Characterisation of nucleoid and interactions of a nucleoid associated DNA binding protein from hyperthermophilic archaeon *Sulfolobm acidocaldarius*

A Thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

BY

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CONTENTS

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Declaration

I hereby declare that the work presented in this thesis entitled **"Characterisation of nucleoid and interactions of a nucleoid associated DNA binding protein from hyperthermophilic archaeon** *Sulfolobus acidocaldarius"* is entirely original and was carried out by me in the Department of Biochemistry, University *of* Hyderabad, under the supervision of **Prof. T. Suryanarayana. 1** further declare that to the best of my knowledge this work has not been submitted before for the award of degree or diploma from any University or Institution.

Dated: 25/2/98

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Certificate

This is to certify that this thesis entitled "Characterisation of nucleoid and interactions of a nucleoid associated **DNA** binding protein from hyperthermophilic archaeon *Sulfolobus acidocaldarius"* submitted by Ms. K. Subbalakshmi Jaya for the degree of Doctor of Philosophy to the University of Hyderabad is based on the studies earned out by her under my supevision. This work has not been submitted for any degree or diploma of any University or Institution.

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ABBREVIATIONS

A_{260}, A_{280} and A_{600}	absorbance at 260, 280 and 600 nm respectively
ATP	Adenosine triphosphate
bp	base pairs
BSA	Bovine serum albumin
CM	Carboxy methyl
CNBr	Cyanogen bromide
Da	Daltons
DNA	Deoxyribo nucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
ds DNA	Double stranded DNA
EDTA	Ethylene diamine tetraacetate
g	Centrifugal field
hr	hours
IgG	Immunoglobin G
kb	Kilo base pairs
kDa	Kilodaltons
min	Minutes
mM	Millimolar
MNase	Micrococcal nuclease
MOI	Multiplicity of infection
Mr	Molecular weight
NP-40	Nonidet P-40
PEG	Polyethylene glycol
Poly (A)	Poly adenylic acid
Poly (U)	Poly ribouridylic acid
POPOP	1-4-bis (5-phenyl-2-oxazolyl benzene)
PPO	2-5-diphenyl oxazole
RF	Replicative form
RNase	Ribonuclease
SDS	Sodium dodecyl Sulfate
ss DNA	Single stranded DNA
TCA	Trichloroacetic acid
TEMED	N, N, N', N'-tetramethyl ethylene-diamine
Tris	Tris(hydroxy methyl)aminomethane
TNM	Tetranitromethane
UV	Ultraviolet

Chemicals used

Calf thymus DNA, NP-40, Sucrose, Spermidine-HCl, Alumina, RNase-A, Lysozyme, Micrococcal nuclease, Proteinase-K, BSA, Ovalbumin, Cytochrome-C, Chymotrypsinogen, Chymotrypsin, Coomassie blue R-250, Ethidium bromide, Dimethyl Suberimidate, PPO, POPOP and Ampicillin were purchased from Sigma Chemical Company, U.S.A. Sephacryl S-1000, Sephadex G-50. pBR322 DNA, ϕx174 RF *Hae\U* digest were procured from Pharmacia fine chemicals, Sweden. Acrylamide, N-N'-methylene bisacrylamide and SDS were purchased from Scrva Company, West Germany. Triethanolamine, TPCK-Trypsin were from Merck, West germany. Complete and Incomplete Freund's Adjuvants, Bactotryptone, Yeast extract were purchased from Difco Laboratories, U.S.A. CM-cellulose and DEAE-cellulose were from Whatman, England. Casein acid hydrolysate was from Himedia Chemicals, Bombay. SV40 DNA, *E.coli* DNA polymerase I, *E.coli* RNA polymerase, *Hind* IIIwere procured from New England Biolabs, U.S.A. 1kb DNA ladder and 100 bp DNA ladder were purchased from GIBCO BRL Lifetechnologies, U.S.A. Poly (A), Poly (U), ATP, CTP, GTP, LTP. dATP, dGTP, dCTP, dTTP, S1 nuclease and DNase I were from Boehringer Mannheim, Germany. Anti rabbit lgG peroxidase (goat), Lambda DNA. pBR322 DNA *HaelU* digest, Wheat germ topoisomerase I were purchased from Genei. Bangalore. pH] UTP and pH]dTTP were purchased from BRIT, Bombay. All other chemicals of reagent grade were obtained from local commercial establishments.

CHAPTER

INTRODUCTION

The work carried out in the present dissertation deals with a DNA binding nucleoid protein DBNP-B and characterisation of nucleoid from the thermoacidophilic archaeon *Sulfolobus acidocaldarius*. DBNP-B possesses nucleic acid binding properties such as helix distortion, aggregation of nucleic acids and promotion of renaturation of complementary single stranded DNA. It shows properties similar to recombination proteins like RecA of *E. coli* and shows immunological homology with 10b protein of *Sulfolobus solfataricus*, 11 kDa protein from *Thermoplasmaacidophilum* and T4 gp32 protein. Binding of DBNP-B to double stranded DNA studied by electron microscopy, aggregation and mobility shift assays reveal three distinct complexes at different protein concentrations.

A brief account of the archaea, the histone like proteins in eubacteria and archaea followed by a brief description of several DNA binding proteins which promote aggregation, renaturation and strand transfer are presented in the following pages.

ARCHAEA:

Archaea comprise a domain of life that is distinct from eukarya and eubacteria as revealed by molecular genetic analyses. Archaea have been defined at the molecular level as constituting a third primary kingdom consisting of the methanogens, the extreme halophiles and the sulfur dependent thermophiles. The comparison of catalogues of 16S rRNA (Woese *et al., 1978;* Fox *et al.,* 1980; Woese and Gupta. 1981) supported by the recognition of characteristic features of the membrane lipids (De Rosa *et al.,* 1976) and the cell envelope (Kandler, 1979) and other components of translation apparatus (Matheson and Yaguchi, 1982; Yaguchi *et al.,* 1982; Schmid *et al.*. 1982) and the DNA dependent RNA polymerases (Zillig *et al.,* 1982a, 1982b) has led to the division of prokaryotes into two distinct kingdoms that of eubacteria and archaebacteria. Woese and colleagues (Woese *et al.,* 1990) used 16S rRNA as a molecular chronometer and demonstrated that at the nucleotide sequence level, archaea are different from

eubacteria and eukarya. Archaea are more closely related to eukarya than to eubacteria (Brown and Doolittle, 1995). Archaea were previously referred to as archaebacteria (Woese and Fox, 1977) since they resemble bacteria phenotypically. But phylogenetic trees based on aminoacyl tRNA synthetase, H^+ATPase and elongation factor genes show that archaea and eukarya show a more recent common ancestor than they do with eubacteria.

Archaea lack nuclei and resemble eubacteria in morphology and genomic organisation but their molecular design shares many features with eukarya. Archaea share a number of complex features with eubacteria such as the presence of a single circular chromosome with genes organised into polycistronic operons (Keeling *et al.*, 1994), possession of restriction modification systems, lack of canonical eukaryotic introns in their mRNA (Woese, 1993), presence of anti Shine-Dalgarno sequences in the terminus of 16S rRNA (Stetz, 1978) and arrangement of rRNA genes in the order 16S- 23S- 5S (Neumann *et al.*, 1983). Certain molecular features that suggest a special relationship between archaea and eukarya are the presence of N-linked glycoproteins (Mescher *et al.*, 1976). properties of elongation factors (Kessel and Klink, 1982), DNA dependent RNA polymerases (Schnabel *et al.*, 1982; Huet *et al.*, 1983), DNA polymerases (Zillig *et al.*, 1982a, 1982b), aminoacyl tRNA synthetases (Baumann *et al.*, 1995b), absence of 5' cap on mRNA, presence of introns in tRNA genes (Zillig. 1987), ribosomal A protein homology and shared homology to various antibiotics. The presence of 5' cap on mRNA, use of non-formylated methionine (Bayley and Morton, 1978) and the presence of eIFs in archaea hints that translation may resemble that of eukarya. The transcription systems of archaea and eukarya are fundamentally homologous. Transcription initiation was shown to be mediated by trancription factors and promoters of archaea and eukarya are similar to each other in that they bind transcriptional and regulatory factors prior to binding RNA poKmerase for transcriptional initiation (Baumann *et al.*, 1995b; Langer *et al.*, 1995).

All archaea are considered extremophilic interms of adaptation to high salt. extreme pH, high hydrostatic pressure or extremely high temperatures. Phenotypically archaea are divided into three groups:
i. Methanogens, ii. Halophiles and iii. Sulfur dependent organisms.
Methanogens: They are a very diverse group of archaea with a G + C content from 26- 68 mol% and are not restricted to extreme environments. They exhibit all

known prokaryotic morphologies and inhabit anaerobic environments like sediments, guts of insects, the large bowel of man and animals. They are obligate methane producers growing at pH 6-8.

Halophiles: They include organisms that grow best in 1.5 M to 5 5 M brine. They are predominantly found in salt lakes and are responsible for the water reddening observed during the preparation of salt from sea water.

Sulfur dependent organisms: They include organisms mostly thermophilic but not all. They grow at low pH and metabolise sulfur for generating energy.

Phylogenetically archaea fall into two distinct subkingdoms (Woese *et al.*, 1990) - Crenarchaeota and Euryarchaeota.

Crenarchaeota comprises of sulfur dependent extreme thermophiles.

Euryarchaeota encompasses all methanogens, extreme thermophiles and sulfur reducing organisms.

The division of archaea into these subkingdoms was based on 16S rRNA cataloguing and similarities or differences with respect to eukarya /eubacteria (Wheelis *et al.*, 1992).

Biochemistry and Molecular Biology of Archaea:

Archaeal genome resembles that of bacterial genome and is 1.5-4.0 x 10^6 bp (Klein and Schnorr, 1984) with a single circular chromosome containing genes in polycistronic operons. Archaea exhibit a broad spectrum of G + C contents ranging from 21 to 68 mol%. Extra chromosomal DNAs with very different topological states coexist in several archaea. *Halobacterium salinarium* contains three plasmids with covalently closed circular DNA . Plasmid PHH1 is found in *Halobacterium halobium* (Pfeifer, 1988). Ten plasmids. mostly cryptic like PHe7 and conjugative like PN0B8 and PT1K4 encoding a killer function and plasmids involved in autotrophic growth by sulfur reduction have been found in Sulfolobales. These plasmids range from 4.5 to 40 Kb and occur in high copy number. A plasmid of 15.2 Kb has been found in 7. *acidophilum*. In crenarchaeota four novel virus families are found:

1: Fuselloviridae represented by SS V1 of *S. Shibatae*,

2: The Lipothirixviridae represented by TTV1 to TTV3,

3: Bacilloviridae represented by TTVU & SIRV,

4: Guttaviridae represented by SNDV.

In *Sulfolobus* S1RV, DAFV, SNDV viruses have been found (Zillig *et al,* 1996). Halophilic and methanogenic euryarchaeota have *X* like phages and lambdoid phages of eubacteria. The temperate halobacteriophage ϕH (Stolt and Zillig, 1994) resembles *E. coli* phage P1 in structure, replication and lysogeny (Iwabe *et al.,* 1989).

The complete 1.66 mega base pair genome sequence of *Methanococcus jannaschii* and its 58 Kb & 16 Kb extra chromosomal elements (ECE) were determined by Bult *et al.,* 1996. Out of the 1738 predicted protein coding regions only 38% could be assigned cellular role. Most of the genes involved in transcription, translation and replication are more similar to those found in eukaryotes. Five histone genes were found on the main chromosome and two on the large ECE homologous to eukaryotic histones H2A, H2B, H3 and H4. Putative ATPases belonging to the AAA family proteins were found (Koonin, 1997). In another project over 300 Kb of the 3 mega base pair genome of thermoacidophilic archaeon *Sulfolobus solfätaricus* have been sequenced which suggests clustering of genes by function (Charlcbois *et al.,* 1996).

The cell envelopes of archaea exhibit distinct structural and chemical diversity. They have isopranyl glycerol ether lipids and lack peptidoglycan and muramic acid (Kandler and Konig, 1985; Langworthy, 1985). Glycoproteins are seen on the cell surface. Pseudomurein, a non-sulphated acidic heteropolysaccharide, is present in archaeal cell wall (Schleifer and Kandler, 1972).

Archaeal ribosome is a 70S structure composed of two subunits 50S and 30S similar to eubacterial ribosomes. Each 70S ribosome contains one molecule each of 5S, 16S and 23S rRNA (Visentin *et al.,* 1971). There are two distinct classes of ribosomes within the archaeal kingdom. Extreme halophiles and most methanogens have similar sized ribosomes like eubacteria (White and Bayley, 1972). Ribosomes from sulfur dependent thermoacidophilic branch have a much higher protein content (Cammarano *et al.,* 1986). *Sulfolobus* ribosomes exhibit a typical eukaryotic 1:1 protein/RNA mass ratio of small subunits. Electron microscopic studies show an additional structure the Archaeal "bill" in their 30S subunits which is absent in eubacteria. The archaeal ribosomal proteins show more sequence homology to their eukaryal counterparts (Matheson, 1985).

Unlike eukarya, ribosomal proteins and subunits of DNA dependent RNA polymerases of archaea are not phosphorylated *in vitro* or *in vivo* conditions (Skorko, 1984). Archaeal ribosomes lack binding sites for a number of bacterial 70S antibiotics like chloramphenicol and streptomycin. They have binding sites for certain 80S inhibitors like anisomycin (Pecher and Bock, 1981; Schmid *et al,* 1982) but lack sites for cycloheximide (Sanz *et al.,* 1987).

Archaeal 5S rRNAs are diverse and are found to share similarities with both eukarya and eubacteria. 5S rRNAs of sulfur dependent archaea have triphosphorylated tennini and resemble the cytoplasmic 5S rRNA of eukarya whereas the methanogens and halophiles have monophosphoiylated tennini like eubacteria. Posttrancriptionally modified nucleotides are seen in 5S rRNA of sulfur dependent archaea (Bruenger *et al.,* 1993). Thiolated nucleotides and a variety of modified bases are found in archaeal tRNAs (Best, 1978; Fox *et al.,* 1980; Kilpatrick and Walker, 1981; Edmonds *et al.,* 1991). Nine unique modified nucleotides are found in extremely thermophilic crenarchaeota.

A special class of introns occur in stable rRNA genes of many archaea. An intron was found in the 23S rRNA gene of sulfur dependent thennoproteale *Desulfurococcus mobilis* (Kjems and Garrette, 1985; 1988;1991). This has been shown to encode a site specific DNA endonuclease containing the LAGLIDADG motif that may facilitate intron insertion at homologous sites in intron-alleles and shares many properties with intein endonucleases in other kingdoms (Daniels *et al,* 1985; Dalgaard *et al,* 1993; Lykke-Andersen *et al,* 1994). Leucine and serine tRNA genes in *S. Solfataricus* contain introns (Kaine *et al,* 1983). The first report of introns in the protein coding genes have been described in the DNA polymerase gene of *Thermococcus litoralis* (Perler *et al,* 1992). Later introns have been found in DNA polymerase genes of *Pyrococcus* species GBP and ROD (Southworth *et al.,* 1996).

The transcription systems of archaea and eukarya are similar (Baumann *et al,* 1995b). Archaea contain a single DNA dependent RNA polymerase with 12 or more subunits and is similar in its structural complexity to eukaryotic nuclear polymerases and share immunological crossreactivity, protein sequences (Puhler *et al,* 1989; Zillig *et al,* 1993) and antibiotic sensitivity. The DNA dependent RNA polymerase from archaea was found to be insensitive to rifampicin,

streptolydigin and a amanitin and is stimulated by silybin (Zillig *et al,* 1979). Active transcription depends strictly on a TATA box like sequence 25 bp upstream of the transcription start site resembling the TATA box of eukaryal promoters in sequence (Hausner *et al,* 1991; Palmer and Daniels, 1995). The TATA box having A box motif is required for efficient transcription and start site selection (Reiter *et al,* 1990; Hausner *et al,* 1991). In contrast a number of genes of the archael transcription apparatus are arranged in clusters as in eubacteria (Langer *et al,* 1995). Transcription initiation was shown to be mediated by transcription factors in archaea. Archaeal transcription factor B, aTFB, was shown to interact with the promoter of many archaeal genes. aTFB shows 40% sequence homology to human TATA binding protein (TBP). Eukaryotic TBP can substitute for aTFB from *Methanococcus thermolithotrophicus* in an *in vitro* transcription system thus identifying aTFB as an archaeal TATA binding protein (Wettach *et al,* 1995; Gohl *et al,* 1995). The recent descriptions of archaeal genes coding eukaryal like transcription factors TF11D, TBP, TF11B and the observation that *Pyrococcus woesei* TBP binds specifically to TATA containing DNA suggests that archaea use eukaryal like proteins in the initiation of transcription. TBP and TF11B perform analogous functions in archaea and eukarya (Thomm, 1996).

The gene for archaeal elongation factor EF-2 from *Sulfolobus acidocaldarius* has been cloned and aminoacid sequence comparisons show that it is more closely related to the eukaryotic EF-2 than to the eubactenal EFG (Schroder and Klink, 1991). EF-2 purified from *Sulfolobus* has a structural domain that can be ADP-ribosylated by diptheria toxin (Kessel and Klink. 1980; Siegmund and Klink, 1994). In archaeal systems, EF-2 was found to be insensitive to kirromycin and also to pulvomycin- the eubacterial EF-Tu inhibitors (Cammarano *et al,* 1982). EF-1β (SsEF-β) was purified from *S. Solfataricus* which shows C-terminal homology to its eukaryotic analogue (Raimo *et al,* 1996). A hypusine containing protein similar in molecular mass to eukaryotic eIF-5a has been detected in *S. acidocaldarius* (Bartig *et al,* 1992). Mono ADP ribosylation of proteins is the predominant process compared to poly ADP ribosylation in archaea (Faraone- Mennel *et al,* 1995).

DNA polymerases isolated from archaea are family B -polymerases a like and contain 3'-5' exonuclease activity but not 5'-3' exonuclease activity (Perler *et al,* 1996). A 100 kDa thermostable DNA polymerase was purified from *S.*

acidocaldarius which has neither exonuclease nor primase activities and is insensitive to both N-ethyl maleimide and 2-3-dideoxy ribosyl thyminetriphosphate and resistant to aphidicolin (Elie *et al,* 1989). The genes for DNA polymerase in *S. solfataricus* have been cloned and sequenced. The C-terminal region exhibits Mg^{2+} dependent DNA polymerase activity and the N-terminal domain has Mn^{2+} dependent 3'-5' exonuclease activity (Pusani and Rossi, 1994).

A type II restriction endonuclease *Tha* 1 with a recognition sequence CGCG has been isolated from *Thermoplasma acidophilum* (Mc Connel *et al,* 1978). Another type II restriction endonuclease *Sua* I was purified from *S. acidocaldarius* with a recognition sequence GGCC (Prangishvili *et al.,* 1985). In halobacteria, another enzyme was detected which is a component of the restriction modification system (Daniels and Wais, 1984).

In archaea, a novel type I- 5' topoisomerase I called reverse gyrase was found which catalyses the formation of positively supercoiled DNA in an ATP and Mg^{2+} dependent manner (Kikuchi and Asai, 1984; Forterre *et al.,* 1985; Nadal *et al.,* 1994). The gene coding for reverse gyrase was cloned from *S. acidocaldarius* DSM 639 and is shown to be constituted of two domains - a C-terminal domain related to eubacterial topoisomerase I and *Saccharomyces* topoisomerase II and an N-terminal domain containing several helicase motifs including an ATP binding site which does not show sequence similarity to other known topoisomerases. Reverse gyrase in archaea suggests a model in which positive supercoiling is driven by the concerted action of helicase and topoisomerase in the same polypeptide (Confalonieri *et al.,* 1993). The presence of reverse gyrase in archaea is tightly linked to the high growth temperature of these organisms. A second type I topoisomerase called Dam topoisomerase III, an ATP dependent relaxing enzyme has been purified from *Desulfurococcus amylolylicus* which requires Mg^{2+} for its activity and is specific for single stranded DNA. It relaxes negatively but not positively supercoiled DNA (Slesarev *et al.,* 1991). Yet another topoisomerase I called topoisomerase V, the first DNA topoisomerase which is Mg^{2+} independent was isolated from *Methanopyrus kandleri* which relaxes both positively and negatively supercoiled DNA. It can unlink two strands of a covalently closed circular DNA at high temperatures by relaxing the positive supercoils produced by temperature driven DNA unwinding.

M. kandleri has both reverse gyrase and topoisomerase V. Topoisomerase V is absent in *Sulfolobus* or *Pyrococcus* (Slesarev *et al,* 1994). DNA gyrase activity has not been detected in any archaea. Topoisomerase II has been purified from *S. shibatae* and *Pyrococcus furiosus* which can relax both positively and negatively supercoiled DNA and is more efficient to decatenate interlocked DNA rings than to relax supercoiled DNA (Kikuchi *et al,* 1985) and might be required for chromosome seggregation. This enzyme was found to be resistant to novobiocin. The genes encoding the two subunits of *S. shibatae* topoisomerase II have been cloned and sequenced. No sequence similarity was detected with either eubacterial or eukaryotic topoisomerase II suggesting that this archaeal enzyme could be the first representative of a new topoisomerase II family. This peculiar pattern of DNA topoisomerases in hyperthermophilic archaea is paralleled by a unique DNA topology. Plasmidic DNA in archaea are relaxed to positively supercoiled thus abolishing the need for gyrase activity in these organisms (Forterre *et al,* 1996).

Three ribonucleases have been purified from *S. Solfataricus* referred as P1, P2 and P3. P2 and P3 show sequence similarity with 7d and 7e proteins from *Sulfolobus.* They have a narrow substrate specificity and probably play specific roles in RNA processing (Fusi *et al.,* 1995). RNase P a ribonucleoprotein enzyme that cleaves the precurssor sequences from the 5' ends of the pre tRNAs have been characterised from two archaeal species namely *S. acidocaldarius* and *Haloferax volcanii* (Niewlandt *et al,* 1991; Darr *et al,* 1992; La Grandeur *et al,* 1993). RNase P from *S. acidocaldarius* is predominantly a protein closely resembling eukaryal RNase P and is not sensitive to micrococcal nuclease. The genes coding for the RNAs associated with the RNase P enzymes in *H. volcanii, S. acidocaldarius* and *Methanosarcina barkeri* have been cloned and characterised (Haas *et al,* 1996). The unique feature of the archaeal RNase P enz\Tne is that the RNA components are not by themselves capable of catalysis despite their striking resemblance to their catalytically active eubacterial homologs (Brown and Haas, 1996). *S. acidocaldarius* uses a novel RNA containing endonuclease to excise and mature 16S rRNA from the pre rRNA transcript. It shows sequence and structural similarity to eukaryotic U3 small nucleolar RNA (Potter *et al,* 1995).

A 60 kDa heat shock protein TF55 was found to be induced in *S. shibatae* on exposure to heat shock at 85-90^{0}C (Trent *et al,* 1990) which forms the rosettasome a homo-oligomeric double ring complex. Functionally the archaeal rosettasome has features of bacterial chaperonins (Kagawa *et al,* 1995). Sequence information reveals that they are remarkbly closely related to eukaryotic non heat shock proteins known as TCPIs. These double ring structures were also isolated from *Pyrodictium occuUum* (Phipps *et al,* 1993) and *T. acidophilum* (Waldmann *et al,* 1995). Hsp70 genes were cloned and sequenced in *H. marismortui* (Gupta and Singh, 1992), *Methanosarcinabarkeri* (Bardwell and Craig, 1984), *hi. mazei* (Macario *et al,* 1971). But Hsp70 is conspicuously missing in *Sulfolobus* and other crenarchaeota.

Pyrococcus contains an open reading frame that encodes a polypeptide with high similarity to the *E. coli* din F (DNA damage inducible) gene product suggesting that SOS repair might operate in archaea (Bouyoub *et al,* 1995).

Chromatin Organisation in Eukarya, Eubacteria and Archaea:

In eukarya, chromosomal DNA exists *in vivo* in a condensed, protein associated form- the eukaryotic chromatin. The basic structure of chromatin consists of nucleosomes in which two turns of DNA are wound around an octamer of four histones. The nucleosome core particle is a ubiquitous structure of 11 nm consisting of 146 bp of DNA wrapped around an octamer of the core histones H2A, H2B, H3 and H4. A fifth histone H1 serves to condense the polynucleosomal DNA into higher order structures (Igo-Kemenes *et al,* 1982). The structure of the core histone octamer and the core particle has been determined by X-ray crystallography. The core octamer has a tripartite structure with a central (H3- H4) tetramer that interacts with two H2A-H2B dimers. The nucleosome cores are separated by a variable length of linker DNA that is associated with a single molecule of histone H1 (Pruss *et al,* 1995). Besides its obvious role in DNA packaging this nucleosome structure may also be used in the repression of certain genes by blocking the acess of transcription factors to DNA sequences facing the histone surface or in the potentiation of transcription by creating a static loop which brings together widely seperated sequences.

The specificity of micrococcal nuclease (MNase) for cleavage of DNA in the linker region between nucleosomes has led to the extensive use of the enzyme in the elucidation of the nucleosome structure and higher structural orders of chromatin. The MNase digestion of nuclei generates a repeating pattern of DNA fragments which are 200 bp. When long chromatin stripped of H1 is digested with MNase a 146 bp fragment tightly bound to the octamer of histones-the nucleosome core is obtained.

The structure of chromatin and chromosomes and the regulation of expression of genetic information encoded in the DNA is determined by a variety of interactions between the nucleic acid and protein components of the genome. The proteins contributing to the chromosomal structure and function can be arbitrarily categorised into three general classes of DNA associated proteins:

1: Histones, the predominant proteins.

2: Tight binding proteins, which are highly heterogenous and of relatively low cellular abundance like structural proteins of nuclear matrix and chromosomal scaffold.

3: Enzymes involved in a variety of processes such as replication, transcription, repair and recombination like HMG' s, topoisomerases and polymerases *etc*.

Like eukarya, eubacteria and archaea too compact their DNA within the cell. But there is no clear evidence for a nucleosome like structure in eubacteria and archaea. The organisation of chromatin in these organisms is not clearly understood. The eubacterial and archaeal genome is present in the cell within a complex condensed structure - The Nucleoid (Stonington and Pettijohn, 1971; Worcel and Burgi, 1972; 1974 ; Reddy and Suryanarayana, 1988; 1989). However eubacteria and archaea contain small basic abundant proteins called "histone like proteins" that share some properties with eukaryotic histones. These histone like proteins are believed to compact the intracellular DNA and are thought to be the major protein factors involved in chromatin organisation in eubacteria and archaea.

The eubacterial chromosome is released from the cells by lysis using non-ionic detergents in the presence of 1M NaCl or 5 mM spermidine and can be isolated as a rapidly sedimenting condensed structure (Kornberg *et al.*, 1974). By electron microscopy it has been shown that the bacterial DNA from

gently lysed *E. coli* is organised into a 12 nm filament having an axial repeat of about 13 nm (Griffith, 1976). The nucleoid DNA has been shown to be in a highly supercoiled state (Materman and Van Gool, 1978). Micrococcal nuclease digestion resulted in the formation of 120 bp DNA fragments bound to histone like proteins (Varshavsky *et al,* 1977). However regular ladders of protected fragments such as those found with eukaryotic nucleosomes were not observed (Pettijohn and Sinden, 1985). Topological and electron microscopic investigations indicated that eubacterial DNA is organised into a wrapped chromatin like structure. It has been shown that several different protein components are associated with the nucleoid including RNA polymerase core enzyme, DNA polymerases, several DNA binding proteins and outer, inner membrane proteins both by two dimensional gel analysis and by functional assays (Stonington and Pettijohn, 1971; Portalier and Worcel, 1976; Moriya and Hori, 1981; Moriya *et al,* 1981; Lossius *et al,* 1984; Gaziev *et al,* 1986). Several of the DNA binding proteins of the nucleoid have also been isolated (Yamazaki *et al,* 1984). Folded chromosomes were isolated from *Bacillus subtilis* (Guillen *et al,* 1978) and *Streptomyces hygroscopicus* (Sarfert *et al,* 1983) and their protein composition was analysed.

In archaea nucleosome like structures were found associated to some extent with a portion of chromosomal DNA in the thermoacidophilic archaeon *Thermoplasma acidophilum* (Searcy and Stein, 1980) and halophilic archaeon *Halobactehum salinarium* (Shioda *et al,* 1989). However DNA binding proteins formed some structures that differed from nucleosomes *in vitro* in the thermoacidophilic archaeon *Sulfolobus acidocaldarius* (Lurz *et al,* 1986). Studies of Reddy and Suryanarayana, 1988, 1989 on *S. acidocaldarius* nucleoid showed the presence of several proteins apart from histone like proteins in the archaeal nucleoid. Electron microscopy of *Halobactehum salinarium* shows that the DNA is associated with protein particles with a diameter of 9.4 nm arranged tandemly along the DNA fiber thus resembling that of the eukaryotic chromosome in primary' structure (Shioda *et al,* 1989). The chromatin structure of *H. salinahum* was shown to consist of regions of both protein associated DNA and protein free DNA and these regions are interchangeable depending on the growth phase (Takayanagi *et al,* 1992). Sheared chromosomes obtained from these bacteria in the late exponential phase were separated into two peaks (peak I and peak II) by sucrose density gradient centrifugation. Electron microscopic studies

revealed that the major component of peak I was protein free DNA while the major component of peak II were rugged fibers basically consisting of bacterial nucleosome like structures composed of DNA and protein as demonstrated in experiments like nuclease digestion. The largest amount of DNA is in the peak 1 form in the exponential phase and in peak II form in the stationery phase and the transition between the two forms occurs in the late exponential phase (Takayanagi *et al.,* 1992).

Histone like proteins in Eubacteria:

Four proteins HU, H-NS, HLP1 and H have been identified as eubacterial histone like proteins (Drilica and Rouviere-Yaniv, 1987). In addition two other proteins, the integration host factor (IHF) and factor for inversion stimulation (FIS) have been identified as potentially important proteins in DNA organisation.

In *E.coli* HU or DBP II is the most abundant form which has been thoroughly characterised and is conserved in different eubacteria and also in plant Chloroplast (Drilica and Rouviere-Yaniv, 1987). *E. coli* HU is a small basic 9 kDa protein localised on the transcriptionally active region- the surface of the nucleoid (Duerrenberger *et al,* 1988) and has been shown to stabilise DNA against themial denaturation. HU protein exists as a heterodinier composed of two subunits a and (3 displaying 70% sequence homology. HU has been referred to variously by different groups as H2 (Cukier-Kahn *et al,* 1972), BH2 (Varshavsky *et al,* 1977), NS (Suryanarayana and Subramanian, 1978), HD (Spassky *et al,* 1984). HU interacts with DNA in an aggregated form (Lammi *et al,* 1984). The prominent activity of HU is wrapping DNA into nucleosome like particles (NLSs). These particles have an average of 8-10 dimers of HU and 275 bp DNA as seen by electron microscopy. HU can form NLSs with circular ds DNA which is condensed by a ratio of 2.4 relative to naked DNA (Rouviere-Yaniv *et al,* 1979). HU was found to stimulate RNA synthesis in contrast to histones and can protect DNA from Staphylococcal nuclease digestion (Varshavsky *et al,* 1977). At low protein concentrations, HU induces bends in DNA (Hodges-Garcia *et al,* 1989). A wide variety of site specific DNA protein interactions are stimulated by HU like bacteriophage Mu transposition (Craigie *et al,* 1985; Lavoie and Chaconas, 1990), TN10 transposition, gene inversion (Johnson *et al,* 1986), initiation of DNA

replication at the *E.coli* replication origin (Dixon and Kornberg, 1984; Bramhill and Kornberg, 1988; Hwang and Kornberg, 1992) and modulation of binding specificity of regulatory proteins such as CRP (Flashner and Gralla, 1988), IHF (Bonnefoy and Rouviere-Yaniv, 1992) and Lex A repressor.

Several HU like proteins have been isolated from various bacterial species which include HBs from *Bacillus stearothermophilus*, HBSU and HBST from *Bacillus subtilis*, HPa from *Pseudomonas acroginosa*, HRm from *Rhizobium meliloti*, HCp from *Clostridiumpasteurianum*, HAn from *Anabacna* sp and TF1 from *Bacillus subtilis* phage SP01.

Protein H is a 28 kDa basic protein which was first recognised through its activity as an inhibitor of DNA synthesis *in vitro* (Hubscher *et al*, 1980). Its strength of binding to DNA is low compared to HU and its amino acid sequence resembles eukaryotic histones (Bjornsti *et al*, 1986).

Protein H1 is a 16 kDa neutral protein existing in three different forms H1a, H1b, H1c which differ in their pI (Spassky *et al*, 1984). H-NS is a neutral protein which is one of the major constituents of the nucleoid and has been implicated in the condensation of the chromosome. H-NS is localised within the nucleoid, at the border between the nucleoid and the ribosome rich cytoplasm (Durrenberger *et al*, 1991). Although this protein binds DNA nonspecifically it exhibits specificity for intrinsically curved AT rich DNA with little sequence specificity (Zuber *et al*, 1994; Falconi *et al*, 1996). H-NS does not wrap DNA *in vitro* and does not change the linking number of circular DNA after binding. It directly or indirectly facilitates the initiation of chromosome replication (Katayama *et al*, 1996). H-NS has been implicated not only in the compact organisation but also in global expression of gene expression by negatively regulating a number of apparently unlinked genes thus acting as a global transcriptional repressor (Yeguchi *et al*, 1996), H-NS was shown to inhibit transcription by bringing changes in DNA topology (Tupper *et al*, 1994). H-NS has also been shown to be involved in the post transcriptional regulation of rpo-s gene (Takefumi *et al*, 1995).

TF1 is a histone like protein encoded by the *Bacillus subtilis* bacteriophage SPO1 (Johnson and Geiduschek, 1972). It is a 22 kDa homo-dimer which binds and bends DNA showing preference for DNA that contains hydroxy methyl uracil

instead of thymine (Greene *et al,* 1986b). TF1 recognises specific sites on SPO1 chromosome and binds these sites forming nested complexes on either side through protein - protein interactions (Greene *et al,* 1986a). TF1 is a transcription factor that negatively regulates the expression of several early and middle viral genes and is also involved in phage head morphogenisis (Sayre and Geiduschek, 1990).

HLP-1 is a 17 kDa protein coded by the Fir A gene (Schaffer and Zillig, 1973; Varshavsky *et al,* 1977; Lathe *et al,* 1980). The product of Fir A gene was identified by a mutation that supressed a rifampicin resistant mutation of RNA polymerase. This protein is known to interact with RNA polymerase and is beleived to be involved in transcription and in maintaining the individual domains of supercoiling *in vivo* (Sinden and Pettijohn, 1981).

Integration host factor (IHF) was originally identified as a factor required for bacteriophage A. site specific recombination (Nash and Robertson, 1981). IHF is a 20 kDa dimer composed of an a and a β subunits. It resembles HU in its primary structure and has the ability to wrap DNA around it. IHF recognises and binds to specific DNA sequence unlike HU. Despite the similarities in its primary structure, IHF and HU were shown to form different types of protein complexes with short DNA fragments (Bonnefoy and Rouviere-Yaniv, 1991). IHF participates in a number of activities including gene inversion, transposition, replication, partitioning of replicated DNA molecules and control of transcription. IHF also seems to participate with the λ int protein in forming nucleosome like structures in which the DNA is wrapped in left handed toroidal supercoils (Pollock and Nash, 1983).

Histone like proteins in Archaea:

Archaea express several small DNA binding nucleoid proteins which presumably perform histone like functions or helix stabilising functions in these organisms.

The first archaeal histone like protein identified is HTa from *Thermoplasma acidophilum* (Searcy, 1975; Stein and Searcy, 1978). HTa is a low molecular weight 9.9 kDa basic protein and is acid soluble with a strong net positive charge

composed of 89 amino acids. HTa exists as a tetramer in solution (Searcy, 1986) and when bound to DNA protects it against thermal denaturation by about 40°C. It was found to show significant homology to eukaryotic histones H2A , H3 and to *E. coli* HU protein (Drilica and Rouviere-Yaniv, 1987) and the degree of homology to HU is greater than the histones (De Lange *et al,* 1981). HTa condenses DNA into globular nucleosome like particles containing a core of four HTa subunits and a 40 bp loop of DNA thus protecting it from nuclease digestion (Searcy and Stein, 1980). The localisation of HTa on nucleoid has been shown by immunogold labelling (Bohrman *et al,* 1990). A similar protein called HSa was isolated from *S. acidocaldarius.* It is a small basic protein with a molecular weight of 14.5 kDa. Another nonbasic DNA binding protein of 36 kDa molecular mass has been isolated from *S. acidocaldarius* . Together these proteins protected about 5% of the DNA against nuclease digestion but could not afford protection against thermal denaturation (Green *et al,* 1983). In *S. brierlevi,* five small proteins were identified which are associated with the intracellular DNA (Thomin *et al,* 1982).

Kulms *et al,* (1995) isolated a 9 kDa thermostable DNA binding protein from *S. acidocaldarius* which exhibits RNase as well as DNA binding properties. It was designated as SaRD. It shows an N-terminal sequence homology to 7 kDa and 8.5 kDa RNases from *S. acidocaldarius* (Fusi *et al,* 1993). It exists as a dimer and has a high content of lysine residues arranged in a typical x-k-xxx-k-x-k-xxx-k motif. SaRD protein binds to DNA and in certain cell cycle activity phases acts as a RNase or even functions in RNA processing. SaRD has not been shown to exhibit any DNA aggregating properties.

