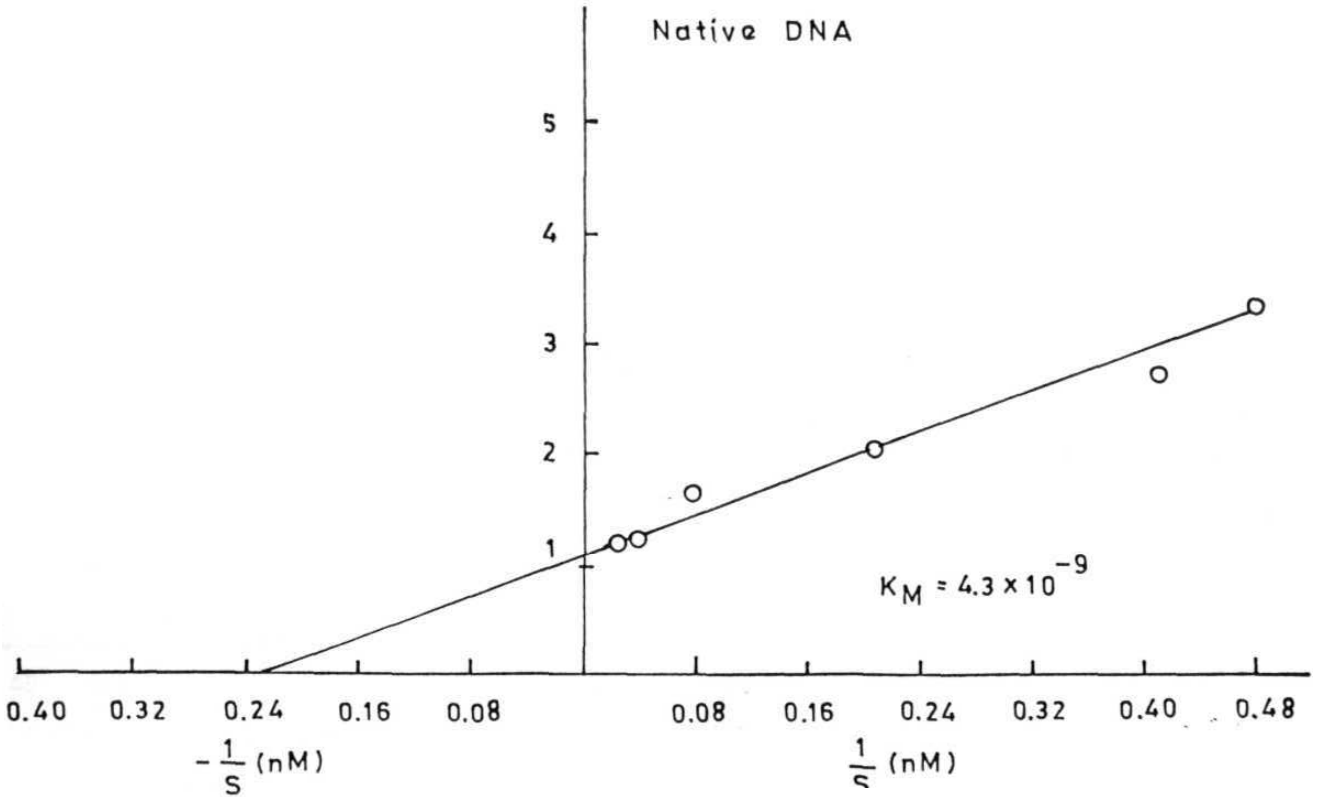


Fig. 13 Line weaver-Burk plot with native DNA as substrate for the rat brain acid/NUV DNase. For the calculation of Km the mol.wt. of calf thymus DNA was taken to be 8.6×10^6 daltons.

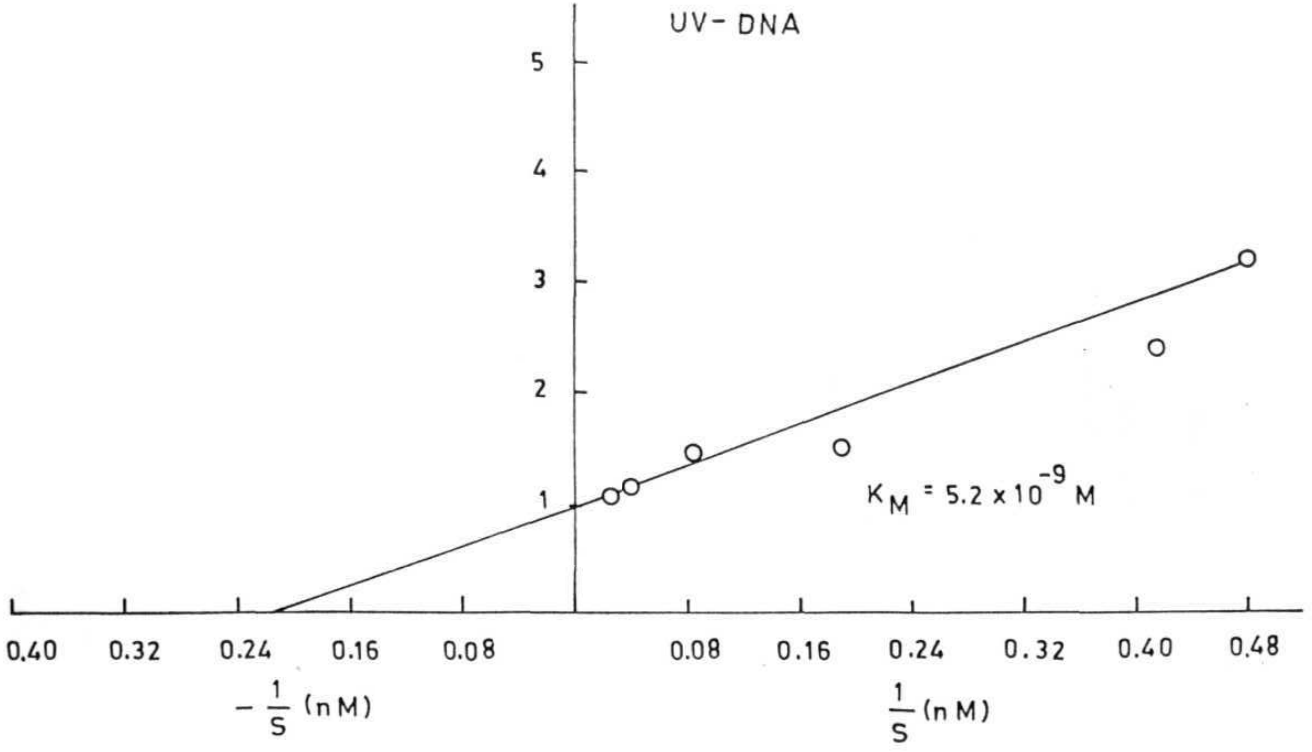


Fig.14 Line weaver-Burk plot with UV-DNA as substrate for the rat brain acid/NUV DNase. For the calculation of Km the mol.wt. of calf thymus DNA was taken to be 8.6 x 10^6 daltons.

Table - 4

Effect of PHMB, EDTA, β-mercaptoethanol, urea, Dithiothreitol & NH_4^+ on purified Acid/NUV DNase activity.

	Addition (mM)	% activity
	Control	100
PHMB	0.05	96
	0.1	91
EDTA	1.0	104
	2.0	131
	4.0	135
	10	**156**
	20	**130**
β-mercaptoethanol	**1.0**	**65**
	5.0	94
Urea	2.0	87
	4.0	75
Dithiothreitol	10	71
	20	110
	50	**104**
NH_4^+	1.0	96
	20.0	80

The details are **ae** in Table **3.**

Table - 5

Effect of various group specific compounds on the purified brain acid/NUV DNase activity

Additive	Additive/enzyme molar ratio	'/. activity
Nil (Control)		100
Iodoacetlc acid	1000	8
	100	12
Iodoacetamlde	1000	51
	100	69
N-bromosuccinimide	100	9
	25	14
Hydrogen peroxide	10000	16
	5000	30
Diisopropyl fluorophosphate	saturated	94

Control value (standard incubation mixture with native DNA as substrate was taken as 100%. All the values are average of 4 experiments.

PHMB, β-mercaptoethanol, dithiothreitol and NH_4^+ ions at the concentration used did not effect the enzyme activity, whereas 0.01 M EDTA was found to activated the enzyme by 156%.

The molecular weight of purified DNase was found to be 62 KD with reference to standard proteins by SDS-PAGE (Fig.15 & 16), gel filtration on sephadex G-100 (Fig.17) and also by HPLC protein pak 300 column. Fig.18 and 19 shows the activity of enzyme on PBR 322 plasmid and also *E.coll* DNA. The enzyme opens covalently closed circular DNA and converts to a linear form with a simultaneous decrease in the molecular weight of *E.coll* DNA revealing that this DNAse is an endonuclease.

Table 5 shows effect of several group specific compounds on the enzyme activity. Iodoacetic acid, N-bromosuccinimide and H_2O_2 were found to be strongly inhibitory, whereas iodoacetamide was slightly inhibitory and diisopropylfluoro phosphate has no effect on enzyme activity. These results revealed the possible involvement of tryptophan and histidine but not of serlne in enzyme activity.

ACTIVITY OF THE PURIFIED DNase ON DAMAGED OR ALTERED DNAs

The enzyme was pretreated with different concentrations of actinomycin D and assayed the enzyme activity, whereas In case of mitomycin C (50 μg) was added as such and mitomycin C treated DNA (Iyer & Szybalski, 1964) was incubated with enzyme and assayed the enzyme activity. Table 6 shows the effect of pretreated

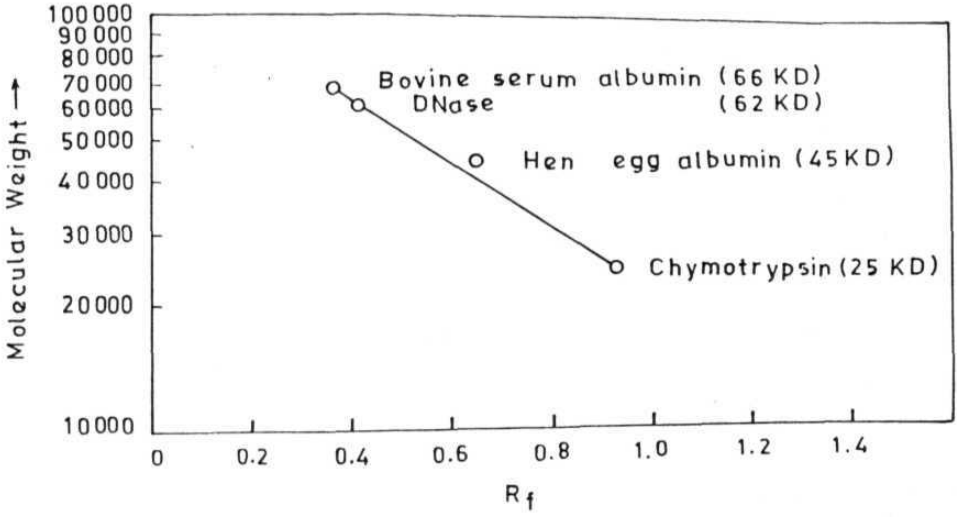


Fig. 16 **Molecular Weight Determination by SDS-PAGE:** Proteins of known mol.wt. and the purified enzyme fraction were run on SDS-PAGE on separate lanes. Rf values of the proteins were calculated and plotted against the corresponding mol.wt. The mol.wt. of purified enzyme fraction band was determined from the plot by its Rf value.

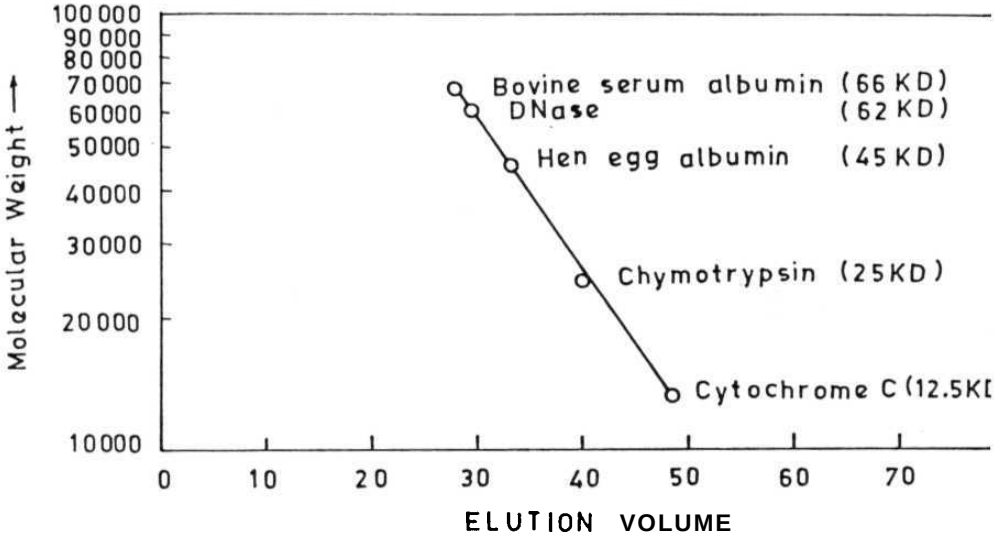


Fig. 17 **Molecular** Weight **Determination by Gel filtration:** Proteins of known **Mol.wt.** & purified **acid/NUV** DNase were loaded on Sephadex **G-100** column of **size** 1.5 x 84 cm. The standard proteins used for mol.wt. determination were Bovine **serum** albumin (66 KD) Hen egg albumin (45 **KD) Chymotrypsin** (25 KD) and Cyto C (12.5 KD). The enzyme activity and absorbance at 280 **nm** was measured in each fraction (2 ml). Elution volume of proteins were calculated and plotted against the corresponding mol.wt. on a **semi** log graph paper. The mol.wt. of the purified enzyme fraction was determined from the plot by **its** elution volume.

Fig.18 Incubation of PBR 322 with acid/NUV DNase for different time intervals.

Lane 1: no enzyme.
Lane 2 to 4 : 5. 10, 15, min of incubation respectively.

Fig.19 Incubation of *E.coli* DNA with acid/NUV DNase for different time intervals.

Lane 1 no enzyme
Lane 2 to 8 - 2, 5, 10, 15, 20, 25, and 30 min of incubation respectively.