MC1 (previously called Hmb) was identified as the most abundant histone like protein in various species of *Methanosarcina.* It is a 14 kDa polypeptide with a marked hydrophilic character (Chartier *et al,* 1985; Chartier *et al,* 1989). It preferentially binds to ds DNA as a monomer in a non-cooperative manner (Culard *et al,* 1993) and protects DNA against thermal denaturation (Chartier *et al,* 1988) and against radiolysis by fast neutrons (Isabelle *et al,* 1993). MC1 stimulates transcription *in vitro* (Chartier *et al,* 1989). Electron microscopic study of MC1-DNA complexes have revealed that MC1 does not form stable repetitive globular structures as do histones which wTap DNA into nucleosome (Toulme *et al,* 1995). However significant changes in DNA structure are observed upon MC1 binding

like formation of DNase 1 hypersensitive sites, sharp DNA bending and compaction of relaxed circular DNA (Laine *et al,* 1991; Teyssier *et al,* 1994; Toulme *et al.,* 1995) suggesting that supercoiling is altered upon MC1 binding. MC1 preferentially binds to negatively supercoiled DNA and bends the DNA upon binding (Teyssier *et al.,* 1996). The amino acid sequence of MC1 significantly differs from eukaryotic histones and from eubacterial histone like proteins and from HTa of *T. acidophilum.*

Another histone like protein Hmf was isolated from the hyperthermophilic archaeon *Methanothermus fervidus* which was found to wrap and compact double stranded DNA into nucleosome like structures NLSs (Sandman *et al,* 1990). Hmf is a mixture of equal amounts of two very similar polypeptides Hmf A and Hmf B (Mr. of 7,468 and 7,667 Da respectively). These polypeptides were 84% identical in their amino acid sequence and closely resemble eukaryal histones at the primary sequence level. Hmf binds to double stranded DNA and increases their resistance to thermal denaturation. The ratio of Hmf A and Hmf B could vary substantially and is growth phase dependent. The homo-dimers of Hmf A and Hmf B have different DNA binding properties consistent with forming NLSs in active and inactive regions of the genome (Sandman *et al,* 1994a). Binding of Hmf to DNA results in positive supercoiling of DNA unlike negative supercoiling as seen in eukaryal nucleosomes. Hmf binding increases the helical periodicity of DNA molecules approximately 11bp/turn and the DNA in the NLSs is constrained in positive toroidal supercoils. Hmf-DNA structure contains between 90-150 bp of DNA wrapped in 1.5 positive toroidal supercoils around a core of four Hmf molecules (Musgrave *et al,* 1991). The three dimensional structure of Hmf B dimer was determined by nuclear magnetic reasonance spectroscopy which confirms that this archael protein has the same overall structure as the histones. The predicted secondary structure is similar to the structures observed for the central domain of eukaryal nucleosome core histones (Starich *et al,* 1996).

Mesophilic methanogen *Methanobacterium formicum* JF-1 has been shown to contain three members of Hmf family of the archaeal histones designated as HFoA1, HFoA2 and HFoB. The encoding genes have been cloned and sequenced. These HFo histones have primary sequences that are 75 to 72% identical to Hmf sequences. The HFo proteins bind and compact DNA into NLSs apparently identical to those formed by the Hmf proteins (Darcy *et al,* 1995).

A 43 kDa histone like protein HAN1 was isolated from the nucleoid of *Thermococcus* species AN 1 which protects DNA from thermal denaturation upto 32°C. The gene for this protein has been cloned and sequenced. HAN1 is shown to be composed of two subunits and compacts DNA in a reversible manner. It shows a high degree of homology at the N-terminus with Hmf and the toroidal wrapping of DNA by HAN1 resembles that described for Hmf (Ronimus and Musgrave, 1996). Two genes designated as HpyA1 and HpyA2 have been cloned and sequenced from a *Pyrococcus* strain GB-3a. They are predicted to encode proteins HpyAl and HpyA2 respectively that are approximately 60% identical to Hmf and Hmt (Sandman *et al,* 1994b).

The euryarchael histone like proteins characterised in *Methanothermus fe nidus* (Hmf), *Methanobacterium thermoautotrophicum* (Hmt), *Methanobacteriunformicicum* (Hfo) and *Thermococcus* sp AN1 (HAN1) have amino acid sequence homology with eukaryal histone consensus sequences. They contain 66-69 amino acids similar in length to the histone fold supporting the notion that archaeal and eukaryal histones are ancestrally related.

S. acidocaldarius contains a number of small basic and abundant DNA binding proteins with molecular weights 7 kDa, 8 kDa and 10 kDa which play a role in DNA compaction and helix stabilisation at high growth temperature of the organism (Kimura *et al,* 1984; Grote *et al,* 1986; Choli *et al,* 1988a). These are organised into three classes on the basis of their molecular weight and their elution pattern from an ion exchange column CM-sepharose CL 6B (Grote *et al,* 1986).

The 7 kDa class shows microheterogeneity and is composed of five isoforms referred individually as Sac7a-Sac7e. The high degree of sequence similarity between the 7 kDa proteins suggests that these proteins might have probably evolved through gene amplification. The 8 kDa and 10 kDa class consists of two proteins each (8a, 8b & 10a, 10b). These proteins have no apparent homology to any of the histones. Electron microscopic studies of protein-DNA complexes of 7, 8 and 10 kDa proteins showed that they bind ss DNA and ds DNA to form compact structures (Lurz *et al,* 1986). Similar kinds of proteins from *S. solfataricus* have also been identified (Kimura *et al,* 1984; Choli *et al,* 1988a).

Sac7a, b, d, e have been sequenced. The genes for Sac7d and Sac7e have been cloned into *E. coli* and sequenced (McAfee *et al,* 1995). They contain a characteristic N-terminal sequence of alternating lysine residues in the order x-k-x-k-x-k-x-k (Choli *et al,* 1988b). Sac7d and Sac7e differ by six amino acid residues and are coded by distinct genes. Sac 7a and Sac7b are carboxy truncated forms of Sac7d (McAfee *et al.,* 1995). The native Sac7 proteins are all monomethylated at specific lysine residues to some extent. Studies of Baumann *et al,* 1994 suggest that methylation of lysine residues is related to the thermal stability of these proteins. None of the Sac7 proteins contain cysteines and they lack metal binding sites.

The structure of Sac7d has been extensively studied by Baumann *et al.,* 1994; 1995a; McAfee *et al.,* 1995 and Edmondson *et al.,* 1995. Sac7d protein has been shown to be globular with dimensions approximately 20 x 20 x 30 A^0. The protein has extensive regions of β sheet structure and an a helix at the C-terminus. (Edmondson *et al.,* 1995). The thermodynamics of binding of Sac7d to ds DNA has been characterised using spectroscopic signals from both the protein and the DNA (McAfee *et al.,* 1996). The salt dependence on the DNA binding affinity of Sac7d indicates that the predominant interaction is electrostatic and the binding is temperature dependent. Lysine monomethylation has no effect on the DNA binding function of Sac7d but appears to be important primarily in increasing the stability of the protein (McAfee *et al.,* 1995).

The structure of the 7 kDa protein from *S. solfataricus* Sso7d has been determined by NMR spectroscopy (Baumann *et al,* 1995a) and has been shown to contain a similar structural motif like Sac7d with an extensive region containing (3 sheets. This structure is very similar to those of eukaryotic src homology 3 (SH3) domains suggesting that this folding motif has been conserved in the domains of archaea and eukarya (Woese *et al,* 1990). The DNA binding surface of Sso7d was identified by NMR . A model of DNA-protein complex suggests that Sso7d interacts with the DNA major groove (Baumann *et al,* 1995a). An increase in Sso7d methylation occurs upon heat shock indicating that methylation may be directly related to protein stability (Baumann *et al,* 1994). The binding site size of Sso7d was estimated to be 3-6 bp using fluorescence quenching. It was shown that Sso7d promotes renaturation of complementary single strands in a homology

dependent manner indicating that Sso7d may be a component of the protein machinery devoted to **the** coupling of DNA stability to metabolic flexibility at high growth temperatures (Guagliardi *et al,,* 1997).

Protein 10b from *S. acidocaldarius* was shown to be able to bind to rRNA also with greatly reduced strength and it differs significantly from other proteins. It is characterised by its ability to form three different well defined nucleoprotein structures which show some relationship to complexes made by RecA . Thus 10b might play a role similar to that of RecA in recombination /repair (Lurz *et al,* 1986).

A 7 kDa protein was identified from *S. solfataricus* that exhibited high affinity for homologous DNA. This protein was able to protect DNA from denaturation and DNase I digestion in a dose dependent manner (Faraone and Farina, 1995). The amino acid composition of this protein showed a high content of lysine and glutamic acid residues. This protein has also been shown to be methylated at lysines. The partial amino acid sequence of the protein suggests that it could belong to the 7 kDa family of proteins of *S. acidocaldarius* (Kimura *et al.,* 1984; Choli *et al.,* 1988a, 1988b). It has been shown that this protein can be ADP ribosylated (Faraone *et al,* 1995).

Reddy and Suryanarayana, (1988) isolated nucleoid for the first time from *S. acidocaldarius* and purified four low molecular weight acid soluble DNA binding proteins. They were named HSNP (Helix stabilising nucleoid protein) -A (12 kDa), HSNP-C (9 kDa), HSNP- C' (7 kDa) and DNA binding nucleoid protein B (DBNP-B) of 10 kDa molecular mass. Three of these proteins HSNP-A, C, C' showed strong helix stabilising properties and protected DNA against thermal denaturation. DBNP-B although binds strongly to ds and ss DNA does not protect DNA against thermal denaturation. HSNP-A, C, C' are present exclusively on the ribosome free genomic DNA of the cell suggesting that they could be responsible for DNA compaction and genome organisation (Bohrmann *et al,* 1994).

Electron microscopic studies of HSNP-C' and DNA complexes show that the protein strongly binds to DNA and compacts it into globular structures. HSNP-C' exists in aggregated multimeric structures. The protein binds cooperatively to DNA (Francina and Suryanarayana, 1995). The involvement of aromatic amino

acids in the DNA binding domain has been indicated by modification of specific amino acid residues (Francina, 1996). HSNP- C' was shown to be identical to Sac7d with immunological homology to HMG-1 class of proteins from rat liver. It is involved in the condensation of DNA, promotion of renaturation of complementary' single stranded DNA, aggregation of DNA and shows RNase activity (Gaun, 1997). The nucleic acid binding properties of DBNP-B, which is related to 10b reported by Grote *et al.,* (1986) and Lurz *et al.,* (1986) have been studied in detail. The protein forms different types of novel complexes with DNA at different protein to DNA ratios (Sreenivas, 1994; Sreenivas *et al,* 1998).

Proteins that promote aggregation, renaturation and recombination of **DNA:**

Pairing of homologous single strands is a key step in all fundamental processes involving nucleic acids such as replication, recombination, transcription and repair. Homologous recombination observed in bacteria and eukaryotes is used fry cells for DNA repair and generation of genetic diversity. The central step in homologous recombination is reannealing of the two complementary interacting DNA molecules, then searching for homology and exchanging the DNA strands between them. Proteins participating in such reactions have been reported both from prokaryotes and eukaryotes and on the basis of their ATP utilization have been classified as ATP dependent and ATP independent types (Eggleston and Kowalczykowski, 1991). *E. coli* RecA and *Saccharomyces cerevisiae* Rad51 proteins are the archaetypal members of two related families of proteins that play a central role in homologous recombination

E. coli shows an absolute requirement for RecA, an inducible protein that also functions as an activator of the SOS response to DNA damage (Walker, 1984). RecA functions as a coprotease in the cleavage of Lex A repressor (autoproteolysis) and phage A. repressor (Roberts *et al.,* 1978) and Umu D protein (Craig and Roberts, 1981; Nohmi *et al.,* 1988). *In vitro* RecA promotes a variety of reactions like ss DNA and ds DNA dependent ATPase activity (Ogawa *et al.,* 1979; Weinstock *et al.,* 1981a, 1981b). RecA is a 37.8 kDa protein which binds to ss DNA in a cooperative manner and promotes the renaturation of complementary single stranded DNA molecules in an ATP and Mg^{2+} dependent manner (Weinstock *et al.,* 1979; Mc Entee, 1985; Menge and Bryant, 1992a, 1992b) and

ATP dependent strand exchange between a wide range of molecules showing nucleotide sequence complementarity (Kowalczykowski, 1987; Radding, 1989; Cox, 1994) The pairing reaction requires ATP but not its hydrolysis (Kowalczykowski *et al,* 1994). In the absence of DNA RecA can self assemble into a variety of multimeric forms like a highly aggregated structure.

Sequences of RecA genes from three distantly related thermophilic bacteria *Thermus aquaticus, Thermotaga maritma* and *Aquifex pyrophilis*were cloned into *E. coli,* expressed and purified to homogeneity. These proteins are thermoresistant upto 90°C and are ss DNA dependent ATPases with high temperature optimum (Wetmur *et al,* 1994). RecA like proteins from *Salmonella typhimurium* (Pierre and Paoletti, 1983), *Proteus mirablis* (Lovett and Roberts, 1985) were reported which show considerable homology to *E.coli* RecA and complement UV sensitivity of *E.coli* strains containing RecA mutations (Miller and Kokjohn, 1990).

Genetic recombination process results in regions of single stranded DNA as intermediate products. Pairing of DNA is inhibited by secondary structures in ss DNA (Studier, 1969). ss DNA binding or helix destabilising proteins such as *E.coli* SSB. T4 Gene 32 and λ β protein can play an acessory role in RecA mediated strand invasion and strand exchange reaction. In addition these proteins can catalyse a RecA independent strand annealing reaction that may be involved in recombination.

E. coli single strand binding protein (SSB) an 18.8 kDa protein binds cooperatively only to ss DNA and RNA as a tetramer (Sancar *et al.,* 1981, Lohman and Overman. 1985). SSB shows stimulator)' effects on many *in vitro* reactions of RecA by nonspecifically promoting the binding of RecA to ss DNA and stimulates strand transfer activity of RecA by melting the secondary structures in ss DNA (Tsang *et al,* 1985). SSB has been demonstrated *in vitro* to be involved in a number of replicative, DNA renaruration and protective functions. SSB catalyses ATP independent renaturation of complementary single stranded DNA in the presence of Mg^{2+} and excess of protein at pH 5.5. The renaturation was negligible at pH 7.0 and appears to be polyamine dependent between pH 5.5 and 7.5 (Christiansen and Baldwin, 1977). SSB can interact with exonuclease I to enhance the DNA deoxyribo phosphodiesterase activity suggesting that SSB may have an

important role in DNA base excision pathway (Sandigursky and Franklin, 1994). SSB also enhances helix destabilisation by DNA helicase and protects ss DNA from nuclease digestion (Williams and Kaguni, 1995). SSBs have been purified from *S. cerevisiae* (YRpA), and humans (HRpA) (Heyer and Kolodner, 1989; Alani *et al*, 1992). YRpA, a 115 kDa heterotrimeric protein, stimulates yeast SEP1 mediated strand exchange and RecA strand exchange. HRpA stimulates HPP 1 mediated strand exchange.

The protein encoded by the red (3 gene of bacteriophage *X* , a 28 kDa protein promotes Mg^{2+} dependent, ATP independent renaturation of complementary single stranded DNA at an optimum of pH 6.0 (Kmeic and Holloman, 1981). β protein stimulates the formation of joint molecules by RecA protein by destabilising the secondary structure in ss DNA and also promotes the joining of complementary ss ends of phage A, DNA (Muniyappa and Radding, 1986). P protein resembles RecA in its strand annealing activity and SSB in its ability to reduce secondary structures in ss DNA. β protein also protects ss DNA from digestion by pancreatic DNase.

T4 gene 32 protein is a 35 kDa protein synthesised by T4 phage. It binds cooperatively to ss DNA and is shown to be essential for DNA replication and recombination. T4 gp32 binds cooperatively to ss DNA and it catalyses DNA denaturation and renaturation at physiological conditions *in vitro* in a Mg^{2+} dependent and ATP independent manner (Alberts and Frey, 1970). T4 gp32 is analogous to *E. coli* SSB in stimulating many *in vitro* reactions of RecA protein (Egner *et al.*, 1987).

The bacteriophage UvsX protein is a 39 kDa strand transferase that resembles RecA in function and size and catalyses homologous pairing of ss and ds DNA (Yonesaki and Minagawa, 1985). UvsX binds cooperatively to ss DNA and ds DNA (Formosa and Alberts, 1986), coaggregates ss and ds DNA and has a ss DNA dependent ATPase activity. It promotes renaturation of heat denatured ds DNA and assimilates linear ss DNA into homologous superhelical duplexes to produce D loops. UvsX catalyses strand transfer from ds DNA to homologous ss DNA in an ATP and Mg^{2+} dependent manner to generate both paranemic and

plectonemic joints (Birkenkanp and Kemper, 1996). T4 gp32 was found to stimulate many reactions promoted by UvsX protein (Hams and Griffith, 1988; Kodadek, 1990).

Rec1 protein from eukaryote *Ustilago maydis*, a 70 kDa protein is similar to Rec A and promotes ATP dependent renaturation of complementary DNA and strand transfer between circular ss DNA and homologous ds DNA (Kmeic and Holloman, 1983; 1984; 1986; Kmeic *et al*, 1985). The protein was found to link two supercoiled molecules in the presence of topoisomerase.

The RecO gene product of *E. coli* is a 20 kDa monomer in solution and is required for the RecF pathway mediated recombination and repair. It binds cooperatively to both ss DNA and ds DNA and promotes renaturation of complementary ss DNA in a Mg^{2+} dependent and ATP independent manner (Luisi-Deluca and Kolodner, 1994). RecO protein promoted renaturation is also similar to renaturation promoted by phage A. β protein. RecO has no detectable exonuclease or endonuclease activity on ss or ds DNA, ATPase activity or topoisomerase activity and it cannot mediate joint molecule formation without RecA (Umezu *et al.*, 1993). The accumulation of high molecular weight products with increasing times of incubation in the RecO promoted renaturation reaction is similar to the ATP dependent RecA promoted reaction.

The RecT gene product of *E. coli*, is a 33 kDa DNA pairing protein which works in concert with RecE exonuclease in the RecE pathway of recombination (Hall and Kolodner, 1994). RecT binds to ss DNA and promotes ATP independent renaturation of complementary ss DNA and joint molecule formation between homologous ds DNA and ss DNA in the presence of exonuclease VIII. RecT strand transfer is independent of ATP and requires ss DNA ends on linear duplex which are complementary to sequences in the circular ss DNA partner for strand transfer to occur (Thresher *et al*, 1995). The mechanism of pairing by RecT is similar to SEP1, HPP1, RrP1 and different from RecA family of proteins (Bortner *et al.*, 1993).

The three best characterised proteins with homologous pairing and strand transfer activity in eukaryotes - the human HPP1, yeast SEP1 and Drosophila RrPl proteins all contain an intrinsic or tightly associated exonuclease activity

which may be required for the initial synapsis step and do not require ATP for pairing.

Moore and Fischel, 1990 first reported a 120 kDa mammalian protein from human T cell acute leukemic rumour cell line which is capable of homologous pairing and strand exchange. HPP1 catalyses homology dependent transfer in a Mg^{2+} dependent and ATP independent manner. The nature of pairing catalysed by HPP1 is similar to that of RecA protein although the exact mechanism of strand transfer may be different. Human SSB (HRpA) stimulates the homologous pairing by HPP1 (Moore *et al,* 1991). The Herpes simplex virus type 1 (HSV-1) encodes a 138 kDa protein called 1CP8 that preferentially binds to ss DNA and promotes renaturation and strand exchange in a Mg^{2+} dependent and ATP independent manner (Hernandez and Lehman, 1990). ICP8 also stimulates DNA replication *in vitro* and is shown to be involved in nuclear localisation of the HSV-1 replication machinery (Bortner *et al,* 1993).

Drosophila recombination repair protein 1 (Rrp1) purified from Drosophila embryos is a 105 kDa protein which has four tightly associated activities- DNA strand transfer, ss DNA renaturation, 3' exonuclease and apurinic endonuclease. The protein interacts with ss DNA and can promote renaturation in a Mg2+ dependent and ATP independent manner and strand transfer (Sander *et al,* 1991). The strand transfer requires homologous DNA and proceeds by a strand displacement. RrP1 is also involved in repair of oxidative and alkylation damage to DNA which generates apurinic sites and are cleaved by RrP1. The C-terminal of RrP1 is homologous to *E. coli* exonuclease III, *S. pneumoniae* exonuclease 1 and is responsible for nuclease activity. The N-terminal which is lysine and glutamate rich is not related to any of the proteins in the database and performs DNA strand transfer and ss DNA renaturation (Sander *et al,* 1993). The exonuclease activity of RrP1 has a role *in vitro* DNA strand transfer reactions carried out by RrP1 (Sander and Benhaim, 1996) but is not coupled to strand transfer. *In vivo* role of RrP1 may be to facilitate recombination repair of DNA damage at or adjacent to abasic sites.

SEP1, a 175 kDa protein is characterised from *S. cerevisiae* which promotes DNA aggregation, renaturation of ss DNA and homologous DNA strand exchange in the absence of ATP (Heyer *et al,* 1988). In addition to strand

exchange SEP1 contains an intrinsic exonuclease that is active on ss and ds **DNA** with preference to ss DNA (Johnson and **Kolodner, 1991).** SEP1 catalyses heteroduplex DNA formation and branch migration *in vitro* and the initial pairing reaction requires limited exonucleolytic digestion of ds DNA by the intrinsic 5'- 3' exonuclease of SEP1 (Dykstra *et al,* 1990; Johnson and Kolodner, 1994). The stable joint molecule formation by SEP1 requires ds DNA with ss DNA ends. SEP1 is stimulated by SF1 (stimulatory factor 1) from yeast. SF1 is a 32 kDa single strand binding protein which in the absence of SEP1 promotes the renaturation of homologous ss DNA in a Mg^{2+} dependent manner suggesting that it might act in some phase of the strand exchange reaction. SF1 does not show any stimulation on RecA promoted strand exchange (Norris and Kolodner, 1990).

The product of Rad 10 gene in *S. cerevisiae* which is required for incision step of excision repair of UV damaged DNA and mitotic recombination (Schiestl and Prakash, 1988), is a 25 kDa DNA binding protein (Reynolds *et al.,* 1985) which binds strongly to ss DNA and catalyses ATP independent renaturation of complementary single stranded DNA (Sung *et al.,* 1992, Tomkinson *et al.,* 1993). Rad10 does not promote joint molecule fomiation and has no DNA dependent ATPase activity or DNA helicase or exonuclease activity. Rad1 and RadlO proteins associate in a 1:1 stoichiometry and this complex degrades single stranded DNA endonucleolytically and also supercoiled duplex DNA is converted to nicked relaxed form (Tomkinson *et al.,* 1994). Rad1/Rad10 endonuclease is a junction specific nuclease that uniquely cleaves the 3' extension at the duplex/ SS junction (Bardwell *et al.,* 1994) indicating that Rad1-Rad10 promotes the 5' incision event in nucleotide excision repair. Radl and RadlO are required for intrachromosomal recombination between direct repeats and affects the integration of linear DNA molecules and circular plasmids into genomic sequences (Davies *et al.,* 1994). The endonuclease activity is dependent on Mg^{2+} and salt and has a pH optimum of 8.5 and specifically degrades ss circular DNA but not linear DNA (Reynolds *et al.,* 1985). This endonuclease activity' is presumably required to remove non homologous regions of ss DNA during mitotic recombination between repeated sequences.

S. cerevisiae Rad51 gene is required for genetic recombination and recombinational repair of DNA strand breaks (Sung and Stratton. 1996). Rad51 gene is characterised by Shinohara *et al.,* 1992 and the protein sequence is similar

to that of RecA (Aboussekhra *et al,* 1992). The protein filaments are indistinguishable from RecA protein filaments. (Ogawa *et al,* 1993). Rad 51 protein has ATP dependent ss DNA and ds DNA binding and ATP independent ss DNA binding activities and promotes homologous pairing and strand exchange *in vitro* (Sung, 1994; Sung and Robertson, 1995). Rad51 is conserved in most of the eukaryotes and homologs in archaea have been identified. Rad51 has a DNA dependent ATPase activity and catalyses ATP dependent pairing and strand exchange between homologous DNA (Sung and Stratton, 1996).

The human Rad51 protein (HsRad51) was purified which is homologous to RecA. It binds to ss DNA and exhibits DNA dependent ATPase activity. It promotes homologous pairing and strand exchange reactions *in vitro* using ss circular and homologous linear duplex DNA. Joint molecule formation is dependent on ATP hydrolysis (Baumann *et al,* 1996). The stoichiometry of binding to DNA and rate of renaturation of complementary strands were similar in both RecA and HsRad51 (Gupta *et al,* 1997). HsRad51 forms stable joint molecules by transfer of the 5' end of the complementary strand of the linear duplex to the ss DNA. The polarity of the strand transfer is opposite to that observed with RecA (Baumann and West, 1997).

Using PCR process primed by oligonucleotides designed to encode regions of the proteins showing the greatest degree of identity, DNA from three archaea *S. solfataricus, Halofcrax volcanii* and *Methanococcus jannaschii* were examined for sequences encoding proteins similar to RecA and Rad51. Putative RecA and Rad51 homologues (called RadA proteins) found in all the three organisms are similar to Rad51 family (40% amino acid homology) than to RecA family. The levels of RadA mRNA do not increase after treatment with U.V. irradiation in *S. solfataricus* indicating that it is a constitutively expressed gene. It is likely that RadA genes in archaea will have a role in DNA repair and possibly in homologous recombination (Steven *et al,* 1996).

The Pk-rec gene encoding a RecA /Rad51 homologue from the hyperthermophilic archaeon *Pyrococcus* sp KODl was cloned and sequenced by Rashid *et al,* 1996. The entire protein basically corresponds to the essential central domain of its counterparts RecA, Rad51, Dmc 1. The Pk-rec gene was expressed in *E. coli* and purified to homogeneity and was shown to be in a dimeric

form. A striking property of the recombinant Pk-rec gene product was the unusual DNase activity on both ss and ds DNA along with ATPase activity with different metal ion requirements for both the activities. The reaction product of the DNase activity was mononucleotides at an optimum pH of 8-8.5 at 60°C. (Rashid *et al.*, 1997).

OBJECTIVES AND SCOPE OF THE PRESENT INVESTIGATION

Sulfolobus acidocaldarius, a sulfur dependent hyperthermophilic archaeon is unique in its survival at extreme conditions of temperature (75^0C-80^0C) and acidic pH of 3.0. It lacks a rigid cell wall (Brock *et al,* 1972) and its intracellular concentration is low (Green *et al,* 1983). The G + C content of its DNA is 40%, thus reinforcing the need for intracellular factors that confer stability to DNA in this organism. Phylogenetically archaea are found to be closer to eukarya than to eubacteria (Brown and Doolittle, 1995). In eukarya, the organisation of DNA has been extensively studied and the nucleosome was derived as the basic unit of organisation. The structural organisation of intracellular DNA in archaea is still not well understood. The archaeal genome is present in the cell within a complex condensed structure, the nucleoid (chromatin). The ease with which chromatin can be isolated from archaea, eubacteria and eukaryotes is strikingly different. There is no standard and effective procedure yet to isolate chromatin from archaea. This inturn obscures the certainity as to which of the proteins are instrumental, if any in packaging of DNA. So we intended to isolate nucleoid from *S. acidocaldarius* in pure form without any contamination and observe if there are any changes in the nucleoid depending on the growth phase of the organism or on storage at -80^0C. The nucleoid proteins which bind to the DNA were characterised and nuclease digestion experiments of nucleoid were performed with both DNase 1 and micrococcal nuclease to observe if the pattern of digestion resembles that of eukaryotic chromatin.

Reddy and Suryanarayana (1988, 1989) purified nucleoid from *S. acidocaldarius* and identified four low molecular weight DNA binding proteins HSNP-A, DBNP-B, HSNP-C and HSNP-C' from the nucleoid. Three of these proteins were found to stabilise DNA against thermal denaturation and showed strong helix stabilising properties. The fourth protein DBNP-B (DNA binding nucleoid protein-B) present in abundant amounts fails to protect DNA against thermal denaturation but binds strongly to ss DNA, ds DNA and RNA. DBNP-B forms novel complexes with DNA at different DNA:protein concentrations and promotes renaturation of complementary single stranded DNA, aggregation of DNA (Sreenivas, 1994; Sreenivas *et al,* 1998).

The present study on DBNP-B shows some important properties of the protein like aggregation of DNA which increases the effective concentration of DNA thereby facilitating reactions such as complementary strand pairing. DBNP-B shows a nonspecific nicking activity dependent on Mg^{++} and protein concentration. The solution state of DBNP-B was studied by various crosslinking studies and by gel filtration chromatography. The interaction of DBNP-B with DNA as well as RNA was studied by gel mobility shift assays, fluorescence titrations and other assays. The effect of DBNP-B on the activities of enzymes like DNA topoisomerase I, DNA topoisomerase II, DNA polymerase I and RNA polymerase were studied which give information on the possible role of the protein in the cell. The influence of chemical modification of the protein on DNA aggregation, DNA renaturation and nicking activity of the protein can be studied to assess the amino acid functional groups involved. The crystalline aggregation of DNA by DBNP-B may facilitate effective utilisation of DNA template by enzymes acting on it as seen by stimulation of both DNA polymerase and RNA polymerase activities. In addition, DBNP-B promotes renaturation of complementary ss DNA. These two activities are important intermediate reactions involved in the recombination process. A detailed study of the archaeal proteins involved in recombination /repair will help in understanding the evolution of recombination and repair systems.

CHAPTER 2

MATERIALS AND METHODS

Organisms used in the present study:

Sulfolobus acidocaldarius strain DSM 639 was obtained from Deutche Sammulung Von Mikroorganisemen, Gottigen, Germany. *Escherichia coli* JM109 containing pUC19 plasmid was obtained from the laboratory of Dr. A.R.Subramanian, MaxPlanck Institute for Molecular Genetics, Berlin, Germany.

Growth of the organisms:

Sulfolobus acidocaldarius was grown at 75^0C for 40-48 hrs with vigorous aeration in a medium containing 0.1% yeast extract, 0.1% bactotryptone, 0.1% casein acid hydrolysate, 0.1% glucose, 0.02% sodium chloride, 0.13% ammonium sulphate, 0.03% pottasium dihydrogen phosphate, 0.025% magnesium sulphate, 0.07% calcium chloride and the pH was adjusted to 2.8 with 1M sulfuric acid (Kikuchi and Asai, 1984). The growth of the cells was arrested by neutralising the culture with 1M Tris base solution. The cells were harvested by centrifugation at 6,000 x g for 15 minutes. The cell pellets were finally suspended in 10 mM Tris-Cl (pH 7.6), 50 mM KCl, 10 mM magnesium acetate and 7 mM β–mercaptoethanol and centrifuged at 6,000 x g for 20 minutes. The cell pellets obtained were stored at -80^0C until further use.

E. coli was grown in enriched medium at 37^0C with good aeration (Minks *et al*, 1978).

Isolation of M13 ss DNA and RF DNA:

M13 ss DNA and RF DNA were isolated according to Messing, (1983). *Escherichia coli* JM109 was grown at 37^0C on a 2% agar plate containing M9 medium with glucose as a carbon source. A single colony was picked and inoculated to 2X YT medium and allowed to grow to an A_{600} of 0.3. The cells

were then infected with M13 mp7 phage particles with an MOI of 10. Incubation was continued at 37^0C for another 6 to 8 hours. The titer was usually 10^{11} to 2 x 10^{12} per ml.

Infected cells were collected by centrifuging the culture at 6,000 x g for 10 minutes at 4°C. The supernatant containing M13 phage particles was centrifuged again to remove any bacterial cells. The supernatant was made 0.5 M in NaCl and 6% in PEG 6000 and incubated at 4^0C for 60 minutes. The turbid solution was centrifuged at 10,000 x g for 15 minutes and the supernatant was removed carefully without disturbing the M13 phage pellet. The pellet was then suspended in 0.3 volumes of 10 mM Tris-Cl (pH 7.6) and 0.1 mM Na_2-EDTA. The resuspended phage solution was suspended again in 6% PEG 6000 and 0.5 M NaCl and left at 4°C for 60 minutes and the pellet was resuspended in 0.15 volumes of 10 mM Tris-Cl (pH 7.6) and 0.1 mM Na_2-EDTA. The M13 ss DNA from the collected phage particles was isolated by extracting the phage solution once with buffered phenol followed by buffered phenol:chloroform. The M13 ssDNA was concentrated by precipitation with ethanol and the DNA was dissolved in a small volume of 10 mM Tris-Cl (pH 7.6) and 0.1 mM Na_2-EDTA.

M13 RF DNA from the cell pellet was isolated by the procedure of Sambrook *et al,* (1989). The cell pellet containing RF was suspended in 50 mM glucose, 10 mM Na_2-EDTA and 25 mM Tris-Cl (pH 8.0). To this cell suspension, 0.5 ml of freshly made 20 mg /ml lysozyme in the above buffer was added, mixed and incubated on ice for 10 minutes. Then, 5 ml of 0.2 M NaOH and 1% SDS were added, mixed gently and incubated on ice for 10 minutes. Potassium phosphate (4 ml) was added to the above mixture and the suspension was mixed gently by swirling. The suspension was kept on ice for 10 minutes and centrifuged at 12,000 x g for 15 minutes. The clear supernatant was taken out carefully and incubated with RNase A (20 μg /ml) at 37^0C for 30 minutes. The DNA was extracted with equal volume of buffered phenol followed by buffered phenol-chloroform. The aqueous phase was collected, mixed with one tenth volume of 3M sodium acetate (pH 4.8) and 2.5 volumes of ethanol. The DNA was collected by centrifugation and washed once with 70% ethanol and dissolved in a small volume of 10 mM Tris-Cl (pH 7.6) and 0.1 mM Na_2-EDTA. The RF DNA was linearised by incubating DNA with *Eco R*\ followed by phenol-chloroform extraction and precipitation with ethanol.

Isolation of pUC19 supercoiled DNA:

pUC19 supercoiled DNA was isolated as described by Wang and Rossman, (1994). LB plates containing 25 μg /ml ampicillin were streaked with *E. coli* JM109 containing pUC19 plasmid and the culture was grown for 24 hours at 37°C. A single colony was picked from these plates and inoculated into 25 ml LB medium, supplemented with 1% glucose and 25 μg /ml ampicillin. The culture was grown with vigorous shaking at 37°C until late logarthmic phase (A_{600} = 0.3). 250 ml prewarmed LB medium containing ampicillin, glucose was inoculated with 2.5 ml of the above culture and grown at 37°C for 16 hours. The bacterial cells were harvested by centrifugation at 5,000 x g for 10 minutes at 4°C. The bacterial pellet obtained was resuspended in 10 ml lysis buffer (50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM Na_2-EDTA and 5 mg /ml lysozyme). This cell suspension was incubated at RT for 5 minutes and 20 ml of freshly prepared alkaline solution (0.2N NaOH, 1% SDS) was added, mixed and placed on ice for 10 minutes. 12.5 ml of saturated ammonium acetate solution was added, mixed well by swirling and placed on ice for 10 minutes. It was centrifuged at 12,000 x g for 10 minutes at 4°C and to the supernatant obtained 0.7 volumes of ice-cold isopropanol was added and placed on ice for 20 minutes. The DNA precipitate was collected by centrifugation at 12,000 x g for 15 minutes at 4°C. The DNA pellet was redissolved in 6 ml of acidic extraction solution (0.75 M NaCl, 10 mM Na_2-EDTA, 0.3M Na acetate (pH 4.2)). The DNA solution was left on ice for 15 minutes, an equal volume of water-saturated phenol was added and continuously inverted for 2 minutes. The upper aqueous phase was separated by centrifugation at 12,000 x g for 10 minutes at 4°C. The organic phase was reextracted with an equal volume of acidic extraction solution. 3.5 ml of reverse extraction buffer (1.5 M Trizma base, 5 mM Na_2-EDTA) and 3.5 ml of chloroform were added to the organic phase and this mixture was continuosly inverted for 5 minutes and centrifuged at 12,000 x g for 10 minutes. The upper aqueous phase obtained was carefully transferred to another tube and the DNA in the aqueous phase was precipitated by adding 0.1 volumes of 3M sodium acetate (pH 4.2) and 0.7 volumes of ice-cold isopropanol. The DNA pellet obtained was washed twice with 70% ethanol and dissolved in TE buffer (10 mM Tris-Cl (pH 7.6) and 0.1 mM Na_2-EDTA).

Isolation of pBR322 supercoiled DNA:

E. coli DH5a cells containing pBR322 were grown and supercoiled DNA was extracted according to Wang and Rossman, (1994) as described above.

pBR322 and pUC19 supercoiled DNA were linearised by incubating with *Hind* III followed by phenol -chloroform extraction and precipitation with ethanol.

Preparation of ss and **ds DNA-cellulose:**

ss DNA and ds DNA-cellulose were prepared according to the procedure described by Alberts and Herrick, (1971). The cellulose was activated by washing several times with boiling ethanol to remove pyridine, then quickly washed at room temperature sucessively with 0.1M NaOH, 1 mM Na_2-EDTA and 10 mM HC1 solutions respectively. It was later washed with water to neutrality, dried and used for coupling of DNA.

For ds DNA-cellulose, a solution of 1-3 mg /ml DNA in 10 mM Tris-Cl (pH 7.4), 1 mM Na_2-EDTA (Tris-EDTA) is taken in a glass beaker. Activated dried cellulose was added with ocassional stirring with a flat bottomed glass rod until the paste thickens (~ lg cellulose /3 ml). This lumpy mixture was dried by spreading on a petriplate and left at room temperature covered with a gauze. The thoroughly dried cellulose powder was suspended in 20 volumes of Tris-EDTA and left overnight at 4^0C. After a quick wash to remove the free DNA, the DNA' cellulose is stored as a frozen slurry in Tris-EDTA buffer containing 0.15 M NaCl. The efficiency of coupling is checked by measuring A_{260} of supernatant obtained after centnfugation of an aliquote of the DNA-cellulose suspension incubated at 100^0C for 20 minutes.

For ss DNA-cellulose the DNA is denatured at 100°C in 10 mM potassium phosphate and 1 mM Na_2-EDTA. After rapid cooling the DNA is made upto 3 mg /ml concentration with Tris-EDTA buffer and coupled to activated cellulose as described above.