Fig.18

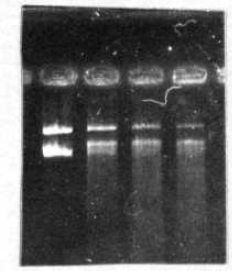


Fig.19

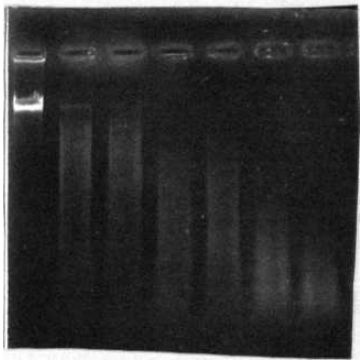


Table - 6

Effect of Actlnomycin 0 and Mitomycin C on purified rat brain acid/NUV DNase

Antibiotic (μg)	% activity
Control	100
Mitomycin (50)	92
Mitomycin C **pretreated** DNA	30
Actlnomycin D pretreated DNA (25)	82
Actlnomycin D pretreated DNA (50)	66
Actlnomycin D pretreated DNA (100)	41
Actinomycin D pretreated DNA (200)	39

Activity of **enzyme with** standard reaction mixture **with** native DNA as substrate was taken as 100% Other values are expressed as percentage of control **value.Results** are average from four different experiments.

actinomycin D and mitomycin C DNA on enzyme activity. When the mitomycin C was added at the time of the reaction no effect was seen. However, when the substrate DNA was pretreated with mitomycin C, it was found to be strongly inhibitory. Actlnomycln D inhibited the activity, the effect being more 20-70'/. with increasing concentration of the antibiotic (25-200 μg/ml).

DNA treated with different damaging agents to produce various types of damage, were used to check the preferential substrate for the purified enzyme UV-irradiation causes the formation of thymine dimers, photo products, and conformational distortions in DNA. The results are presented in Table 7. The enzyme showed almost equal specific activity towards double stranded native DNA and UV DNA. It was also found that this enzyme has no activity towards depurinated DNA and single stranded DNA or denatured DNA. To know more about the substrate specificity this enzyme, the activity of this enzyme was tested towards both native as well as UV-irradiated *E.coli* ^{3}H-DNA. The enzyme showed activity on both the substrates (Fig.20 and 21). To find out whether or not this enzyme has any affinity to a pyrimidine dimer in DNA, the products of the reaction were analyzed as follows (Carrier and Setlow, 1966).

ASSAY FOR ABILITY TO EXCISE *PYRIMIDINE DIMERS* FROM DNA:

This assay measures the ratio of radioactivity in thymine containing diners to that in thymine in acid insoluble fraction of reaction mixture. The reaction mixture (60 μl) consisting of 10 μg of *E.coli* ^{3}H DNA (70,000 DPM) irradiated at a dose of 2 x 10

Table - 7

Specific activity of acid/NUV DNase with different substrates

Purification Step	Native DNA	UV DNA	Denatured DNA	Apurinic DNA
Brain Extract	22.1	23.4	4.7	4.1
Ammonium Sulfate precipitation	98	92	1.6	7.8
Sephadex G-100	150	146	1.7	10.0
Affinity Chromatography	4840	4910	1.2	18.1

Specific activity is expressed as µg of acid soluble DNA-P liberated/2 hrs/mg protein. The values are the average of four experiments.

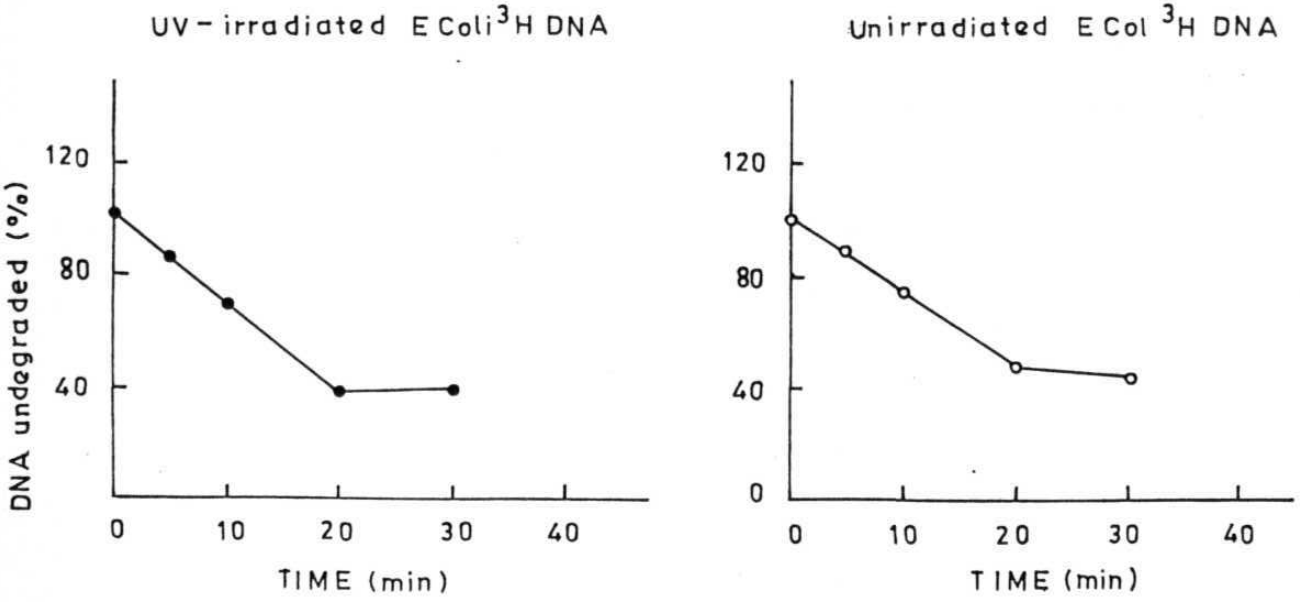


Fig. 20 & 21 Activity of enzyme on UV-irradiated E.coli ^{3}H DNA and unirradiated E.coli ^{3}H DNA at different timeintervals. Assay was performed and radioactivity in acid insoluble PPt was counted and radioactivity of '0' time control was taken as 100%.

Table - 8

Excision of thymine diners from UV-irradiated *E. coli* ^{3}H DNA by Rat Brain acid/NUV DNase

0 min PPt			30 min PPt		
DPM			DPM		
Monomer	Dimer	D/M ratio	Monomer	Dlmer	D/M ratio
53006	2973	0.056	35916	2050	0.057
37713	2439	0.064	36248	1739	0.047
92966	4859	0.052	73919	3118	0.052

Values of 0 mln ppt and 30 mln ppt are expressed as mean ± SD

0 mln ppt	30 mln ppt
0.057 ± 0.0049	0.052 ± 0.004

J/m^2 at 254 nm using philips TUV 8 15 w germicidal lamp, 0.1 M sodium acetate buffer pH 5.1 and 1.25 µg of pure enzyme was incubated for 30 min at 37°C. The reaction was terminated by addition of 1.4 N PCA. The precipitate was collected by centrlfugatlon, hydrolyzed in 97% formic acid and chromatographed in n-butanol-acetic acid and water (80:12:30, V/V/V/) on Whatman no 1 paper, the thymine and thymine containing pyrimidine dimer regions were cut out eluted with water and counted for radioactivity in Beckman LS-1800 scintillation counter. Table 8, shows the excision of dimers from the UV irradiated DNA by enzyme. The value at '30' min precipitate was not different statistically from '0 min' precipitate, which indicates that enzyme does not excise dimers from UV-irradiated DNA, but it might be acting around the pyrimidine dimers in UV irradiated DNA.

SUBSTRATE SPECIFICITY OF PURIFIED ENZYME:

The specific activity of purified DNase for various substrates are shown in Table 9. A double stranded co polymer poly (dA-dT).Poly (dA-dT) was rapidly degraded by DNase, but double stranded co polymer poly (dG-GC).poly(dG-dC) was degraded considerably more slowly. The enzyme was active on native DNA and native UV irradiated DNA but not on apurnlc DNA as well as denatured DNA poly(dA), poly d(A-T) poly dA.Oligo dT_{12-18}, poly dA-dT. Poly dA, poly A, poly I, poly U and poly (dG-dC) are scarcely susceptible to hydrolysis by DNase.

The amino acid composition of HPLC purified rat brain DNase was shown in Table 10. For the sake of comparison, the amlno acid

Table - 9

Substrate Specificity of Purified Rat Brain acid/NUV DNase

Substrate	'/. activity
Native DNA	100
UV-irradiated DNA	115
Denatured DNA	5
Apurlnic DNA	19
Poly (dA).(dT)	6.1
Poly (dA-dT).Poly(dA-dT)	181
Poly (dA).Ollgo (dT)-12-18	42
Poly d(A-T)	18
Poly dA	5.1
Poly A	4.6
Poly I	6.5
Poly U	7.5
Poly (dG-dC)	5.5
Poly(dG-dC) . Poly (dG-dC)	22

All the values are the average of four experiments The activity of the enzyme on native DNA was considered as 100'/..

Table - 10

Amino Acid Analysis of Brain acid/NUV DNase and Spleen DNase **(DNaseII)**

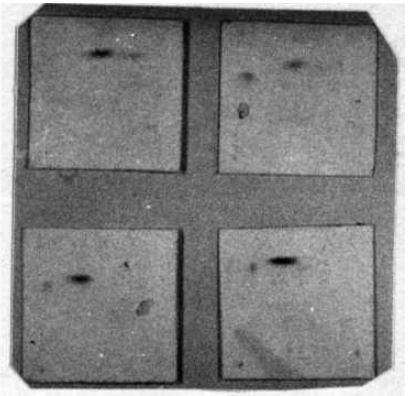
Amino acid	Brain DNase Mole *V.*	Spleen DNase Mole *V.*
Asp	5.7	9.9
Glu	8.9	10.8
Ser	14.2	8.4
Gly	14.6	4.0
His	2.9	2.1
Arg	0.8	5.3
Thr	3.7	5.5
Ala	4.4	4.6
Pro	7.2	6.8
Tyr	2.9	5.1
Val	23.4	3.3
Met	4.0	1.5
Cys	0.9	2.1
Ile	1.4	2.4
Leu	2.4	10.7
Phe	1.1	6.2
Lys	1.5	6.7
Total	100	97.8
	Asp + Glu - 14.6 Lys + Arg - 2.3	Asp + Glu - 20.7 Lys + Arg - 11.9

Rat brain DNase amino acid composition was analysed by HPLC Pico-Tag amino acid analysis system. The spleen DNase amino acid composition was taken from the data of Bernardi *et.al.*(1971).

Fig.22 Partial sequencing of Rat brain Acid/NUV DNase: HPLC analysed pure enzyme was taken and manual sequencing was performed by using Diethylaminoazobenzene isothiocyanate. For experimental details please see materials and Methods section of this of chapter.

II A line drawing of 22.

Fig 22



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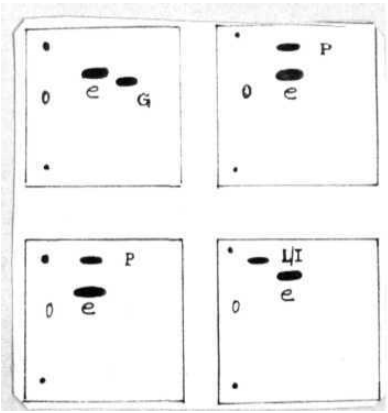


pattern of classical DNase (DNase II) as reported by Bernardl *et al.,* (1971) was also presented. Brain DNase contained highest content of valine followed by glycine and serine residues. Whereas in spleen DNase has highest content of leucine, Aspartlc and glutamic acid residues.

Partial sequencing of brain DNase was performed by dethylaminobenzene isothiocyanate (Fig.22). The results indicate that glyclne as the N-terminal amino acid followed by prollne, prollne and leuclne or isoleucine.

CHARACTERIZATION OF **ANTISERUM:**

Rabbit polyclonal antibodies against DNase from young rat brain were characterized for their mono specificity against brain DNase. The ochterlony double immuno diffusion plates of antiserum against pure and partially purified DNase preparations showed a single immnunoprecipitin line (Fig.23). The monospecificity of antiserum against DNAse was further confirmed by counter immunoelectrophoresis of DNase which also resulted in single precipitin line (Fig.24).

DISCUSSION

The precise physiological role of DNases in mammalian tissue is far from clear. It is possible that these enzymes may play a major role in several aspects of DNA metabolism including its synthesis, degradation, recombination and repair. Hanawalt *et al.,* (1978, 1979)) Bernstein (1981) Freidberg *et al.,* (1981)

Fig.23 Ochterlony double immuno diffusion to examine the specificity of antiserum towards rat brain acid/NUV DNase the central well in agar plate was filled with antiserum (AS) while peripheral wells were filled with pure enzyme, b, c & d DNase preparation from crude brain extracts after ammonium sulfate fraction and sephadex G-100 respectively.

Fig.24 Counter immunoelectrophoretic analysis of purified DNase.a & b contains pure enzyme.

FIG.23

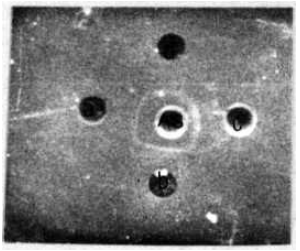
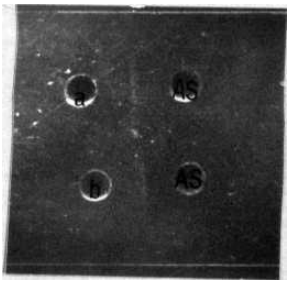


FIG.24



Seeberg & Kleppe (1981) and Sutherland (1981) have gathered enough evidences to show direct involvement of DNases in DNA repair in prokaryotlc systems. However, the role of various DNases, found in mammalian tissues remained largely conjectural. The picture was more gloomy with respect to brain in spite of its central role in influencing directly or indirectly all other bodily functions.

The results presented in this chapter represent an effort to examine the biological functions of one of the two major DNases found in brain earlier in this laboratory and where changes with age suggested a role in DNA-replication linked repair. (Subba Rao, 1986). It was also an objective to see whether this enzyme, with an acidic pH optimum is the same as classical DNaseII, the spleen DNase.