Preparation of ss **DNA** by heat denaturation:

ds DNA of known concentration was taken and heated in a boiling water bath for 3 minutes. It was then immediately chilled in ice-cold water for 5 minutes, again boiled at 100^0C for 5 minutes and chilled for 10 minutes in ice-cold water. The concentration of the resulting ss DNA is almost the same as that of ds DNA.

Preparation of ss DNA by alkaline denaturation:

To the ds DNA, 0.1 M NaOH was added and incubated on ice for 20 minutes and precipitated with 2.5 volumes of ethanol. The DNA precipitate was collected by centnfugation, washed with 70% ethanol and dissolved in TE buffer (10 mM Tris-Cl (pH 7.4) and 1 mM Na_2-EDTA).

Isolation of Nucleoid:

Nucleoid from *S. acidocaldarius* was isolated by a procedure described by Reddy and Suryanarayana, (1988). Cells (2 gm) harvested in midlogarthmic phase were suspended in 4 ml of 10 mM Tris-Cl (pH 7.6) containing 1% NP-40, 2 mM spermidine-HCl, 10 mM Na_2-EDTA and incubated at 10^0C for 30 minutes. The lysate was centrifuged at 10,000 x g for 10 minutes. The cleared viscous supernatant was layered on a 30% sucrose cushion in 10 mM Tris-Cl (pH 7.6), 3 mM magnesium chloride and centrifuged at 10,000 x g for an hour. The concentrated nucleoid pellet obtained was dissolved in 3 ml of 20 mM Tris-Cl (pH 7.6). 150 mM KCl, lmM Na_2-EDTA and 6 mM β–mercaptoethanol and the A_{260} was measured.

Microeoccal nuclease digestion of nucleoid:

This was performed following the procedure of Owen-Hughes and Workman, (1996). Nucleoid (1 μg) fraction was incubated with 0.1 units of micrococcal nuclease in 20 mM Tris-Cl (pH 8.8), 50 mM NaCl, 50 mM $MgCl_2$ and 1 mM $CaCl_2$ at 37^0C for different time intervals. The reaction was stopped by adding SDS and EDTA to 1% and 25 mM final concentrations respectively. The

reaction products were analysed on a 1.4% agarose gel. The optimum conditions for MNase digestion were determined by incubating the nucleoid with increasing concentrations of MNase for different time intervals.

Sephacryl S-1000 column chromatography of ***Sulfolobus acidocaldarius*** nucleoid:

The nucleoid isolated was gently sonicated and centrifuged at 3,000 x g for 10 minutes to remove the contaminating cell debris. This cleared and sheared nucleoid was chromatographed on a sephacryl S-1000 gel filtration column. A sephacryl S-1000 column of 100 ml bed volume was packed and equilibrated with 20 mM Tris-Cl (pH 7.6), 150 mM KCl, lmM Na_2-EDTA and 6 mM β–mercaptoethanol (nucleoid buffer). The sheared nucleoid was loaded on the S-1000 column and eluted with two bed volumes of the nucleoid buffer. Fractions (2 ml) were collected at a flow rate of 25 ml /hr and the absorbance was measured at 260 nm. Fractions (50 µl of each) were analysed on a 15% SDS-polyacrylamide gel. DNA in the fractions was analysed on a 0.8% agarose gel. The DNA concentration in the fractions was measured by ethidium bromide fluorescence assay.

Isolation of DNA from the sephacryl S-1000 nucleoid fractions: -

DNA in the sephacryl S-1000 fractions was isolated by phenol-chloroform extraction. Equal volume of buffer-saturated phenol was added to the sephacryl S-1000 fractions and gently mixed for 10 minutes and centrifuged at 5,000 x g for 5 minutes. The upper aqueous phase was removed and the phenolisation step was repeated. Subsequently, the aqueous phase was extracted twice with an equal volume of phenol:chloroform (1:1) by gently mixing for 10 minutes and centrifuging at 5,000 x g for 5 minutes. The upper aqueous phase was collected and extracted twice with chlorofornrisoamyl alcohol (24:1) by mixing gently for 10 minutes and centrifuging at 5,000 x g for 5 minutes. The aqueous phase containing DNA was precipitated with 2.5 volumes of etlianol in the presence of one tenth volume of 3M potassium acetate (pH 5.0) and left overnight at -20^0C. The DNA pellet collected was washed twice with 70% ethanol and dissolved in a small volume of TE buffer (10 mM Tns-Cl (pH 7.6) and 1 mM Na_2-EDTA).

Sephadex G-50 column chromatography of the sephacryl S-1000 peak-II nucleoid proteins:

Sephacryl S-1000 peak-II fractions were pooled, dialysed against 20 mM Tris-Cl (pH 7.6), 50 mM NaCl, 5 mM $MgCl_2$ and 7 mM β–mercaptoethanol and subjected to DNase I digestion by adding 200 μg DNase I and incubating at 37^0C for 1 hr and 65^0C for 3 hours. It was then concentrated to 1 ml by lyophilisation. dialysed against column buffer (20 mM Tris-Cl (pH 7.6), 1 mM Na_2-EDTA, 150 mM KC1 and 7 mM p-mercaptoethanol) and loaded on a 100 ml sephadex G-50 column preequilibrated with the column buffer. The column was eluted with the same buffer and 4 ml fractions were collected at a flow rate of 5ml /hr. The absorbance of the fractions was measured at 280 nm and these fractions were analysed on a 15% SDS-polyacrylamide gel (Laemmli, 1970).

DNA-cellulose column chromatography of sephadex G-50 nucleoid fractions:

A ds DNA-cellulose column of 4 ml bed volume was packed and equilibrated with column buffer (20 mM Tris-Cl (pH 7.6), 50 mM KCl, lmM Na_2-EDTA and 7 mM p-mercaptoethanol). Sephadex G-50 peak fractions were pooled and diluted to 50 mM KCl in the column buffer without KCl and loaded onto the DNA cellulose column. The column was washed with 5 bed volumes of the column buffer and eluted with a stepwise gradient of 100 mM, 200 mM, 300 mM, 400 mM, 500 mM and 700 mM KCl in column buffer. Fractions (1.5 ml) were collected and the A_{280} was measured. These fractions were analysed by 15% SDS-PAGE.

DNA-cellulose **column** chromatography **of** sephacryl S-1000 peak-I nucleoid fractions:

Sephacryl S-1000 peak-I nucleoid fractions were pooled, the KC1 concentration was adjusted to 50 mM with 20 mM Tris-Cl (pH 7.6), 1 mM Na_2-EDTA and 7 mM β–mercaptoethanol and subjected to DNase I digestion by treating with 200 μg DNase 1 at 37^0C for 1 hr. The DNase I digested nucleoid fraction was dialysed against the column buffer (20 mM Tris-Cl (pH 7.6), 50 mM KCl, 1 mM Na_2-EDTA, 7 mM p-mercaptoethanol) and incubated at 65°C for 3 hrs. It was then loaded on a 5ml ds DNA-cellulose column preequilibrated with

the column buffer. The column was washed with 5 bed volumes of the column buffer and eluted with a stepwise gradient of 100 mM, 200 mM, 300 mM, 400 mM and 700 mM KCl in the column buffer. 1 ml fractions were collected and the A_{280} was measured. These fractions were analysed by 15% SDS-PAGE.

DEAE-cellulose column chromatography **of** sephacryl S-1000 peak-II nucleoid fractions:

Preswollen DEAE-cellulose was suspended in a buffer containing 20 mM Tris-Cl (pH 7.6), 150 mM KC1, 1 mM Na_2-EDTA and 7 mM β–mercaptoethanol (column buffer). The gel was packed into a column of 6 ml bed volume and equilibrated with the column buffer. Sephacryl S-1000 peak-II nucleoid fractions were pooled and loaded onto the DE-52 column, washed with 5 bed volumes of the column buffer and eluted with 1500 mM KCl containing column buffer. 1 ml fractions were collected and the A_{280} was measured. These fractions were analysed on a 15% SDS-PAGE, the DNA concentration was estimated by ethidium bromide fluoresence assay and subjected to DNase I digestion.

DNase I digestion of DEAE-cellulose nucleoid fractions:

DNase 1 digestion of the nucleoid fractions, obtained from the DEAE-cellulose column was performed by incubating with DNase I at a ratio of 10:1 (DNA:DNase I) in 10 mM Tris-Cl (pH 7.6), 50 mM NaCl, 5 mM $MgCl_2$ and 1 mM DTT at 37^0C for 30 seconds. The reaction was terminated by the addition of SDS and EDTA to 1% and 25 mM final concentrations respectively. The reaction products were analysed on a 0.8% agarose gel. The gel was stained with ethidium bromide and photographed under UV illumination.

Isolation of ribosomes and **post-ribosomal** supernatant fraction **(S-100)** from ***Sulfolobusacidocaldarius*:**

This was carried out according to Minks *et al,* (1978) at 0-4°C. Cells harvested at mid logarthmic phase were ground with double the weight of alumina until soft and sticky, extracted with buffer (3ml /gm cells) containing 20 mM Tns-

Cl (pH 7.6), 50 mM KCl, 10 mM magnesium acetate, 7 mM p-mercaptoethanol and 2 µg DNase I (RNase free) per gram cells and centrifuged at 30, 000 x g for 30 minutes at 4°C to obtain cell extract (S-30). The S-30 was centrifuged at 1,00,000 x g for 4 hrs (Beckman L8-80, Ti-60 rotor) to pellet the ribosomes. The upper two thirds of the supernatant (S-100) was collected and dialysed immediately against buffer containing 20 mM Tris-Cl (pH 7.6), 50 mM KG, 10 mM magnesium acetate, 7 mM p-mercaptoethanol and 10% glycerol and stored at -80°C. The crude ribosomal pellet was rinsed once with the above buffer and suspended in the same buffer containing 1M ammonium chloride. The ribosomes in 1M ammonium chloride buffer were pelleted by centrifugation at 1,00,000 x g for 4 hrs and the supernatant (ammonium chloride wash) was collected. The ribosomal pellet was suspended in 10 mM Tris-Cl (pH 7.6), 50 mM KCl, 10 mM magnesium acetate, 7 mM p-mercaptoethanol, 10% glycerol and stored frozen at -80^0C.

Isolation of acid soluble proteins:

Acid soluble proteins from the concentrated nucleoid or post-ribosomal supernatant (S-100) were extracted with one tenth volume of 2.7 M sulfuric acid, added dropwise to the sample on ice. The acid treated samples were stirred for 4 hours at 4^0C and the acid soluble proteins were collected by centrifugation at 15,000 x g for 30 minutes. The clear acid soluble supernatant was dialysed against buffer containing 20 mM Tris-Cl (pH 7.6), 50 mM KC1, 1 mM Na_2-EDTA and 7 mM p-mercaptoethanol for 20 hours and clarified at 10,000 x g for 20 minutes.

Purification of DBNP-B:

DBNP-B was purified according to the procedure of Reddy and Suryanarayana. (1989) with some modifications. DBNP-B was purified from the acid extract of nucleoid or S-100 by a single CM-cellulose chromatographic step.

Pre swollen CM-cellulose was suspended in a buffer containing 20 mM Tris-Cl (pH 7.6), 125 mM KCl, lmM Na_2-EDTA and 7 mM p-mercaptoethanol (column buffer). The gel was packed into the column and equilibrated with the above buffer. The acid extracts of post-ribosomal supernatant or nucleoid

containing DBNP-B were dialysed against CM-cellulose column buffer and applied onto the column. The column was washed thoroughly with 250 mM KCl containing column buffer and the proteins were eluted in 500 mM KCl in the column buffer. Fractions (4 ml) were collected at 30 ml /hr flow rate and aliquots (20 µl) from alternate fractions were analysed by 15% SDS-PAGE. In order to remove the salts, the 20 µl aliquots were diluted to 1 ml with water, made to 10 percent in TCA and incubated at 0°C for 2 hours. The samples were centrifuged and the pellets were washed with acetone and dissolved in SDS gel loading buffer.

Concentration **of purified DBNP-B:**

The fractions containing DBNP-B were pooled and diluted to 125 mM KCl and applied onto a small (2ml bed volume) CM-cellulose column equilibrated with column buffer. After washing the column with 250 mM KCl in the column buffer, the protein was eluted with 800 mM KCl in the column buffer. Protein containing fractions were pooled and immediately dialysed against 10 mM Tris-Cl (pH 7.6) or 10 mM sodium acetate (pH 5.0) and stored frozen until further use at -80^{0}C. The purity of the concentrated protein was checked by electrophoresing 10 µg protein on SDS-polyacrylamide gel followed by silver staining .

Raising antibodies to DBNP-B:

Antibodies to purified DBNP-B were raised as described by Stoffler and Wittman, (1971). About 200 µg of DBNP-B was emulsified with Freund's complete adjuvant and injected subcutaneously into the rabbit at multiple sites. After four weeks, booster doses each of 50 µg of protein in Freund's incomplete adjuvant were given subcutaneously every week till the 6th week. The rabbit was bled after the third booster injection through the pinna vein. Blood collected from the rabbit was first kept at room temperature for 2 hours and then at 4^{0}C for 12-16 hours. It was then centrifuged at 6,000 x g for 15 minutes to remove the clot. The supernatant obtained was recentrifuged at 10,000 x g for 30 minutes. The resulting supernatant was incubated at 56°C for 30 minutes followed by centrifugation at 10,000 x g for 15 minutes. The supernatant obtained (antiserum) was stored frozen in aliquots until further use.

Isolation of IgG from the antiserum:

Antiserum was fractionated by ammonium sulphate precipitation. IgG fraction obtained from 50-60% ammonium sulphate saturation, was dialysed against 70 mM sodium phosphate (pH 6.3) and loaded onto a 5 ml DEAE-cellulose column. The column was eluted with 70 mM sodium phosphate (pH 6.3) and 2 ml fractions were collected. The protein content was analysed by measuring the absorbance at 280 nm. The flowthrough fractions showing a high absorption at 280 nm were pooled and precipitated by adding equal volume of saturated ammonium sulphate solution and centrifuged at 8,000 x g for 40 minutes. The resulting pellet was dissolved in phosphate buffered saline, dialysed against the same buffer and stored frozen in small aliquots at - 80°C.

Gel filtration chromatography of **DBNP-B:**

Preswollen sephadex G-50 was deaereated and packed into a thin long column of 100 ml bed volume and equilibrated with 20 mM Tris-Cl (pH 7.6), 50 mM KCl, 1 mM Na_2-EDTA and 7 mM β–mercaptoethanol. DBNP-B (200 μg) was loaded on to the column and eluted with the same buffer. Fractions (2 ml) were collected from the column at a flow rate of 5 ml /hr and alternate fractions were analysed for the presence of the protein by both SDS-PAGE and absorbance measurments at 280 nm. Simultaneously protein molecular weight markers *viz*- bovine serum albumin (66 kDa), ovalbumin (44 kDa) and cytochrome-C (12 kDa) were also chromatographed on the sephadex G-50 column for the determination of the molecular weight of the native state of DBNP-B.

Gel mobility shift assay:

Gel mobility shift analysis of DBNP-B nucleic acid complexes was carried out as described by Lohman *et al.,* (1986). The reaction was carried out in 30 μl reaction volume in 10 mM Tris-Cl (pH 7.6), 0.1 mM Na_2-EDTA and 25 mM NaCl. DBNP-B-nucleic acid complexes were formed at different protein to nucleic acid ratio, incubated for 15 minutes at 37^0C and electrophoresed on a 0.8% agarose gel in TAE buffer (40 mM Tris-acetate (pH 7.8) and 1 mM Na_2-EDTA). 3 μl loading dye (50% (v/v) glycerol, 0.05% (w/v) bromophenol blue, 0.05% (w/v)

xylene cyanol) was added to each sample and loaded directly onto the agarose gel. The gels were then stained with 0.5 μg /ml ethidium bromide in TAE buffer and photographed under UV illumination.

Chemical and proteolytic cleavage **of DBNP-B:**

DBNP-B was cleaved chemically with CNBr and proteolytically with V8 protease, trypsin and chymotrypsin.

Cyanogen bromide cleavage:

DBNP-B was treated with cyanogen bromide according to Giorginis and Subramanian, (1980) with slight modifications. DBNP-B (0.25 mg /ml) in 90% formic acid was treated at room temperature under nitrogen with CNBr (10mg /ml) in 90% formic acid in a protein:reagent ratio of 1:20 for different time intervals. The products of CNBr cleavage were vacuum dried, dissolved in SDS-gel loading buffer and analysed by 15% SDS-PAGE.

V8 protease cleavage:

V8 protease digestion of DBNP-B was performed according to Cleveland *et al.*, (1977). DBNP-B (1mg /ml) in 125 mM Tris-Cl (PH 6.8), 10% glycerol and 0.5% SDS, was boiled at 100^0C for 2 minutes, cooled and digestion was carried out at 37^0C by the addition of protease at a concentration of 10:1 (protein:enzyme) for different time intervals. The reaction was stopped by adding β–mercaptoethanol and SDS to final concentrations of 10% and 2% respectively and boiling the samples for 2 minutes at 100°C. The reaction products after V8 protease cleavage were analysed by 15% SDS-PAGE.

Trypsin and chymotrypsin cleavage:

DBNP-B in 10 mM Triethanolamine (pH 8.0) was reacted with 20:1 protein:enzyme concentration of TPCK-trypsin or chymotrypsin at 37^0C for different time intervals. At different time intervals aliquots were taken out and the reaction was quenched by the addition of TCA to 10% final concentration and incubated at 0^0C for 30 minutes. The precipitates were collected by

centrifugation, washed with acetone, dissolved in SDS gel loading buffer containing 10 mM DTT and electrophoresed on a 15% SDS-polyacrylamide gel.

Cross linking studies:

DBNP-B crosslinking experiments were performed with three different reagents *viz.* formaldehyde (HCHO), dimethyl suberimidate (DMS) and difluoro dinitrobenzene (DFDNB).

Formaldehyde erosslinking:

DBNP-B crosslinking with formaldehyde as a crosslinking agent was performed according to Jackson, (1978) with some modifications. DBNP-B in 20 mM Triethanolamine-HCl (pH 7.5) or 10 mM sodium acetate (pH 5.0) was reacted with 200 mM HCHO, pH 7.5 and 5.0 respectively in the presence or absence of 10 mM magnesium chloride at 37^0C, 65°C and 80°C for different time intervals. At different time intervals small aliquots were taken out and the crosslinking was quenched by the addition of TCA to 10% final concentration. The precipitates were collected by centrifugation, washed with acetone and dissolved in electrophoresis sample buffer containing 0.1% SDS but devoid of β–mercaptoethanol /DTT and electrophoresed on 12.5% or 15% SDS-polyacrylamide gels. HCHO crosslinking of DBNP-B was also performed at different concentrations of NaCl (50, 100, 150, 200 and 500 mM) for different time intervals at 37^0C.

Dimethyl suberimidate crosslinking:

DBNP-B was crosslinked with DMS following the procedure of Thomas and Kornberg, 1975. DBNP-B in 20 mM Triethanolamine (pH 8.5) was reacted with 0.2 mg /ml DMS (in 20 mM Triethanolamine (pH 8.0)) and incubated at 37°C and 65°C in the presence or absence of 10 mM magnesium chloride. At different time intervals, small aliquots were taken out and the crosslinking was stopped by the addition of TCA to 10% final concentration. The precipitates were collected by centrifugation after incubation at 0^0C for 30 minutes. The precipitates were washed with acetone and dissolved in electrophoresis sample buffer containing

0.1% SDS and devoid of β–mercaptoethanol / DTT. These samples were analysed by 12.5% SDS-PAGE.

Fluoresence **titrations** of **DBNP-B** with **RNA:**

All fluorescence measurments were obtained using JASCO FP-777 spectrofluorimeter. The measurments were performed in 10 mM Tris-Cl (pH 7.6), lmM DTT and different NaCl concentrations at room temperature (25°C). The excitation band width was 5 nm and the emission band width was 10 nm. Reverse titrations were performed by adding lattice (RNA) to the ligand (DBNP-B). Small volumes of (2-3 μl) concentrated polynucleotide solution (Poly (A) or poly (U) were added to DBNP-B (22 μg) in the buffer and the decrease in emission fluorescence intensity was recorded after each addition. After each addition the reactants were mixed gently and left undisturbed for 60 seconds. The fluorescence readings were recorded after another 60 seconds which was the time usually taken for the stabilisation of the fluorescence signal. Usually three readings (at 10 second intervals) were taken for each titration point and the average of these readings were used for the analysis. The fluorescence intensity measured in arbitrary units was corrected for the dilution during titrations. In our experimental conditions the inner filter effect was very insignificant. The excitation was at 274 nm and the emission fluorescence intensity was measured at 304 nm. The initial protein fluorescence was taken to be 100% and all other measurments were made with reference to the initial fluorescence.

The strength of the binding of RNA to the protein was measured by adding increasing concentrations of 4M NaCl to DBNP-B-RNA complexes at saturation in aliquots and the dissociation of the RNA-protein complexes due to increase in ionic strength in the reaction medium was followed by observing the recovery of the protein fluorescence.

Tetranitromethane modification of DBNP-B:

This was performed following the procedure of Riordan and Vallee, (1972). DBNP-B (0.3 mg /ml) in 50 mM Tris-Cl (pH 8.1) and 100 mM KCl was reacted with 5 mM tetranitromethane (in methanol) at 21^0C for 30 minutes. The reaction

was terminated by dialysing exhaustively against 100 volumes of 10 mM Tris-Cl (pH 7.5), 30 mM NaCl and 7 mM β–mercaptoethanol for 16 hours. The TNM modified DBNP-B was analysed by 15% SDS-PAGE .

ss **DNA-cellulose** chromatography **of TNM** modified **DBNP-B:**

TNM modified DBNP-B was passed through a 1 ml ss DNA-cellulose column equilibrated with 10 mM Tris-Cl (pH 7.6), 125 mM KC1, 1 mM Na_2-EDTA and 7 mM p-mercaptoethanol. The column was washed with 150 mM KG containing above buffer and eluted with a stepwise gradient of 300 mM and 500 mM KC1 in 10 mM Tris-Cl (pH 7.6), lmM Na_2-EDTA and 7 mM p-mercaptoethanol. Fractions (0.2 ml) were collected and analysed for the presence of protein on a 15% SDS-polyacrylamide gel.

Renaturation assays:

DNA renaturation assays were carried out according to Sung *et al,* 1992 with slight modifications. The reaction was carried out in a 20 µl sample volume in 10 mM sodium acetate (pH 5.0), 12 mM magnesium chloride, 1 mM DTT with heat denatured DNA or alkaline denatured DNA and increasing concentrations of DBNP-B. The reaction mixtures without DNA were preincubated at 37°C for 5 minutes. The reaction was then started by the addition of denatured DNA and incubation was continued for 15 minutes at 37^0C. The reaction was stopped by the addition of SDS and EDTA to final concentrations of 1% and 25 mM respectively. The renaturation products were analysed by electrophoresis on 0.8-1.6% agarose gels or on 7.5% native polyacrylamide gels. Extent of DNA renaturation by DBNP-B was also followed by measuring the resistance of the renaturation products to S1 nuclease digestion. The dependence of renaturation on temperature and pH was followed at different temperatures and pH . Time course was carried out to determine the optimum time for renaturation.

S1 nuclease digestion of DNA renatured in the presence of **DBNP-B:**

S1 nuclease digestion assay was carried out to see the extent of pairing promoted by DBNP-B. Renaturation assay with increasing amounts of DBNP-B and heat denatured *X* DNA in 10 mM sodium acetate (pH 5.0), 12 mM

magnesium chloride and 1 mM DTT was performed for 30 minutes at 37^0C in duplicates. After deproteinisation of the reaction products with 1% SDS, one set of the reaction products was directly loaded on a 0.8% agarose gel while the other set was subjected to S1 nuclease digestion in S1 nuclease digestion buffer (10X: 500 mM sodium acetate (pH 4.7), 1500 mM NaCl and 10 mM zinc chloride) at 37°C for 5 minutes (2 units of S1 nuclease /μg DNA). The enzyme digestion was quenched by the addition of EDTA and SDS to 50 mM and 1% final concentrations respectively and the reaction products were analysed by electrophoresis on a 0.8% agarose gel. The gel was stained with ethidium bromide and photographed under UV illumination.

DNase I sensitivity of pUC19 supercoiled DNA in the **DBNP-B-DNA** complexes:

DBNP-B in increasing amounts was incubated with 400 ng of pUC19 supercoiled DNA in 10 mM Tris-Cl (pH 7.6), 50 mM NaCl, 5 mM $MgCl_2$ and 1 mM DTT at 37^0C for 45 minutes. DNase I was added to the reaction mixtures to 10:1 ratio (DNA:DNase I) and incubated at 37°C for 1 minute. The digestion was quenched by deproteinising with 1% SDS and 25 mM EDTA. The reaction products were analysed on a 0.8% agarose gel or on a 2.5% acrylamide and 0.5% agarose composite gel.

DNA aggregation assay:

DNA aggregation by DBNP-B was studied by the extent of light scattering monitored by the increase in absorbance at 320 nm spectrophotometrically. Increasing amounts of DBNP-B in aliquots were added to a fixed amount of λ DNA (2 μg) in 1 ml reaction buffer containing 10 mM sodium acetate (pH 5.0) and 1 mM Na_2-EDTA. The formation of DNA-protein aggregates was assayed by measuring the increase in absorbance at 320 nm. The influence of salt and magnesium on DNA aggregation by DBNP-B was tested by performing this assay at different sodium chloride and magnesium chloride concentrations at room temperature (25°C).

Nicking assay:

The nicking activity of DBNP-B was tested by incubating DNA containing both supercoiled and relaxed forms or only relaxed form in a reaction volume of 20 µl of 50 mM Tris-Cl (pH 7.5), 50 mM KCl, 0.1 mM Na_2-EDTA, 0.5 mM DTT and 10 mM $MgCl_2$ with different concentrations of the protein at 37^0C and 65°C for different time intervals. The reaction was terminated by the addition of SDS to 1% final concentration and the samples were digested with 2 µl of 0.8 mg /ml proteinase K for 30 minutes at 37^0C. Then, 3 µl of loading buffer containing 0.05% bromophenol blue, 0.05% xylene cyanol, 10 mM Tris-Cl (pH 8.0) and 50% glycerol was added and the reaction products were electrophoresed on a 1% agarose gel. The same reaction was performed at different pH and also the influence of magnesium and ATP on the nicking activity of DBNP-B was studied.

DNA polymerase assay:

Reaction mixtures (100 µl) contained 40 mM Tris-Cl (pH 7.9), 8 mM $MgCl_2$, 1 mM DTT, 4 mM ATP, 100 µM each of dATP, dGTP, dCTP, 25 µM dTTP, 1 µCi of [^{3}H] dTTP, 5 µg of activated calf thymus DNA and 0.5 units of *E.coli* DNA polymerase I. After incubation for 20 minutes at 37^0C the reaction was terminated by adding 50 µg of denatured calf thymus DNA and 1 ml of ice-cold 10% TCA with 1% sodium pyrophosphate and kept on ice for 30 minutes. Precipitates were collected on whatman GF/C filters, washed thrice with 3 ml each of 10% TCA containing 1% sodium pyrophosphate, washed thrice with ethanol and dried. Acid insoluble radioactivity on the GF/C filters was measured in a Beckman liquid scintillation counter after adding 5ml of toluene scintillation fluid. The effect of DBNP-B on the activity of DNA polymerase I was studied by adding DBNP-B at different DNA:protein concentrations and incubating at 37^0C for 15 minutes prior to the addition of DNA polymerase I.

RNA polymerase assay:

Reaction mixtures (500 µl) contained 25 mM Tris-Cl (pH 7.9), 10 mM $MgCl_2$, 1 mM Na_2-EDTA, 0.2 mM each of CTP, GTP, ATP, 0.05 mM UTP, 5 µCi of pH] UTP, 6 µg of activated calf thymus DNA, 1 mM K_2HPO_4 (pH 7.0), 1

mM DTT and 50 μg of bovine serum albumin. The final NaCl concentration was adjusted to 0.15 M for each assay and 2 units of *E.coli* RNA polymerase was added, incubated at 32°C for 30 minutes. The reaction was stopped with 6 ml of 10% TCA containing 1% sodium pyrophosphate after adding 0.25 mg calf liver RNA and kept on ice for 30 minutes. Precipitates were collected on Whatman GF/C filters, washed thrice with ice cold 10% TCA containing 1% sodium pyrophosphate, thrice with ethanol and dried. The acid insoluble radioactivity was measured in a Beckman liquid scintillation counter. The effect of DBNP-B on the activity of RNA polymerase was studied by adding DBNP-B at different DNA:protein concentrations and incubating at 32°C for 15 minutes prior to the addition of RNA polymerase.

Topoisomerase I assay:

Wheat germ topoisomerase I was assayed in a 20 μl reaction volume containing 50 mM Tris-Cl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM Na_2-EDTA, 0.5 mM DTT, 30 μg /ml bovine serum albumin, 0.5 μg pBR322 supercoiled DNA and 2 units of wheat germ topoisomerase I. The reaction mixtures were incubated at 37°C for 1 hour and the reaction was terminated by the addition of SDS and EDTA to 1% and 25 mM final concentrations respectively. The reaction products were digested by adding 2 μl of 0.8 mg /ml proteinase K for 30 minutes at 45^0C. Then 3 μl of loading buffer containing 0.05% bromophenol blue, 0.05% xylene cyanol in 10 mM Tris-Cl (pH 8.0) and 50% glycerol was added and the reaction products were analysed on a 1% agarose gel. The effect of DBNP-B on the wheat germ topoisomerase 1 activity was observed by adding DBNP-B at different DNA:protein concentrations and incubating these complexes at 37^0C for 15 minutes prior to the addition of topoisomerase I.

Topoisomerase II assay:

Rat testis topoisomerase II was purified as described by Galande and Muniyappa, (1996). The Rat testis topoisomerase II relaxation assay was carried out in a 20 μl volume buffer containing 10 mM Tris-Cl (pH 8.0), 50 mM NaCl, 50 mM KG, 5 mM $MgCl_2$, 0.1 mM Na_2-EDTA, 30 μg /ml bovine serum albumin, lmM ATP, 400 ng pBR322 supercoiled DNA and 100 nM topoisomerase II. The

reaction mixtures were incubated at 37^0C for 30 minutes. The reaction was terminated by the addition of SDS and EDTA to 1% and 25 mM final concentrations respectively. These samples were digested with 2 µl of 0.8 mg /ml proteinase K for 30 minutes at 45°C. Then, 3 µl of loading buffer containing 0.05% bromophenol blue, 0.05% xylene cyanol in 10 mM Tris-Cl (pH 8.0) and 50% glycerol was added and the reaction products were electrophoresed on a 1% agarose gel. The effect of DBNP-B on the relaxation activity of topoisomerase II was studied by adding DBNP-B at different DNA:protein concentrations and incubating at 37°C for 15 minutes prior to the addition of topoisomerase II.

SDS-PAGE:

Protein samples were analysed by electrophoresing on 15% or 12.5% polyacrylamide gels containing SDS as described by Laemmli, (1970). The ratio of acrylamide to bisacrylamide was 30:0.8. Resolving gel consisted of 15% acrylamide, 0.4% N-N' methylene bisacrylamide or 12.5% acrylamide, 0.33% N-N' methylene bisacrylamide in 0.375 M Tris-Cl, 0.1% SDS, pH 8.8. The stacking gel consisted of 6% acrylamide, 0.16% N-N' methylene bisacrylamide in 0.125 M Tris-Cl, 0.001% SDS, pH 6.8. Samples were reconstituted in loading buffer (0.1% SDS, 10 mM DTT, 10% glycerol, 62.5 mM Tris-Cl, 0.05% bromophenol blue, pH 6.8), boiled for 2 mins at 100°C, cooled and loaded. Electrophoresis was carried out at 120V for 6 hrs in electrode buffer (0.05 M Tris-Cl, 0.38 M glycine, 0.1% SDS, pH 8.3).

Staining of polyacrylamide gels:

Coomassie blue staining:

Gels were first washed with 7.5% acetic acid for 30 minutes. The gels were stained with coomassie blue R-250 (0.1% in 50% methanol, 7.5% acetic acid) for 60 minutes at room temperature and destained in 5% methanol, 7.5% acetic acid.

Silver staining:

Gels were stained according to Blum and Gross, (1987). Gels were first fixed in 50% methanol, 12.5% acetic acid overnight, washed with 50% ethanol twice each for 20 minutes and once with 30% ethanol for 20 mins. Gels were then pretreated with $Na_2S_2O_3$. 5 H_2O (0.2 g /lit) for 1 min, rinsed with H_2O for 30 seconds thrice. The gels were impregnated with $AgNO_3$ (2g /lit) and 0.75 ml 37% HCHO /lit for 20 minutes, and rinsed with water for 30 seconds thrice. Gels were developed with $Na_2S_2O_3$. 5 H_2O (4 mg /lit), 0.5 ml 37% HCHO /lit and Na_2CO_3 (60g /lit) for about 10 minutes. Developing was stopped by washing thrice in H_2O for 2 minutes and soaking in 50% methanol and 12.5% acetic acid for 10 minutes. The gels were finally washed with 50% methanol.

Western Blotting:

Western blotting was performed according to Towbin *et al*, (1979) with slight modifications. Proteins were separated on a 15% SDS-Laemmli polyacrylamide gel, and transferred onto nitrocellulose membranes (0.45 μm pore size) electrophoretically for a period of 3-5 hours. The transfer buffer employed contained 25 mM Tris, 192 mM glycine, 0.1% SDS and 20% methanol at pH 8.3. After the completion of the transfer, the blots were air dried briefly and incubated in the blocking buffer (10 mM Tris-Cl (pH 7.6), 150 mM NaCl, 0.1% Tween-20) for 2 hours. The blots were then incubated in the cognate primary antibody (blocking buffer containing 0.1% IgG) for 2 hours. After the incubation, the blots were washed for 1 hour with the blocking buffer containing 0.1% Tween-20 (with three changes of buffer). The blots were finally incubated with the secondary antibody (anti-rabbit IgG from goat coupled to horse radish peroxidase at a dilution of 1:500) for 2 hours. This was followed by thorough washing of the blots for 1 hour with three changes at intervals of 20 minutes. The final washing was carried out in 10 mM Tris-Cl (pH 7.6), 150 mM NaCl. The colour development was done in freshly prepared solution of 0.03% 4-chloro-1-napthol and 0.03% (v/v) H_2O_2 in 10 mM Tris-Cl (pH 7.6) and 150 mM sodium chloride.

Agarose gel electrophoresis:

DNA was analysed by electrophoresis on 0.8% to 1.6% agarose gels. The electrophoresis was carried out in 40 mM Tris-acetate (pH 7.8) and 1 mM Na_2-EDTA buffer. After electrophoresis the gels were stained in 0.5 μg /ml ethidium bromide in electrophoresis buffer and photographed under UV illumination.

Acryiamidc- agarose composite gel electrophoresis:

DNase I digested products in the DNA-DBNP-B complexes were separated on composite gels containing 2.5% acrylamide and 0.5% agarose. The ratio of acrylamide to bisacrylamide in the gel was 40:0.4 in TAE buffer (40 mM Tris-acetate (pH 7.8) and 1 mM Na_2-EDTA). 1% agarose in TAE was dissolved by boiling, and brought to 50^0C, then mixed with equal volume of acrylamide solution and the gel was casted. The electrophoresis running buffer employed was TAE. The gel was stained with 0.5 μg /ml ethidium bromide in TAE buffer and photographed under UV illumination.

Nucleic acid and Protein estimation:

The concentration of DNA in the nucleoid fractions was determined by an ethidium bromide fluoresence assay following the procedure of Morgan *et al,* (1979). DNA was assayed by exploiting the enhanced fluoresence of ethidium intercalated into duplex regions of DNA. Increasing amounts of calf thymus DNA was added to 1 ml buffer containing 0.5 μg /ml ethidium bromide, 5 mM Tris-Cl (pH 8.0), 0.5 mM Na_2-EDTA. The fluorescence emission was measured at 600 nm by exciting at 525 nm with a band width of 20 nm in a JASCO FP-777 spectrofluorimeter. Nucleoid fractions were added to 1 ml buffer and the fluorescence was measured as above. The amount of DNA **in** the nucleoid fractions was calculated from the calf thymus DNA standard graph.

The concentration of poly (U) and poly (A) were determined spectrophotometrically using molar absorption coefficients of 9800 and 9350 at 258 and 260 nm respectively.

Protein concentration was determined by folin reagent (Lowry *et al.,* 1951) using bovine serum albumin as a standard.

CHAPTER 3

Results

Chapter 3.1. Characterisation of *Sulfolobus acidocaldarius* nucleoid:

Growth of *Sulfolobus acidocaldarius:*

Sulfolobus acidocaldarius was grown at 75°C as described in methods section. The growth was followed by measurment of absorbance at 650 nm after every 6 hr intervals (Fig. 1).

Isolation of nucleoid:

Nucleoid from *Sulfolobus acidocaldarius* was isolated according to Reddy and Suryanarayana, (1988) with some modifications. Freshly harvested cells (2gm) grown upto midlogarthmic phase were gently lysed using non-ionic detergents and the lysate was centrifuged through a 30% sucrose cushion to separate the rapidly sedimenting DNA-protein complex, nucleoid (chromatin). The nucleoid pellet was dissolved in 3 ml of 20 mM Tris-Cl (pH 7.6), 150 mM KCl, 1 mM Na_2-EDTA and 6 mM β-mercaptoethanol (nucleoid buffer). The nucleoid obtained by this method contained a lot of membrane contamination. So the nucleoid was purified by chromatography on a sephacryl S-1000 column.

Sephacryl S-1000 column chromatography of *S. acidocaldarius* nucleoid:

The crude nucleoid obtained from 2 gms of freshly harvested cells grown upto midlogarthmic phase was gently sonicated to shear the chromatin and centrifuged at 3,000 x g to remove the contaminating cell debris that is still present in the chromatin. This sheared chromatin was further purified by passing it through a sephacryl S-1000 column equilibrated with the nucleoid buffer as described in methods section. The sheared chromatin was separated into two distinct peaks as shown in Fig.2A (peak-I and peak-II) suggesting that chromatin probably consists of two forms differing in quality. The protein composition of the peak fractions of the two peaks was analysed by SDS-polyacrylamide gel electrophoresis (Fig.2B). Peak-I contains mostly high molecular weight proteins and low amount of low molecular weight proteins. Peak-II contains histone like

Fig. 1: Growth curve of *Sulfolobus acidocaldarius*:

The bacteria was grown upto 75 hours at 75^0C and the absorbance was measured at 650 nm.

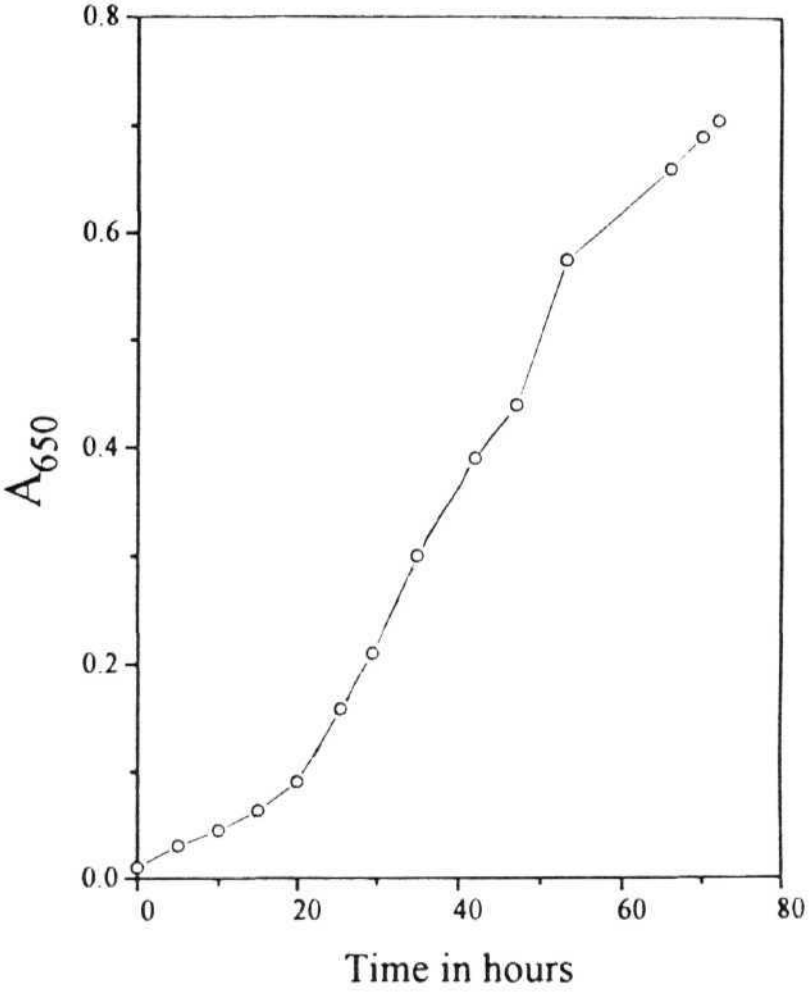

Figure 1

Fig. **2A:** Sephacryl S-1000 column chromatographic elution profile of *S. acidocaldarius* nucleoid:

Fractions (50 µl each) obtained from sephacryl S-1000 column chromatography of nucleoid isolated from freshly harvested midlogarthmic phase cells was measured at 260 nm in 1 ml of nucleoid buffer. DNA concentration in 50 µl of each fraction was estimated by ethidium bromide fluorescence assay as described in materials and methods section.

Fig. 2B: SDS-PAGE analysis of fractions obtained from sephacryl S-1000 chromatography of nucleoid:

Nucleoid isolated from freshly harvested cells grown upto midlogarthmic phase was chromatographed on a sephacryl S-1000 column. Fractions (50 µl each) were analysed on a 15% SDS-polyacrylamide gel followed by coomassie blue staining.

Lane 1-5: Peak I fractions 16, 17, 18, 19 and 21 respectively.
Lane 6-11: Peak II fractions 25, 26, 27, 28, 29 and 30 respectively.

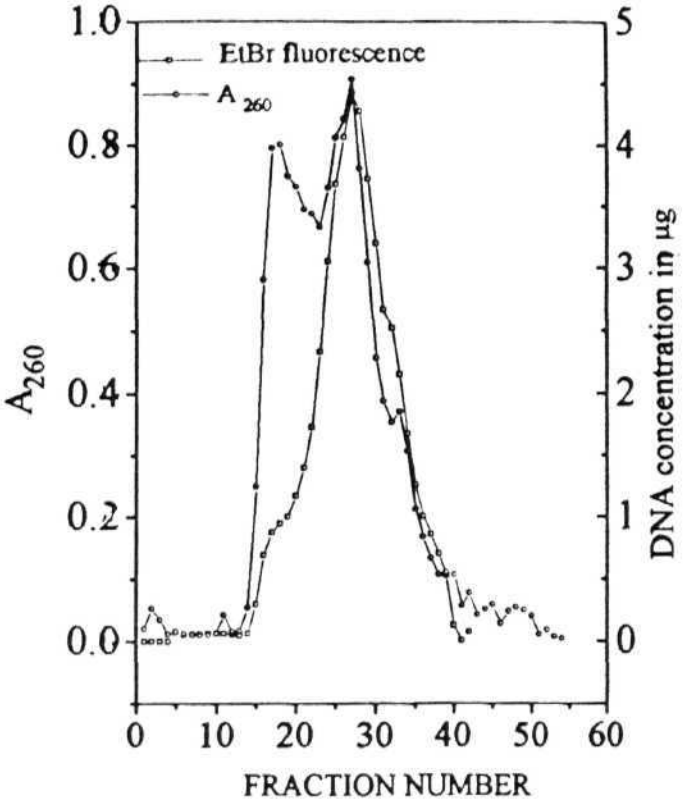

Figure 2A

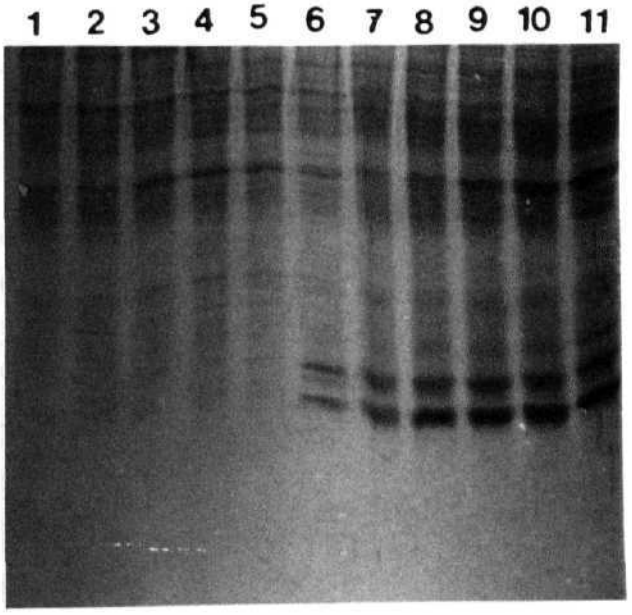

Figure 2B

proteins in abundance (Fig.2B) along with high molecular weight proteins. The DNA content in peak-I is relatively low when compared to that of peak-II as measured by ethidium bromide fluorescence assay. Peak-II contained a significant amount of DNA (Fig.2A). The DNA in both peak-I and peak-II sephacryl S-1000 fractions was analysed by agarose gel electrophoresis without deproteinisation. As seen in Fig.3A the DNA present in peak-I has a high molecular weight than the DNA present in the peak-II as indicated by slower migration. The DNA in peak-I is seen in the slot of the agarose gel whereas the DNA in peak-II migrated as a broad smear (Fig.3A). The DNA in both peak-I and peak-II was isolated by phenol extraction as described in methods section. As can be seen from Fig.3B very little DNA in the peak-I is retained in the slot and DNA in peak-II fractions migrated as a broad smear.

Nucleoids isolated from cells grown upto midlogarthmic phase and stored at -80°C and from freshly harvested cells grown upto early logarthmic phase (30 hrs) were chromatographed on the sephacryl S-1000 column to observe if the separation of nucleoid into two peaks is growth phase dependent and if any changes occur on storage of cells at -80^0C.

Nucleoid was isolated as described in methods section from cells grown upto midlogarthmic phase and stored at -80°C. This nucleoid was chromatographed on a sephacryl S-1000 column. The fractions obtained were analysed for the DNA content by measuring absorbance at 260 nm and ethidium bromide fluorescence assay (Fig.4A). The sheared chromatin from midlogarthmic phase cells stored at -80^0C also separated into two distinct peaks as seen in Fig.4A. The protein composition of the peak fractions of the two peaks was analysed by SDS-PAGE (Fig.4B). The DNA content in peak-I is also relatively very low compared to that of peak-II as measured by ethidium bromide fluorescence assay (Fig.4A). The DNA-protein complex in both peak-I and peak-II sephacryl S-1000 fractions was analysed by agarose gel electrophoresis without deproteinisation (Fig.5A). The DNA in both peak-I and peak-II was isolated by phenol extraction and analysed on a 0.8% agarose gel (Fig.5B). The DNA in both peak fractions was treated with 1% SDS and then subjected to proteinase K digestion at 45^0C for 1 hr and analysed on a 0.8% agarose gel. As can be seen from Fig.5C DNA in peak-I fractions migrated as a high molecular weight sharp band (slightly faster migration than in Fig.5A) with relatively low

Fig. 3: Agarose gel electrophoresis of fractions obtained from sephacryl S-1000 column chromatography of nucleoid:

Fig. 3A: Fractions (20 μl each) obtained from sephacryl S-1000 chromatography of nucleoid from freshly harvested cells grown upto midlogarthmic phase were analysed on a 0.8% agarose gel.

Lane 1-5: Peak I fractions 16, 17, 18, 19 and 21 respectively.
Lane 6-12: Peak II fractions 25, 26, 27, 28, 29, 30 and 31 respectively.

Fig. 3B: DNA from 20 μl of each fraction obtained from sephacryl S-1000 chromatography of nucleoid from freshly harvested cells grown upto midlogarthmic phase was isolated by phenol extraction, precipitated with ethanol and analysed on a 0.8% agarose gel.

Lane 1-5: Peak I fractions 16, 17, 18, 19 and 21 respectively.
Lane 7-11: Peak II fractions 25, 26, 27, 28 and 29 respectively.

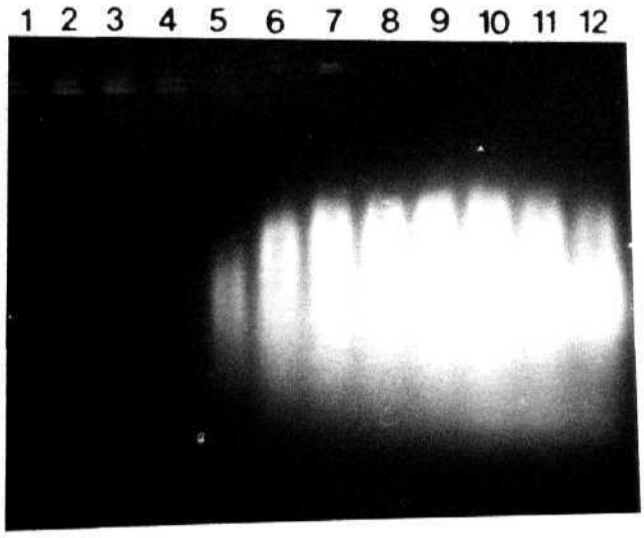

Figure 3A

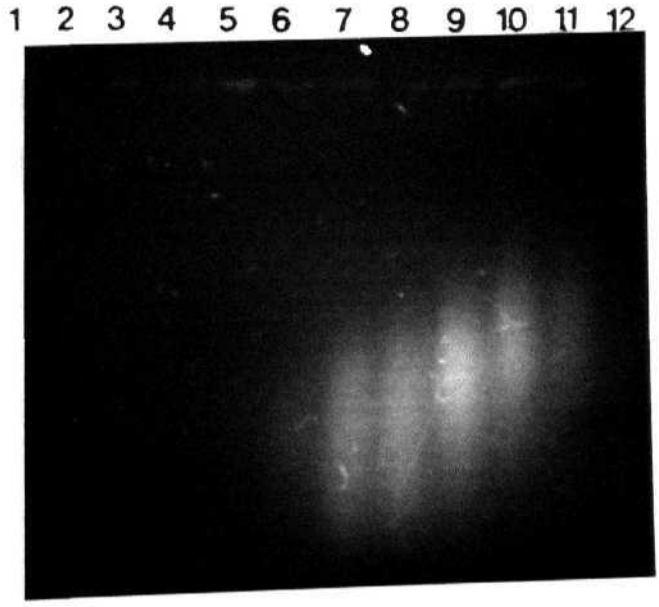

Figure 3B

Fig. 4A: Sephacryl S-1000 column chromatographic elution profile of *S. acidocaldarius* nucleoid:

Fractions (20 µl each) obtained from sephacryl S-1000 column chromatography of nucleoid isolated from cells harvested at midlogarthmic phase and stored at - 80^{0}C was measured at 260 nm in 1 ml of nucleoid buffer. DNA concentration in 50 µl of each fraction was estimated by ethidium bromide fluorescence assay as described in materials and methods section.

Fig. 4B: SDS-PAGE analysis of fractions obtained from sephacryl S-1000 chromatography of nucleoid:

Nucleoid isolated from cells grown upto midlogarthmic phase and stored at - 80^{0}C was chromatographed on a sephacryl S-1000 column. Fractions (50 µl each) were analysed on a 15% SDS-polyacrylamide gel followed by coomassie blue staining.

Lane 1-5: Peak I fractions 13, 14, 15, 17 and 21 respectively.
Lane 6-12: Peak 11 fractions 25, 27, 29, 31, 33, 35 and 37 respectively.

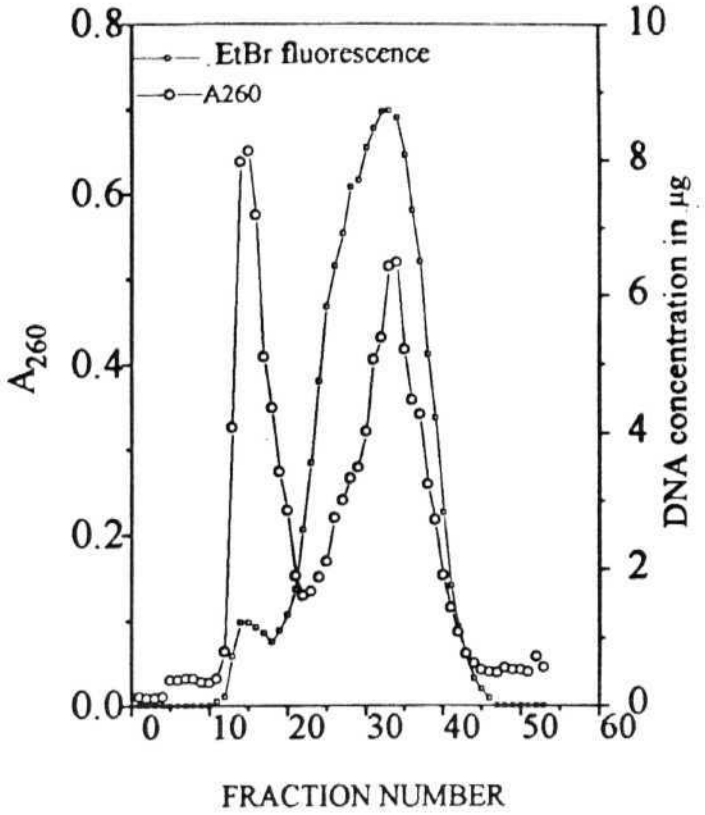

Figure 4A

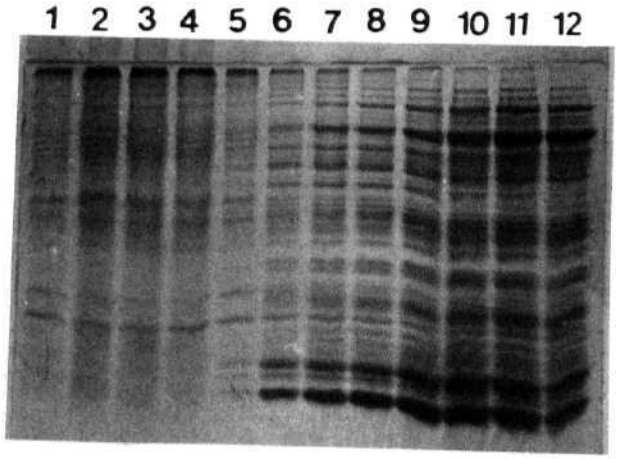

Figure 4B

Fig. 5: Agarose gel electrophoresis of fractions obtained from S-1000 chromatography of nucleoid from cells harvested at midlogarthmic phase and stored at -80^{0}C:

Fig. 5A: Fractions (20 µl each) were directly electrophoresed.

Lane 1-5: Peak I fractions 13, 14, 15, 17 and 21 respectively.
Lane 6-12: Peak II fractions 25, 27, 29, 31, 33, 35 and 37 respectively.

Fig. 5B: DNA from 20 µl of each fraction was extracted and was analysed on a 0.8% agarose gel after phenol extraction.

Lane 1-4: Peak I fractions 13, 15, 17 and 21 respectively.
Lane 5-11: Peak II fractions 25, 27, 29, 31, 33, 35 and 37 respectively.

Fig. 5C: Fractions (20 µl each) were treated with 1% SDS and then subjected to proteinase K digestion by incubating at 45^{0}C for 1 hr and analysed on a 0.8% agarose gel.

Lane 1-4: Peak I fractions 14, **15,** 17 and 21 respectively.
Lane 5-11: Peak II fractions 25, 27, 29, 31, 33, 35 and 37 respectively.

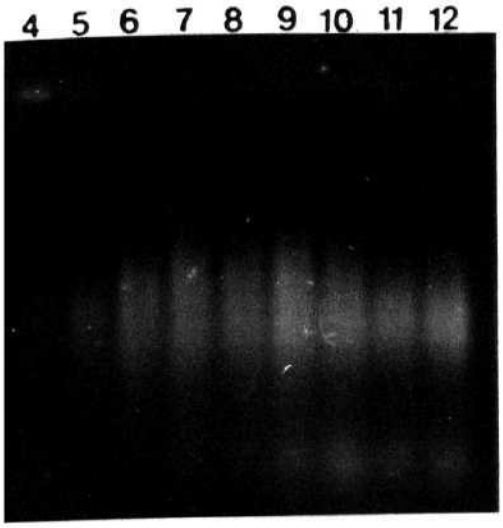

Figure 5A

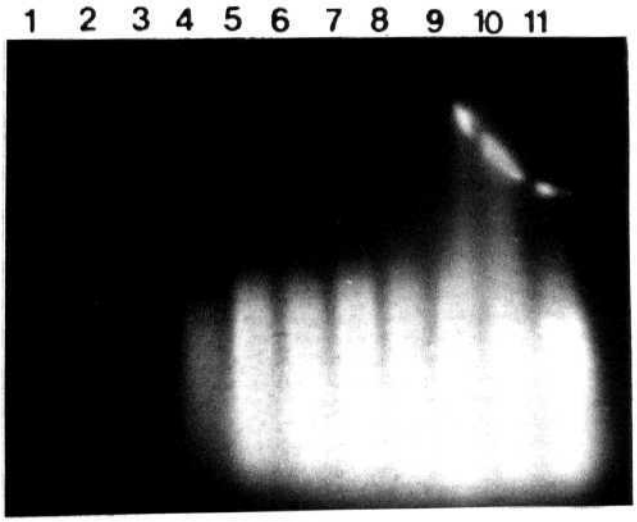

Figure 5B

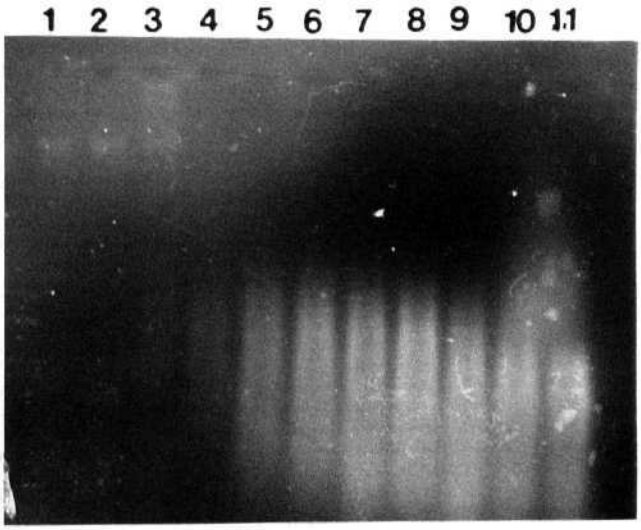

Figure 5C

amount of low molecular weight DNA (Fig.5C lanes 1-4) and the DNA in peak-II migrated as a broad smear (Fig.5C lanes 5-11) indicating that some of the DNA appearing in the slot in peak-I fractions may be bound by protein.

Nucleoid isolated from freshly harvested early logarthmic phase cells (grown upto 30 hrs) was chromatographed on a sephacryl S-1000 column. The fractions obtained were analysed for the DNA content by measuring absorbance at 260 nm and by ethidium bromide fluorescence assay (Fig.6A). The sheared chromatin from freshly harvested early logarthmic phase cells also separated into two peaks as seen in Fig.6A. The DNA content in peak-I is also relatively very-low compared to that of peak-II as measured by ethidium bromide fluorescence assay. The protein composition of the peak fractions of both peaks was analysed by SDS-PAGE (Fig.6B) and it shows a similar pattern to that of sephacryl S-1000 nucleoid fractions isolated from freshly harvested cells grown upto midlogarthmic phase. The DNA content in both peak-I and peak-II was analysed by agarose gel electrophoresis (Fig.7). DNA in peak-I fractions has a relatively high molecular weight as can be seen in Fig.7 (lanes 1-4) when compared to that of DNA in peak-II fractions (Fig.7 lanes 5-10) which migrated as a broad smear.

These results indicate that the chromatin in *S. acidocaldarius* consists of two components with peak-I and peak-II in early logarthmic phase, midlogartlimic phase and midlogartlimic phase cells stored at -80°C.

Sephadex G-50 column chromatography of sephacryl S-1000 peak-II nucleoid fractions:

Nucleoid from freshly harvested midlogarthmic phase cells chromatographed on a sephacryl S-1000 column as described in methods section was separated into two peaks, peak-I and peak-II. Peak-II fractions were pooled, treated with DNase I. These pooled fractions were concentrated by lyophilisation and loaded on a sephadex G-50 column. The column was eluted with 20 mM Tris-Cl (pH 7.6), 1 mM Na_2-EDTA, 150 mM KCl and 7 mM β-mercaptoethanol containing buffer. These fractions were analysed for protein by measuring absorbance at 280 nm (Fig.8A) and electrophoresed on a 15% SDS-polyacrylamide gel (Fig.8B). Peak fractions contained all the nucleoid proteins as seen in Fig.8B lanes 2-7. The tail fractions (Fig.8B lanes 8-11) contained high

Fig. 6A: Sephacryl S-1000 column chromatographic elution profile of *S. acidocaldarius* nucleoid:

Fractions (50 µl each) obtained from sephacryl S-1000 column chromatography of nucleoid isolated from freshly harvested cells grown upto 30 hrs (early logarthmic phase) was measured at 260 nm in 1 ml of nucleoid buffer. DNA concentration in 150 µl of each fraction was measured by ethidium bromide fluorescence assay as described in materials and methods section.

Fig. 6B: SDS-PAGE analysis of fractions obtained from sephacryl S-1000 chromatography of nucleoid:

Nucleoid isolated from freshly harvested cells grown upto early logarthmic phase (30 hrs) was chromatographed on a sephacryl S-1000 column. Fractions (100 µl each) were analysed on a 15% SDS-polyacrylamide gel followed by coomassie blue staining.

Lane 1- 4: Peak I fractions 11, 12, 13, and 14 respectively.
Lane 5-10: Peak II fractions 25, 26, 27, 28, 29 and 30 respectively.
Lane 11: DBNP-B.

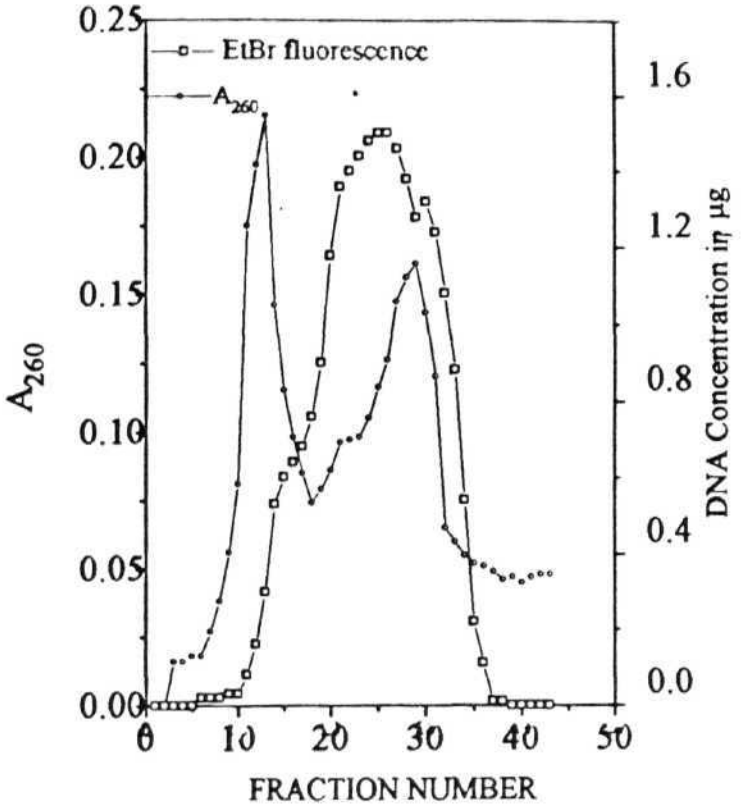

Figure 6A

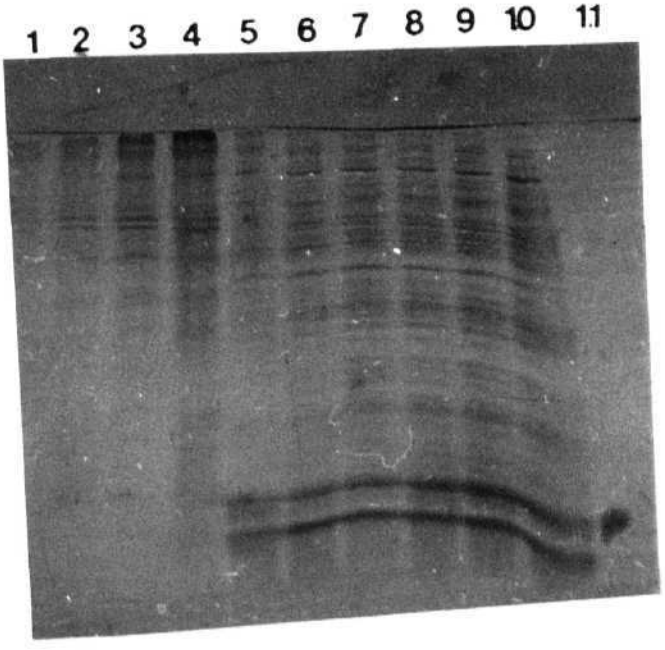

Figure 6B

Fig. 7: Fractions (40 μl each) obtained from sephacryl S-1000 chromatography of nucleoid from freshly harvested cells grown upto early logarthmic phase (30 hrs) were analysed on a 0.8% agarose gel.

Lane 1-4: Peak I fractions 11, 12, 13 and 14 respectively.
Lane 5-10: Peak II fractions 25, 26, 27, 28, 29 and 30 respectively.

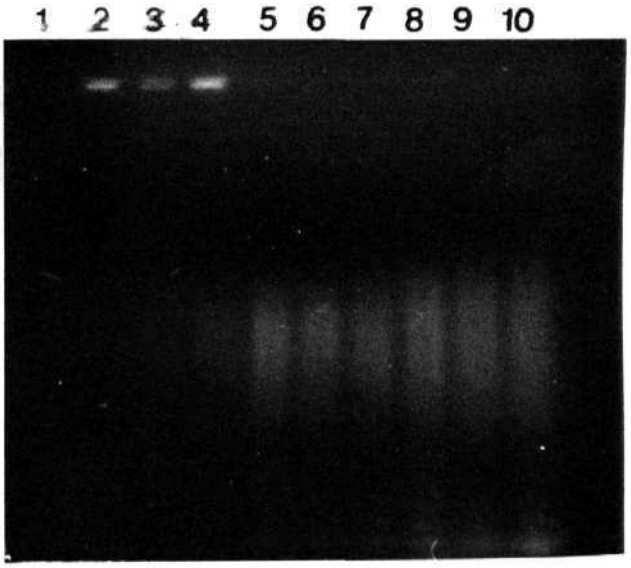

Figure 7

Fig. 8A: Sephadex G-50 column chromatographic elution profile of *S. acidocaldarius* nucleoid:

The pooled sephacryl S-1000 peak-II nucleoid fractions were chromatographed on a sephadex G-50 column as described in materials and methods. Fractions (50 µl each) were measured at 280 nm in 1 ml of column buffer.

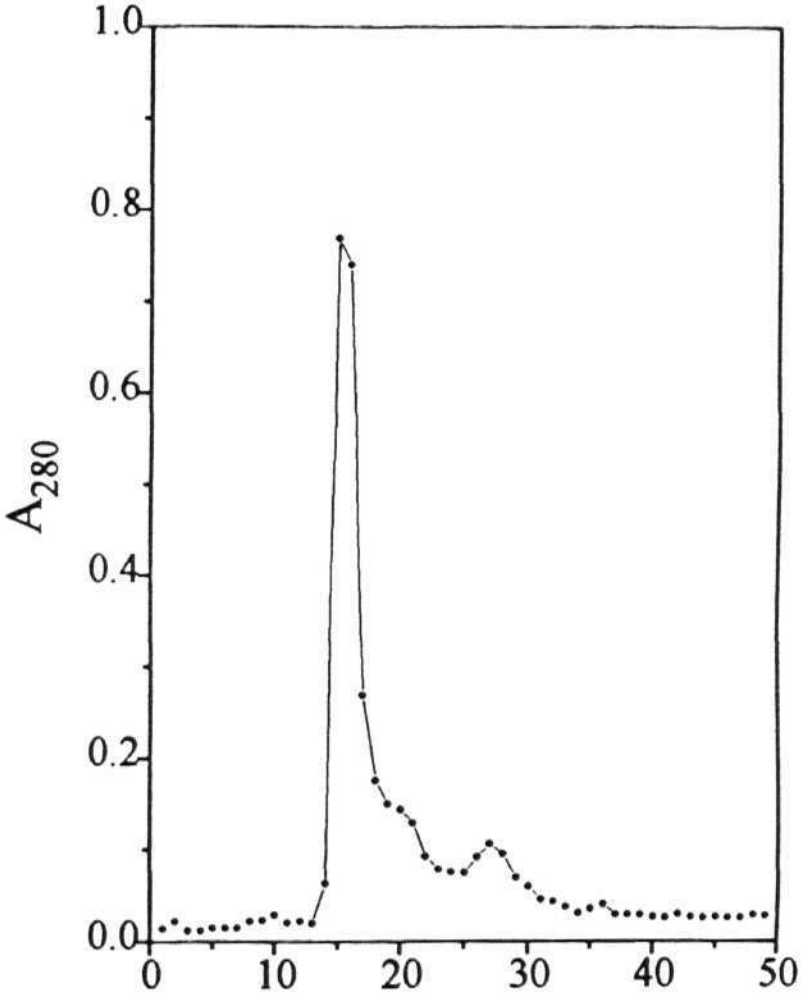

Figure 8A

molecular weight smear at the top of the gel along with some amount of low molecular weight nucleoid proteins. These fractions containing high molecular weight complexes were electrophoresed on a 15% SDS-PAGE. As can be seen in Fig.8C lanes 2-4 the high molecular weight complexes are stained by ethidium bromide. Thus the tail fractions obtained from the G-50 column contained proteins complexed with DNA. These G-50 fractions were electrophoresed, transferred onto nitrocellulose and probed with anti-HSNP-A IgG, anti-DBNP-B IgG and anti-HSNP-C IgG. The results of the immunoblotting indicate that G-50 peak shows cross reaction with anti-DBNP-B (fractions 15-22) and anti-HSNP-A (fractions 15-19). The tail fractions (fractions 19-23) show cross reaction with HSNP-C. Thus peak fractions contain HSNP-A and DBNP-B in abundance and HSNP-C' in low amounts where as tail fractions contain DNA with HSNP-C in abundance and DBNP-B in low amounts. Thus, the sephadex G-50 column chromatography resulted in the separation of nucleoid proteins into two groups.

DNA-cellulose column chromatography of sephadex G-50 nucleoid fractions:

In order to see which of the nucleoid associated proteins, other than histone like proteins bind to DNA, the sephadex G-50 column peak fractions 14-18 were pooled, diluted to 50 mM KCl in column buffer and chromatographed on a ds DNA-cellulose column as described in methods section. The fractions obtained were analysed for the presence of protein by measuring the absorbance at 280 nm (Fig.9A) and by electrophoresis on a 15% SDS-polyacrylamide gel (Fig.9B, Fig.9C). As can be seen from Fig.9B and Fig.9C, 200 mM KCl fractions contain HSNP-A in abundance along with DBNP-B, HSNP-C, an ~15 kDa protein and an ~25 kDa protein. Fractions eluted with 300 mM and 400 mM KCl contain HSNP-C and an ~ 25 kDa protein in abundance. The presence of these proteins is also confirmed by immunoblotting of these gradient fractions with the respective anti IgG's.

DNA-cellulose column chromatography of sephacryl S-1000 peak-I nucleoid proteins:

The sephacryl S-1000 peak-I fractions obtained by chromatography of nucleoid isolated from freshly harvested *S. acidocaldarius* midlogarthmic phase cells were pooled, diluted to 50 mM KCl in the column buffer, subjected to

Fig. 8B: SDS-PAGE analysis of fractions obtained from sephadex G-50 column chro matography of sephacryl S-1000 peak II nucleoid fraction:

Fractions (150 µl each) were TCA precipitated, washed with acetone and dissolved in SDS-gel loading buffer and electrophoresed on a 15% SDS-polyacrylamide gel followed by silver staining.

Lane 1: Sample loaded.
Lane 2-14: Fraction numbers 14-26 respectively.
Lane 15: HSNP-C.
Lane 16: DBNP-B.

Fig. *SC:* SDS-PAGE analysis of fractions obtained from sephadex G-50 column chro matography of sephacryl S-1000 peak II nucleoid fraction:

Fractions (150 µl each) were TCA precipitated, washed with acetone and dissolved in SDS-gel loading buffer and electrophoresed on a 15% SDS-polyacrylamide gel followed by ethidium bromide staining.

Lane 1-4: Fraction numbers 20-23 respectively.

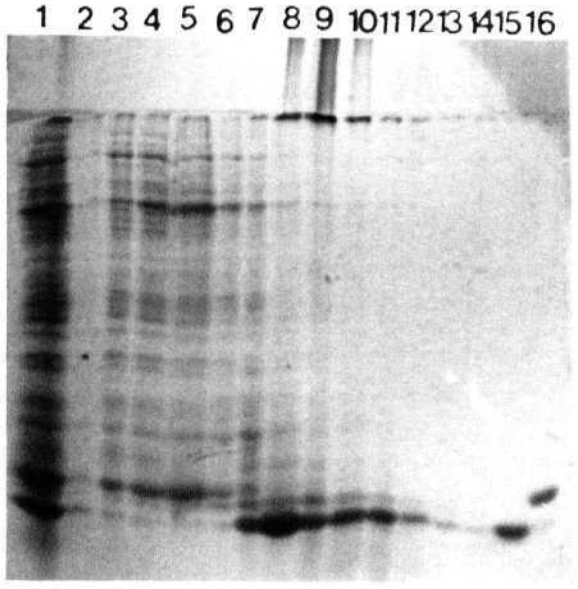

Figure 8B

Figure *SC*

Fig. 9A: DNA-cellu!ose chromatographic elution profile of sephadex G-50 nucleoid fraction:
The pooled sephadex G-50 nucleoid peak fractions were chromatographed on a ds DNA-cellulose column as described in materials and methods. The absorbance of 1 ml of each fraction was measured at 280 nm.

Fig. 9B: SDS-PAGE analysis of fractions obtained from dsDNA-cellulose chromatography of sephadex G-50 nucleoid fraction:
Fractions (500 µl each) were TCA precipitated, washed with acetone, dissolved in SDS-gel loading buffer and analysed on a 15% SDS-polyacrylamide gel.

Lane 1-2: Flowthrough fractions.
Lane 3-4: Fraction numbers 1 and 5 respectively obtained from the 50 mM KCl buffer elution.
Lane 5-8: Fraction numbers 18, 19, 22 and 25 respectively obtained from the 100 mM KCl buffer elution.
Lane 9-12: Fraction numbers 29, 31, 33 and 36 respectively obtained from the 200 mM KCl buffer elution.
Lane 13: Fraction number 40 obtained from the 300 mM KCl buffer elution.
Lane 14: DBNP-B.
Lane 15: Molecular weight markers (66 kDa- BSA, 45 kDa -Ovalbumin, 25 kDa-Chymotrypsinogen, 20.1 kDa -Trypsin inhibitor and 12.3 kDa-Cytochrome-C).