In the present study, the purified DNase from rat brain showed comparable activity towards double stranded native DNA and UV irradiated native DNA and did not require divalent cations for its activity. While some of its properties with respect to inhibition by some anlons group specific compounds and heavy metals and being an endonuclease are similar to that of DNase II,it differed in many important respects from the spleen acid DNase. Thus, the present enzyme from brain has a different size, amino acid composition and N-terminal aminoacids from that of DNase II Moreover, the present enzyme exhibited a preference for dA-dT sequence, DNase II was reported to prefer dG-dC sequence (Doskocil and Sorm, 1961). From these considerations, it may be regarded that the present enzyme may be a different gene product having some common properties with the classical DNase II.

To our knowledge, this is the first time that a brain DNase with an ability to attack both native and UV damaged native DNA, but ineffective on single stranded, and depurinated DNA. It thus appears that this enzyme recognized a double stranded DNA with some distortions in its native structure. The reason for native but depurinated DNA not being recognized, in spite of its supposed double strandedness, could be due to the single strand breaks that might have resulted following the depurination. It is interesting to note the low activity of the enzyme on DNA pretreated with either Mitomycin C or Actinomycin D, both known to cause adducts and cross linkings in DNA. It would therefore appear that the brain DNase attacks only such native double stranded DNA suffering from a sequence based distortion. At this point one might argue that why the native calf thymus DNA is a good substrate which has apparently no damage inflicted on its base sequence. The only speculative answer that can be returned to this question is that no isolated DNA, no matter how efficient and careful the procedure has been, is completely native and some distortions in its conformation could have occurred which are related in nature to pyrimidine dimers to other photo products resulting from UV-irradiation of DNA.

Studies on the mechanism of action of the enzyme on UV-irradiated DNA suggested that this enzyme does not excise pyrlmldine dimers from UV irradiated DNA (Table 8). Two other possibilities may be considered to explain the action of this enzyme on UV DNA. (1) The enzyme cuts phosphodiester bond near the pyrlmidlne dimer site, but dimers are not excised which is

similar to that of endonuclease purified from *Micrococus luteus* which has activity towards UV-irradiated DNA (Carrier & Setlow, 1970). (2) The site of action of this enzyme on UV-lrradlated DNA is a photo product other than pyrimidine dimers like that of the endonuclease purified from calf thymus acting on irradiated DNA (Bachetti & Rob Benne, 1975), or the real substrate to this enzyme may not be either pyrlmldlne dlmers or other photo products but a locally denatured open regions in a double helical DNA. The exact chemical structure of the damage recognized by this enzyme remains unknown.

Brent *et al.*, (1975) reported the enzyme activity from human lymphoblasts active against UV-lrradlated or unlrradlated native DNA but is not specific for UV-irradiated DNA.

Since the brain DNase under study shows an acidic pH optimum and attacks native and UV damaged DNA. We now refer to this enzyme as acid/NUV (native and UV damaged DNA) DNase. In fact, this author would like to omit the word acid and call this enzyme simply as NUV DNase.

CHAPTER IV

Purification of Native and UV irradiated DNA Specific Deoxyribonuclease (acid DNase) from Old Rat Brain.

CHAPTER - IV

PURIFICATION OF NATIVE AND UV IRRADIATED DNA SPECIFIC DEOXYRIBONUCLEASE (ACID DNase) FROM OLD RAT BRAIN

INTRODUCTION:

All biological reactions in the body are catalyzed by enzymes. They are therefore essential for various functions of the body. Hence structural alterations in enzyme molecules during aging may alter the functional ability of an organism (Reiss, 1977). A considerable amount of data has accumulated which show that the levels of certain enzymes decrease and of a few other increase and several other enzymes do not show any change with age. A number of enzymes have been found to be 'altered' in old animals (Sharma, et aJ., 1978, 1980[a,b]; Rothsteln, 1977, 1979). The common result of altered enzymes is a reduced specific activity of the enzyme based upon activity/unit of antiserum or activity/mg pure enzyme. The enzymes may become 'altered' by either errors in sequence or modifications after their synthesis and during 'cell life' (Sharma & Rothstein (1980),Rothstein (1984) have provided substantial evidence that the modification involving only a change in conformation after synthesis is responsible for the observed alterations in properties of 'old' enzymes.

Previous studies from this laboratory from chick and rat brains showed that the acid DNase activity was highest during early embryonic stages and decreased during postnatal ages

(Shrlvastav & Subba Rao, 1975; Subba Rao & Subba Rao, 1982). Chanda *et al.*, (1975) showed similar type of results in rat cerebellum. Subsequent studies of Subrahmanyam (1989) on acid DNase in brain cells showed the presence of this enzyme in nuclei of neurons, astrocytes and oligodendrocytes in substantial amounts. The specific activities of acid DNase in nuclear fraction decrease with age in neurons. A decrease or increase in enzyme activity could be due to various reasons. The enzyme protein may be synthesized in altered amounts which means the protein synthetic machinery vis-a-vis the enzyme may be affected at the genetic level or at the translation level. Alternatively the enzyme protein synthesized may be the one that is, altered, either in its primary structure or in conformation due to post translatlonal modification as a result of which the catalytic activity of the enzyme may be changed.

The present work deals with the purification of acid/NUV DNase from old rat brain, in order to gain some insight into the reasons for its decreased activity in aging rat brain. Some physico chemical properties of purified enzyme were studied. The results indicate that conformational change in the 'old' enzyme molecules may be the reason for the decreased specific activity.

MATERIALS AND METHODS:

The general materials and methodology for assay of acid/NUV DNase is described in Chapter II and III.

IMMUNOTITRATION:

The monospecific antibodies of pure young acid/NUV DNase (Fig. 23 & 24) was used to ascertain the levels of DNase at different ages by lmmunotltration. The acld/NUV DNase preparations were incubated with increasing amounts of antlsera at 4 C. The final volume of reaction was then brought to 0.5 ml with 0.01 M sodium acetate buffer pH 5.0. The incubation was carried out for 24 hrs after which the reaction mixture was centrifuged for 30 min. at 5000 xg. A control experiment was run simultaneously using non-immune serum.

PURIFICATION OF NUV/ACID DNase FROM OLD RAT BRAIN:

Brains of 2 year old albino rats were taken and homogenized in ice cold double distilled water containing 10 mM PMSF to make 10% homogenates. The procedure for enzyme extraction, purification, enzyme and protein assay was similar as described in Chapter II and III.

POLYACRYLAMIDE GEL ELECTR0PH0RESIS:

PAGE, SDS-PAGE, staining and destaining of the gel was carried out essentially as described in Chapter **III.**

DETERMINATION OF N-TERMINAL AMINOACID:

The N-terminal aminoacid of old rat brain DNase was

determined by the procedure of Chang (1983). The procedure was described in Chapter III.

RESULTS

Immunotltratlon of pure acid/NUV DNase obtained from young rat cerebrum against the antiserum prepared to young purified DNase was shown in Fig.25. The results indicated that the pure young enzyme (2 μg) required 40 μl antlserum to inhibit 50'/. of its activity. The initial experiment was conducted to find out a suitable amount of antlserum which could be directly used for the immunotitration acid/NUV DNase activity present in brain homogenates of different ages.

Fig.26 shows immunotitration of acid/NUV DNase activity in homogenates of young and old rat brains. The initial activity was adjusted to 5 units in both young and old samples before the addition of antiserum. These results point out that the old enzyme required more antiserum per unit of activity compared to young enzyme. In other words, the old enzyme required 370 μl of antlserum to inhibit 50% of its activity, whereas young enzyme required 130 μl of antiserum to inhibit 50% of its activity. These results suggest the presence of considerably higher concentration of enzyme antigen per unit of enzymatic activity in old compared to young enzyme. The acid/NUV DNase was purified from old rat brain by employing same procedure followed for the purification of young enzyme.

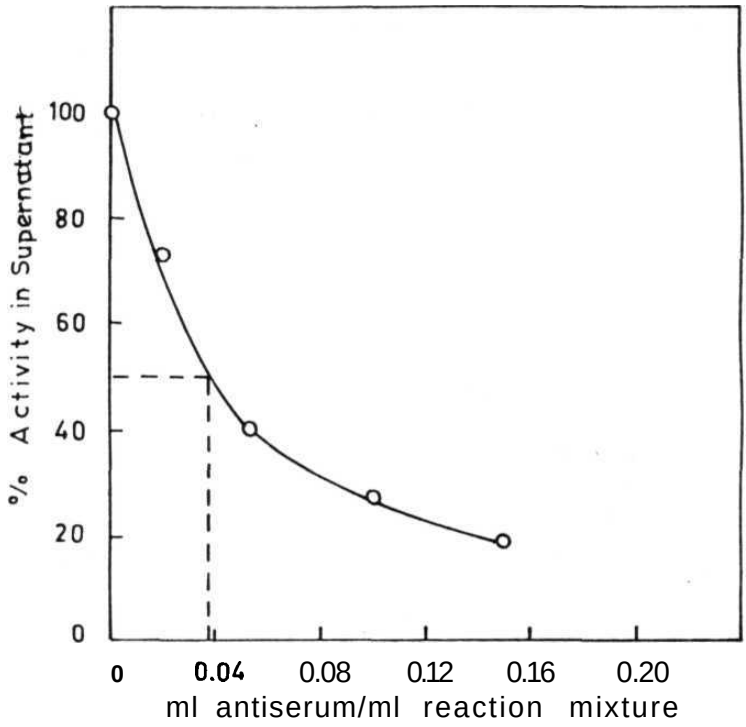


Fig.25 Immunotitration of purified acid DNase obtained from young rat cerebrum against the antiserum prepared against the same enzyme purified from young brain. A fixed amount of acid/NUV DNase (10 units) was mixed with different amounts of antiserum in 1 ml of 0.01 M sodium acetate buffer pH 5.0 and the mixture was incubated at 4°C for 24 hr. The immunocomplex was separated by centrlfugatlon at 5,000 x g for 30 min. The acid/NUV DNase activity was assayed in supernatant.

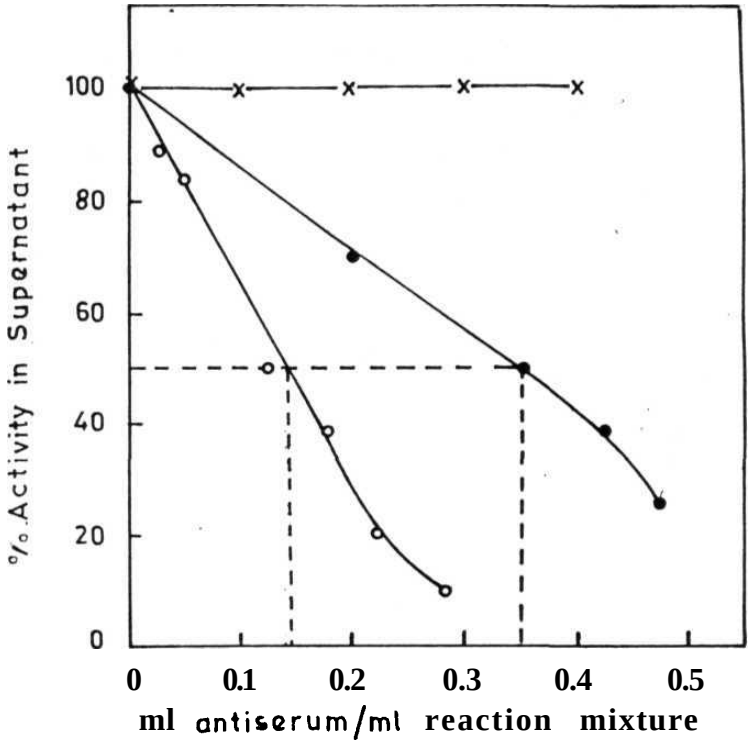


Fig.26 Immunotltratlon of acid DNase obtained from 'young' and 'old' rat brain homogenate against the antiserum prepared to young purified DNase. The acid/NUV DNase from both young and old brain homogenates were subjected to immunotltration by mixing with different amounts of young acid/NUV DNase antlserum and the mixtures were incubated for 24 hrs at 4 C. The initial activity was adjusted to 5 units in both 'young' and 'old' samples before the addition of antiserum. The acid/NUV DNase activity was determined in the supernatant. o-o 'young' acid/NUV DNase ●-● old acid/NUV DNase (incubation time overnight at 4°C) x-x acid/NUV DNase activity in the presence of control serum and each point represents averge of 3 experiments.

Fig.27 shows the elution profile of old acid/NUV DNase from sephadex G-100. The elution profile is similar to that of young acid DNase (See Fig.2, Chapter III). The active fractions were pooled and loaded on to the affinity column of DNA bound Ecteola-cellulose Fig.28 shows the elution profile of acid/NUV DNase from affinity column. The elution profile is similar to that of young enzyme (see Fig.3 Chapter III). The active fractions were pooled and concentrated and used for further studies.

The purification schedule of old rat brain acid/NUV DNase was shown in Table 11. As can be seen 110 fold purification with *ZV.* yield was achieved. It may be noted that the specific activity of enzyme from old brain is only one fourth of that from young brain in the initial extracts, whereas after the affinity chromatography step the enzyme preparation from old brain exhibited only one eighth of the specific activity as that of corresponding young enzyme. It can be noted from Table 2 that the young enzyme showed a specific activity of about 4840 while purified preparation from old brain showed a specific activity of 614 only.

The final preparation showed a single band on SDS-polyacrylamide gel electrophoresis (Fig.29). The molecular weight of old NUV DNase was found to be 62 KD (Fig.30) by plotting Rf against the corresponding molecular weight of marker proteins on semi-log graph paper.