Fig. 9C: SDS-PAGE analysis of fractions obtained from dsDNA-cellulose chromatography of sephadex G-50 nucleoid fraction:
Fractions (500 µl each) were TCA precipitated, washed with acetone, dissolved in SDS-gel loading buffer and analysed on a 15% SDS-polyacrylamide gel.

Lane 1-2: Fraction numbers 42 and 44 respectively obtained from the 300 mM KCl buffer elution.
Lane 3-7: Fraction numbers 52, 54, 56, 57 and 61 respectively obtained from the 400 mM KCl buffer elution.
Lane 8-11: Fraction numbers 64, 66, 68 and 70 respectively obtained from the 500 mM KCl buffer elution.
Lane 12-13: Fraction numbers 77 and 79 respectively obtained from the 700 mM KCl buffer elution.
Lane 14: DBNP-B.
Lane 15: Molecular weight markers as in Fig.9B

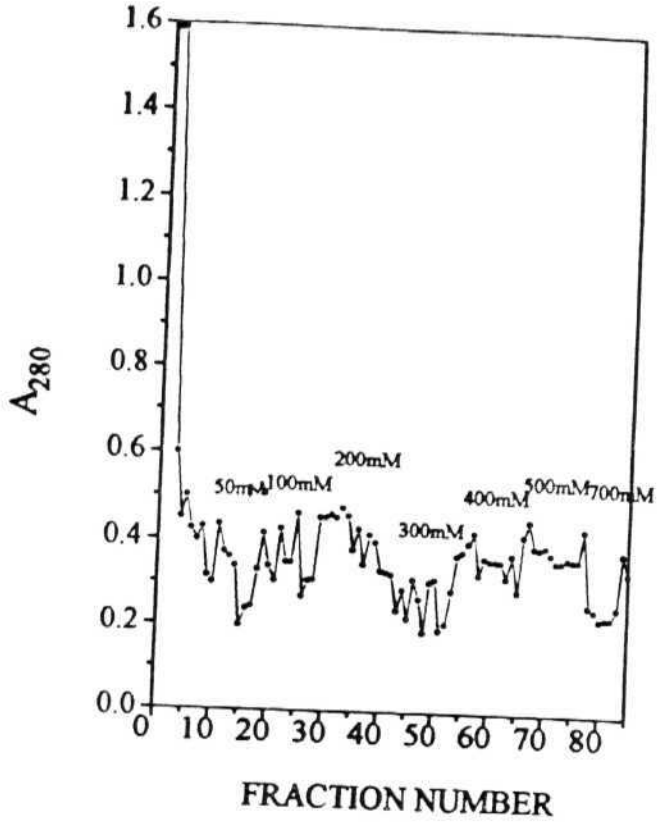

Figure 9A

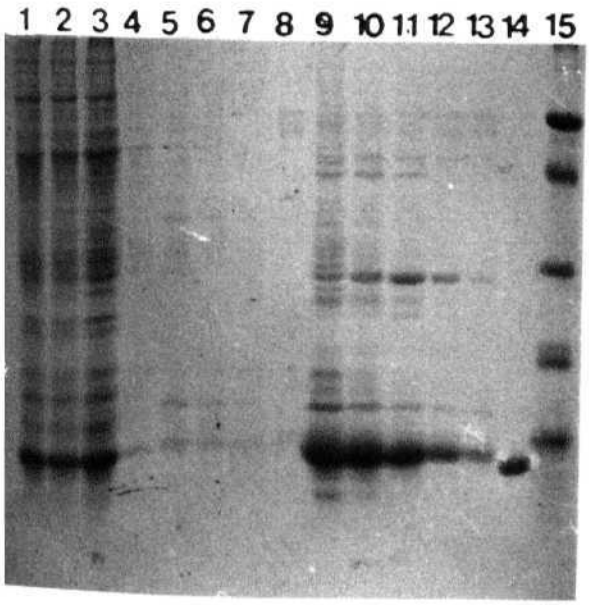

Figure 9B

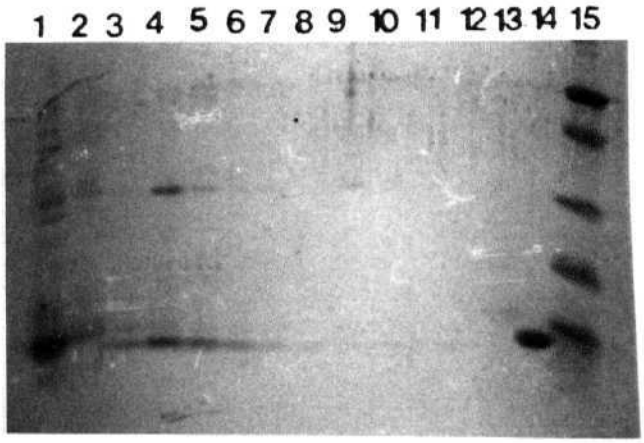

Figure 9C

DNase I digestion and loaded on a ds DNA-cellulose column as described in methods section. The fractions obtained were analysed for the presence of protein by measuring the absorbance at 280 nm (Fig.lOA) and electrophoresed on a 15% SDS-polyacrylamide gel (Fig. 1 OB). As can be seen from Fig. 1 OB, 200 mM KC1 fractions (Fig.lOB lanes 7-9) contain HSNP-A in abundance along with HSNP-C, HSNP-C', an ~15 kDa protein and an ~ 25 kDa protein. The 300 mM KCl fractions (Fig.lOB lanes 10-12) contain DBNP-B and HSNP-C in abundance along with HSNP-A, an ~ 15 kDa protein and an ~ 25 kDa protein. 700 mM KCl fractions contain DBNP-B.

DEAE-cellulose chromatography of sephacryl S-1000 peak-II nucleoid proteins:

The peak-II fractions obtained from sephacryl S-1000 chromatography of nucleoid from freshly harvested cells grown upto midlogarthmic phase were pooled and chromatographed on a DEAE-cellulose column as described in methods section to obtain DNA-protein complexes with strongly bound proteins. The fractions obtained were analysed for protein by measuring the absorbance at 280 nm. (Fig. 11 A). The DNA concentration in the fractions was measured by ethidium bromide fluorescence assay. These fractions were analysed on a 15% SDS-polyacrylamide gel (Fig. 11B). As can be seen from Fig.llB HSNP-C did not bind to the DE-52 column under these conditions and is eluted in the flowthrough fraction. Other nucleoid associated proteins bound to the column were eluted with 1.5M KCl containing buffer. The nucleoid isolated from DE-52 column contained HSNP-A, DBNP-B, 15 kDa protein and other proteins in the molecular weight range 25 to 35 kDa. The proteins associated with DNA in the nucleoid obtained from DE-52 column may represent proteins which are tightly bound to *S. acidocaldarius* DNA. Hence this DNA-protein complex was further analysed by nuclease digestion.

DNase I digestion of DEAE-cellulose nucleoid fraction:

Nucleoid fraction obtained from DEAE-cellulose chromatography of pooled sephacryl S-1000 peak-II fractions was subjected to DNase I digestion at a ratio of DNA:DNase 1 of 10:1 at 37°C for 30 seconds. The reaction was terminated by the addition of SDS and EDTA to 1% and 25 mM final

Fig. 10A: DNA-cellulose chromatographic elution profile of sephacryl S-1000 peak-I nucleoid fraction:

The pooled sephacryl S-1000 peak-I nucleoid fractions were chromatographed on a DNA cellulose column as described in materials and methods. The absorbance of 1 ml of each fraction was measured at 280 nm.

Fig. 10B: SDS-PAGE analysis of fractions obtained from dsDNA-cellulose column chromatography of sephacryl S-1000 peak I nucleoid fraction:

Fractions (20 µl each) were electrophoresed by 15% SDS-PAGE followed by silver staining.

Lane 1: Flow through fraction.
Lane 2-3: Fraction numbers 1 and 3 respectively eluted with 50 mM KCl containing buffer.
Lane 4-6: Fraction numbers 10, 12 and 15 respectively eluted with 100 mM KCl containing buffer.
Lane 7-9: Fraction numbers 22, 24 and 27 respectively eluted with 200 mM KCl containing buffer.
Lane 10-12: Fraction numbers 30, 32 and 35 respectively eluted with 300 mM KCl containing buffer.
Lane 13: Molecular weight markers (66 kDa- BSA, 45 kDa -Ovalbumin, 25 kDa-Chymotrypsinogen, 20.1 kDa -Trypsin inhibitor and 12.3 kDa-Cytochrome-C).
Lane 14: Molecular weight markers (66 kDa- BSA, 45 kDa -Ovalbumin, 25 kDa-Chymotrypsinogen, 20.1 kDa -Trypsin inhibitor, 12.3 kDa-Cytochrome-C and 11 kDa- DBNP-B).

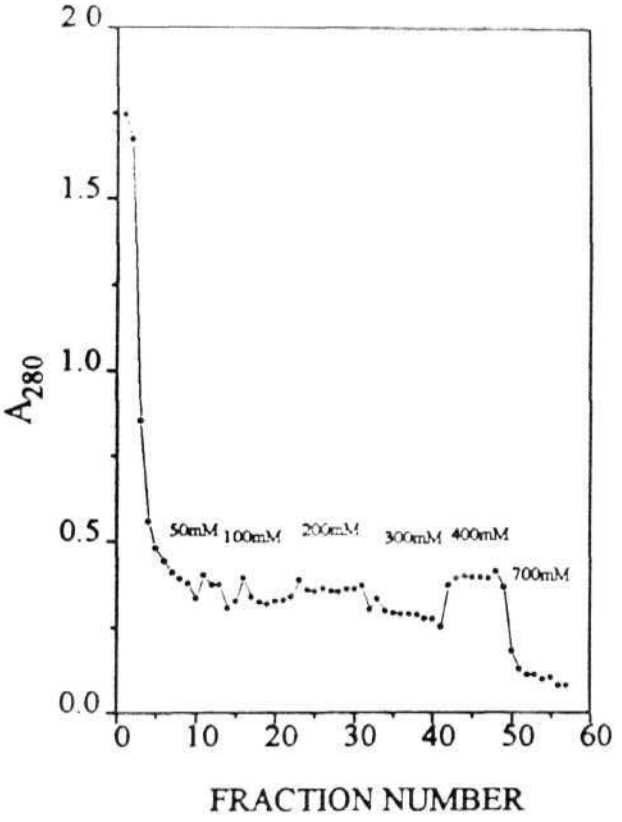

Figure 10A

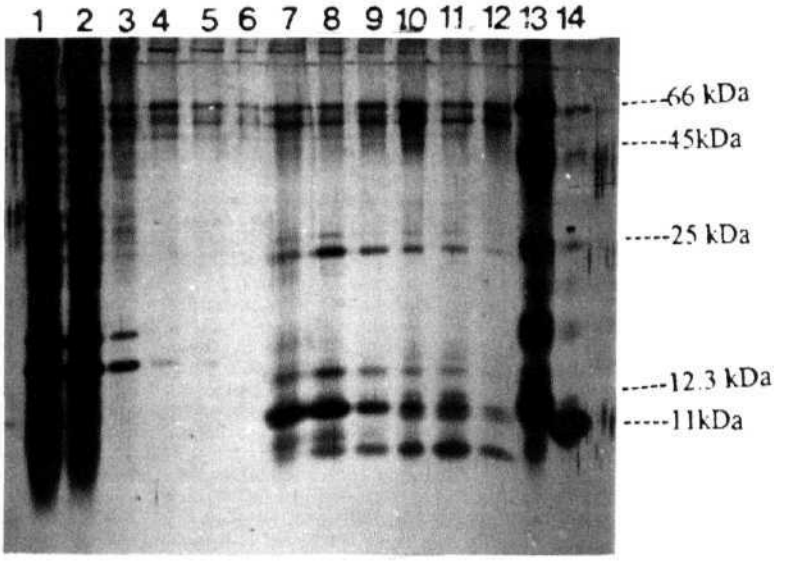

Figure 10B

Fig. 11 A: DEAE-cellulose chromatographic elution profile of sephacryl S-1000 peak-II nucleoid fraction:

The pooled sephacryl S-1000 peak-II nucleoid fraction was chromatographed on a 6 ml DE-52 column as described in materials and methods. The absorbance of 1ml of each fraction was measured at 280 nm. DNA concentration in 150 μl of each fraction was estimated by ethidium bromide fluorescence assay.

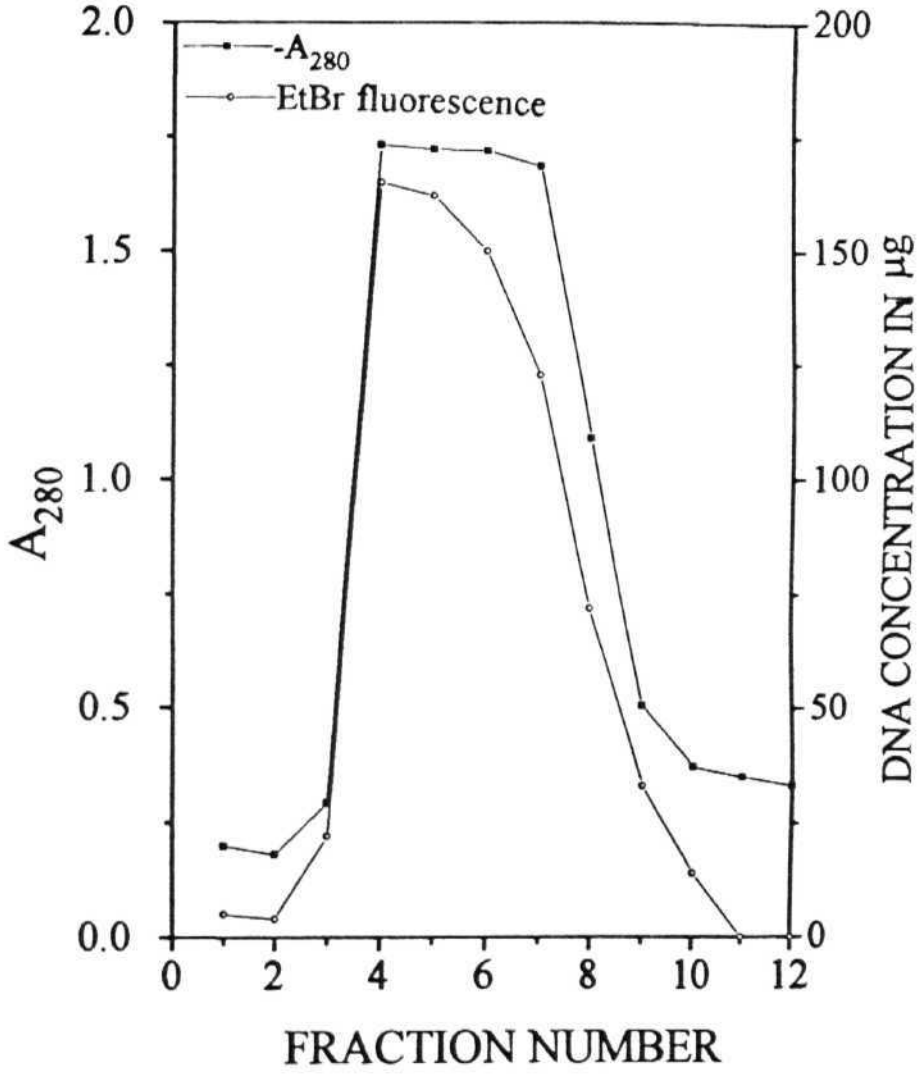
2.0
1.5
1.0
0.5
0.0
A_{280}
-A_{280}
EtBr fluorescence
200
150
100
50
0
DNA CONCENTRATION IN µg
0
2
4
6
8
10
12
FRACTION NUMBER

Figure 11A

Fig. **11B:** SDS-PAGE analysis of fractions obtained from De-52 column chromatography of sephacryl S-1000 peak II nucleoid fraction:

Fractions (20 µl each) were electrophoresed by 15% SDS-PAGE followed by silver staining.

Lane 1-2: Flowthrough fractions.
Lane 3-8: Fractions 3, 4, 5, 6, 7 and 9 respectively.
Lane 9: DBNP-B.
Lane 10: Molecular weight markers (66 kDa- BSA, 45 kDa-Ovalbumin, 25 kDa-Chymotrypsinogen, 20.1 kDa-Trypsin inhibitor, 12.3 kDa-Cytochrome-C).

Fig. 12: DNase I digestion of DEAE-cellulose nucleoid fraction:

Nucleoid fraction obtained from DEAE-cellulose column chromatography of pooled sephacryl S-1000 peak II nucleoid fraction was subjected to DNase I digestion at a ratio of DNA:DNase I of 10:1 at 37^0C for 30 seconds and analysed on a 0.8% agarose gel.

Lane 1: Nucleoid control (5µg).
Lane 2: Nucleoid (5µg) treated with DNase I.
Lane 3: Nucleoid control (3µg).
Lane 4: Nucleoid (5µg) treated with DNase I.
Lane 5 Nucleoid control (2µg).
Lane 6: Nucleoid (2µg) treated with DNase I.

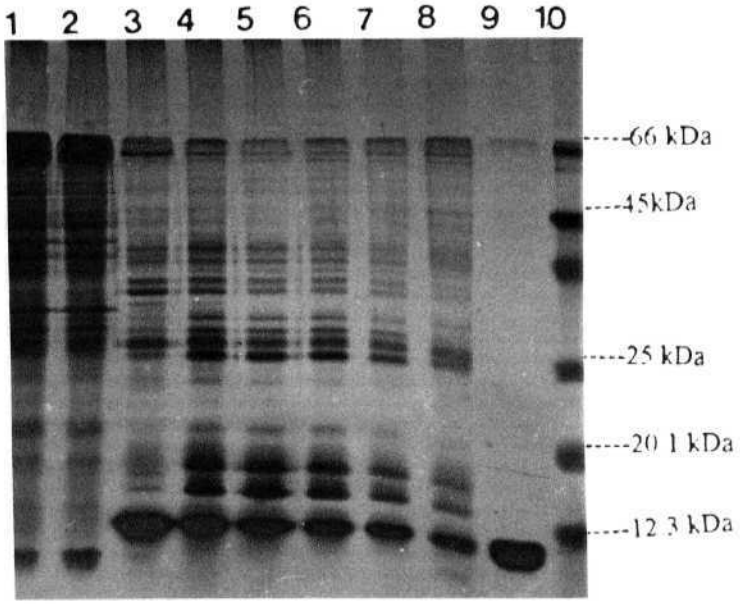

Figure 11B

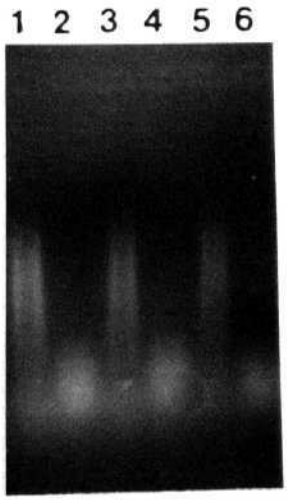

Figure 12

concentrations respectively and analysed on an agarose gel. As can be seen in Fig. 12 DNase 1 digestion generated small DNA fragments which appear as a smear at the bottom of the gel.

Micrococcal nuclease digestion of *S. acidocaldarius* nucleoid:

Micrococcal nuclease (MNase) digestion of *S. acidocaldarius* nucleoid isolated from freshly harvested midlogarthmic phase cells was also performed as described in methods section and the products were analysed by electrophoresing on an agarose gel (Fig. 13A to Fig.l3E). MNase digestion was performed with different concentrations of the enzyme for different time intervals to determine the optimal conditions for MNase digestion of *S. acidocaldarius* nucleoid. In all the cases MNase digestion results in ~ 600 bp to 1500 bp of DNA (as seen in Fig. 13A-E). The control nucleoid (in the absence of MNase) when loaded on **the** gel without SDS treatment is retained in the slot as seen in Fig. 13C lane 8 whereas nucleoid treated with MNase and loaded on the gel without SDS treatment is seen as a broad smear from top to the bottom of the gel along with some DNA retained in the slot (Fig. 13C lanes 6,7)- MNase digestion of nucleoid with increasing concentrations of the enzyme generates DNA fragments ranging from 600 bp-1500 bp seen as a smear on the gel (Fig. 13D and 13E) with strong intensity of bands at 600 bp and 1200 bp. The 600 bp DNA may correspond to the DNA length in the MNase digested core.

Fig. 13A: Micrococcal nuclease digestion of nucleoid:

Nucleoid isolated from freshly harvested cells was subjected to MNase digestion for different time intervals, deproteinised with 1% SDS and electrophoresed on a 2% agarose gel.

Lane 2-3: Nucleoid 1μg and 2 μg respectively (control).

Lane 5-7: 1 μg nucleoid digested with 0.1U MNase for 10 mins, 30 mins and 60 mins respectively.

Lane 8-10: 2 μg nucleoid digested with 0.2U MNase for 10 mins, 30 mins and 60 mins respectively.

Fig. 13B:

Nucleoid isolated from freshly harvested cells was subjected to MNase digestion with increasing concentrations of MNase for different time intervals, deproteinised with 1% SDS and electrophoresed on a 1.4% agarose gel.

Lane 1-2: Nucleoid 1μg and 2 μg respectively (control).

Lane 3-6: 1μg nucleoid incubated with 0.1U, 0.2U, 1U and 2 units MNase respectively for 60 mins.

Lane 7: 1 μg nucleoid incubated with 2 units of MNase for 30 mins.

Lane 8-11: 2 μg nucleoid incubated with 0.1U, 0.2U, 1U and 2 units MNase respectively for 60 mins.

Lane 12: 2 μg nucleoid incubated with 2 units of MNase for 30 mins.

Fig. 13C:

Nucleoid (2μg) isolated from freshly harvested cells was subjected to MNase digestion with increasing concentrations of MNase at 37°C for 1 hr, deproteinised with 1% SDS and electrophoresed on a 1.4% agarose gel.

Lane 1: Nucleoid (control).

Lane 2-5: Nucleoid incubated with 0.1U, 0.2U, 1U and 2 units MNase respectively .

Lane 6-7: Nucleoid incubated with 1U and 2 units of MNase respectively and loaded onto the gel without SDS treatment.

Lane 8: Nucleoid (control) loaded directly without SDS treatment.

Lane 10-11: Molecular weight markers.

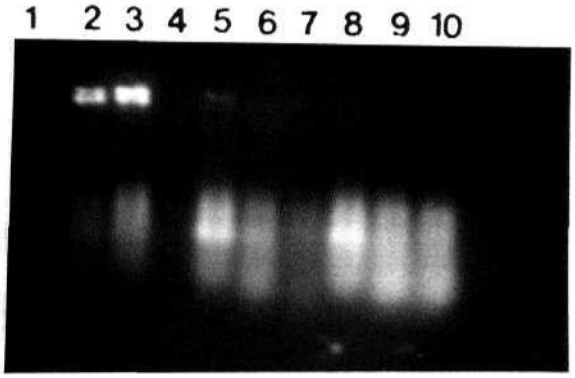

Figure 13A

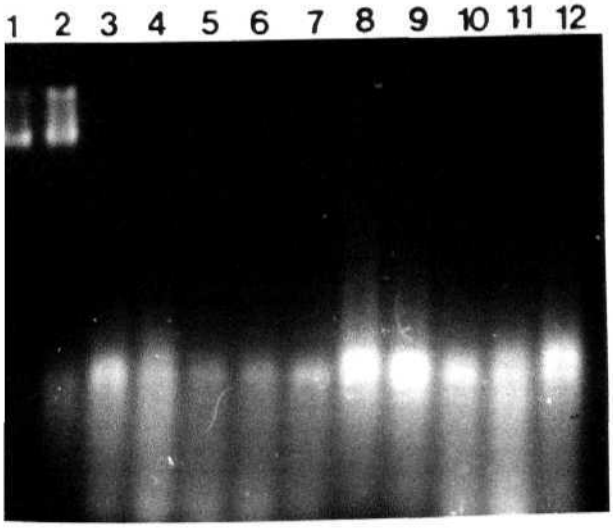

Figure 13B

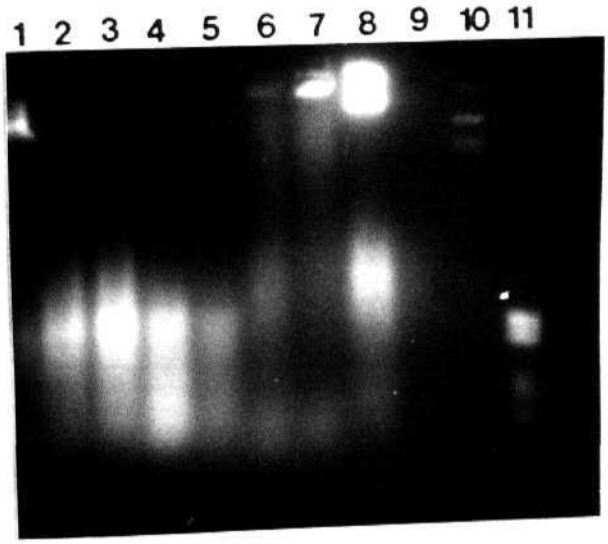

Figure 13C

Fig. 13D:

Nucleoid (1μg) isolated from freshly harvested cells was subjected to MNase digestion with increasing concentrations of MNase at 37^0C for 1 hr, deproteinised with 1% SDS and electrophoresed on a 1.4% agarose gel

Lane 1: Nucleoid (control).
Lane 2-5: Nucleoid incubated with 0.1U, 0.2U, 0.5 U and 1U MNase respectively.
Lane 6: Molecular weight markers (lOObp ladder consisting of 2072bp, 1500bp to lOObp).

Fig. 13E:

Nucleoid (1μg) isolated from freshly harvested cells was subjected to MNase digestion with increasing concentrations of MNase at 37^0C for 1 hr, deproteinised with 1% SDS and electrophoresed on a 1.4% agarose gel

Lane 1: Nucleoid (control).
Lane 2-6: Nucleoid incubated with 0.1U, 0.2U, 0.5 U, 1U and 2U MNase respectively.
Lane 8: Molecular weight markers (lOObp ladder) as in Fig. 13D.

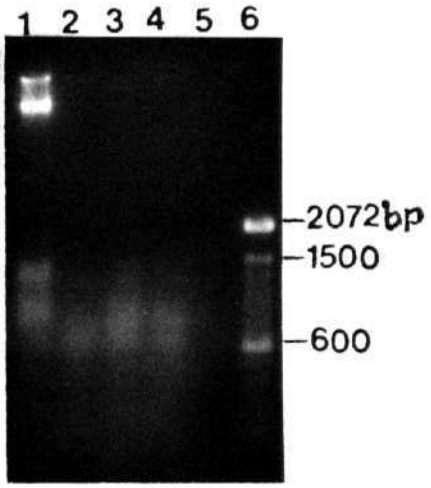

Figure 13D

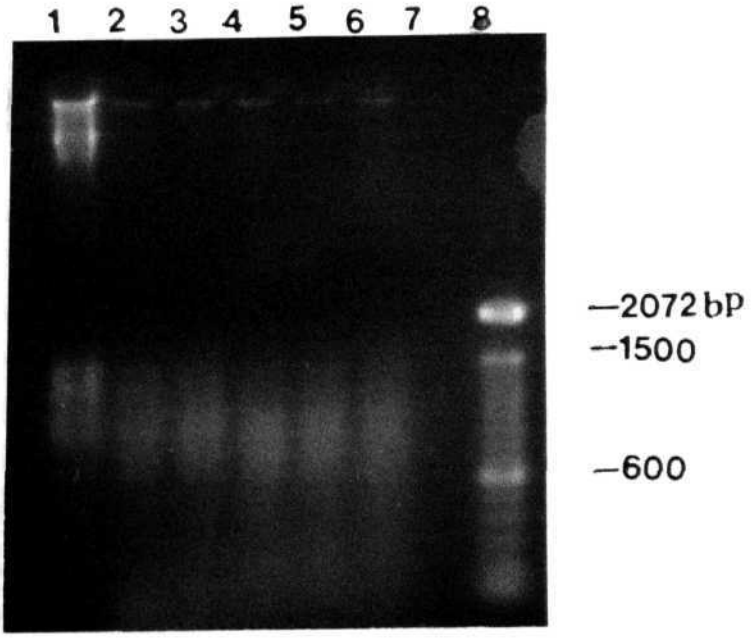

Figure 13E

Chapter 3.2. Purification and properties of DBNP-B:

The nucleoid of *S. acidocaldarius* contains HSNP-A, DBNP-B and HSNP-C' in abundance in the order HSNP-A<DBNP-B<HSNP-C'. Studies from our laboratory have shown that HSNP-C is one of the components of *S. acidocaldahus* nucleoid (Bohrmann *et al.,* 1994) and it condenses DNA to form compact structures (Francina and Suryanarayana, 1995; Francina, 1996). DBNP-B which is next to HSNP-C in abundance has been isolated in our laboratory and its nucleic acid binding properties were studied (Sreenivas *et al,* 1992; Sreenivas, 1994; Sreenivas *et al,* 1998). In tbe present study protein chemical, nucleic acid binding and functional properties of DBNP-B are presented.

Purification of **the DNA** binding nucleoid protein-B **(DBNP-B)** from *Sulfolobus acidocaldarius:*

DBNP-B, a 11 kDa DNA binding protein from *Sulfolobus acidocaldarius* was purified following the procedure of Reddy and Suryanarayana, (1989) with some modifications. The acid extract of post-ribosomal supernatant (S-100) or nucleoid was subjected to CM-cellulose chromatography as described in methods section. The proteins bound to the CM-cellulose column were eluted with 0.5 M KCl containing buffer and the fractions were analysed on a 15% SDS-polyacrylamide gel. As can be seen from Fig. 14A lanes 8-10 and Fig. 14B lanes 2-12 only DBNP-B was eluted from the column. These pure protein containing fractions were pooled, diluted to decrease the KCl concentration to 125 mM and applied onto a small CM-cellulose column (2ml bed volume). The column was eluted with 800 mM KCl containing buffer. The fractions containing concentrated protein were pooled, dialysed against 10 mM Tris-Cl (pH 7.6) and 1 mM DTT or 10 mM sodium acetate (pH 5.0) and 1 mM DTT and stored at -80°C The protein concentration was determined according to Lowry *et al,* 1951. The purity of the concentrated protein was checked by electrophoresing 10 μg protein on SDS-PAGE followed by silver staining (Fig. 14C). The protein was found to be more than 99% homogeneous.

Fig. 14: SDS-PAGE analysis of fractions obtained from CM-cellulose chromatography of acid treated post-ribosomal supernatants of *S.acidocaldarius*:

Protein in the alternate fractions (50 μl) was precipitated with 10% TCA and dissolved in SDS-gel loading buffer and electrophoresed on a 15% SDS-polyacrylamide gel.

Fig. 14 A:

Lane 1-10: Fraction numbers 5, 7, 9, 11, 13, 15, 17, 19, 21 and 23 respectively.
Lane 11: Post-ribosomal supernatant.
Lane 12: Acid treated post-ribosomal supernatant.

Fig. 14B:

Lane 2-12: Fractions numbers 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, and 47 respectively.

Fig. 14C: SDS-PAGE analysis of purified DBNP-B on a 15% polyacrylamide gel followed by silver staining:

Lane 1: Purified DBNP-B- 10 μg.
Lane 3: Molecular weight markers (66 kDa-BSA, 45 kDa-Ovalbumin, 25 kDa-Chymotrypsinogen, 20.1 kDa-Trypsin inhibitor and 12.3 kDa-Cytochrome-C).

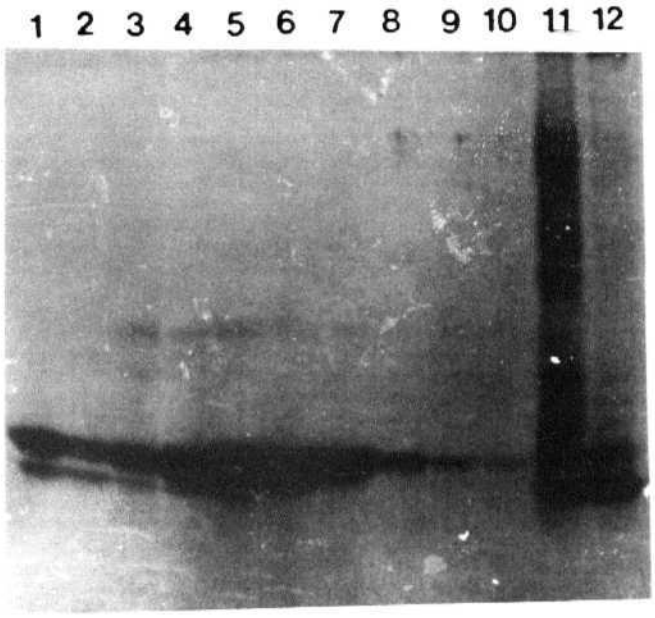

Figure 14A

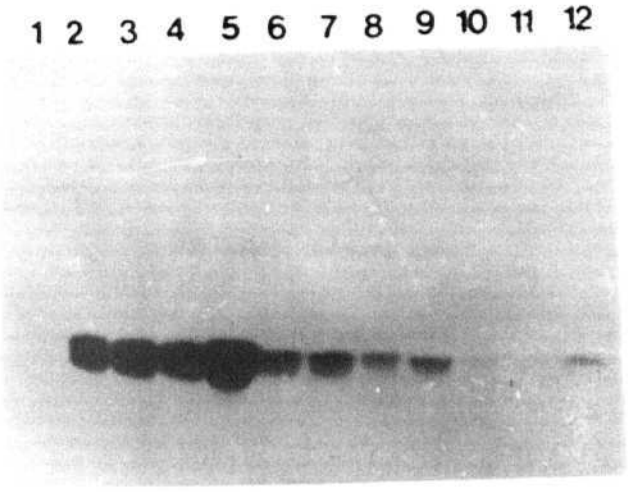

Figure 14B

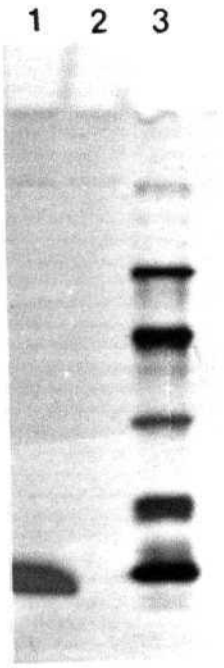

Figure 14C

Protein-Protein crosslinking:

Crosslinking was employed as a tool to study the solution state of DBNP-B. Crosslinking experiments were performed with bifunctional reagent DMS **and** also with zero level crosslinker, HCHO at three different temperatures and **pH.**

Formaldehyde crosslinking of DBNP-B:

Crosslinking for different time intervals was carried out at 37°C in 20 mM Triethanolamine (pH 7.5) or 100 mM sodium tetraborate (pH 8.2). Crosslinking in 20 mM Triethanolamine (pH 7.5) resulted in the formation of predominantly a dimer along with some trimeric, tetrameric and high molecular weight aggregates at all time intervals as seen in Fig.l5A lanes 1-5. Crosslinking for different time intervals at 37^0C in 100 mM sodium tetraborate (pH 8.2) also resulted in the formation of dimers along with tetramers, pentamers and high molecular weight aggregates (Fig.l5A lanes 7-11). The effect of NaCl concentration **on the** crosslinking pattern of DBNP-B was studied at 37°C. As seen in Fig .15B lanes 1-5 crosslinking of DBNP-B in the presence of NaCl resulted in a new crosslinked species with molecular weight of about 100 to 120 kDa optimally at 150-250 mM NaCl compared to the crosslinking in the absence of NaCl in 20 mM Triethanolamine (pH 7.5) at 37^0C.

Crosslinking of DBNP-B with HCHO at 37^0C in 10 mM sodium acetate (pH 5.0) however showed the formation of only dimeric product (Fig. 15C lanes 1-14) at all time intervals. Addition of $MgCl_2$ during crosslinking did not change the crosslinking pattern as only dimers are formed both in the presence of $MgCl_2$ (Fig. 15C lanes 2-7) and in the absence of $MgCl_2$ (Fig. 15C lanes 8-13) at 37^0C in pH 5.0 buffer. $MgCl_2$ has no effect on the crosslinking pattern in pH 5. 0 buffer as only dimers are formed both in the presence (Fig. 15C lanes 2-7) and absence (Fig. 15C lanes 8-13) of $MgCl_2$.

Crosslinking of DBNP-B with HCHO at 65°C in 20 mM Triethanolamine (pH 7.5) resulted in the formation of dimeric, trimeric, tetrameric, pentameric, hexameric and high molecular weight aggregates (Fig .16A). $MgCl_2$ seems **to** enhance the crosslinking of DBNP-B into high molecular weight aggregates **with**

Fig. 15A: SDS-PAGE analysis of DBNP-B crosslinked with HCHO at 37^0C:

HCHO crosslinking was performed with 5 μg of DBNP-B for different time intervals in 20 mM Triethanolamine (pH 7.5) or 0.1 M sodium tetraborate (pH 8.2) at 37°C and loaded on a 15% SDS-polyacrylamide gel followed by silver staining.

Lane 6: DBNP-B (control).
Lane 1-5: Crosslinking with HCHO for 1/2 hr, 1 hr, 2hrs, 3hrs and 6 hrs respectively in 20 mM Triethanolamine (pH 7.5).
Lane 7-11: Crosslinking with HCHO for 1/2 hr, 1 hr, 2hrs, 3hrs and 6 hrs respectively in 0.1 M sodium tetraborate (pH 8.2).

Fig. 15B: SDS-PAGE analysis of DBNP-B crosslinked with HCHO at 37°C:

HCHO crosslinking was performed with 5μg of DBNP-B for different time intervals at different NaCl concentrations in 20 mM Triethanolamine (pH 7.5) at 37^0C and loaded on a 15% SDS-polyacrylamide gel followed by silver staining.

Lane 6: DBNP-B (control).
Lane 1-5: Crosslinking with HCHO in 50 mM, 100 mM, 150 mM, 200 mM and 500 mM NaCl in 20 mM Triethanolamine (pH 7.5} respectively for 30 minutes.
Lane 7-11: Crosslinking in 20 mM Triethanolamine (pH 7.5) for 30 mins, 20 mins, 15 mins, 10 mins and 5 mins respectively.