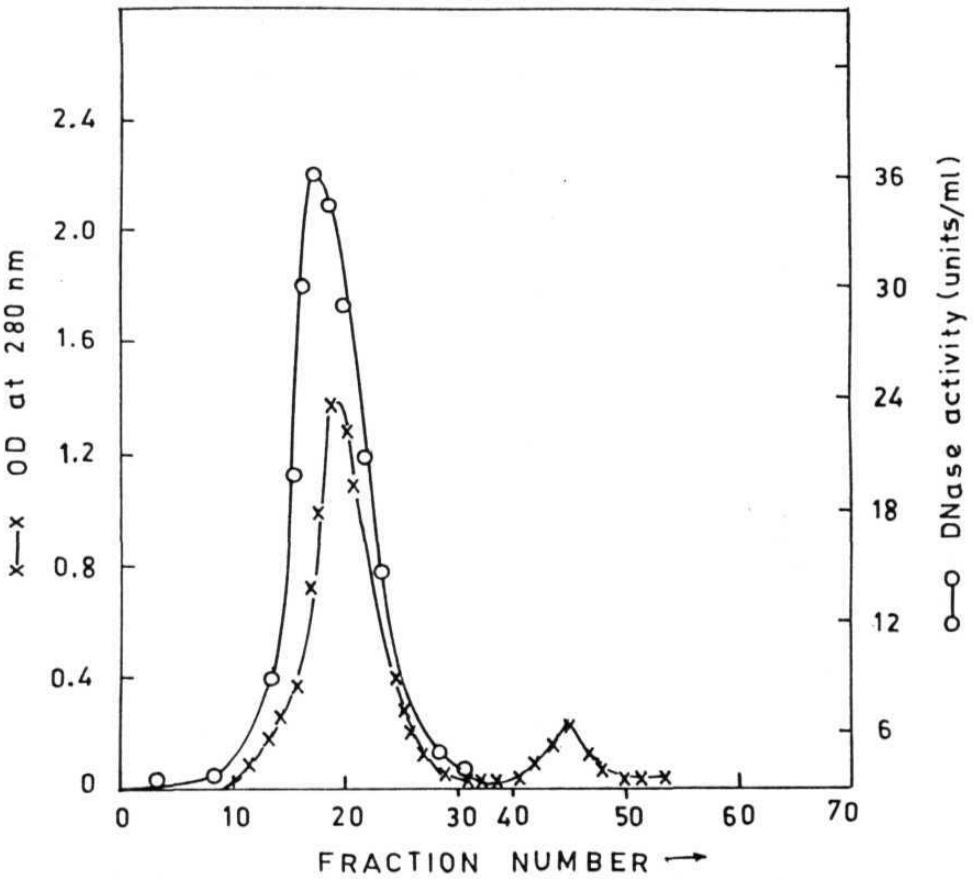


Fig.27 Elution profile of 'old' acid/NUV DNase activity from Sephadex-G-100: The 95"/. ammonium sulphate precipitate containing the activity was dialysed and loaded on 1.5 Cm x 84 Cm column of sephadex G-100. The column was preequilibriated with 10 mM sodium acetate buffer pH 5.0 and developed with same buffer at a flow rate of 15 ml per hour. 2 ml fractions were collected. Fractions 15 to 25 were pooled for further purification. Elution profile was monitored by taking OD at 280nm of each fraction (x-x) An aliquot of each fraction was taken for the enzyme assay.(0-0) Activity with native DNA as substrate.

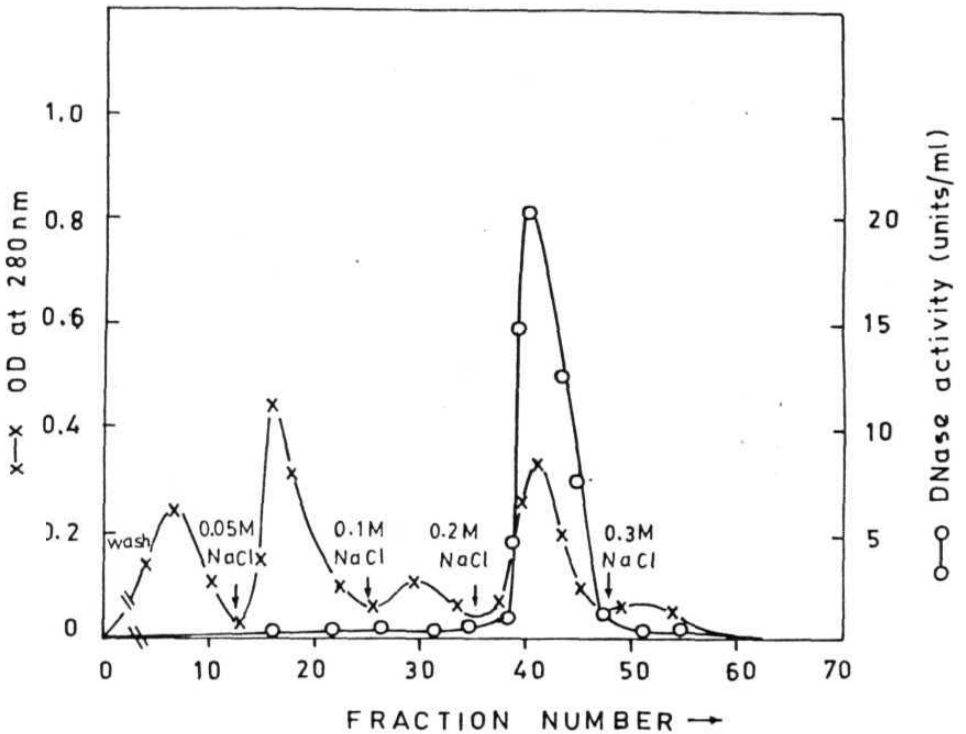


Fig 28 Elution profile of 'old' Acid/NUV DNase from DNA bound Ecteola cellulose affinity column: The active fractions from the sephadex G-100 column were loaded on 2 Cm x 7 Cm affinity column. The column was preequillbriated and developed with 10 mM sodium acetate buffer pH 5.0. The unbound proteins were washed out. The bound proteins were eluted by NaCl step wise gradient in the buffer. The concentrations of NaCl used were 0.05, 0.1, 0.2 and 0.3M. Other details are shown in Fig. 27.

Table - 11

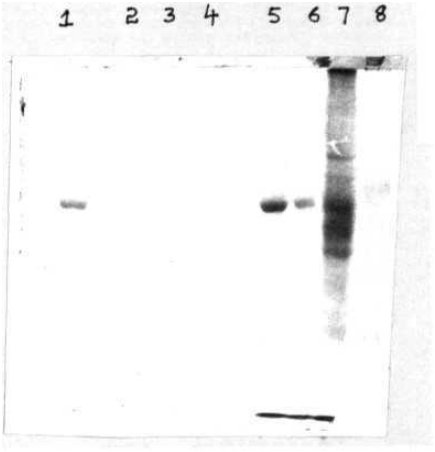
Purification of Acid/NUV DNase from 'old' Rat Brain

Purification step	Vol. ml.	Units/ ml.	Protein mg/ml	Total pro.	Total act.	Sp.act	Yield %
Brain homogenate	320	50	8	2560	16000	6.2	100
60% Supernatant	270	35	2.5	675	9450	14	59
95% PPt	30	102	6	180	3060	17	19
Sephadex G-100	13	69	1.39	18	897	50	6
Affinity Chromatography	2	166	0.27	0.54	332	614	2

Specific activity is expressed as µg of acid soluble DNA-P liberated/2 hrs/mg protein. Other details are as in Table 2.

Fig. 29 10% SDS-PAGE.
Lane 1 to 4 Affinity **Chromatography** fraction
Lane 5 Sephadex **G-100**
Lane 6 Ammo. **Sul.** fraction.
Lane 7 Brain **homogenate**
Lane 8 Std. Proteins, BSA Egg albumin,
Glyceraldehyde-3-phosphate dehydogenase & **chymotrypsin**.

FIG.29



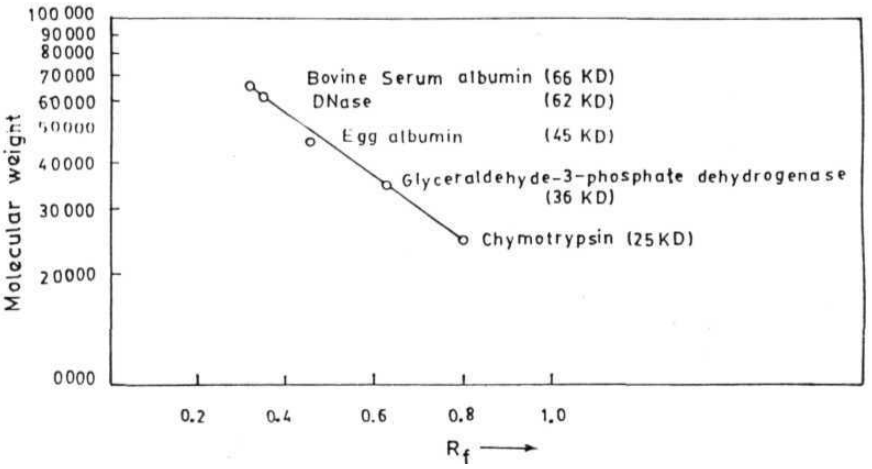


Fig. 30 Molecular Weight Determination by SDS-PAGE: Proteins of known mol.wt. and the purified enzyme fraction were run on SDS-PAGE on separate lanes. Rf values of the proteins were calculated and plotted against the corresponding mol.wt. The mol.wt. of purified enzyme fraction band was determined from the plot by its Rf value.

Fig.31 N-terminal amino acid of pure old acid/NUV DNase was determined by using *DABITIC* method (Chang, 1983). The details are desribed in Materials and Methods.

Fig.31[a] A line drawing of Fig 31.

(e) Internal marker(G) Glyclne.

FIG.31

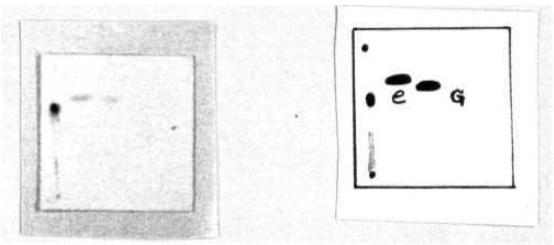


FIG.31

The N-terminal amino acid of old NUV/acid DNase was found to be glyclne (Fig.31) by DABITIC method which is same as that observed with young enzyme.

Thermal inactivation studies were performed by assaying acid/NUV DNase activity at different temperatures. Acid/NUV DNase in 0.01 M sodium acetate buffer pH 5.0 from young (2-5 µg protein) and old (10-15 µg of protein) rat brain was incubated in a water bath at 55 C and 65 C and the enzyme activity was assayed at different time intervals. Initial activity of enzyme was adjusted to 10 units in both ages. Fig. 32 shows the effect of the incubation at 55 and 65 C on the stability of purified DNase. Pre-incubation of purified NUV DNase "young" or "old" at the temperatures mentioned for different time periods, resulted in considerable loss of activity. It is also clear from Fig.32 that the loss of activity is more in case of old enzyme compared to young. Thus, it would appear that aging rat brain may contain partially denatured DNase molecules.

The inactlvatlon of young and old acid/NUV DNase by pronase E (Streptonyus griseus) was shown in Fig.33. The initial activity of acld/NUV DNase was adjusted to 100 units/100 µl for both the enzyme preparations. To this 1.5 units of pronase E (sp.acty 6 units/mg) was added and incubated in 10 mM Tris HC1 pH 7.5 at 37°C. Aliquots of 10 µl were withdrawn at different time periods. Proteolysis was terminated by the addition of 4 µl of 0.01 M PMSF in acetonitrile and residual enzyme activity was determined. Fig.33 shows the pronase E digestion studies of young and old

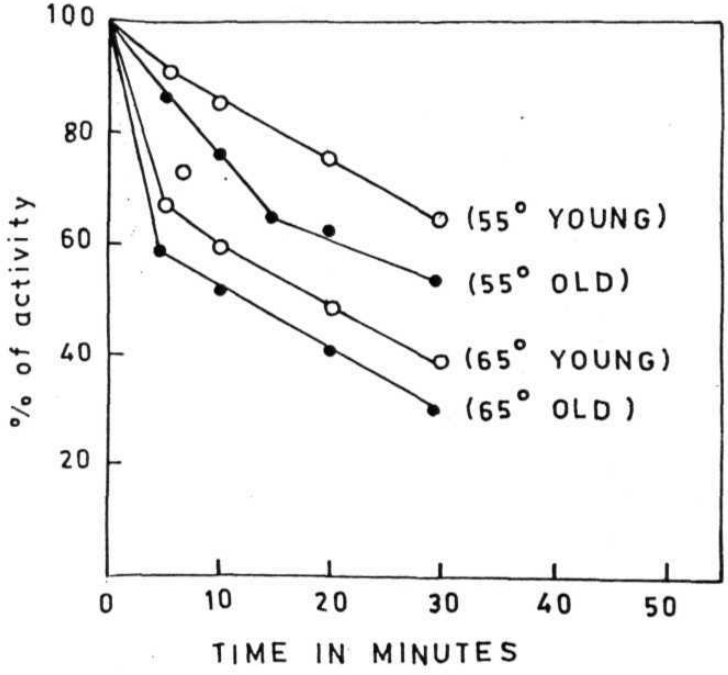


Fig 32 Effect of prelncubatlon at different temperatures for varying periods on the purified acid/NUV DNase prepared from 'young' and 'old' rat brain. Each point represents the average of three experiments.

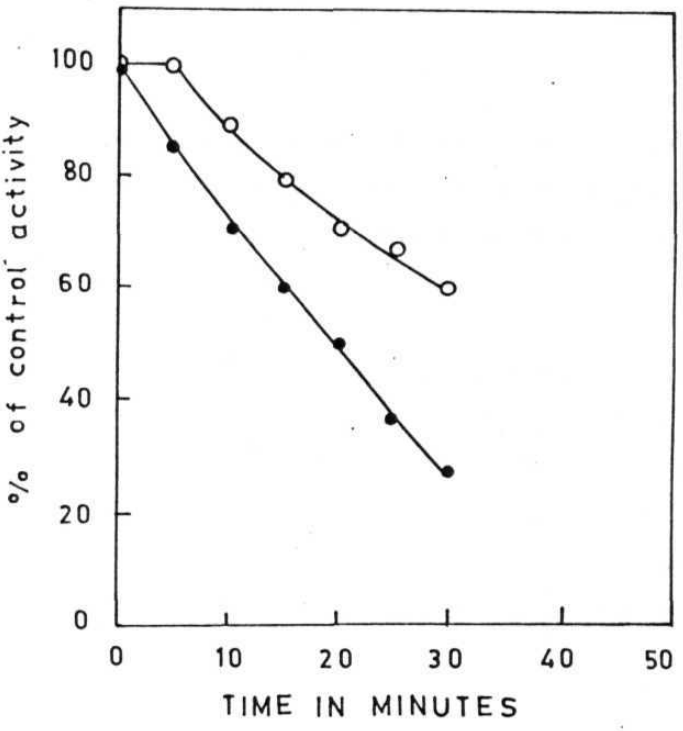


Fig.33 Inactivation of DNase by pronase E. : (0-0) 'young' DNase **(●-●)** old DNase. In the control experiment (enzyme with no protease present) there was no significant change in the activity over the indicated time period. Each point represents average of three experiments.

acid/NUV DNase. These results suggest that the old enzyme was more susceptible to pronase E digestion compared to its young counterpart.