Fig. 15C: SDS-PAGE analysis of DBNP-B crosslinked with HCHO at 37^0C in pH 5.0 buffer:

HCHO crosslinking was performed with 5μg of DBNP-B for different time intervals in 10 mM sodium acetate (pH 5.0) and in the presence or absence of 12 mM $MgCl_2$ at 37^0C and loaded on a 15% SDS-polyacrylamide gel followed by silver staining.

Lane 1: DBNP-B (control) in 10 mM Na acetate (pH 5.0), 12 mM $MgCl_2$.
Lane 2-7: Crosslinking for 10 mins, 15 mins, 30 mins, 60 mins, 120 mins and 180 mins respectively in the presence of 12 mM $MgCl_2$.
Lane 14: DBNP- B (control) in 10 mM Na acetate (pH 5.0).
Lane 8-13: Crosslinking for 10 mins, 15 mins, 30 mins, 60 mins, 120 mins and 180 mins respectively in the absence of 12 mM $MgCl_2$.
Lane 16: Molecular weight markers (66 kDa-BSA, 45 kDa-Ovalbumin and 29-kDa-Carbonic anhydrase).

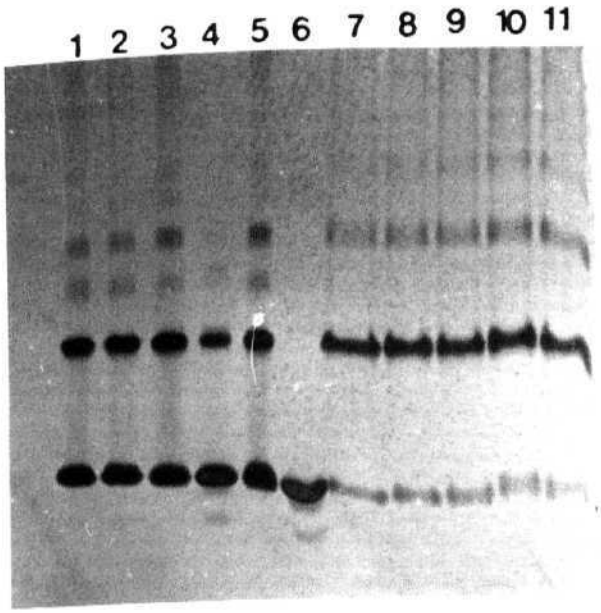

Figure 15A

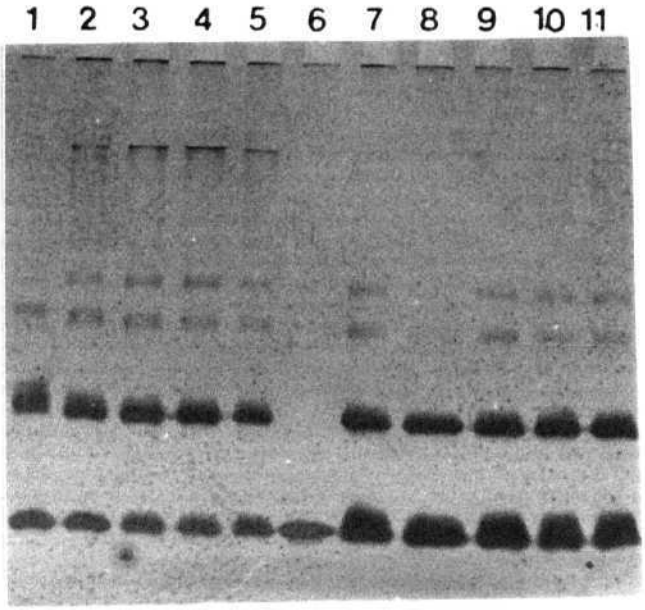

Figure 15B

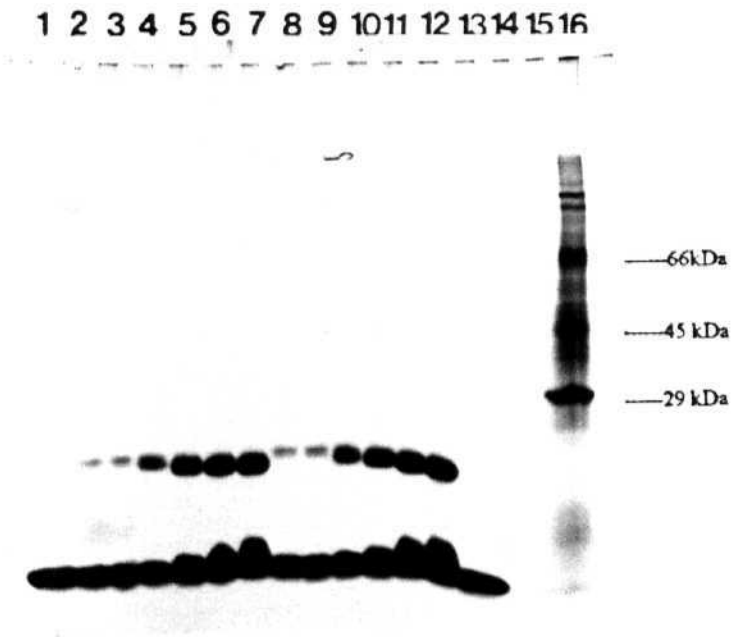

Figure 15C

Fig. **16A:** SDS-PAGE analysis of DBNP-B crosslinked with HCHO at 65^0C in pH 7.5 buffer:

HCHO crosslinking was performed with 5μg of DBNP-B in 20 mM Triethanolamine (pH 7.5) in the presence or absence of 12 mM $MgCl_2$ at 65^0C for different time intervals and loaded on a 15% SDS-polyacrylamide gel followed by silver staining.

Lane 8: DBNP-B (control).
Lane 2-7: Crosslinking for 10 mins, 15 mins, 30 mins, 60 mins, 120 mins and 180 mins respectively in the absence of $MgCl_2$.
Lane 9-14: Crosslinking for 10 mins, 15 mins, 30 mins, 60 mins, 120 mins and 180 mins respectively in the presence of $MgCl_2$.
Lane 16: Molecular weight markers (66 kDa- BSA, 45 kDa-Ovalbumin, 25 kDa-Chymotrypsinogen, 20.1 kDa -Trypsin inhibitor and 12.3 kDa-Cytochrome-C).

Fig. **16B:** SDS-PAGE analysis of DBNP-B crosslinked with HCHO at 65°C in pH 5.0 buffer:

HCHO crosslinking was performed with 5μg of DBNP-B in 10 mM sodium acetate (pH 5.0) in the presence or absence of 12 mM $MgCl_2$ at 65^0C for different time intervals and loaded on a 15% SDS-polyacrylamide gel followed by silver staining.

Lane 8: DBNP-B (control).
Lane 2-7: Crosslinking for 10 mins, 15 mins, 30 mins, 60 mins, 120 mins and 180 mins respectively in the absence of $MgCl_2$.
Lane 9-14: Crosslinking for 10 mins, 15 mins, 30 mins, 60 mins, 120 mins and 180 mins respectively in the presence of $MgCl_2$.
Lane 16: Molecular weight markers (66 kDa- BSA, 45 kDa -Ovalbumin, 25 kDa-Chymotrypsinogen, 20.1 kDa-Trypsin inhibitor and 12.3 kDa-Cytochrome-C).

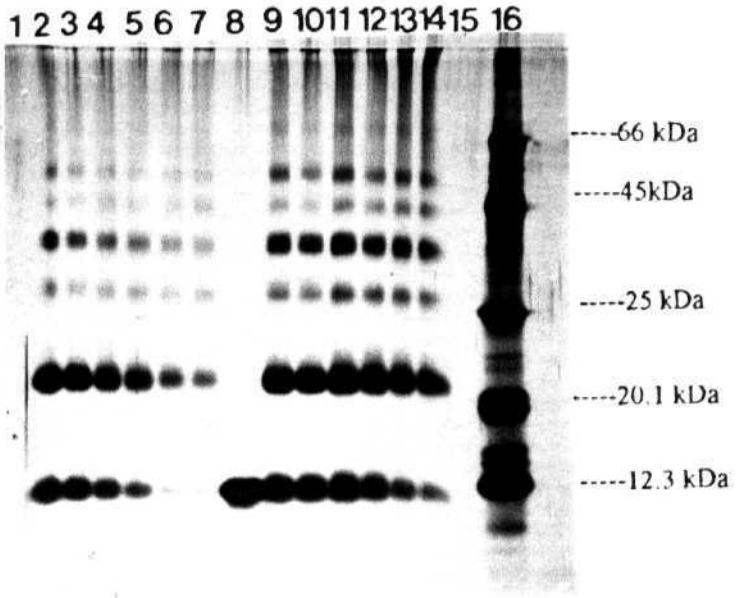

Figure 16A

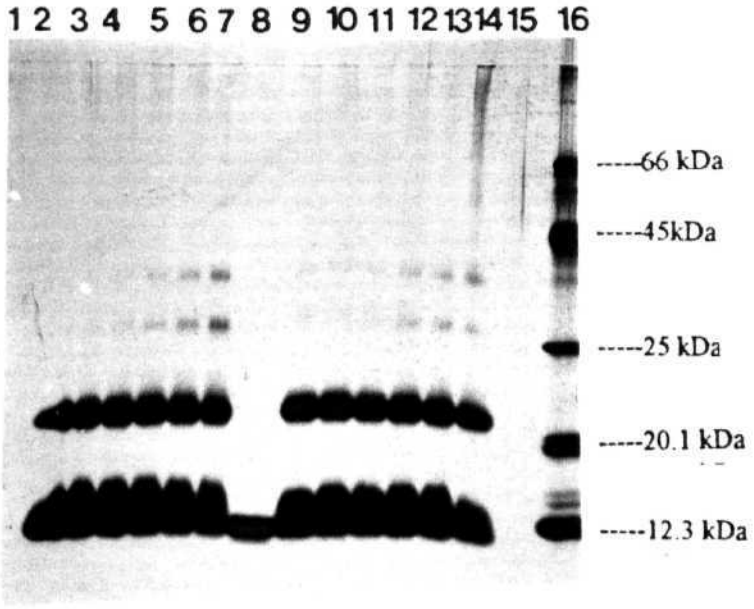

Figure 16B

increased tetrameric form as seen in Fig.l6A lanes 9-14 (presence of $MgCl_2$) and Fig. 16A lanes 2-7 (absence of $MgCl_2$).

Crosslinking of DBNP-B with HCHO at 65^0C in 10 mM sodium acetate (pH 5.0) (Fig.l6B) resulted in the formation of a 22kDa dimeric form along with low amounts of trimeric and tetrameric forms progressively with time. $MgCl_2$ seems to enhance the formation of high molecular weight aggregates with prolonged incubation at 65°C even in pH 5.0 buffer (Fig.l6B lanes 9-14).

As the crosslinking of DBNP-B is enhanced by elevated temperature and $MgCl_2$, crosslinking of DBNP-B with HCHO was performed at 80^0C in the presence of $MgCl_2$ both at pH 5.0 and 7.5. At pH 7.5 there was formation of dimers, trimers, tetramers, pentamers, hexamers and high molecular weight aggregates within 2 minutes and longer incubation for > 30 minutes resulted in the formation of high molecular weight aggregates with disappearance of lower molecular weight aggregates (Fig. 17 lanes 1-7). In 10 mM sodium acetate (pH 5.0) buffer at 80^0C crosslinking resulted in the formation of trimers, tetramers, pentamers, hexamers and high molecular weight aggregates with incubation for longer time periods (> 30 mins) (Fig. 17 lanes 9-15).

These results indicate that DBNP-B exists predominantly in a dimeric state in pH 5.0 containing buffer at 37^0C and 65°C and it probably exists as oligomeric forms at 80°C in the presence of $MgCl_2$.

As a control, HCHO crosslinking was performed with a low molecular weight histone like DNA binding protein H-NS from *E. coli*. Crosslinking of H-NS with HCHO at 37°C in 20 mM Triethanolamine-HCl (pH 7.5) for different time intervals upto 2 hrs resulted in the formation of predominantly a tetramer (Fig. 18) which is the form in which H-NS has been shown to exist *in vivo* (Falconi *et al.*, 1988).

Dimethyl suberimidate (DMS) crosslinking of DBNP-B:

Crosslinking of DBNP-B with DMS resulted in the formation of a crosslinked product of 22 kDa (dimeric form) after 24 hrs at 65°C (Fig. 19 lanes 1 and 2).

Fig. 17: SDS-PAGE analysis of DBNP-B crosslinked with HCHO at 80^0C:

HCHO crosslinking was performed with 5μg of DBNP-B in 20 mM Triethanolamine (pH 7.5) or 10 mM sodium acetate (pH 5.0) in presence of 12 mM $MgCl_2$ at 80°C for different time intervals and loaded on a 15% SDS-polyacrylamide gel followed by silver staining.

Lane 8: DBNP-B (control).
Lane 1-7: Crosslinking for 2 mins, 5 mins, 10 mins, 15 mins, 30 mins, 60 mins and 120 mins respectively in 20 mM Triethanolamine (pH 7.5).
Lane 9-15: Crosslinking for 2 mins, 5 mins, 10 mins, 15 mins, 30 mins, 60 mins and 120 mins respectively in 10 mM sodium acetate (pH 5.0).
Lane 16: Molecular weight markers (66 kDa- BSA, 45 kDa-Ovalbumin, 25 kDa-Chymotrypsinogen, 20.1 kDa-Trypsin inhibitor and 12.3 kDa-Cytochrome-C).

Fig. 18: SDS-PAGE analysis of *E. call* H-NS crosslinked with HCHO at 37^0C:

HCHO crosslinking was performed with 10 μg of H-NS in 20 mM Triethanolamine (pH 7.5), 12 mM $MgCl_2$ and 50 mM NaCl at 37^0C for different time intervals and loaded on a 15% SDS-polyacrylamide gel followed by silver staining.

Lane 1-4: Crosslinking for 15 mins, 30 mins, 60 mins and 120 mins respectively.
Lane 6: H-NS (control).

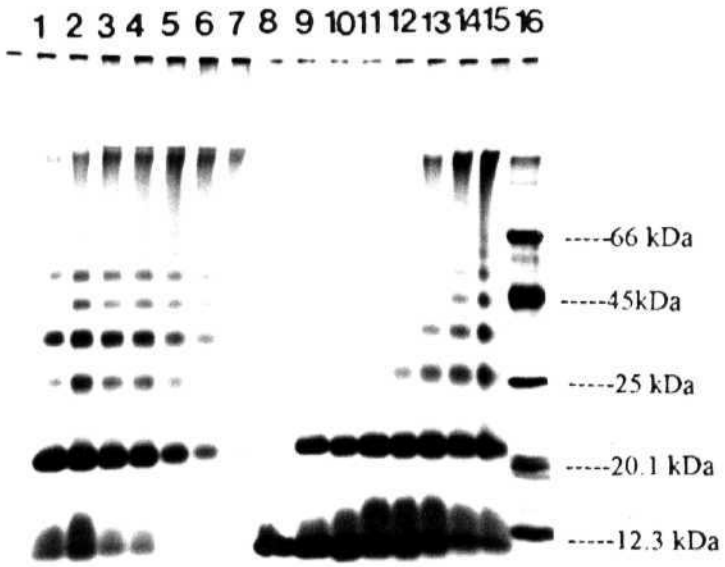

Figure 17

Figure 18

Fig. 19: SDS-PAGE analysis of DBNP-B crosslinked with DMS:

DMS crosslinking was performed as described in methods section with 5μg of DBNP-B for different time intervals at 65^{0}C and loaded on a 15% SDS-polyacrylamide gel followed by silver staining.

Lane 3: DBNP-B (control).
Lane 1-2: Crosslinking with DMS for 24 hrs and 16 hrs respectively.
Lane 7: Molecular weight markers (66 kDa-BSA, 45 kDa-Ovalbumin, 25 kDa-Chymotrypsinogen, 20.1 kDa-Trypsin inhibitor and 12.3 kDa-Cytochrome-C).

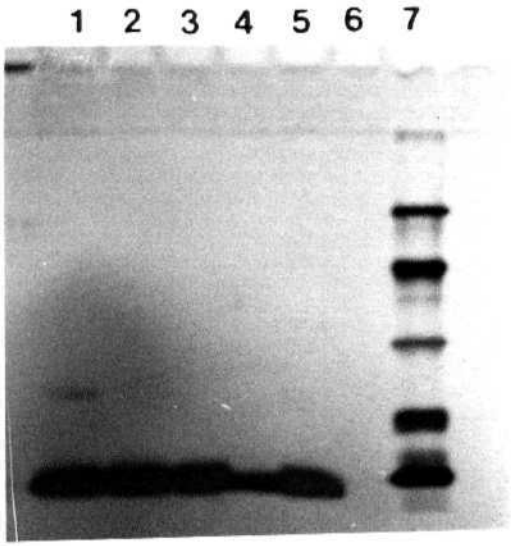

Figure 19

Gel filtration chromatography of DBNP-B:

The solution state of DBNP-B was also studied by gel filtration chromatography at room temperature. A sephadex G-50 column (100 ml bed volume) was equilibrated with buffer containing 20 mM Tris-Cl (pH 7.6), 50 mM KCl, 1 mM Na_2-EDTA and 7 mM β-mercaptoethanol. DBNP-B was loaded and the column was eluted with the above buffer. Similarly molecular weight marker proteins were also chromatographed. DBNP-B eluted as aggregates corresponding to dimer predominantly along with some tetrameric protein (Fig.20).

Fluorescence spectrum of **DBNP-B:**

DBNP-B in 10 mM sodium acetate (pH 5.0) showed a low fluorescence emission at 304 nm when excited at its excitation maximum of 274 nm (Fig.21A spectrum 1) compared to fluorescence emission which has increased several fold on addition of $MgCl_2$ to a final concentration of 12 mM (Fig.21A spectrum 2). The fluorescence intensity of DBNP-B was maximum on addition of 50 mM NaCl in 10 mM sodium acetate (pH 5.0), 12 mM $MgCl_2$ buffer (Fig.21A spectrum 3). DBNP-B in 10 mM Tris-Cl (pH 7.6) (Fig.21B spectrum 1) resulted in significant increase in its fluorescence emission although not to the same extent as at pH 5.0 (Fig.21B spectrum 2 and spectrum 3). This increase in fluorescence emission of DBNP-B upon addition of $MgCl_2$ was nullified when EDTA is added (Fig.21C) suggesting that Mg^{++} probably binds to the protein.

These results indicate conformational changes in DBNP-B upon addition of $MgCl_2$. The effect was more pronounced at pH 5.0, which is the optimal pH for renaturation of complementary single stranded DNA promoted by DBNP-B, in the presence of Mg^{++}. DNA aggregation by DBNP-B studied by light scattering at pH 5.0 also showed a 10 mM $MgCl_2$ optimum (see chapter 3.3).

Chemical and proteolytic cleavage of **DBNP-B:**

DBNP-B was cleaved chemically with cyanogen bromide and proteolytically with V8 protease, trypsin and chymotrypsin.

Fig. 20: Gel filtration chromatography of DBNP-B in native state:

DBNP-B (200 μg) was loaded on a sephadex G-50 column (100 ml bed volume) and eluted with 20 mM Tris-Cl (pH 7.6), 50 mM KCl, 1 mM Na_2-EDTA and 7 mM β-mercaptoethanol. Fractions (2 ml) collected were analysed by measuring the absorbance of 1 ml fraction at 280 nm. Similarly protein molecular weight markers (66 kDa-BSA, 45 kDa-Ovalbumin and 12.3 kDa-Cytochrome-C) were loaded on the column, eluted and analysed at 280 nm.

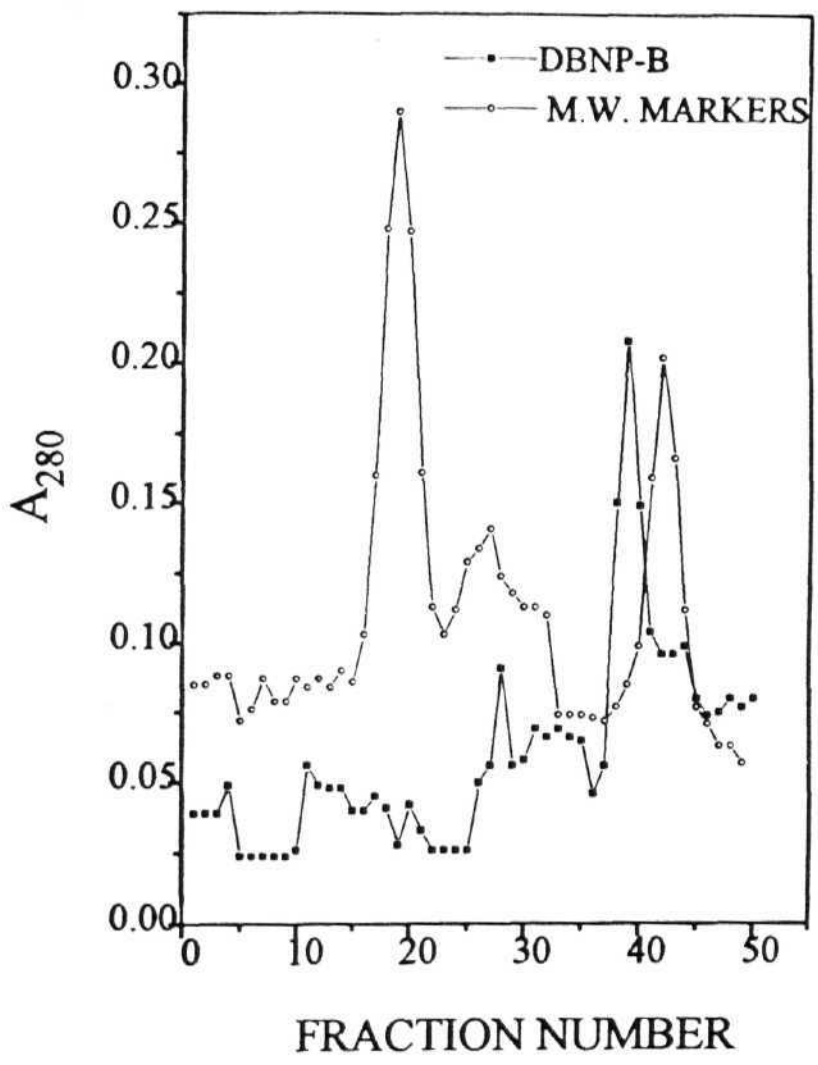
DBNP-B
M.W. MARKERS
0.30
0.25
0.20
0.15
0.10
0.05
0.00
A_{280}
0
10
20
30
40
50
FRACTION NUMBER

Figure 20

Fig. 21 A: Fluorescence emission spectrum of DBNP-B in 10 mM Na acetate (pH 5.0):

DBNP-B (5 μg) in 1ml of 10 mM Na acetate (pH 5.0) was excited at 274 nm and the emission spectrum was recorded.

Spectrum 1: DBNP-B in 10 mM sodium acetate.
Spectrum 2: DBNP-B in 10 mM sodium acetate, 12 mM $MgCl_2$.
Spectrum 3: DBNP-B in 10 mM sodium acetate, 12 mM $MgCl_2$ and 50 mM NaCl.

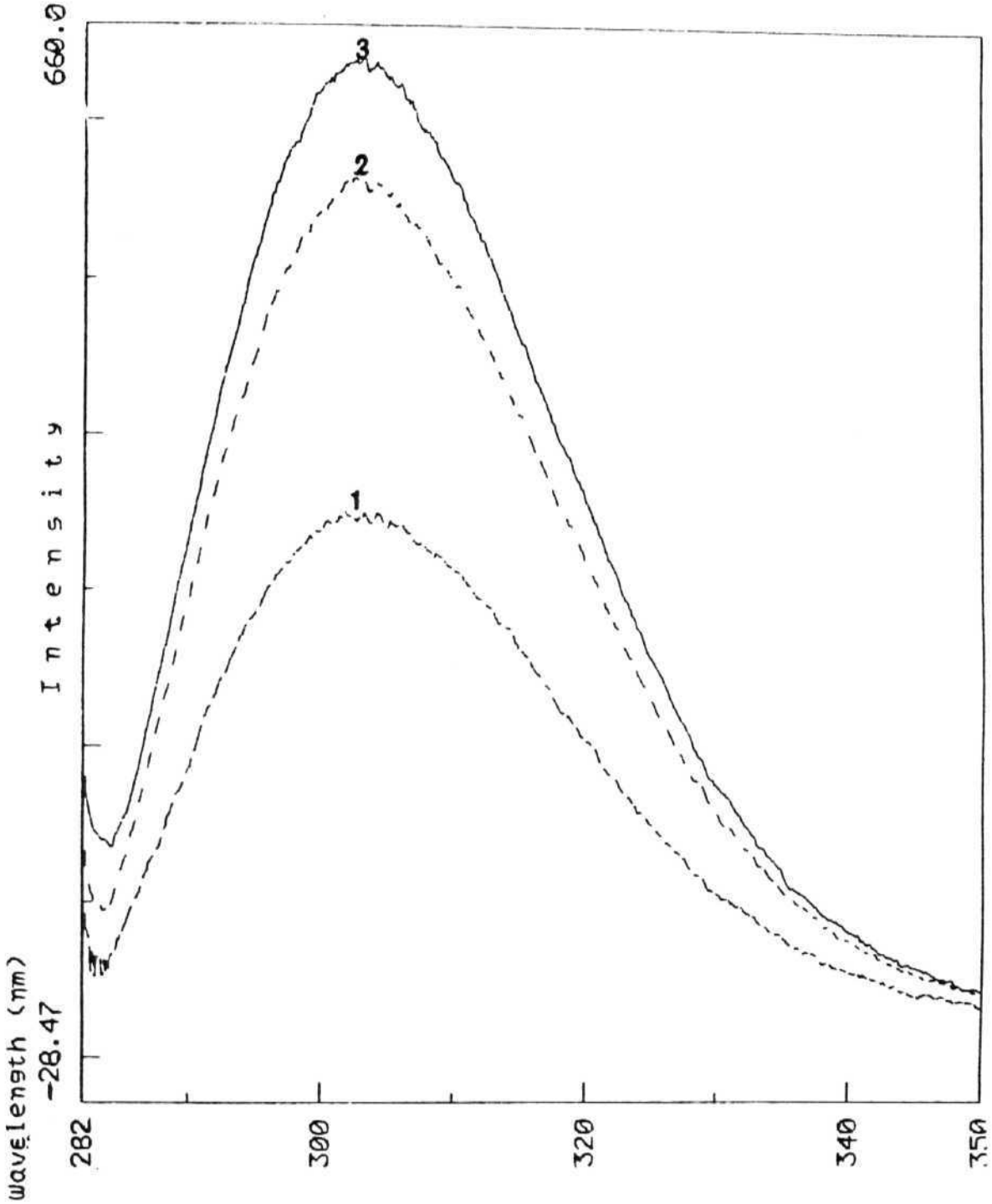

Figure 21A

Fig. **21B:** Fluorescence emission spectrum of DBNP-B in 10 mM Tris-Cl (pH 7.6):

DBNP-B (5 μg) in 1ml of 10 mM Tris-Cl (pH 7.6) was excited at 274 nm and the emission spectrum was recorded.

Spectrum 1: DBNP-B in 10 mM Tris-Cl (pH 7.6).
Spectrum 2: DBNP-B in 10 mM Tris-Cl (pH 7.6), 12 mM $MgCl_2$.
Spectrum 3: DBNP-B in 10 mM Tris-Cl (pH 7.6), 12 mM $MgCl_2$ and 50 mM NaCl.

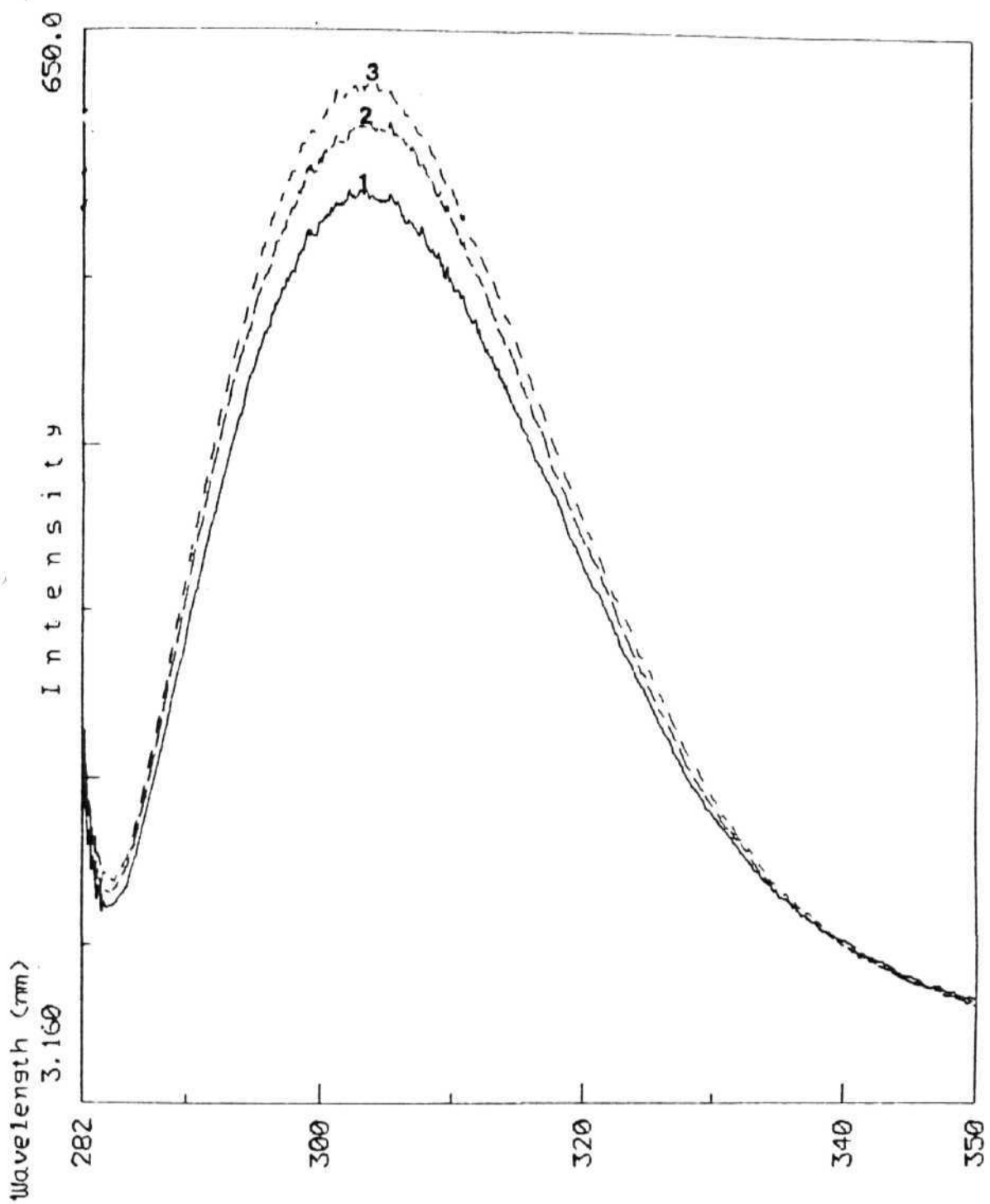

Figure 21B

Fig. **21C:** Fluorescence emission spectrum of DBNP-B in 10 mM Na acetate (pH 5.0):

DBNP-B (10 μg) in 1ml of 10 mM Na acetate (pH 5.0) was excited at 274 nm and the emission spectrum was recorded.

Spectrum 1: DBNP-B in 10 mM sodium acetate.
Spectrum 2: DBNP-B in 10 mM sodium acetate, 12 mM $MgCl_2$.
Spectrum 3: DBNP-B in 10 mM sodium acetate, 12 mM $MgCl_2$ and 50 mM Na_2EDTA.

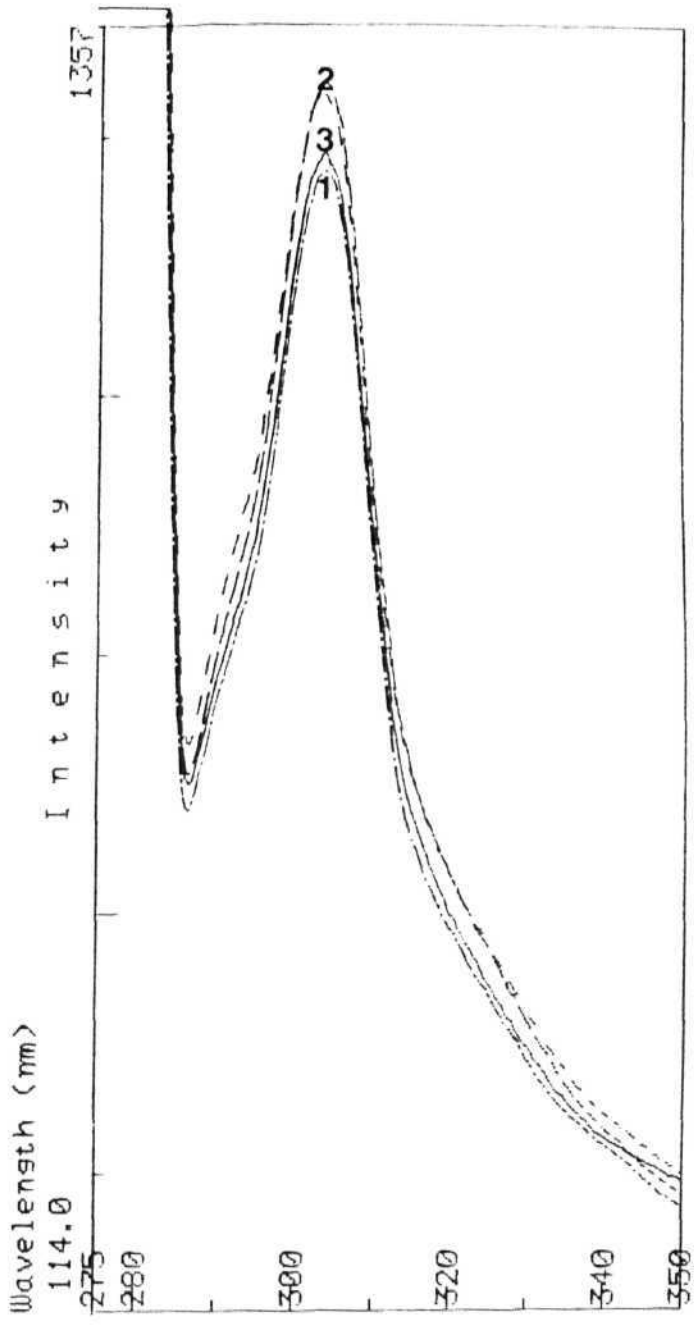
Wavelength (nm)
114.0
275
280
300
320
340
350
Intensity
1357
1
2
3

Figure 21C

V8 protease cleavage of DBNP-B:

V8 protease cleaves peptide bonds specifically on the carboxyl terminal side of either aspartic acid or glutamic acid. Results presented in Fig.22A lanes 2-6 show that within 5 minutes a fragment with an apparent molecular weight of 8 kDa appears in abundance which is stable upto 2 hrs of incubation with V8 protease. Upon further incubation, this 8 kDa fragment is entirely displaced by an ~ 6 kDa fragment (Fig.22B lanes 2-6). These results suggest that atleast two aspartic acid or glutamic acid residues are present in DBNP-B either at the N-terminal or C-terminal region of the molecule.

Cyanogen bromide cleavage **of DBNP-B:**

Cyanogen bromide specifically cleaves proteins at the carboxyl end of their methionine residues. The products after cyanogen bromide cleavage as analysed on a 15% SDS-polyacrylamide gel (Fig.23) led to the formation of two polypeptides with a Mr of ~ 8 kDa and 6 kDa. Both these fragments appear by 12 hours of treatment, the larger one in abundance. In 24 hours some of the protein intensity corresponding to the 8 kDa fragment decreases and it is probably degraded into the smaller fragment as the intensity of the protein band corresponding to the 6 kDa fragment increases. After 24 hours both these fragments seem to be stable as they are resistant to further degradation even after 48 hour treatment with cyanogen bromide. These results suggest the presence of atleast two methionine residues in the protein which are either at the N-terminal or C-terminal region of DBNP-B.

Trypsin and Chymotrypsin cleavage of **DBNP-B:**

Results presented in Fig.24 (lanes 1-4) show that DBNP-B is resistant to trypsin even after 6 hrs of treatment with trypsin even at a protein:enzyme ratio of 20:1 at 37^0C.

Treatment of DBNP-B with chymotrypsin results in an ~ 10 kDa fragment after 1 hr of incubation. There seems to be no further proteolytic cleavage (Fig.24 lanes 6-9) even after 6 hrs of incubation with chymotrypsin.

Fig. 22: SDS-PAGE analysis of DBNP-B digested with V8 protease:

DBNP-B in 125 mM Tris-Cl (pH 6.8), 10% glycerol and 0.5% SDS was digested with 10:1 ratio of DBNP-B: V8 protease for different time intervals. At different time intervals aliquots were taken out and the reaction was stopped by adding 10% (3-mercaptoethanol and *2%* SDS and the reaction products were analysed on a 15% SDS-polyacrylamide gel followed by silver staining.

Fig.22A:

Lane 7: DBNP-B (control).
Lane 8: Molecular weight markers (66 kDa- BSA, 45 kDa -Ovalbumin, 25 kDa-Chymotrypsinogen, 20.1 kDa -Trypsin inhibitor, 12.3 kDa-Cytochrome-C and 7 kDa- HSNP-C').
Lane 2-6: DBNP-B treated with V8 protease for 120 mins, 60 mins, 30 mins, 15 mins and 5 mins respectively.

Fig. 22B:

Lane 7: DBNP-B (control).
Lane 9: Molecular weight markers (66 kDa-BSA, 45 kDa -Ovalbumin, 25 kDa-Chymotrypsinogen, 20.1 kDa -Trypsin inhibitor, 12.3 kDa-Cytochrome-C and 7 kDa-HSNP-C).
Lane 2-6: DBNP-B treated with V8 protease for 6 hrs, 4 hrs, 2 hrs, 1 hr and 1/2 hr respectively.

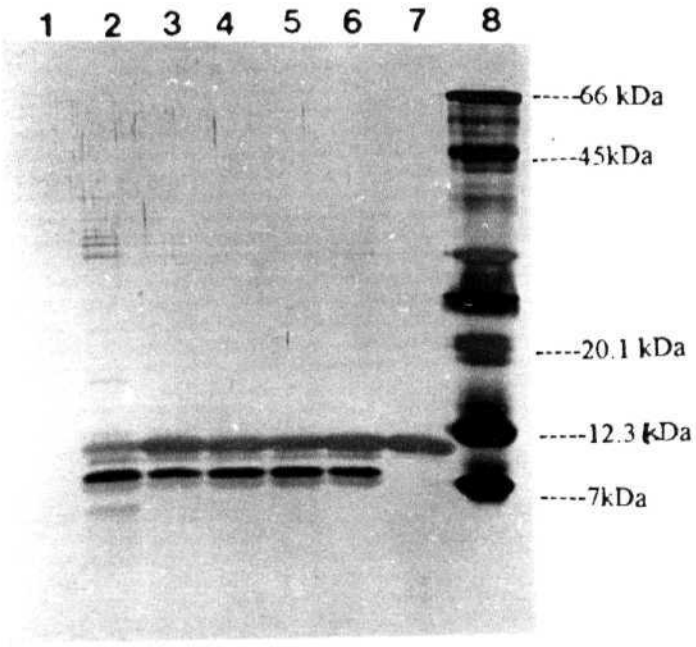

Figure 22A

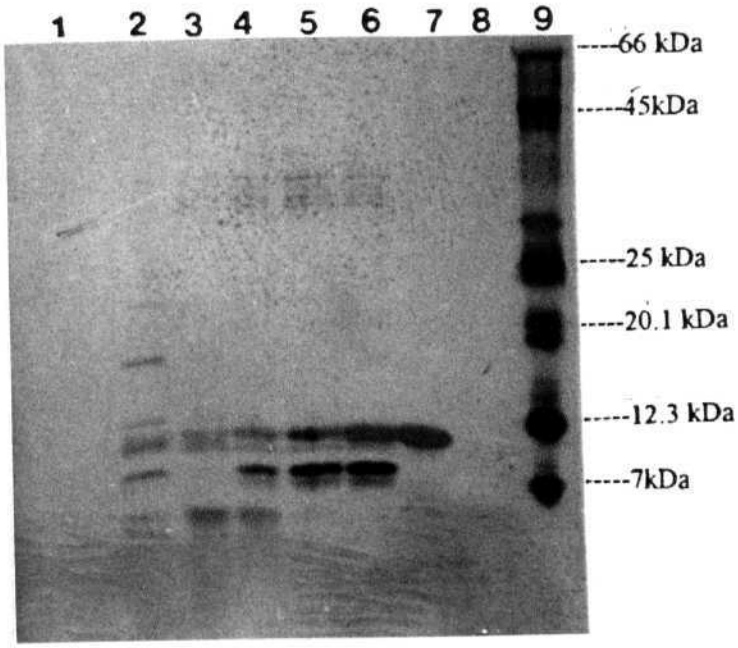

Figure 22B

Fig. 23: SDS-PAGE analysis of DBNP-B cleaved with cyanogen bromide:

DBNP-B in 90% formic acid was reacted with cyanogen bromide under N_2 for different time intervals. At different time intervals aliquots were taken out, vacuum dried and dissolved in SDS-gel loading buffer. These reaction products were analysed on a 15% SDS-polyacrylamide gel followed by silver staining .

Lane 10: Molecular weight markers (66 kDa- BSA, 45 kDa -Ovalbumin, 25 kDa-Chymotrypsinogen, 20.1 kDa -Trypsin inhibitor, 12.3 kDa-Cytochrome-C and 7 kDa- HSNP-C').
Lane 8: Native DBNP-B (control).
Lane 2-5: DBNP-B treated with CNBr for 12 hrs, 24 hrs, 36 hrs and 48 hrs respectively.

Fig. 24: SDS-PAGE analysis of DBNP-B cleaved with trypsin and chymotrypsin:

DBNP-B in 10 mM Triethanolamine (pH 8.0) was reacted with 20:1 protein:enzyme concentration of TPCK-trypsin or chymotrypsin at 37^0C for different time intervals. The reaction was stopped by precipitating with 10% TCA and dissolved in SDS-gel loading buffer and analysed on a 15% SDS-polyacrylamide gel followed by silver staining.

Lane 5: DBNP-B (control).
Lane 10: Molecular weight markers (66 kDa-BSA, 45 kDa-Ovalbumin, 25 kDa-Chymotrypsinogen, 20.1 kDa-Trypsin inhibitor, 12.3 kDa-Cytochrome-C and 7 kDa-HSNP-C').
Lane 1-4: DBNP-B treated with TPCK-trypsin for 1 hr, 2hrs, 4 hrs and 6 hrs respectively.
Lane 6-9: DBNP-B treated with chymotrypsin for 6hrs, 4hrs, 2hrs and 1 hr respectively.

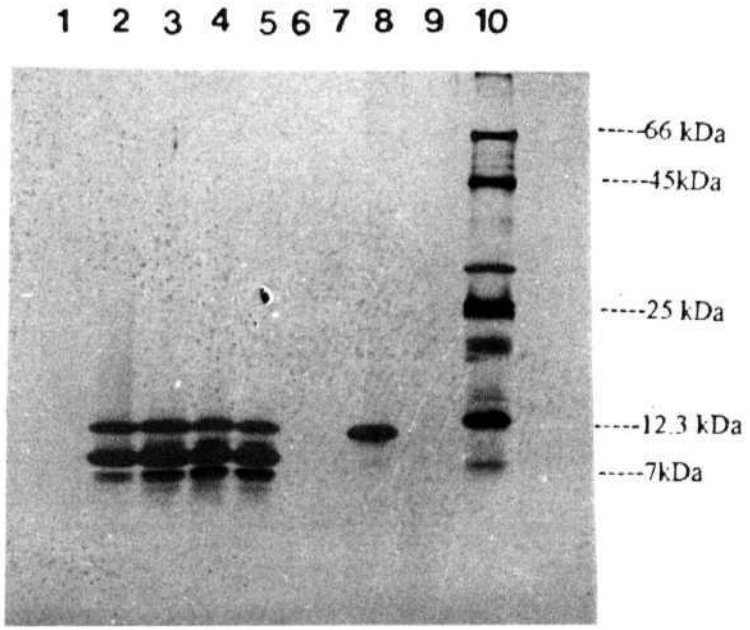

Figure 23

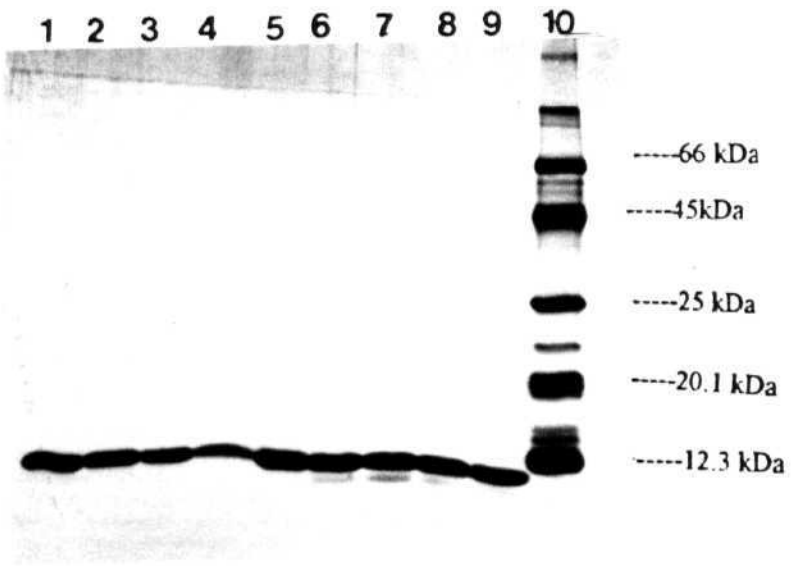

Figure 24

Chapter 3.3. Nucleic acid binding properties of DBNP-B:

Several techniques have been employed to study the interaction between DBNP-B and DNA as well as RNA involving mobility shift assays, fluorescence titrations, DNA aggregation, DNA renaturation and other assays.

Gel **mobility shift** assays:

The binding of DBNP-B to different DNAs was analysed by gel mobility shift analysis on agarose gels. DBNP-B binding to pUC19 supercoiled DNA, SV40 DNA, pBR322 DNA and pBR322 relaxed form of DNA are shown in Fig. 25A, 25B, 25C and 25D respectively.

The mobility of DBNP-B-pUC19 supercoiled DNA complexes was retarded (Fig.25A lanes 2-13) compared to the DNA in the absence of DBNP-B (Fig.25A lane 1). Retardation of DBNP-B-DNA complexes increases with increasing amounts of DBNP-B. Upto a ratio of protein to DNA of 24, the retardation of the DNA was low and progressive. Above this ratio highly retarded complexes were formed.

DBNP-B binding to SV40 DNA is shown in Fig.25B. The mobility of DBNP-B-SV40 DNA (containing both relaxed and supercoiled forms) is retarded in the gel (Fig.25B lanes 2-11). The retardation of nucleoprotein complexes increases with increasing amounts of the protein. At higher protein to DNA (>30 w/w) these complexes fail to enter the gel and remain in the wells of the agarose gel indicating aggregation of both relaxed and supercoiled forms of SV40 DNA.

DBNP-B binds to both supercoiled and relaxed forms of pBR322 DNA and the mobility of the DBNP-B-pBR322 DNA complexes is retarded in the gel (Fig.25C lanes 2-11). The retardation increases with increasing amounts of the protein. At higher protein to DNA (>32 w/w) the complexes fail to enter the gel and remain in the wells of the agarose gel indicating aggregation.

Binding of DBNP-B to relaxed form of pBR322 DNA was also tested by gel mobility shift assay (Fig.25D). DBNP-B-DNA complexes are also retarded

Fig. 25 Gel mobility shift analysis of DBNP-B-DNA complexes:

DNA was incubated with increasing amounts of DBNP-B and analysed on a 0.8% agarose gel.

Fig. 25A: pUC 19 supercoiled DNA (300 ng).

Lane 1: DNA incubated in the absence of DBNP-B (control).
Lane 2-13: DNA incubated with 0.3µg, 0.9µg, 1.8µg, 2.4µg, 3µg, 4.8µg, 6µg, 7.2µg, 9.6µg, 19.2µg, 37.5µg and 75µg of DBNP-B respectively.

Fig. 25B: SV40 DNA (300 ng) containing both supercoiled and relaxed fonns.

Lane 1: DNA incubated in the absence of DBNP-B (control).
Lane 2-11: DNA incubated with 0.3µg, 0.9µg, 1.8µg, 2.4µg, 3µg, 4.8µg, 6µg, 7.2µg, 9.6µg and 19.2µg of DBNP-B respectively.

Fig. 25C: pBR322 DNA (500 ng) containing supercoiled and relaxed fonns.

Lane 1: DNA incubated in the absence of DBNP-B (control).
Lane 2-11: DNA incubated with 0.5µg, 1.5µg, 3µg, 4µg, 5µg, 8µg, 10µg, 12µg, 16µg and 32µg of DBNP-B respectively.

Fig. 25D: pBR322 DNA (500 ng) containing relaxed form.

Lane 1: DNA incubated in the absence of DBNP-B (control).
Lane 2-8: DNA incubated with 0.3µg, 0.9µg, 1.8µg, 2.4µg, 3µg, 4µg and 6µg of DBNP-B respectively.

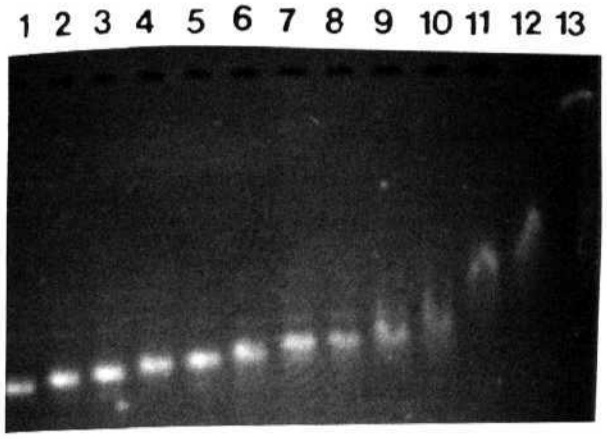

Figure 25A

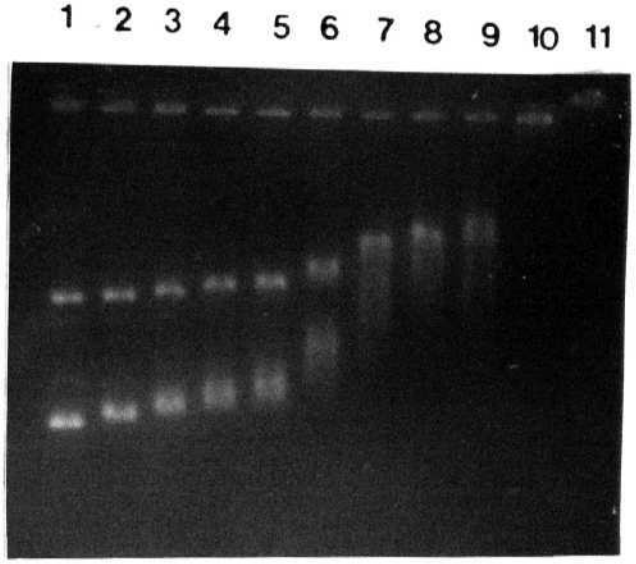

Figure 25B

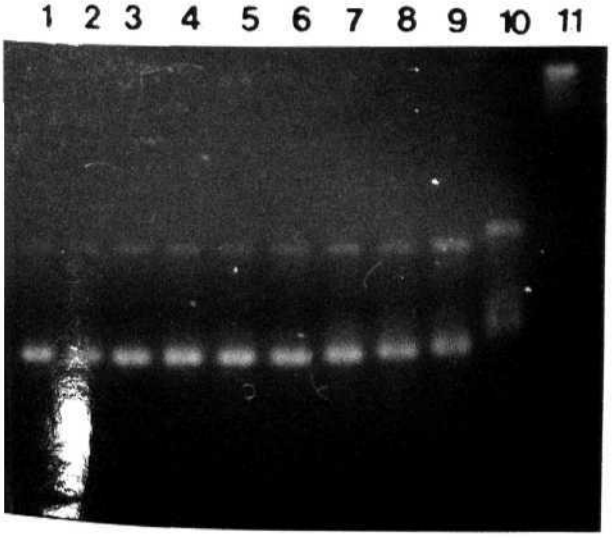

Figure 25C

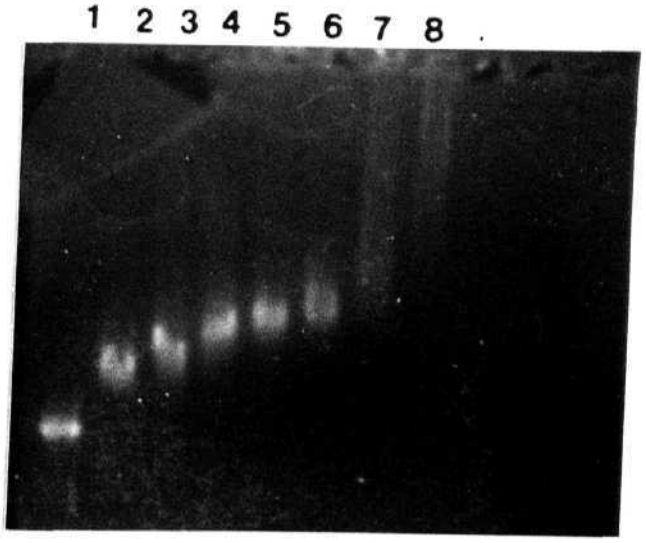

Figure 25D

(Fig.25D lanes 2-8) compared to the DNA in the absence of protein (Fig.25D lane 1) and the retardation of these nucleoprotein complexes increases with increase in protein concentration.

Fluorescence titrations of **DBNP-B** with RNA:

Protein-nucleic acid interactions can be studied by measuring the change in the intrinsic fluorescence of the protein (reverse titration) or the extrinsic fluorescence of the nucleic acid (direct titration). In an earlier study (Sreenivas, 1994; Sreenivas *et al*, 1998) fluorescence titrations were carried out using ss DNA and ds DNA and the binding characteristics were determined. The protein binds with relatively higher affinity to ss DNA than to ds DNA with a site size of 4 nucleotides /monomer. In the present study, reverse titrations were performed by adding lattice (RNA) to the ligand (DBNP-B) to understand the RNA binding properties of DBNP-B. When nucleic acid binds to the protein, the fluorescence of the aromatic residues in the protein is quenched. Characterisation of the binding interaction was done by titrating the protein with increasing concentrations of poly(U) and poly(A) at different salt concentrations. DBNP-B was excited at the absorption maxima of tyrosyl residues (274 nm) and the fluorescence was measured at the emission maxima (304 nm). The titrations were performed as described in methods section. As can be seen from Fig.26 and Fig.27, the addition of either poly(U) or poly(A) to DBNP-B causes quenching of the protein fluorescence and a saturation state in binding is reached. Further addition of RNA at this stage showed no more change in fluorescence.

DBNP-B binds to both poly(U) and poly(A) with differing affinities. The extent of maximum quenching Qmax for poly(U) and poly(A) is determined. A Qmax value of 72% for poly(U) and 67% for poly(A) were obtained. Data for titrations at 50 mM and 100 mM NaCl are very similar (Fig.26 and Fig.27) and are useful for establishing the site size (n), *i.e* the average number of nucleotides bound by a protein monomer. The ratio of RNA to protein at the intersection point of initial and final slope of the fluorescence quenching corresponds to the site size. A site size of 4 and 4.5 is obtained for poly(U) and poly(A) respectively.

RNA-protein interactions due to electrostatic interactions are sensitive to the ionic strength of the reaction medium. The strength of the binding of poly(U)

Fig.26: Reverse titrations of DBNP-B with poly (U):

DBNP-B (2μM) in 1 ml buffer containing 10 mM Tris-Cl (pH 7.6), lmM DTT and different NaCl concentrations was titrated with increasing concentrations of poly (U) and the fluorescence emission was measured at 304 nm by exciting at 274 nm. The ratio of intensity of fluorescence (F in arbitrary units) measured at each addition and the initial fluorescence (F_0) was plotted against RNA /protein (mole ratio).

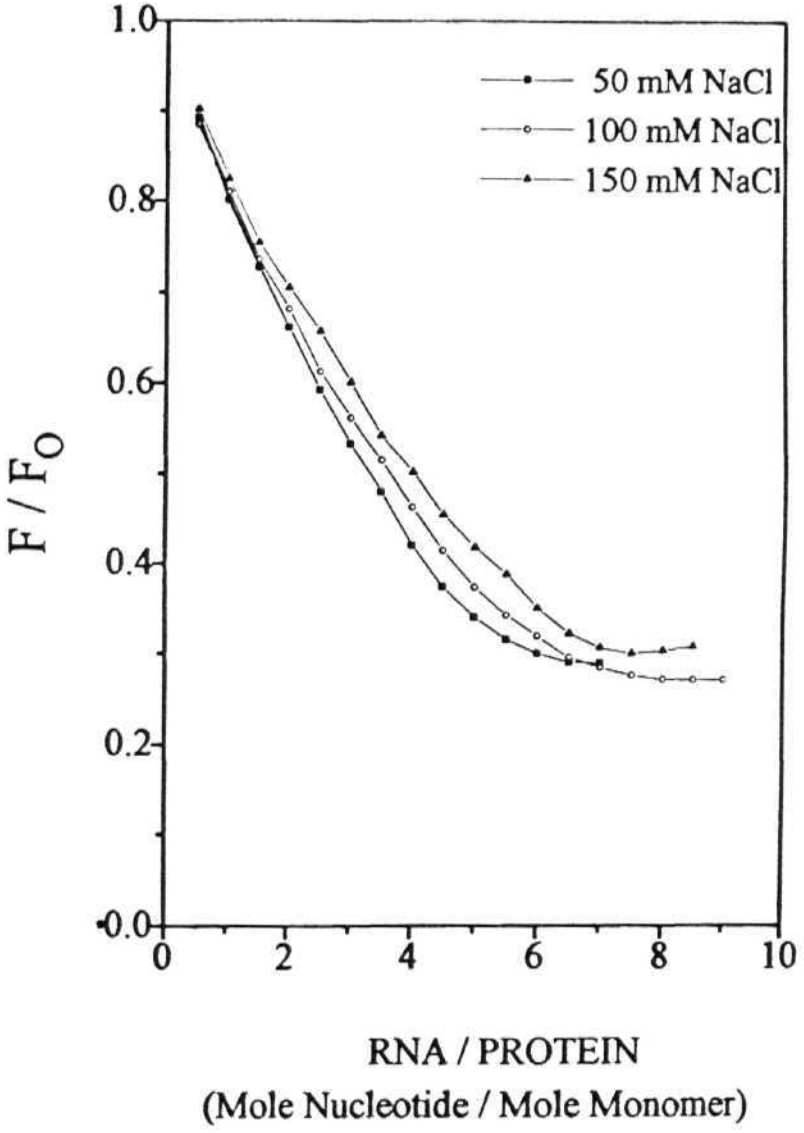
1.0
0.8
0.6
0.4
0.2
0.0
F / F$_O$
50 mM NaCl
100 mM NaCl
150 mM NaCl
0
2
4
6
8
10
RNA / PROTEIN
(Mole Nucleotide / Mole Monomer)

Figure 26

Fig. 27: Reverse titrations of DBNP-B with poly (A):

DBNP-B (2μM) in 1 ml buffer containing 10 mM Tris-Cl (pH 7.6), lmM DTT and different NaCl concentrations was titrated with increasing concentrations of poly (A) and the fluorescence emission was measured at 304 nm by exciting at 274 nm. The ratio of intensity of fluorescence (F in arbitrary units) measured at each addition and the initial fluorescence (F_0) was plotted against RNA /protein (mole ratio).

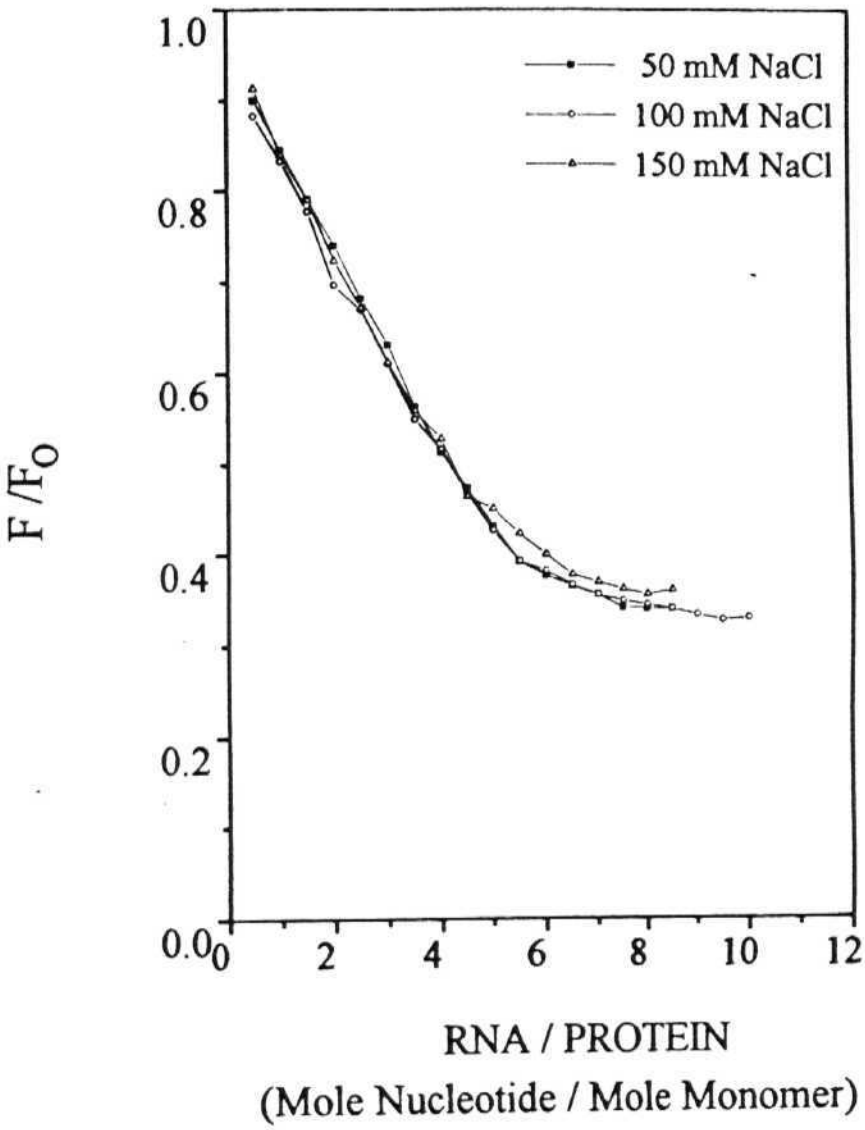
1.0
0.8
0.6
0.4
0.2
0.0
F /F0
0
2
4
6
8
10
12
50 mM NaCl
100 mM NaCl
150 mM NaCl
RNA / PROTEIN
(Mole Nucleotide / Mole Monomer)

Figure 27

and poly(A) to DBNP-B was measured by adding increasing concentrations of 4M NaCl to DBNP-B-RNA complexes at saturation in aliquots and the dissociation of the complexes due to increase in ionic strength of the reaction medium was followed by observing the recovery of the protein fluorescence. Both poly(U)-DBNP-B and poly(A)-DBNP-B complexes are sensitive to salt. Poly(U)-DBNP-B complexes are little more stable as they required ~ 400 mM NaCl for complete dissociation compared to poly(A)-DBNP-B complexes which were completely dissociated at ~ 350 mM NaCl concentration (Fig.28). From our earlier studies, the relative affinity of DBNP-B to different nucleic acids is ss DNA > ds DNA > RNA.

DNA aggregation by DBNP-B:

DBNP-B aggregates DNA at protein to DNA (w/w) ratios of 2.0 to 5.0 at pH 5.0. Aggregation of DNA is observed at both pH 5.0 and 7.6 and increases with the increase in DBNP-B concentration. Aggregation of DNA at pH 7.6 requires higher protein concentration (Sreenivas, 1994). Formation of DNA-protein aggregates was studied by light scattering monitored as increase in absorbance at 320 nm (Fig.29 and Fig30). DNA aggregation by DBNP-B was performed at different concentrations of NaCl and $MgCl_2$. The aggregation of DNA is dependent on protein concentration and increases with increasing protein concentration. Increasing NaCl concentrations decrease the extent of DNA aggregation by DBNP-B (Fig.29). In 500 mM NaCl containing buffer, the DNA aggregation by DBNP-B is inhibited to a larger extent. Aggregation of DNA by DBNP-B was monitored at different $MgCl_2$ concentrations to determine the requirement of Mg^{++} for aggregation. Results presented in Fig.30 show that 10 mM Mg^{++} is the optimum concentration required for DNA aggregation by DBNP-B at all NaCl concentrations. However DNA aggregation by DBNP-B is observed even in the absence of Mg^{++} to a certain extent at very high protein concentration.

DNA renaturation by **DBNP-B:**

DBNP-B promotes renaturation of complementary single stranded DNA in an ATP independent, Mg^{++} dependent and protein concentration dependent manner and shows an optimum pH of 5.0. The optimum Mg^{++} concentration for renaturation is 12 mM. Optimal renaturation is seen at a DNA:DBNP-B

Fig. 28: Back titration of RNA-DBNP-B complexes with salt:

Aliquots of 4M NaCl were added at Qmax and the increase in fluorescence intensity was measured at each NaCl concentration as described in materials and methods.

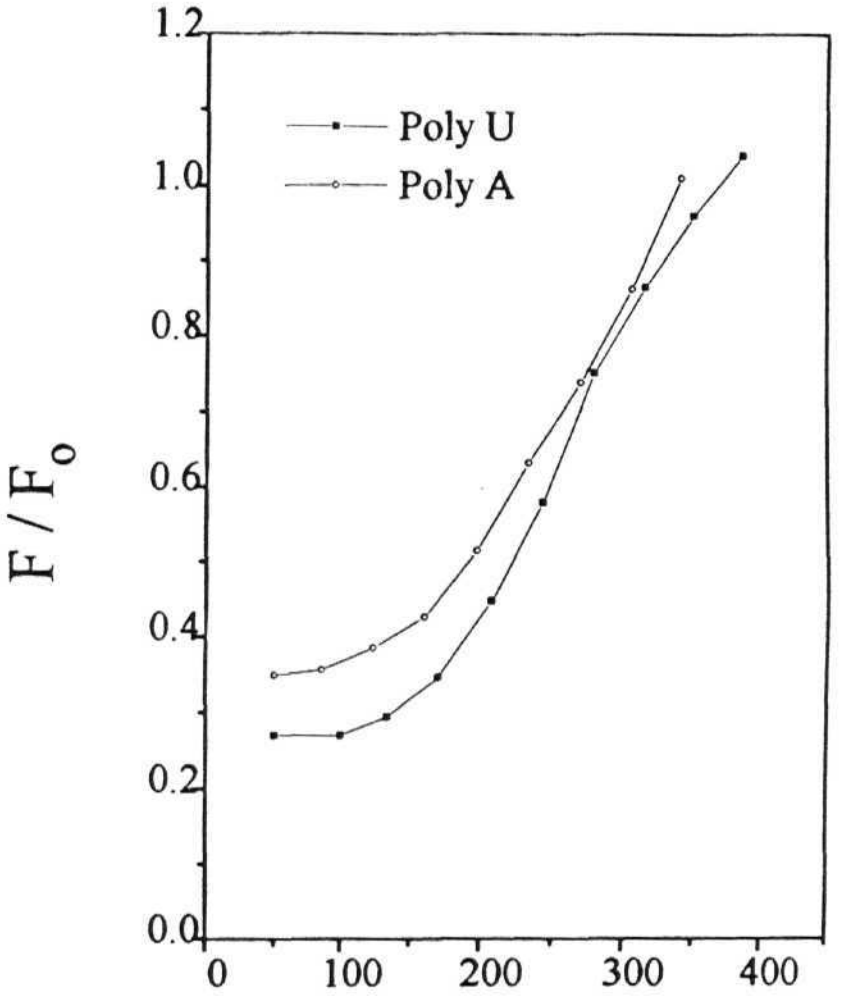
1.2
1.0
0.8
0.6
0.4
0.2
0.0
F / F0
Poly U
Poly A
0
100
200
300
400
NaCl concentration (mM)

Figure 28

Fig. 29: Effect of salt on DNA aggregation by DBNP-B:

DNA aggregation by DBNP-B at different concentrations of NaCl in 10 mM Na acetate (pH 5.0) and 10 mM $MgCl_2$ buffer was studied by measuring the increase in absorbance at 320 nm.

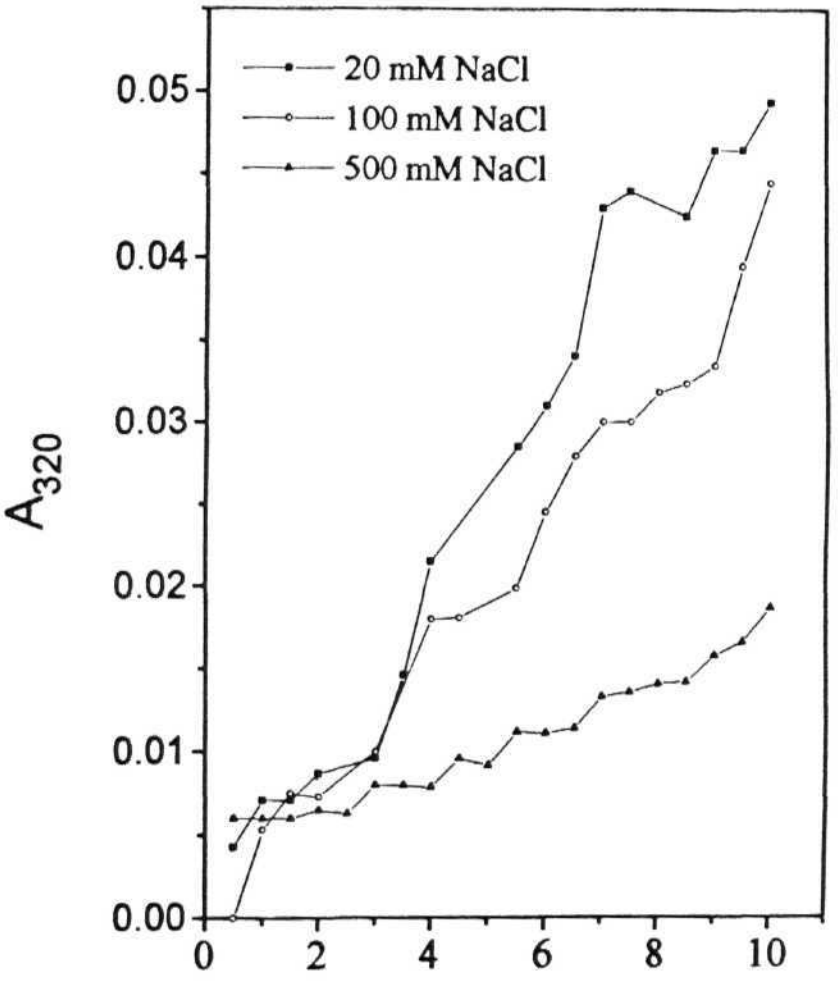
20 mM NaCl
100 mM NaCl
500 mM NaCl
A_{320}
0.05
0.04
0.03
0.02
0.01
0.00
0
2
4
6
8
10
PROTEIN / DNA

Figure 29

Fig. 30: Effect on Mg^{++} on DNA aggregation by DBNP-B:

Fig. 30A:

DNA aggregation at different concentrations of $MgCl_2$ in 10 mM Na acetate (pH 5.0) and 20 mM NaCl buffer.

Fig. 30B:

DNA aggregation at different concentrations of $MgCl_2$ in 10 mM Na acetate (pH 5.0) and 100 mM NaCl buffer.

Fig.30C:

DNA aggregation at different concentrations of $MgCl_2$ in 10 mM Na acetate (pH 5.0) and 500 mM NaCl buffer.

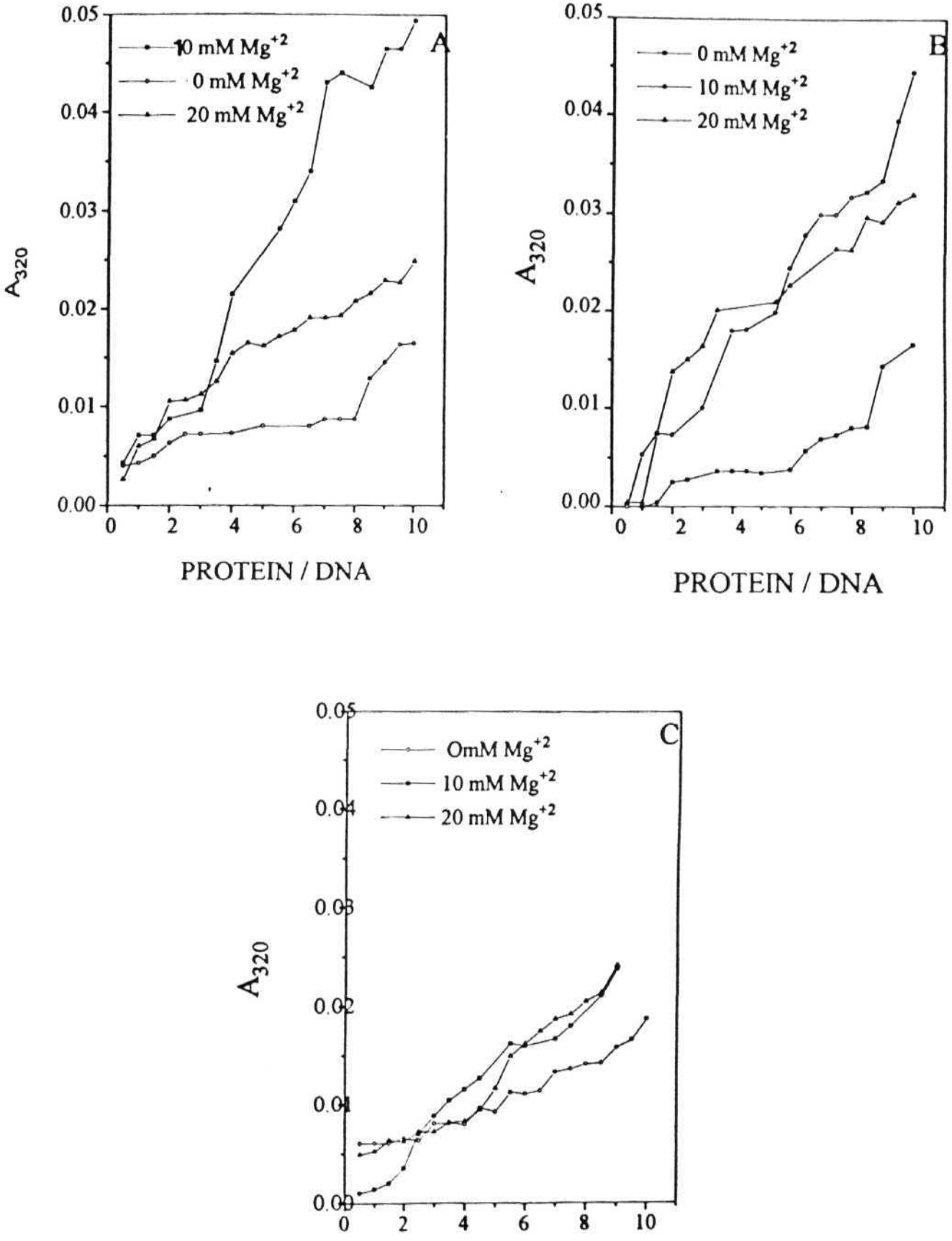
A
10 mM Mg+2
0 mM Mg+2
20 mM Mg+2
A320
PROTEIN / DNA
B
0 mM Mg+2
10 mM Mg+2
20 mM Mg+2
A320
PROTEIN / DNA
C
OmM Mg+2
10 mM Mg+2
20 mM Mg+2
A320
PROTEIN / DNA

Figure 30

concentration of 1:3 (Sreenivas, 1994). To characterise the right substrates and the reaction conditions for the renaturation reaction promoted by DBNP-B, renaturation of complementary ss DNA was carried out with heat denatured or alkali denatured DNA from different sources under different reaction conditions. Renaturation was carrried out according to Sung *et al,* (1992) with slight modifications. Heat denaturation or alkali denaturation of double stranded DNA generated fragments of complementary ssDNA and renaturation of this fragmented heterogeneous single stranded DNA forms networks of high molecular weight renatured DNA with lots of single stranded gaps. This renatured DNA fails to enter the agarose gel compared to unpaired DNA. This type of assay to follow renaturation of complementary ss DNA has been widely used (Sung *et al,* 1992; Menge and Bryant, 1992; Weinstock *et al.,* 1979; Heyer *et al,* 1988; Bryant *et al,* 1989). This assay was used in the present work to follow DNA renaturation by DBNP-B. The renaturation products were deproteinised by treatment with SDS or proteinase K before electrophoresis on agarose gels.