DISCUSSION:

The acid/NUV DNase old rat brain was purified to apparent homogeneity, where pure enzyme showed a single band on SDS-polyacrylamide gel electrophoresis. The molecular weight of enzyme was calculated to be 62,000 daltons.

Throughout the purification schedule the enzymes from young and old brain behaved similarly in their elution pattern and mobility in electric field. These results revealed an important fact that young and old acid/NUV DNases are similar in size and charge. These results also indicated that there is no gross difference between primary structure of young and old acid/NUV DNase.

The N-terminal amino acid was found to be glycine in both young and old acid/NUV DNase preparations. This gives an idea that altered properties of old enzyme are not the results of 'proteolysis'. This gives an additional support that there are no major differences in the primary structures of acid/NUV DNase preparations obtained from young and old rat brain. If the error catastrophe theory of Orgel (1963) were to hold good, then it is to be expected that several wrong amino acid would be incorporated into the enzyme molecules in aging brain and should behave

differently with respect to various criteria mentioned above. Thus, the present observations do not support the error catastrophe theory.

However, the present data also show that the activity of acid/NUV DNase from aging brain is markedly decreased. In fact, the extent of reduction is so marked (85%) it is not known whether there is any other enzyme suffering loss of activity at this magnitude with age. So far, purified superoxide dismutase (Reiss *et al.*, 1976) lsocltrte lyase (Reiss and Rothsteln, 1975) seem to be the enzymes most affected during agiing, and even here there was only a 60% decrease in the activity. The question then arises, "What is the cause for loss of enzyme activity in acld/NUV DNase molecules from aging brain? The answer to this question is porvided, atleast to some extent, by some of the immunological, thermal inactivation and protease digestion studies. Immunotitration experiments with young and old acid/NUV Dnase showed that the latter requires more antiserum per unit of activity (Flg.26). This suggest that the enzyme molecules synthesized in aging brain are catalytlcally "defective" and therefore can be considered as "altered" or different from the molecules elaboratd by embryonic brain.

The next question that would arise is what is the "difference" or "alteration" that the acld/NUV DNase molecules from old rat brain suffer from as compared to the molecules synthesized by embryonic and developing brain. Thermal stability as well as protease digestion experiments provide some clues to this question. The old enzyme was more sensitive to heat. The

increase in thermolability of the enzymes in old age have been attributed to subtle post translational modifications of amino acid side chains and not to substitution of amlno acids. The same pattern of findings were reported in case of enzymes of free living nematode, *Turbatrix aceti* (Sharma, 1976; Rothstein, 1979). A significant observation of the present investigation is that the old enzyme was also more susceptible than the young enzyme to neutral protease digestion (Fig.33). It is increasingly becoming clear now that covalent post translational modification of a protein may serve as a marker/signal for proteases to act upon (Reviewed by Stadtman, 1990). This means such of those proteins in the cell that are covalently modified would serve as better substrates for the neutral proteases leading to the accelerated turnover of such proteins. If in aging tissue a number of proteins lose their biological activity due to post translational covalent modifications (as is the conclusion of present and several other earlier studies) it must also follow by a rapid degradation of such altered molecules by the neutral proteases. Indeed our experiments with pronase E adduce support for such a contention as the acid/NUV DNase from old brain was degraded more efficiently (Fig.33). But then why an aging tissue should accumulate such altered molecules in spite of being better substrates for degradation? Either the concerned protein is synthesized much faster than the degradation or the proteolysis is slowed down in aging tissue as a result of decreased levels of proteases. Evidence is accumulating to supports the latter possibility as it is shown by Starke-Reed and Oliver (1989) that the intracellular accumulation of catalytically inactive or less

active forms of several enzymes which occurs during aging is correlated with an age dependent decrease in the intracellular levels of neutral-alkaline proteases.

Immunological, spectral, thermal and protease digestion studies on purified enolase from *Turbatrix aceti* by Sharma & Rothstein (1978, 1980[a]) provide substantial evidence to suggest that the enzyme from old organism suffers conformational change possibly due to post translational modification. The same workers also provide evidence for the conformational alteration in the phosphoglycerate klnase of aging rat liver and brain (Sharma *et al.*, 1980[b]). The present studies with purified acid/NUV DNase from embryonic and old rat brain also point out conformational alteration due to post translational modifications of amino acids in enzyme molecules as a possible reason for the loss of activity in the old enzyme.

It is not, however clear today precisely what is the alteration or modification that makes the enzyme from old animals less active catalytically.

This should obviously form one of the future directions of aging research.

CHAPTER V

Partial Puriication of Acid/NUV DNase from Rat Brain Nuclei

CHAPTER - V

PARTIAL PURIFICATION OF ACID/NUV DNase FROM RAT BRAIN NUCLEI

INTRODUCTION:

Studies of various DNases in both bacterial and mammalian cells indicated that some may have an important role in cellular metabolism i.e. DNA repair, DNA recombination, DNA synthesis and DNA degradation (Lehman, 1967; Lesca, 1971). If so, it would be logical to expect their presence in the nucleus, where most of the DNA is located. Indirect support for this hypothesis came from comparitive studies of DNases (Alfrey & Mirsky, 1952; Coordinnier & Bernard!, 1968) which suggested a correlation between the activity of acid DNase and the capacity of certain tissues to divide. By using histochemical methods, Swingle and Code (1964) detected DNase II activity in rat liver nuclei directly. Lesca (1968) also demonstrated the presence of that enzyme in nuclear fraction of mouse liver cells. He was able to conclude that the nuclear DNase II activity was not due to lysosomal contamination. The presence of acid DNase in the nuclear fraction was also established in the nuclei of calf thymus. Hela S$_3$ cells, neurons, astrocytes and oligodendroglial cells of chick brain (Slor & Lev, 1971; Stamblova *et al.*, 1973). Slor (1973) ruled out the possibility that the presence of nuclear DNase activity is caused by lysosomal contamination of the nuclear fraction or by preferential binding of lysosomal or cytoplasmic DNase II to nuclear DNase during the purification of nuclear fraction.

Previous studies from this laboratory also reported the presence of acid/NUV DNase activity in nuclear fraction of neurons, astrocytes and oligodendroglial cells of rat brain (Subrahmanyam, 1989). Recently (Suvarchala *et al.*, unpublished data) also found the presence of acld/NUV DNase activity in the nuclei of chick brain cells. Therefore a study has been undertaken to purify atleast partially from the nuclei of rat brain the acld/NUV DNase and compare its properties with that obtained from whole homogenate (Chapter III).

MATERIALS AND METHODS:

Highly polymerized calf thymus DNA, sephadex G-100 were purchased from Sigma Chemical Company, USA. Sucrose (ExcelaR grade) was purchased from Glaxo Laboratories (India) Ltd., Nylon cloth (80 μ pore size) was purchased from small parts Inc., Miami, Florida, USA. All other chemicals used were of analytical grade.

ISOLATION OF NUCLEI:

The nuclei were isolated from the rat brain according to the procedure of Lovertrup-Rein & Mc Ewen (1966).

Brains of 21 days old rats were removed and washed in ice cold homogenization medium (0.32 M sucrose, 1 mM MgCl and 1 mM potassium phosphate buffer pH 6.4). Menlnges and blood clots were removed and the tissue was homogenzied by hand (20 strokes) in

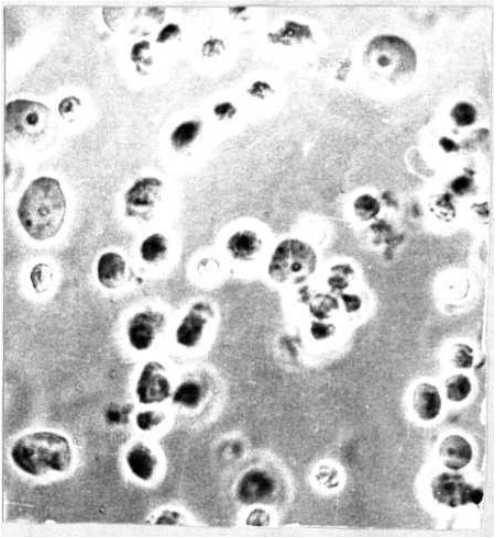
loose fitting potter elevehjem homogenizer with teflon pestle in the homogenizing medium (14 ml/g of tissue). The homogenate was filtered through 80 μ nylon mesh and centrlfuged at 850 xg for 10 min in refrigerated centrifuge. The sediment was washed once again with the homogenizing medium and centrlfuged at 600 xg for 10 mln. The crude nuclear pellet thus obtained was suspended in buffer containing 2.0 M sucrose, 1 mM $MgCl_2$, 1 mM potassium phosphate pH 6.4. The suspension was centrlfuged for 45 mln in SW 28 rotor of the Beckman L8-80 M ultra centrifuge at 55,000 xg. The nuclear pellet obtained was washed once with homogenization buffer and centrlfuged at 1000 xg for 10 mln at 4°C. The nuclear pellet was then suspended for 5 mln in 0.14 M NaCl, 1 mM MgCl , 2 mM potassium phosphate buffer pH 6.4 to which 0.5%. Triton X-100 was added and centrlfuged at 1000 xg for 10 mln. The pellet contains pure nuclei triton X-100 removes the outer nuclear membrane and also decrease the cytoplasmic contamination. The pure nuclei were homogenized in ice cold double distilled water containing 10 mM PMSF in a potter elvehjem homogenlzer. The nuclear homogenate was centrifuged at 10,000 xg for 10 min at 4 C The supernatant was taken as enzyme source. All purification steps were carried out in cold room.

RESULTS:

Fig.34 shows the phase contrast photomicrograph of pure nuclei, where no contamination of other organelles could be seen. The acid/NUV DNase was extracted and purified from such pure nuclei. The enzyme extract from pure nuclear pellet (2 ml) was

Fig.34 Phase contrast micrograph of brain nucler fraction.

FIG.34



loaded on to Sephadex-G-100 column of 1.5 cm x 42 cm. The column was preequl libra ted with 0.01 M sodium acetate buffer pH 5.0 and developed with same buffer at a flow rate of 8 ml/hour. Fig. 35 shows the elutlon profile of acid/NUV DNase. The fractions 12 to 24 containing the major activity were pooled and dlalysed against 0.01 M sodium acetate buffer pH 5.0, concentrated and used for further studies.

Table 12 shows the purification schedule of acld/NUV DNase from brain nuclear extract. As can be seen 50 fold purification with 2'/. yield was achieved. The partially purified final preparation showed almost equal activity towards native DNA and native DNA exposed to UV light.

The optimum pH for nuclear acid/NUV DNase was determined and found to be 5.0 (Fig.36). The DNase activity of nuclear DNase was also determined by assaying the enzyme activity in 10 mM potassium phosphate buffer pH 7.0 as well as in 10 mM sodium acetate buffer pH 5.0, with addition of various concentrations of NaCl. Under the conditions of low ionic strength (0.02 M NaCl) the enzyme showed considerable amount of activity at pH 7.0 (Fig.37).

The effect of various metal ions on the nuclear acid/UV DNase is shown in Table 13. The compounds tested were magnesium chloride, calcium chloride, sodium sulphate, magnesium sulphate and sodium dlhydrogen phosphate. There was no significant change of activity in the presence of Mg^{+2} and Ca^{+2} ions upto a conc. of 10 mM whereas the enzyme activity was significantly inhibited by

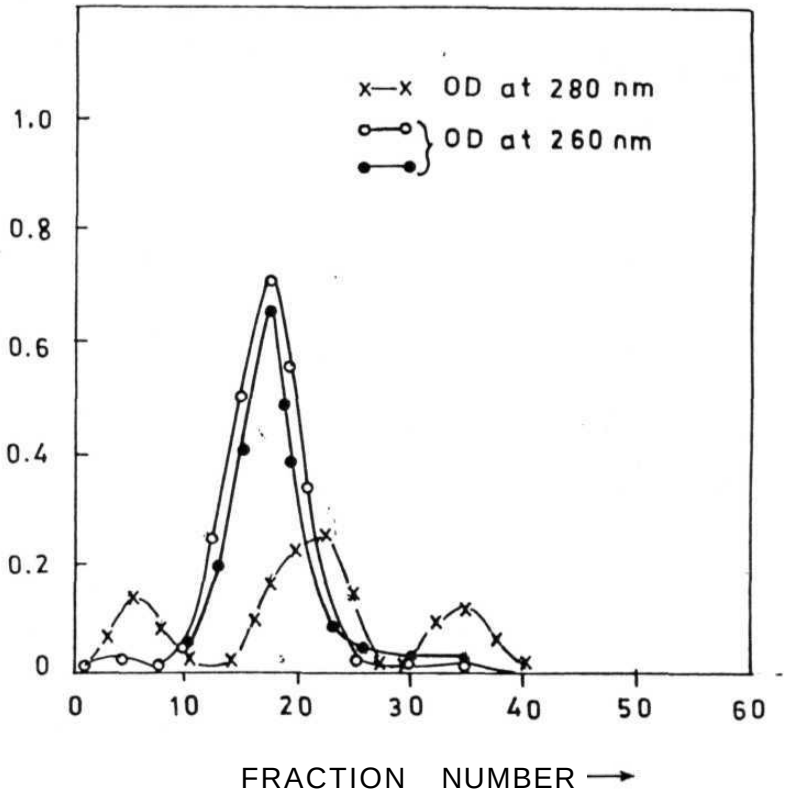


Fig.35 Elution profile nuclear DNase from Sephadex, G-100: Pure nuclear fraction containing the enzyme activity was loaded on 1.5 Cm x 42 Cm column of Sephadex G-100. The column was pre- equilibriated with 10 mM sodium acetate buffer pH 5.0 and developed with same buffer at a flow rate of 8 ml/hr. 2 ml fraction were collected. Elution profile was monitored by taking OD at 280 nm of each fraction (x - x). An aliquot of each fraction was taken for acid/NUV DNase assay. 0-0 activity with native DNA as a substrate o-o activity with UV DNA as a substrate.

Table - 12

Purification of Acid/NUV DNase from Rat Brain nuclei

Purification	Tot.Act.	Tot. Protein (mg)	Sp.Act	Yield %
Brain extract				
Native DNA	5430	900	6.03	100
UV-DNA	5280		5.8	
Crude nuclear pellet				
Native DNA	461	73	6.3	8.4
UV-DNA	448		6.1	
Pure nuclear pellet				
Native DNA	268	7.9	33.9	4.9
UV-DNA	240		30.3	
Sephadex G-100				
Native DNA	120	**0.4**	300	2
UV-DNA	115		287	

Specific activity is expressed as µg of acid soluble DNA-P liberated/2 hrs/mg protein. Total activity is obtained by multiplying the specific activity with the protein content of the tissue in mg. For the assay of NUV DNase the reaction mixture consisted of 200 µg of native or UV DNA, 0.1 M sodium acetate buffer pH 5.0, and enzyme in a total volume of 0.6 ml.At the end of 2 hr incubation at 37°C the reaction was stopped by adding 0.4 ml of 1.4 N PCA and immediate chilling. The whole reaction mixture was centifuged at 4,000 rpm for 10 min and the OD of supernatant was read at 260 nm against appropriate blank.

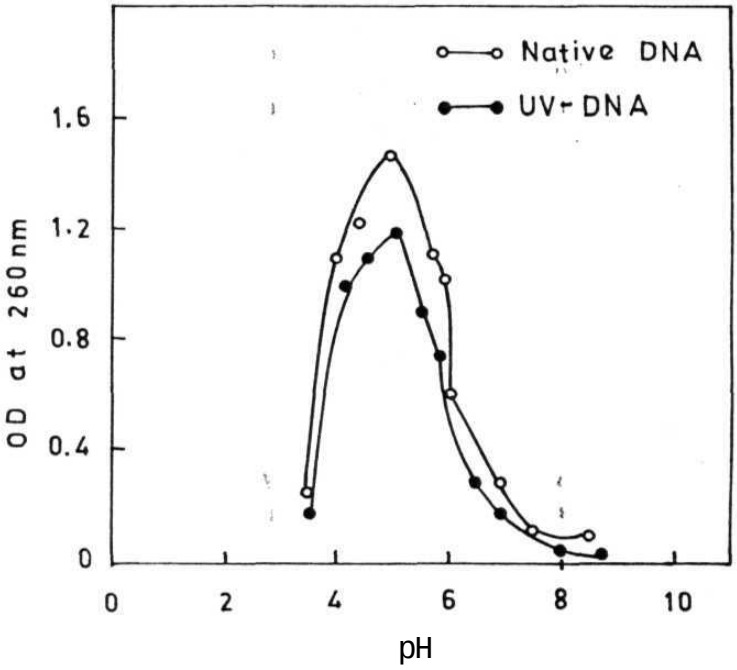


Fig.36 Effect of pH on purified nuclear **enzyme.** The buffers used were sodium acetate buffer pH (3- 5.5) potassium phosphate buffer pH (6-7.5) and **Tris** HC1 buffer (pH 8-9.5) Where each point represents average of three individual **experiments.**

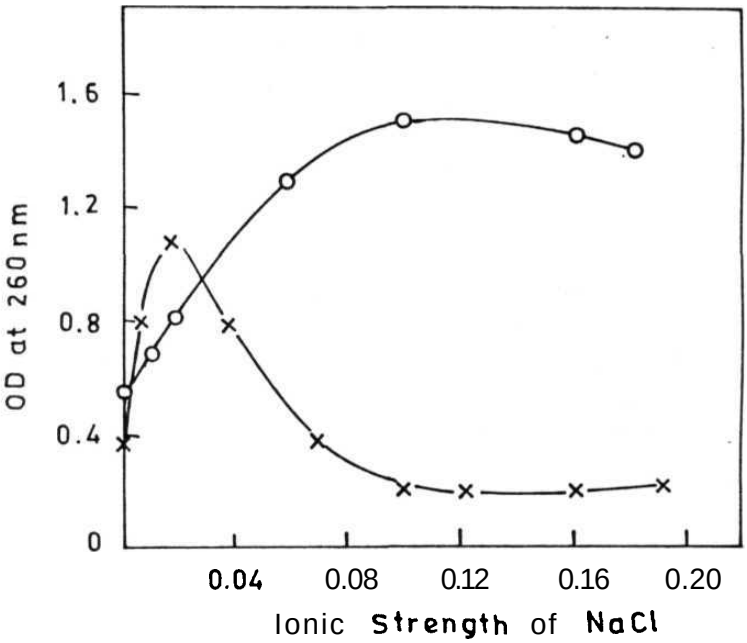


Fig.37. Nuclear acid/NUV DNase activity at pH 5.0 and 7.0 as a function of ionic strength. The activity (o-o) at pH 5.0 was assayed in 0.01M sodium acetate buffer pH 5.0 and addition of various quantities of NaCl. The activity, (x-x) at pH 7.0 was assayed in 10 mM potassium phosphate buffer pH 7.0 with various quantities of NaCl. DNase activity expressed as OD at 260 nm of acid soluble nucleotides/hr at 37°C/10 µg partially purified enzyme.

Total - 13

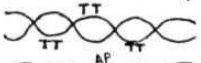
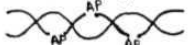
Effect of various metals/inorganic ions on the Nuclear acid/NUV DNase activity

Cations & anions	'/. activity			
	5 mM	10 mM	20 mM	100 mM
MgCl	105	114	55	3.2
$CaCl_2$	104	112	129	22
Na_2SO_4	64	50	22	5
MgSO	74	60	45	4
NaH_2Po_4	52	48	22	10

All the values are the average of three individual experiments. Activities are expressed as **µg** of acid soluble DNA-P liberated/2 hrs at 37 C. Activities are expressed as percentages where control value is taken as **100%.**

Table - 14

Properties of Acid/NUV DNase from Total Brain Homogenate and Nucleus

	Enzyme purified from total brain homogenate	Partially purified enzyme from Nuclear fraction brain cells
1. pH	5.0	5.0
	7.0 (0.02 M NaCl)	7.0 (0.02 M NaCl)
2. Metal ion		
Mg^{+2}, Ca^{+2} (5 mM)	<——>	<——>
3. SO_4^{-2}, PO_4^{-2}	↓	↓
4. Heat Stability	65°C	65°C
5. Substrate		
	+	+
	−	−
	+	+
	−	−
6. Physiological function	DNA repair/ DNA replication	DNA repair/ DNA replication

<——>, No change, ↓ activity inhibited significantly.

anlons like SO_4^{-2} and PO_4^{-2} ions, even at a concentration of 5mM.

Table 14 shows comparision of some of the properties of acid/NUV DNase from total brain homogenate and nuclei. These results revealed the enzyme from nuclear fraction is very much similar to that purified from brain homogenate (Chapter III).

DISCUSSION

The acid/NUV DNase was purified 50 fold from pure brain nuclear fraction. The enzyme does not require divalent cations for its activity, whereas the enzyme was inhibited by anions like SO. and PO . The final preparation showed almost equal specific activity towards native DNA and UV DNA.

Looking at the properties of the partially purified acid/NUV DNase from nuclei, it is quite apparent that this enzyme is identical with that purified to homogeneity from brain homogenates. From this it may be concluded that the acid/NUV DNase is located partly in nucleus. Considering its activity at pH 7.0 under different ionic conditions, the enzyme seems to be a likely candidate for carrying out the incision step that is necessary for DNA excision repair either during the DNA replication or under quiescent conditions. However, all our earlier work with respect to changes in the activity of this enzyme with age, point out, a role for this enzyme in replication linked to DNA repair possibly the post replicational repair.

CHAPTER VI

Regulation of acid/NUV DNase activity by an Endogenous Protein Inhibitor

CHAPTER - VI

REGULATION OF ACID/NUV DNase ACTIVITY BY AN ENDOGENOUS PROTEIN INHIBITOR

INTRODUCTION:

Any substance that reduces the velocity of an enzyme catalyzed reaction can be considered to be an "Inhibitor". The inhibition of enzyme activity Is one of the major regulatory devices of living cells. Inhibition studies often tell us about the specificity as well as regulation of an enzyme in the cell. The inhibitors are very useful tools to know much about the behaviour as well as physiological role of enzymes *in vitro,* we may begin to think about the more challenging problems of understanding the complex structural situation of these enzymes *in vivo.*

Different types of inhibitors have been described for various DNases. Two DNases of bacterial origin were reported to be inhibited by RNA (Lehman, 1962). Lindberg (1966) reported protein inhibitors of neutral DNases, Cooper *et al.,* (1950) found a specific inhibitor for bovine pancreatic DNase I. Lindberg and coworkers characterized the inhibitor (Lindberg, 1967[a]; Lindberg & Snook, 1970) which is a major component of the cell protein and identified it as monomeric actin (Lazardies & Lindberg, 1974; Hitchcock *et al.,* 1976). Preliminary and incomplete evidence suggests the existence of a natural inhibitor for the class of acid DNases. (Laskowski, 1961). Later Lesca (1968, 1976) has

actually purified an endogenous inhibitor for acid DNase from mouse liver.

In order to know more about the possible function and regulation of activity of acid/NUV DNase, an attempt has been made to see whether there is a natural inhibitor for this enzyme in brain cells. Indeed an inhibitor was found in brain extracts. This Chapter deals with the isolation and property studies of such an inhibitor.

MATERIALS AND METHODS:

CM-Sephadex, hydroxylapatite, RNase (bovine pancreas) Trypsin (Type 1, from bovine pancreas), Soya bean trypsin inhibitor (Type II S), pancreatic DNase, Spleen DNase were purchased from Sigma Chemical Company, St. Louis, MO, USA. The methodology of purification of acid/NUV DNase was described in Chapter III.

ASSAY OF INHIBITOR ACTIVITY:

The assay medium consisted of 100 mM sodium acetate buffer pH 5.0, 50 µg of calf thymus DNA, enzyme fraction 1-2 µg (sp.act. 4.8 x 10 units/mg) and inhibitor fraction in a final volume of 0.6 ml. The incubation was for 2 hrs at 37 C. After the incubation the tubes were transferred to ice and 400 µl of 1.4 N perchloric acid was added. After keeping for 10 min on ice, the tubes were centrifuged and the supernatants were trasnferred to

another set of tubes and the OD was measured at 260 nm in a Hitachi 150-20 Spectrophotometer. The unit of inhibitor activity was defined as the amount of the protein responsible for a 50% decrease in the acid/NUV DNase activity.

RESULTS

PURIFICATION OF INHIBITOR:

Brains of 20 days old albino rats were taken for purification. All steps were carried out at 4 C and all solutions contained 0.01 M β-mercaptoethanol. Brains were dissected out and washed in ice cold double distilled water. Brains were homogenized in 0.14 M NaCl in potter elvehjem homogenizer to make 10% homogenate. The homogenate was centrifuged at 30,000 xg for 30 min in high speed Kubota refrigerated centrifuge. Most of the activity was present in the supernatant. To the supernatant ammonium sulphate was added slowly, while stirring, to make 60-95% saturation and the solution was centrifuged at 20,000 xg for 20 mln. The pellet which contained activity was taken for further purification. The pellet was then dissolved in double distilled water and dlalyzed exhaustively against 0.05 M phosphate buffer pH 6.0. The dialysate was centrifuged at 10,000 xg for 10 mln. The supernatant was loaded on carboxy methyl-Sephadex (CM-Sephadex A-50) column (2 x 17 cm) equilibriated with 0.05 M phosphate buffer pH 6.0. The column was eluted with continuous gradient between 0.05 M phosphate buffer pH 6.0 and 0.5 M, pH 7.0. Fig. 38 shows the elution profile of inhibitor from CM-Sephadex.

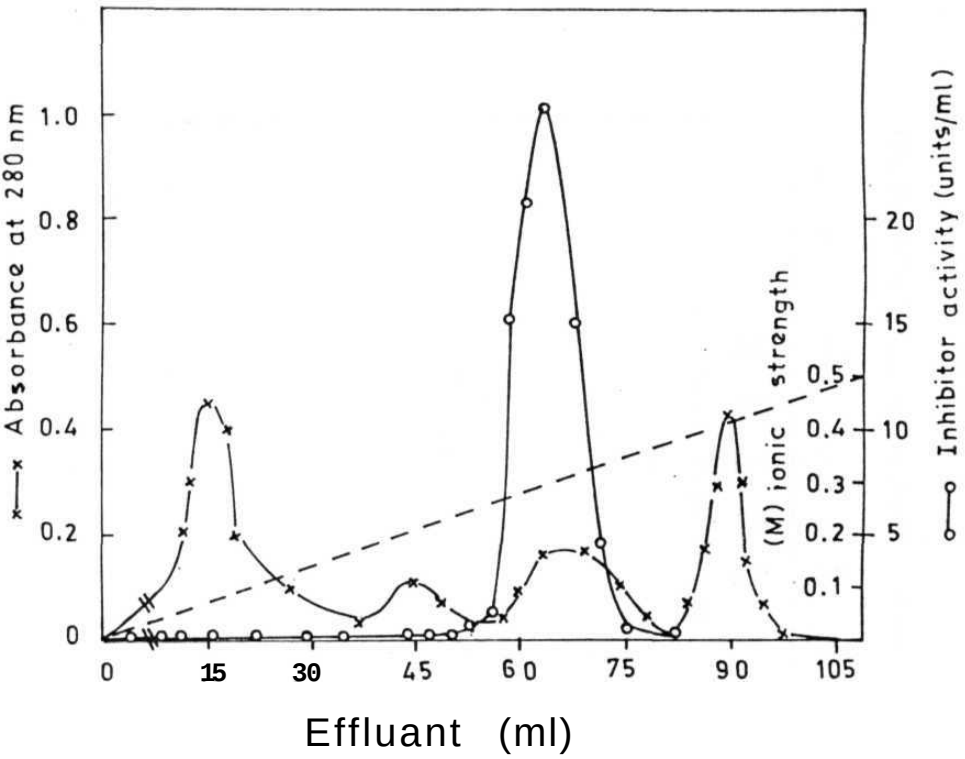


Fig. 38 Elution profile of inhibitor from CM-Sephadex: The 95% ammonium sulphate precipitate was dialysed against 0.05 M phosphate pH 6.0 and loaded on the 2.5 x 17 Cm column of CM-Sephadex. Elution profile was monitored by taking OD at 280 nm of each fraction (x-x) An aliquot of each fraction was for the inhibitor assay. o-o Inhibitor with 50µg calf thymus DNA as a substrate. The unit of inhibitor activity is defined as amount of protein required for 50% inhibition of NUV DNase activity under standard conditions.

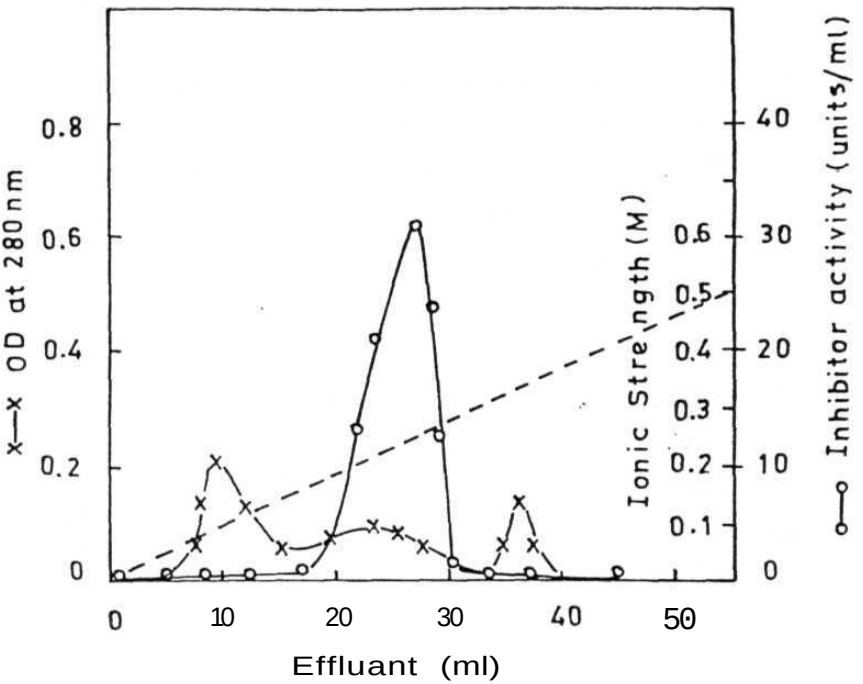


Fig.39 Elution profile of inhibitor from hydroxylapatite column: The inhibitor active fractions from the CM-Sephadex were loaded on 1 Cm x 8 Cm hydroxylapatite column. The column was preequilibrated with 0.05 M phosphate buffer pH 6.8, and eluted with 0.05 M and 0.5 M phosphate buffer pH 6.8. Other details are shown in Fig.38.

Fractions containing inhibitoractivity were eluted between 0.27 M pH 6.5 and 0.34 M pH 6.7. The active fractions were pooled and concentrated and loaded on to a hydroxylapatite column (1 x 8 cm).

The hydroxylalpatite column was preequilibriated with 0.05 M phosphate buffer pH 6.8 and eluted with a continuous gradient between 0.05 M and 0.5 M phosphate buffer pH 6.8. Fractions containing inhibitor activity were eluted between 0.2 and 0.25 M phosphate buffer. Fig.39 shows the elution profile of inhibitor from hydroxylapatite column.

The extent of purification and yield were difficult to ascertain, since the brain homogenate contains unspecific inhibitors of the enzyme such as histones and RNA, which are removed during the purification procedure. The specific activity after the hydroxylapatite chromatography was around 250 units/mg. This partially purified inhibitor was used for further studies.

Fig.40 shows the effect of inhibitor concentration on purified acid/NUV DNase. 5 units of purified acid/NUV DNase (1 μg) were taken and different concentrations of inhibitor was added. The results indicate that 4 μg of inhibitor was needed to decrease 50% of the activity.

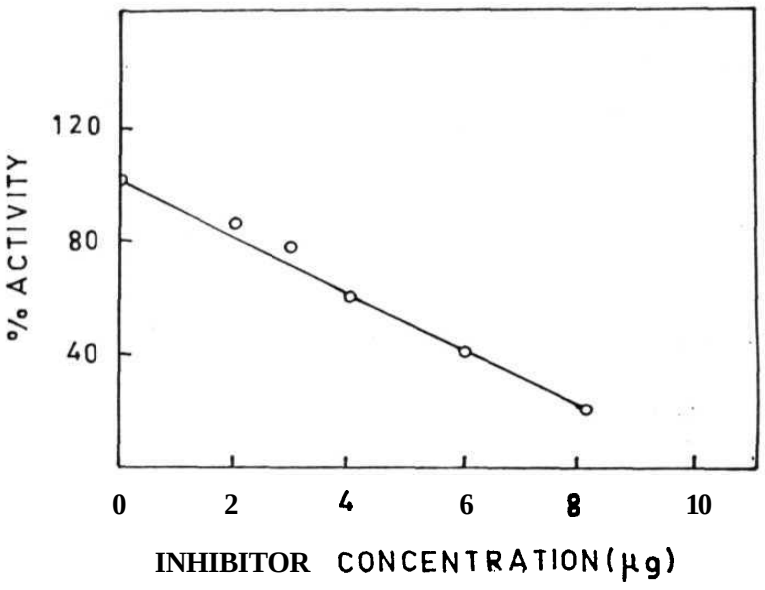


Fig.40 Effect of inhibitor concentration on activity of purified DNase. Each point represents average of three experiments.

PROPERTIES OF INHIBITOR:

ULTRAVIOLET SPECTRUM:

The UV spectrum of purified acid/NUV DNase inhibitor was taken between 220 and 400 nm. The purified inhibitor had an absorption spectrum such as can be expected from a protein mixture devoid of nucleic acids (Fig.41).

In order to ascertain the protein nature of inhibitor its inactivation by a proteolytic enzyme was investigated. In the experiment shown in Table 15.8 µg of acid/NUV DNase inhibitor was treated with trypsin (3 mg/ml) and incubated at 37 C in a volume of 100 µl of 50 mM Tris HC1 pH 8.1. After 30 min the samples were placed in ice and 0.1 mg of soya bean trypsin inhibitor was added to stop the trypsin action. An aliquot of this reaction mixture was taken out and its activity to inhibit the acid/NUV DNase activity was tested asusal. The results (Table 15) clearly show that trypsin did effectively inactivate the acid/NUV DNase inhibitor. These results indicate that the acid/NUV DNase inhibitor is a protein in nature.

Table 16 shows the effect of pretreatment of inhibitor with pancreatic RNase on its subsequent ability to inhibit the acid/NUV DNase activity. These results revealed that the inhibitor activity was retained after pre treatment with pancreatic RNase indicating that the inhibition was not due to an associated RNA in the preparation.

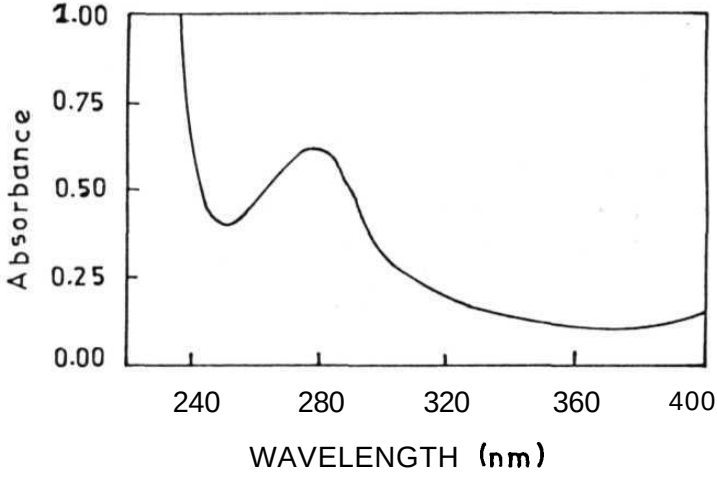


Fig.41 Ultraviolet spectrum of brain acid/NUV DNase inhibitor

Table - 15

Reversal of Acid DNase inhibition after Trypsin treatment

Addition to complete incubation mixture	Activity units/ml
None	14 ±1.5
8 μg Inhibitor	5.2 ± 0.8
Trysin treated Inhibitor	12.6 ±2.3

The inhibitor was treated with trypsin (3 mg/ml) for 30 min. at 37°C. in 0.05 M Tris HC1 pH 8.1 and then trypsininhibitor was added to stop the reaction and then the potency of inhibitor to inhibit the DNase activity was assayed. The values are expressed as mean + SD from 5 experiments.

Table - 16

Treatment of Inhibitor with Pancreatic RNase

Addition to complete incubation mixture •	**Activity** units/ml
Control	12 + 2
Inhibitor (5 μg)	7.3 ± 1.2
Pancreatic RNase treated Inhibitor	7.7 ± 0.8

The assay conditions were same as in Table 15. The inhibitor was treated with Pancreatic RNase at a conc. of 100 μg/ml in 0.1M NaCl for 30 min. at 37°C. and then the inhibitor activity was assayed. The values are expressed as mean ± S.D from 5 experiments.

Table - 17

Effect of Inhibitor on Other Deoxyribonucleases

DNases	Without inhibitor	With inhibitor
Brain acid/NUV DNase	35+1.0	11.8+1.4
Spleen DNAse	34+4.9	16 + 0.6
DNase I (Pancreas)	32+1.2	30 +2.8
Alkaline DNase (brain)	25 + 2.2	24 +1.0

The brain acid/NUV DNase and spleen DNase were incubated in 0.1 M sodium aceate buffer pH 5.0. Whereas alkaline DNase and panreactic DNase were incubated with 5 units of inhibitor in 0.05 M Tris HC1 buffer pH 8.0 and the inhibitor activity was assayed. The values are expressed as mean ± S.D. from 5 experiments.

Table 17 shows effect of inhibitor on other DNases. The DNases purified from other sources along with brain acid/NUV DNase were incubated with the inhibitor (5 U/ml). The DNases from other sources were, DNase I from bovine pancreas, alkaline DNase from rat brain. DNase II from spleen. The alkaline and pancreatic DNases were incubated with inhibitor in 50 mM. Tris HC1 pH 8.0, whereas in the case of spleen DNase and brain acid/NUV DNase, the incubation was in 100 mM sodium acetate buffer pH 5.0. The results indicate that the inhibitor was active on brain acld/NUV DNase and spleen DNase, but has no activity either on brain alkaline DNase or on pancreatic DNase.

There are two possible ways in which the inhibitor might be exerting its action. (a) The inhibitor might bind the enzyme and thereby inactivate it. (b) the inhibitor might bind the substrate and may thus prevent the interaction between the substrate and enzyme.

Fig.42 shows the effect of increasing acld/NUV DNase concentration on the inhibitor activity. In the absence of inhibitor the activity of enzyme was proportional to the amount of acld/NUV DNase added, while in the presence of inhibitor (4 μg and 8 μg) the activity became linear only after an initial lag. After this initial lag, however, the activity increased in a linear fashion with the increasing amounts of DNases, upto 8 μg per reaction mixture. This result is interpreted as an indication of the inhibitor binding to the enzyme directly through a protein-protein interaction.

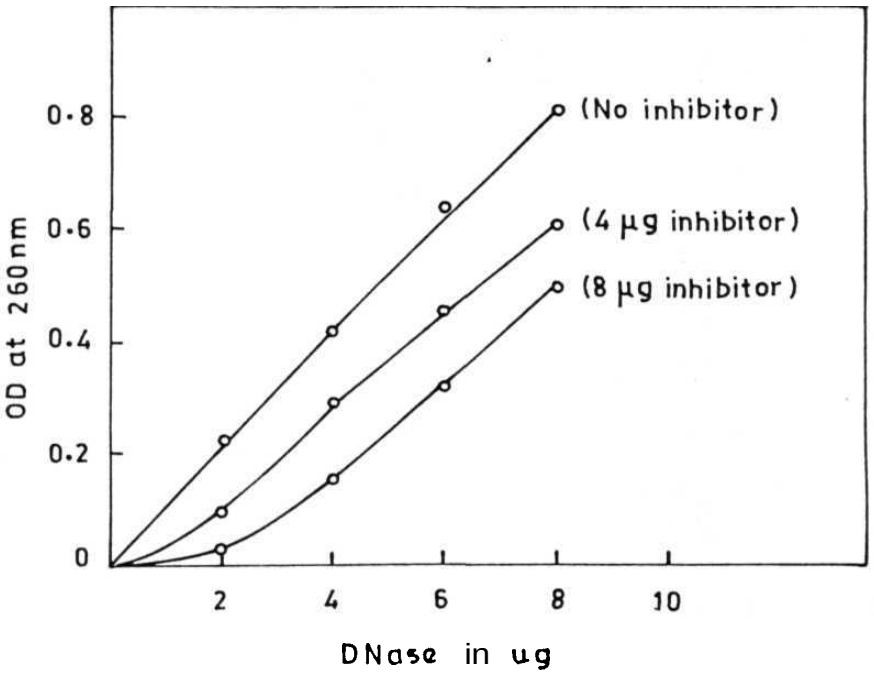


Fig.42 Effect of enzyme concentration on inhibitor activity. Each point represents average of three experiments.

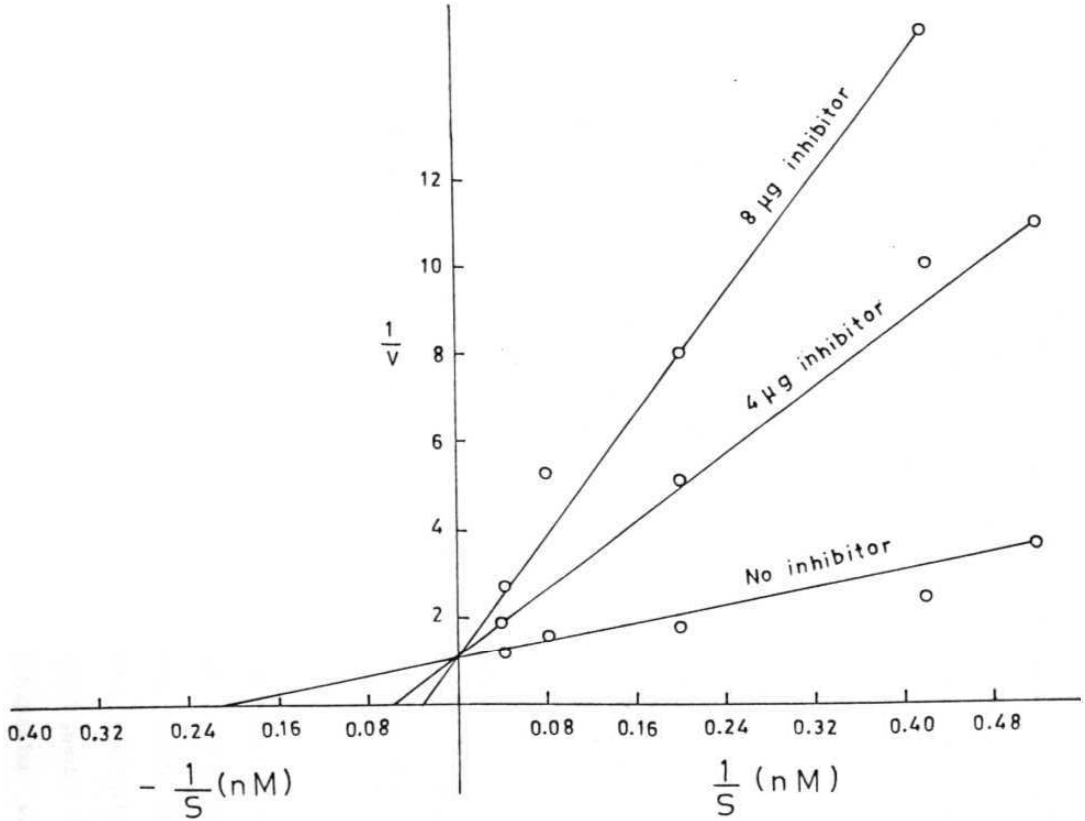


Fig. 43 Line weaver-burk plot to show the nature of inhibition of the acid/NUV DNase inhibitor.

In the next experiment, the potency of the inhibitor with fixed amount of acid/NUV DNase but varying concentrations of substrate was examined (Fig.43). The LineWeaver-Burk plot at the two levels of the inhibitor (4 and 8 fig) revealed a competitive nature of the inhibition. Then results along with those shown in Fig. 43 are taken to indicate that both the inhibitor and the substrate DNA are competing for the active site of the enzyme. It thus appears that the inhibitor influence can be relieved either by increasing enzyme or substrate concentration.

DISCUSSION:

The interaction of acld/NUV DNase with protein inhibitor would be of obvious importance in understanding, at the molecular level, such important biological events as DNA replication, recombination and excision after irradiation. Lehman (1962) also suggested that the comparison of the specificity of these DNases in the absence or in the presence of their inhibitors would be of prime interest for this purpose.

The acld/NUV DNase inhibitor was partially purified from rat brain. The properties of inhibitor suggested that it is protein in nature (Fig.4). This idea was further supported by the fact that it could be purified by conventional protein purification procedures and also the inhibitor was inactivated by trypsln. The inhibitor retained its activity even after digestion with pancreatic RNase.

Our kinetic experiments also indicated that inhibitor exerted its inhibition through binding to the catalytic site of enzyme and competing for this purpose with the substrate. This is one of the possible mechanism through which acid/NUV DNase activity is probably regulated in the brain.

CHAPTER VII

General Discussion

GENERAL DISCUSSION

Aging is a highly complex process of genetic and eplgenetlc interactions at all levels of organization. Scientifically aging is one of the most challenging and unresolved problems in biology owing to its highly complex nature. One major notion that has emerged during the last three decades is that functionally almost all biological systems fall progressively with age (Rattan and Clark, 1988). From the findings of several investigators it appears that multiple factors may be involved in the functional deterioration associated with aging. But changes in a single process can also cause a cascade of disturbances in several systems. Several theories have been proposed to explain the process of aging. Among them those theories that explain the process with a genetic basis have attracted many investigators. DNA damage/repair hypothesis (Hart and Setlow, 1974, Bernstein, 1981) is the one among such theories. According to this hypothesis there is a correlation between DNA repair capacity and longevity of an organism.

The environment in which we live posses continuous threat to our genetic material. Ionizing radiation, UV light from the sun and a multitude of chemical agents tend to cause alterations in DNA. In addition, cellular DNA is subjected to spontaneous damage and loss of bases as well as changes in base sequence due to Infidelity of replication or recombination. The DNA repair processes are the cellular responses associated with the restoration of the normal nucleotide sequence of DNA after damage. The enzymatic pathways involved in DNA repair appear to vary considerably with the type of damage introduced but the most well

characterized mechanism is the nucleotide excision repair process responsible for the removal of UV induced damage and bulky chemical adducts.

The first step in the excision repair is recognition and incision of damage. The first enzyme which was supposedly involved in this step was isolated from T infected *E.coli* (T endonuclease V). Several UV specific endonucleases were also purified from various mammalian tissues viz., the endonuclease activity from rat liver (Van Lancker *et.al.*, 1974, Teebor *et al.*, 1977), calf thymus (Bacchetti et ai., 1975), and several types of cultured human cells (Bacchettl et *al.*, 1972, Brent, 1975, 1976 and 1977). Waldsteln et *al.*, (1979) isolated an endonuclease from calf thymus that was specific towards dimers. Several endonucleases that were specific towards apurinic/apyrimidinic sites have been purified from both prokaryotes as well as from eukaryotes, B. Subtllls (Inoue et *al.*, 1978) S. Cerevlslae (Fuchler et *al.*, 1979) calf thymus (Lljungquist et *al.*, 1974, 1975) calf liver (Kubler et *al.*, 1977) Human lymphocytes (Ludwlg et *al.*, 1979) and human placenta (Llnsely et *al.*, 1977).

The second step in the excision repair process involves the activity of an exonuclease that degrades denatured but not native DNA (Kaplan et *al.*, 1971). An exonuclease was purified from human placenta which can excise pyrimidine dimers from incised UV-irradiated DNA (Doniger & Grossman, 1976). A proof reading 3'——> 5' exonuclease, supposed to be a part of DNA polymerase *6* has been described recently by Perrlno and Loeb (1990). The third

step in excision repair is repair replication which is performed by a DNA polymerase and while there are three polymerases in bacteria, there are five (a, β, γ, δ and c) in mammalian cells. (Syvaoja *et al.,* 1990). There is still some uncertainty as to which are the polymerases actually involved in DNA-repair, but it is generally regarded now polymerase a, β and c may participate in repairing different types of DNA-damages (Perrino and Loeb, 1990, Keener and Linn, 1990). The final step in the repair process is ligation. In mammalian cells atleast two types of ligases are known ligase I and ligase II.

Much of the information on enzymology of excision repair has emanated from prokaryotic cells and the mechanism of this process in mammalian cells is poorly understood and there is a general tendency to extrapolate the prokaryotic information. However, it is reasonably clear that the repair pathways in higher organisms is much more complicated. In S. *Cerevlsiae* mutant analysis and gene cloning have revealed the existence of atleast 10 genetic loci collectively designated as RAD 3 epistasis group, that are implicated in excision repair (Friedberg, 1988, Haynes & Kunz, 1981). More than 13 genes are expected to control nucleotide excision in mammalian cells (Hoeijmakers, 1986). Recently Weeda *et al.,* (1990) reported the cloning, and partial characterization of a human gene, designated as ERCC-3 involved in the early steps of the nucleotlde excision repair pathway.

The study of DNA damage/repair assumes greater importance in a post mitotic tissue like brain, because neurons do not possess

the repllcatlve ability. Nevertheless the brain has high metabolic rate and is responsible for bringing about homeostatic integration of all bodily organs. Therefore, the DNA-repair capacity of brain may be a useful pointer to overall ability of the whole organisms to affect DNA-repair, which is suspected to be related to the aging process. In spite of this possibility, studies regarding the DNA repair enzymes in nervous tissue are scanty. A few DNA repair enzymes like uracll DNA glycosylase (Krokan *et al.*, 1983) DNA polymerase β (Waser, et *al.*, 1979, Subrahmanyam & Subba Rao, 1988) and two DNases have been reported in brain tissue. Sung (1968) had described the two DNases as acid and alkaline DNases based on their pH optima. Previous studies from this laboratory also showed two major DNases in brain and the pattern of changes of activities of the two DNases indicated their possible involvement in DNA repair process (Subba Rao, 1986) one of them which showed an optimum pH 5.0 under standard conditions was purified to apparent homogeneity and the results are discussed in Chapter III. This enzyme does not require divalent cations for its activity. This is the first report of a brain DNase acting on both native and native DNA exposed to UV light. However, the enzyme has no activity on apurinic DNA or on denatured DNA. Further the enzyme exhibited preference to attack alternating dA-dT sequence.

The reason for native but not depurinated DNA being recognized in spite of its supposed double strandness of the latter could be due to single stranded breaks that might have resulted following depurination. The enzyme has low activity on

DNA pretreated with mitomycin C or Actinomycin D, both known to cause adducts and cross linklngs in DNA. It would therefore appear that the brain DNase attacks only such native double stranded DNA suffering from a sequence based distortion. The distortion could obviously have been due to the formation of thymidine dimers or a mismatched base pair.

Studies on the mechanism of action of the enzyme on UV irradiated DNA suggested that this enzyme does not excise pyrimidine dimers from UV irradiated DNA but attacks such DNA some what away from the dimer. The exact chemical structure of damage recognized by this enzyme still remains elusive.

A few other endodeoxyribonucleases have been identified in mammalian tissues. They are divided into non specific and specific endonucleases (Laskowski, 1961). Nonspecific endonucleases are the enzymes which do not show a high level of specificity for a given sequence or internucleotide bond split. They are mostly involved in the degradation of polynucleotide chain to a mixture of relatively small oligonucleotlides. However, the specific endonucleases display high level of specificity for a given sequence or distorted area containing mainly thymine dimers apurinic sites and altered bases. The result is one or two nicks in the polynucleotide chain.

The purified brain DNase of present investigation is clearly distinguishable from the other known mammalian DNases (Lindahl *et al.*, 1969[a]). Thus DNase I is specific for double stranded DNA and

has a neutral pH optimum. DNase III is an exonuclease. DNase IV described in rabbit tissues (Lindahl, 1969) is an exonuclease specific for double stranded DNA. The Ca^{+2}, Mg^{+2} activated endonuclease of rat liver (Ishida *et al.,* 1974) has a neutral pH optimum. and prefers double stranded DNA. A brain exodeoxyrlbonuclease with specificity towards single stranded DNA liberating mononucleotides 5'-phosphates was isolated by Ivanov *et al.,* (1983). The DNase specific for ultraviolet or γ-irradiated DNA (Bacchetti & Benne, 1975; Brent, 1973) is specific for photo-products of UV or γ-irradiation with no activity towards unirradiated native DNA. Brain alkaline DNase (Venugopal, 1990) is rather nonspecific in that it acts on native, single stranded, UV-irradiated as well as on depurlnated DNA. DNase VI isolated from human aneuploid cells (Pedrini *et al.,* 1976) is specific for single strand DNA and requires alkaline pH for its activity.

The brain endonuclease described here shares some properties with that of DNase II (Bernard!, 1971). But it also differs in a number of physical and catalytic properties. Brain DNase has different size, amino acid composition and N-terminal amino acid from that of DNase II. Moreover the brain DNase exhibited a preference for dA-dT sequence. DNase II was reported to prefer dG-dC sequence (Doskocll & Sorm, 1961). From these considerations it may be regarded that the present DNase differs from all known mammalian DNases. Since the brain DNase studied here shows an acidic pH optimum under standard conditions attacks native and UV damaged DNA. We propose to call this enzyme as acid/NUV (Native and UV damaged DNA) DNase.

Studies on DNA repair capacity at enzymatic level with advancing age are limitd. Barton and Wang (1975) observed a decrease in DNA polymerase β in spleen and mice with age. While no such decrease occurred in DNA polymerase a and similar type of results were obtained by Muller *et al.,* (1980) in bone marrow cells and in mouse liver during aging (Fry *et al.,* 1984). No age related decrease in 0 alkyl transferase, a repair enzyme was observed (Hall *et al.,* 1985, Lipman *et al.,* 1987). Brain DNA polymerase β (Subrahmanyam & Subba Rao, 1988) and alkaline DNase (a potential DNA repair enzyme) (Subrahmanyam 1989) were found at significant levels even in old age. However, the brain endonuclease under study was found to be decreased with age possibly due to the accumulation of catalytically inactive molecules. This aspect was discussed in Chapter IV.

The reason for decreased DNA repair activity during aging could be due to decrease in potential DNA repair enzymes. It is possible that some other factors needed for DNA-repair might be missing in old age or the rate of accumulation DNA damage is higher than the rate at which the damage can be repaired with the net result being decrease in DNA repair activity during aging.

In order to substantiate the role of brain DNase in DNA repair, the brain DNase was purified from brain nuclei. (Chapter V). The protein present in the nuclei is identical to that of enzyme purified from total brain homogenate (Chapter III). The enzyme showed considerable amount of activity at pH 7.0 under different ionic conditions. The enzyme seems to be a likely

candidate for carrying out the incision step that is necessary forDNA repair that is linked to DNA-replication possibly the recombination repair.

Further studies on the mechanism of action of acid/NUV DNase can give more information regarding the role of this enzyme in DNA repair of brain tissue.

GENERAL SUMMARY

SUMMARY AND CONCLUSIONS

1. A deoxyrlbonuclease (DNase) with a molecular weight of 62 Kd has been purified to homogeneity from young and old rat brain.

2. The purified enzyme is an endonuclease preferring either native DNA or native DNA exposed to UV light as substrate. The enzyme has little activity towards single stranded DNA or depurinated DNA.

3. The enzyme has low activity on DNA pretreated with either mitomycin C or Actinomycin D.

4. The enzyme shows a pH optimum of 5.0 but shows significant activity at pH 7.0 under low ionic conditions.The enzyme does not require divalent cations for its activity.

5. The enzyme shares some properties of classical DNase-II but differs from it in its size, N-terminal aminoacid and substrate specificity.

6. The enzyme exhibits specificity towards alternating dA-dT sequences in the substrate.

7. In view of the properties exhibited by this enzyme it is named by us as acid/NUV-DNase to denote its pH optimum and substrate preferrence.

8. The enzyme from old brain shows low specific activity as compared to the young but many other properties like electrophoretic mobility, size etc. are similar.

9. The enzyme is also purified from pure nuclear fraction and shown to be identical to that from whole homogenate.

10. Immunotitration experiments using antisera raised against pure NUV-DNase from young brain reveal that the low specific activity of the enzyme from old brain is due to accumulation of catalytlcally inactive enzyme molecules which, in its turn, may be due to post-translational modifications.

11. A specific competitive inhibitor (protein in nature) to acid/NUV-DNase was also isolated from brain and it is suspected that the inhibitor may play some role in regulating the aicd/NUV DNase activity under *in vivo* conditions.

12. The acid/NUV-DNase is considered to be a "housekeeping " DNA repair enzyme recognizing base sequence perturbations in native DNA.

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