DNA renaturation was carried out with heat denatured 1 kb DNA ladder consisting of 0.5 kb to 12 kb with different concentrations of protein in pH 5.0 buffer. As seen in Fig.31A maximum renaturation was observed at a protein to DNA ratio of ~3.0 (Fig.31A lane 5). Denatured DNA incubated in the absence of DBNP-B is not renatured (Fig.31A lane 6) as indicated by a smear extending from the bottom of the gel to the middle of the gel.

Renaturation was performed with 1 kb DNA ladder for different time intervals to determine the optimum time required at 37^0C in pH 5.0 buffer at a constant DNA:protein ratio of 1:3 (Fig.31B). By 2 minutes (lane 4) renatured DNA is retained in the well of the agarose gel along with ds DNA fragment bands in the low molecular weight region.

Renaturation of 1 kb DNA marker ladder was also followed at 65^0C for different time intervals at pH 5.0 and a constant amount of DNA:protein ratio of 1:3 to determine the optimum time. Renatured network DNA is seen in the slot within 30 seconds of incubation of denatured DNA with the protein (Fig.31C lane 8) along with ds DNA fragment bands in the low molecular weight range and renaturation is complete within 10 minutes (Fig.31C lanes 5-7). The extent of renaturation is same at 65°C when compared to that at 37°C.

Fig. 31: Renaturation of DNA by DBNP-B:

The renaturation assay was performed as described in materials and methods. Denatured DNA was incubated with increasing amounts of DBNP-B and the reaction products were deproteinised with 1% SDS and loaded on the agarose gel.

Fig. 31 A: DNA renaturation assay with heat denatured 1 kb DNA marker (0.5 kb to 12kb) ladder (500 ng) in presence of DBNP-B was performed in 10 mM sodium acetate (pH 5.0), 12 mM $MgCl_2$ and 1 mM DTT containing buffer at 37°C and electrophoresed on a 0.8% agarose gel.

Lane 3: Native DNA.
Lane 6: Heat denatured DNA incubated in the absence of DBNP-B (control).
Lane 4-5: Heat denatured DNA incubated in the presence of 1μg and 1.5μg of DBNP-B respectively.

Fig. 31B: DNA renaturation assay with heat denatured 1 kb DNA marker ladder (1 Mg) in presence of 3μg of DBNP-B was performed in 10 mM sodium acetate (pH 5.0), 12 mM $MgCl_2$ and 1 mM DTT containing buffer for different time intervals at 37^0C and electrophoresed on a 0.8% agarose gel.

Lane 1: Native DNA.
Lane 3: Heat denatured DNA incubated in the absence of DBNP-B (control).
Lane 4-6: Denatured DNA incubated with DBNP-B for 2 mins, 10 mins and 5 mins respectively.

Fig. **31C:** Heat denatured 1 kb DNA marker ladder was incubated with 3μg of DBNP-B in 10 mM Na acetate (pH 5.0), 12 mM $MgCl_2$ and 1 mM DTT containing buffer at 65°C for different time intervals and electrophoresed on a 0.8% agarose gel.

Lane 2: Native DNA.
Lane 4: Heat denatured DNA incubated in the absence of DBNP-B (control).
Lane 5-8: Heat denatured DNA incubated with DBNP-B for 10 mins, 5 mins, 2 mins and 30 seconds respectively.

Figure 31A

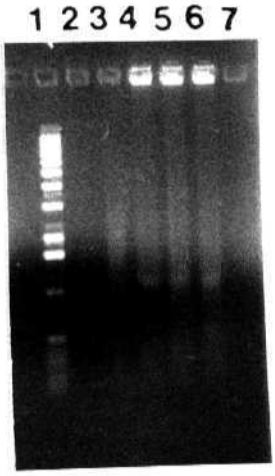

Figure 31B

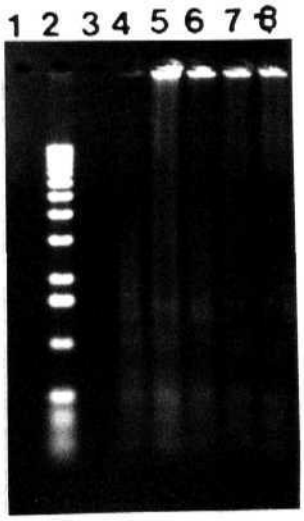

Figure 31C

pUC19 linear DNA (2686 bp) was denatured both by alkali, heat and renaturation reaction was performed with both at a constant DNA:protein ratio of 1:3 at 37°C for 15 minutes in pH 5.0 buffer. Both alkali and heat denatured DNA look alike as a smear and are renatured in the same way as high molecular weight networks which are retained in the well of the gel (Fig.32A). Spontaneous renaturation of denatured pUC19 linear DNA in the absence of DBNP-B is high at pH 5.0. Hence renaturation assay was performed in 10 mM Tris-Cl (pH 7.6) at a constant DNA:protein ratio of 1:3 at 37°C for 15 minutes with both heat denatured and alkali denatured DNA (Fig.32B). Both heat denatured and alkali denatured DNA were renatured into a high molecular weight network which was retained in the well of the agarose gel. At pH 7.6 spontaneous renaturation of heat denatured DNA in the absence of DBNP-B is less compared to that of alkali denatured DNA (Fig.32B lanes 4 and 7). Boiling at 100°C for 2 minutes of the heat denatured linear DNA renatured by DBNP-B generates fragments of denatured DNA which migrated as a smear into the agarose gel indicating pairing of complementary DNA by DBNP-B (Fig.32B lane 5).

Renaturation with equal amounts of denatured M13 RF linear DNA and M13 ss DNA in presence of DBNP-B was performed with increasing concentrations of DBNP-B at a DNA:protein concentration of 1:1, 1:3, 1:5 and 1:10 for 30 minutes at 37^0C in pH 5.0 buffer (Fig.33). With increasing concentrations of DBNP-B there is a progressive increase in the amount of renatured DNA seen in the slot of the agarose gel and a progressive decrease in both ss DNA and denatured linear RF DNA in the gel. Denatured linear RF was seen as a smear on the gel (Fig.33 lane 4).

Since high molecular weight DNA generated renatured network DNA, we studied renaturation promoted by DBNP-B using low molecular weight DNA fragments.

Renaturation of denatured ϕx174 RF *Hae*III digest (72 bp to 1353 bp) was carried out in pH 5.0 buffer at 37^0C for 10 minutes at a DNA:protein ratio of 1:2 and 1:3 (Fig.34). The intensity of ethidium bromide staining of denatured DNA is low compared to native DNA although denatured DNA does not look like a smear on the gel with bands migrating slightly slower than ds DNA fragments. At a

Fig. **32A:** Heat denatured or alkali denatured pUC19 linear DNA (500 ng) was incubated with 1500 ng of DBNP-B in 10 mM Na acetate (pH 5.0), 12 mM $MgCl_2$ and 1 mM DTT containing buffer at 37^0C for 15 minutes and electrophoresed on a 0.8% agarose gel.

Lane 1: Native DNA (1 μg).
Lane 3: Heat denatured DNA (500 ng) incubated in the absence of DBNP-B.
Lane 4: Heat denatured DNA (500 ng) incubated in the presence of DBNP-B.
Lane 5: Alkali denatured DNA (500 ng) incubated in the absence of DBNP-B.
Lane 6: Alkali denatured DNA (500 ng) incubated in the presence of DBNP-B.

Fig. 32B: Heat denatured or alkali denatured pUC19 linear DNA (500 ng) was incubated with 1500 ng of DBNP-B in 10 mM Tris-Cl (pH 7.6), 12 mM $MgCl_2$ and 1mM DTT containing buffer at 37^0C for 15 minutes and electrophoresed on a 0.8% agarose gel.

Lane 1: Native DNA.
Lane 4: Heat denatured DNA incubated in the absence of DBNP-B (control).
Lane 3: Heat denatured DNA incubated in the presence of DBNP-B.
Lane 7: Alkali denatured DNA incubated in the absence of DBNP-B (control).
Lane 6: Alkali denatured DNA incubated in the presence of DBNP-B.
Lane 5: Heat denatured DNA was renatured in the presence of DBNP-B and heated at 100^0C for 3 minutes.

Fig. 33: Heat denatured M13 linear RF DNA (400 ng) and M13 ss DNA (400 ng) was incubated with increasing concentrations of DBNP-B in 10 mM Na acetate (pH 5.0), 12 mM $MgCl_2$ and 1 mM DTT at 37°C and electrophoresed on a 0.8% agarose gel.

Lane 1: Native M13 RF DNA.
Lane 3: M13 ss DNA.
Lane 4: Denatured M13 linear RF DNA.
Lane 5: M13 ssDNA and Denatured linear RF DNA incubated in the absence of DBNP-B.
Lane 6-9: M13 ssDNA and denatured linear RF DNA incubated with 0.8μg, 2μg, 4μg and 8μg DBNP-B respectively.

Fig. 34: Heat denatured ϕx174 RF DNA *Hae*III digest (1μg) was incubated with increasing concentrations of DBNP-B in 10 mM Na acetate (pH 5.0), 12 mM $MgCl_2$ and 1 mM DTT buffer at 37°C and electrophoresed on a 0.8% agarose gel.

Lane 1: Native DNA.
Lane 2: Denatured DNA incubated in the absence of DBNP-B (control).
Lane 3-4: Denatured DNA incubated in the presence of 2μg and 3μg of DBNP-B respectively.

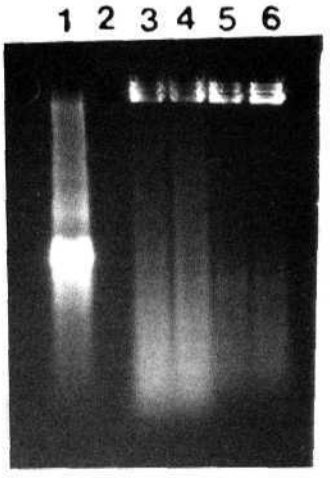

Figure 32A

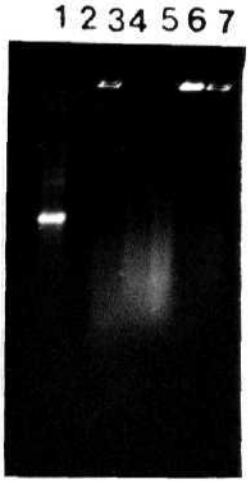

Figure 32B

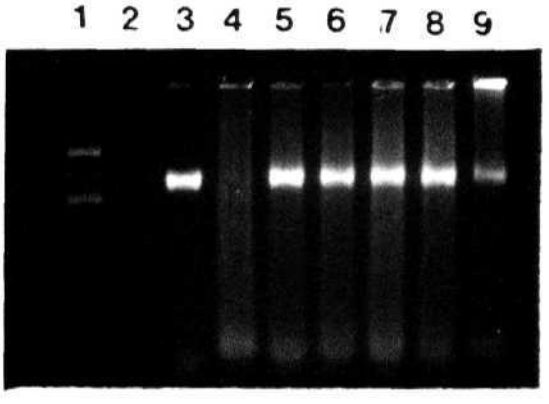

Figure 33

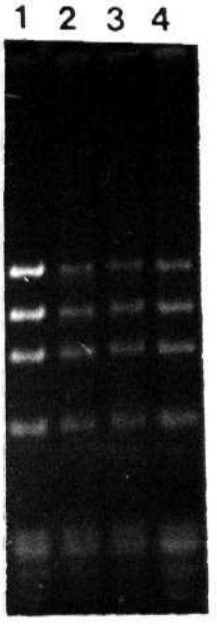

Figure 34

DNA:protein ratio of 1:3 some of the renatured DNA is seen in the slot of the agarose gel (Fig.34 lane 4) and the rest of the renatured DNA is stained brighter with bands migrating exactly like nondenatured DNA compared to the denatured DNA incubated in the absence of DBNP-B (Fig.34 lane 2).

Heat denatured pBR322 *Hae*III digest (7 bp to 587 bp fragments) was incubated with increasing concentrations of DBNP-B in pH 5.0 buffer at 37°C for 15 minutes, deproteinised with 1% SDS and electrophoresed on a 7.5% native polyacrylamide gel (Fig.35A). The denatured DNA incubated in the absence of DBNP-B (Fig.35A lane 2) is stained low with ethidium bromide compared to the native DNA with slightly decreased mobility of the bands (Fig.35A lane 1). The DNA renatured in the presence of DBNP-B is seen as a high molecular weight smear in the polyacrylamide gel (Fig.35A lanes 3-5).

Heat denatured pBR322 *Hae*III digest was incubated with increasing concentrations of DBNP-B in pH 5.0 buffer at 37°C for 15 minutes in duplicates. One set was deproteinised with 5% SDS and electrophoresed on a 7.5% native polyacrylamide gel while the other set was treated with proteinase K after adding 5% SDS before loading on the gel (Fig.35B). The DNA renatured in the presence of DBNP-B is seen as a high molecular weight smear at 1:3 and 1:5 concentrations of DNA:DBNP-B (Fig.35B lanes 5 and 6). In the set treated with proteinase K the renatured product is seen as sharp bands which are stained brighter than the denatured DNA with slightly higher migration of the bands as in the native DNA fragments (Fig.35B lanes 7-9).

Resistance of renatured DNA to S1 nuclease digestion:

Renaturation assay was performed in duplicates at different DBNP-B concentrations with denatured *X* DNA and the reaction was stopped with SDS. One set of the reaction products were loaded directly onto the agarose gel while the other set was incubated with S1 nuclease before loading onto the gel (Fig.36). Increase in DNA renaturation was observed with increase in DBNP-B concentration (Fig.36 lanes 3-6). Denatured λ DNA incubated in the absence of DBNP-B (control) was not renatured (Fig.36 lane 2). In the other set treated with S1 nuclease most of the denatured DNA in the absence of DBNP-B was digested into small fragments (Fig.36 lane 8). Although large amount of DNA is digested

Fig. **35A:** Heat denatured pBR322 *Hae*III digest (1μg) was incubated with increasing concentrations of DBNP-B in 10 mM Na acetate (pH 5.0), 12 mM $MgCl_2$ and 1 mM DTT containing buffer at 37^0C for 15 minutes, deproteinised with 1% SDS and electrophoresed on a 7.5% native polyacrylamide gel.

Lane 1: Native DNA.
Lane 2: Denatured DNA.
Lane 3-5: Denatured DNA incubated with 1μg, 3μg and 5|ig of DBNP-B respectively.

Fig. 35B: Heat denatured pBR322 *Hae*III digest (1 μg) was incubated with increasing concentrations of DBNP-B in 10 mM Na acetate (pH 5.0), 12 mM $MgCl_2$ and 1 mM DTT containing buffer in duplicates at 37^0C for 15 minutes. One set was deproteinised with 5% SDS and loaded on the gel while the other set was deproteinised with 5% SDS, treated with proteinase K and loaded on a 7.5% native polyacrylamide gel.

Lane 1: Native DNA.
Lane 2: Denatured DNA treated with proteinase K.
Lane 3: Denatured DNA.
Lane 4-6: Denatured DNA incubated with 1μg, 3 μg and 5μg DBNP-B respectively.
Lane 7-9: Denatured DNA incubated with 1μg, 3μg and 5μg DBNP-B respectively and subjected to proteinase K treatment.

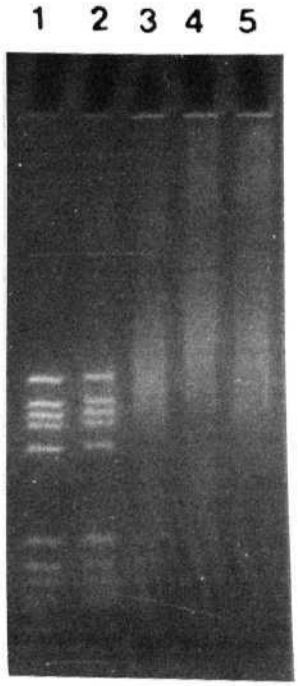

Figure 35A

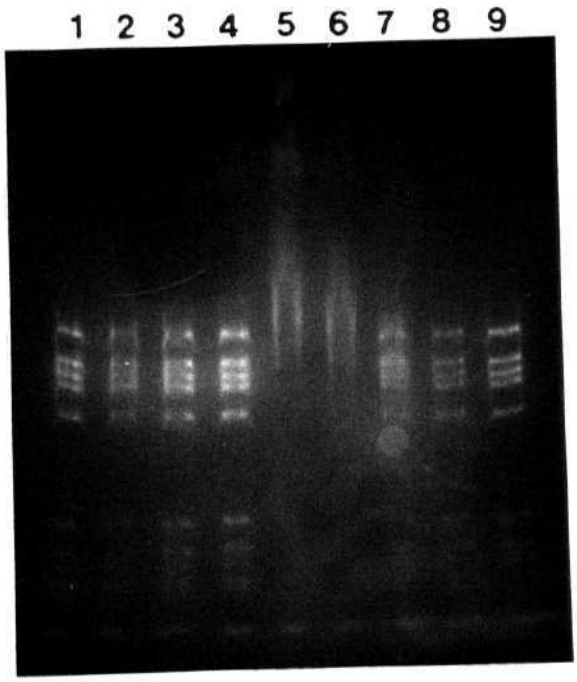

Figure 35B

Fig. 36: Resistance of renatured DNA to S1 nuclease digestion:

Renaturation assay with increasing amounts of DBNP-B (0.5 to 0.8 |ig) and 1μg of heat denatured λ DNA in 10 mM sodium acetate (pH 5.0), 12 mM $MgCl_2$ and 1 mM DTT was performed in duplicates. After deproteinisation with 1% SDS, one set of the reaction products were loaded directly on to the 0.8% agarose gel, while the other set was subjected to S1 nuclease digestion with 2 units of S1 nuclease at 37°C for 5 minutes before loading onto the gel.

Lane 1: Native *X* DNA

Lane 2: Denatured *X* DNA incubated in the absence of DBNP-B (control).

Lane 3-7: Denatured *X* DNA incubated with 0.5μg, 1μg, 3 jig, 4μg and 8μg of DBNP-B respectively.

Lane 8: Denatured *X* DNA incubated in the absence of DBNP-B and subjected to S1 nuclease digestion (control).

Lane 9-12: Denatured *X* DNA incubated with 0.5μg, 1μg, 3 μg, and 4μg DBNP-B respectively, deproteinised with SDS and subjected to S1 nuclease digestion.

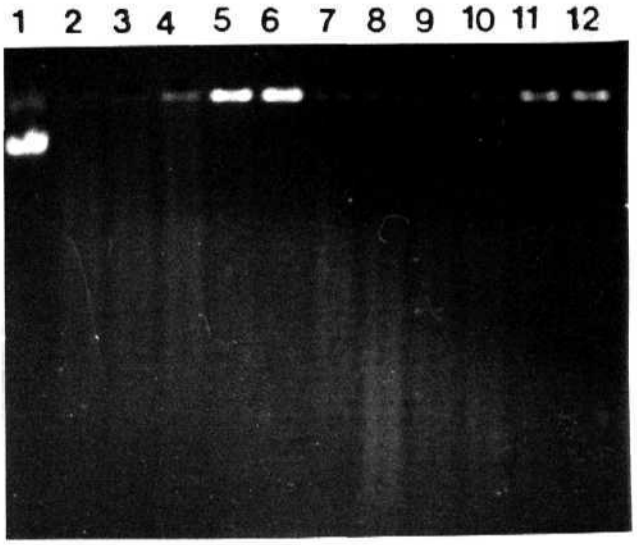

Figure 36

by S1 nuclease, substantial amounts of DNA was resistant to digestion and remained in the wells and lanes of the agarose gel (Fig.36 lanes 9-12). This resistance to S1 nuclease increases as the concentration of DBNP-B was increased and corresponded with the increase in DNA renaturation observed in lanes 3-6. Appearance of S1 resistant DNA smear from top of the gel to the bottom compared to the control (Fig.36 lane 8) indicates the formation of duplex DNA by DBNP-B. At high DBNP-B concentration DNA renaturation is inhibited (Fig.36 lane 7) and the DNA was digested to small fragments by S1 nuclease (Fig.36 lane 13).

Dnase I sensitivity **of** pUC19 supercoiled **DNA in** the **DBNP-B-DNA complexes:**

Preformed DBNP-B-pUC19 DNA complexes at different protein to DNA ratio were incubated with a constant amount of DNase I (DNA /DNase I w/w=10). The reaction was stopped by deproteinising with 1% SDS and the reaction products were analysed by electrophoresing on a 0.8% agarose gel (Fig.37A) or on a 2.5% acrylamide, 0.5% agarose composite gel (Fig.37B).

DNase 1 digestion generated small DNA fragments which appear as a smear below the supercoiled DNA. The DNA in the nucleoprotein complexes was protected from digestion above a certain ratio of protein to DNA (~30 w/w) as seen in Fig.37A lane 10. At intermediate concentration of the protein, the DNA was digested more by DNase I (Fig.37A lanes 7, 8).

Analysis of DNase 1 digested DNA in the DBNP-B-pUC19 supercoiled DNA complexes on a 2.5% acrylamide, 0.5% agarose composite gel (Fig.37B) reveals the formation of different types of DNA-protein complexes at different DBNP-B concentrations as the levels of digestion by DNase 1 vary with the protein concentration. At low protein concentration (Fig.37B lanes 3, 4) the DNA was protected from digestion, at intermediate concentrations the DNA was digested more by DNase I and at high concentrations the DNA was again protected by DBNP-B (Fig.37B lanes12-14).

Fig. 37: DNase I digestion of pUC19 DNA in the DBNP-B DNA complexes:

pUC19 supercoiled DNA (300 ng) was incubated with increasing amounts of DBNP-B. DNase I (DNA /DNase I =10) was added to these DNA-DBNP-B complexes and incubated at 37^0C for 1 minute.

Fig. **37A:** The reaction products were deproteinised with 1% SDS and loaded on a 0.8% agarose gel.

Lane 2: DNA incubated in the absence of DBNP-B and DNase 1 (control).
Lane 3: DNA alone incubated with DNase I.
Lane 5-10: DNA-DBNP-B complexes formed at different protein concentrations (0.3µg, 1.8µg, 2.4µg, 4.8µg, 7.5µg, and 30µg respectively) and incubated with DNase I.

Fig. **37B:** The reaction products were deproteinised with 1% SDS and loaded on a 2.5% acrylamide, 0.5% agarose composite gel.

Lane 1: DNA incubated in the absence of DBNP-B (control).
Lane 2: DNA alone incubated with DNase I.
Lane 3-14: DNA-DBNP-B complexes formed at different protein concentrations (0.075µg, 0.15µg, 0.3µg, 0.6µg, 0.9µg, 1.5µg, 2.4µg, 3µg, 6µg, 9µg, 15µg and 30µg respectively) and incubated with DNase I.

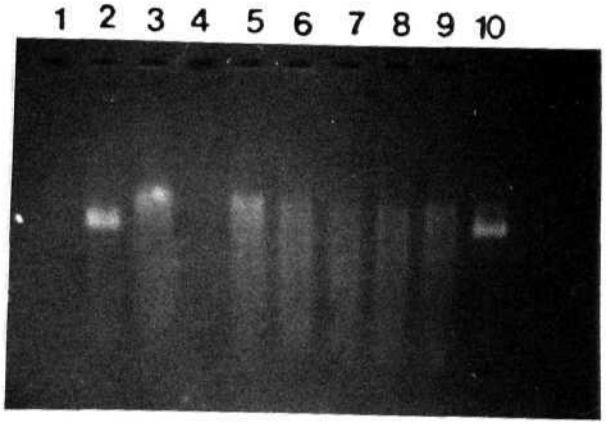

Figure 37A

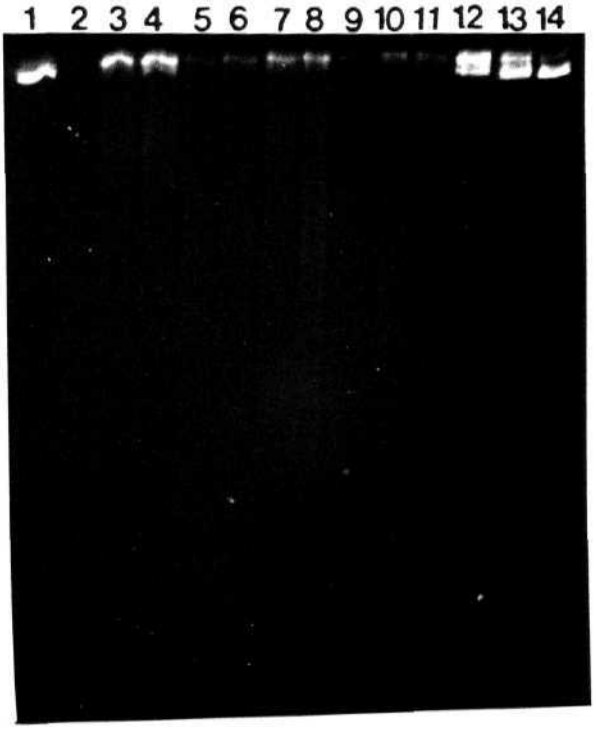

Figure 37B

Nicking activity of DBNP-B:

During mobility shift experiments with pBR322 DNA there was a gradual increase in the intensity of relaxed DNA band suggesting possible nicking of supercoiled DNA by DBNP-B. Nicking activity of DBNP-B was tested by incubating pBR322 DNA containing both supercoiled and relaxed forms as described in methods section with increasing concentrations of DBNP-B at 37°C for 1hr and the products were deproteinised with 1% SDS, subjected to proteinase K digestion and electrophoresed. DNA is converted to a form migrating to the position of linear form at a DNA:protein ratio of 1:3 to 1:8 (Fig.38A lanes 5-7). The optimum concentration of DBNP-B for the convertion of pBR322 DNA to linear form is found to be 1:8 of DNA:DBNP-B. Above this DNA:protein ratio there is no convertion of DNA to linear form. pBR322 DNA containing mostly supercoiled form was incubated with increasing concentrations of DBNP-B at 37°C for 1 hr and electrophoresed after deproteinisation with SDS. Lack of appreciable nicking activity on supercoiled DNA is evident from Fig.38B as the supercoiled DNA looks intact and showed the same mobility as control DNA.

The requirement of Mg^{++} for the nicking activity of DBNP-B was tested by incubating pBR322 DNA in the assay buffer with increasing concentrations of DBNP-B in the absence of $MgCl_2$. As can be seen from Fig.39 both supercoiled andrelaxed forms of DNA are intact at all DBNP-B concentrations implying that Mg^{++} is essential for the nicking activity of DBNP-B.

The influence of ATP on the nicking activity of DBNP-B was tested by incubating pBR322 DNA with increasing concentrations of DBNP-B in 2 mM ATP containing buffer (Fig.40). There is no enhancement in the convertion of pBR322 DNA to linear form at all concentrations of DBNP-B. Hence ATP does not seem to be required for the nicking activity of DBNP-B.

Nicking assay was carried out at different pH conditions to determine the optimum pH for DBNP-B to convert DNA to linear form. As can be seen from Fig.41 lanes 6 and 7, pH 7.0 to 7.5 seems to be optimum at which all the supercoiled form present is converted to relaxed and or linear form. In lane 10 the pBR322 DNA incubated with DBNP-B in the nicking assay buffer for 1 hr, then

Fig. 38A: Nicking activity of DBNP-B:

DBNP-B was assayed for nicking activity by incubating pBR322 DNA (300 ng) containing both supercoiled and relaxed forms as described in materials and methods with increasing concentrations of the protein at 37^0C for 1 hr. The reaction products were deproteinised with 1% SDS, subjected to proteinase K digestion at 37^0C for 30 minutes and electrophoresed on a 1% agarose gel.

Lane 1: DNA incubated without DBNP-B (control).
Lane 2-10: DNA incubated with 0.15µg, 0.3µg, 0.6µg, 0.9µg, 1.8µg, 2.4µg, 3µg, 6µg and 9µg DBNP-B respectively.

Fig. 38B: pBR322 supercoiled DNA (300 ng) was incubated with increasing concentrations of DBNP-B in 50 mM Tris-Cl (pH 7.5), 50 mM KC1, 0.1 mM EDTA, 0.5 mM DTT and 10 mM $MgCl_2$ at 37^0C for 1 hr. The reaction products were deproteinised with 1% SDS and electrophoresed on a 1% agarose gel.

Lane 1: DNA without DBNP-B (control).
Lane 2-11: DNA incubated with 0.15µg, 0.3µg, 0.6µg, 0.9µg, 1.2µg, 1.8µg, 2.4µg, 3µg, 3.6µg and 6µg DBNP-B respectively.

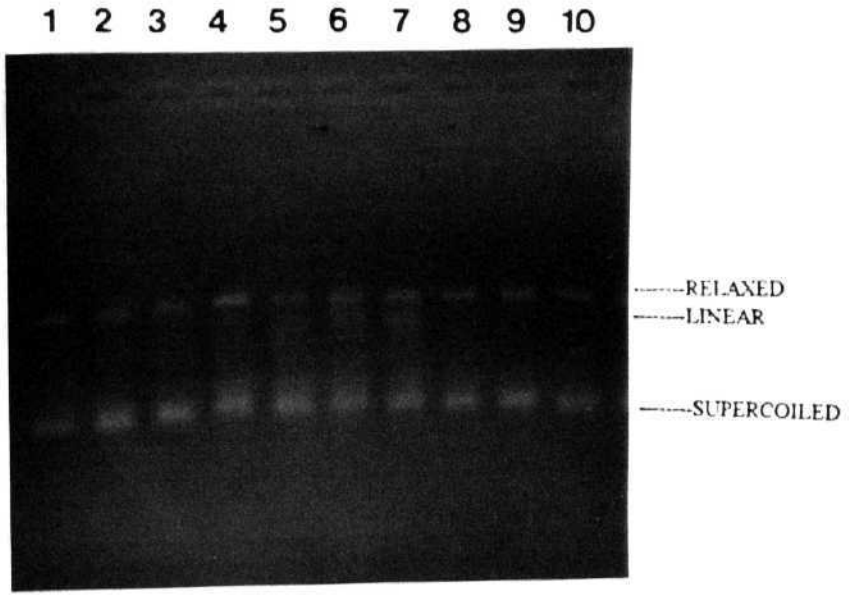

Figure 38A

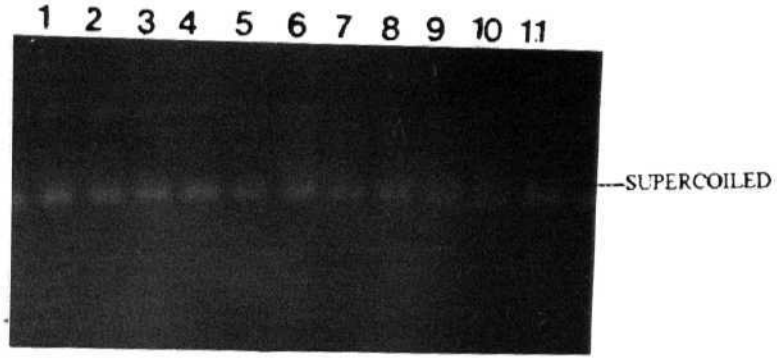

Figure 38B

Fig. 39: Nicking activity of DBNP-B in the absence of magnesium:

pBR322 DNA (300 ng) was incubated with increasing concentrations of DBNP-B in 50 mM Tris-Cl (pH 7.5), 50 mM KCl, 0.1 mM EDTA, 0.5 mM DTT containing buffer at 37°C for 1 hr. The reaction products were deproteinised with 1% SDS and electrophoresed on a 1% agarose gel.

Lane 1: DNA without DBNP-B (control).
Lane 2-11: DNA incubated with 0.15μg, 0.3|ig, 0.6μg, 1.2μg, 2.4μg, 3μg, 6μg, 9μg, 12μg and 15μg DBNP-B respectively.

Fig. 40: Influence of ATP on the nicking activity of DBNP-B:

pBR322 DNA (300 ng) was incubated with increasing concentrations of DBNP-B in 50 mM Tris-Cl (pH 7.5), 50 mM KCl, 0.1 mM EDTA, 0.5 mM DTT and 10 mM $MgCl_2$ buffer containing 2 mM ATP at 37^0C for different time intervals. The reaction products were deproteinised with 1% SDS and electrophoresed on a 1% agarose gel.

Lane 1: DNA incubated without DBNP-B (control).
Lane 2-11: DNA incubated with 0.15μg, 0.3μg, 0.6μg, 1.2μg, 2.4μg, 3μg, 6μg, 9μg, 12μg and 15μg DBNP-B respectively.

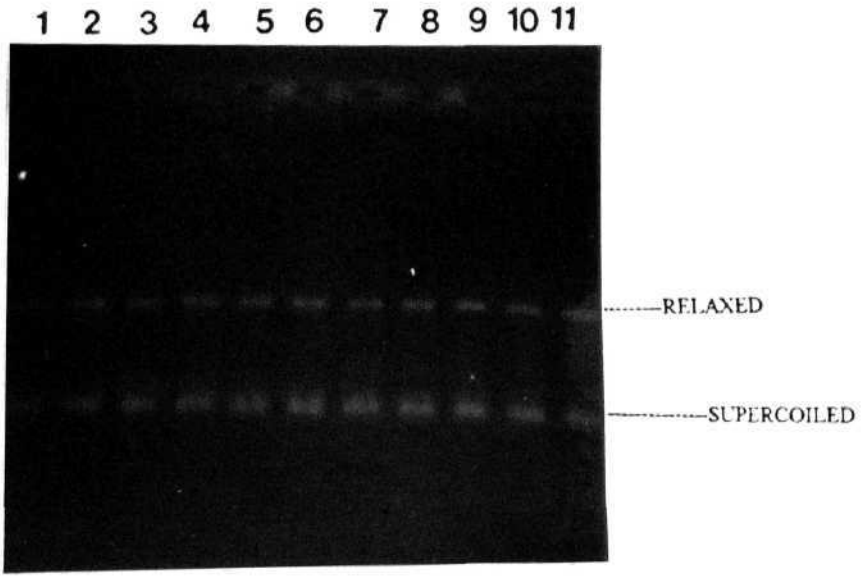

Figure 39

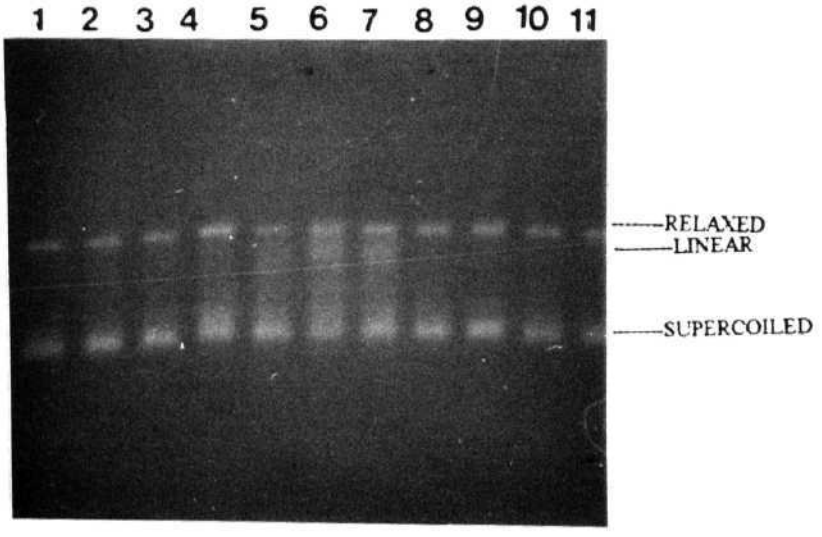

Figure 40

mixed with pBR322 *Hind*III digest containing all the three forms of DNA (supercoiled, relaxed and linear) was loaded. Both pBR322 *Hind*III digest and pBR322 DNA incubated with DBNP-B migrate to the same positions indicating that relaxed form of DNA is converted to linear form by DBNP-B.

pBR322 DNA was incubated with DNA:DBNP-B at a ratio of 1:8 for different time intervals in the nicking assay buffer to determine the optimum time required. As can be seen in Fig.42, by 5 minutes (Fig.42 lane 2) linear form appeared with a progressive increase in the convertion of relaxed DNA to linear DNA upto 1 hr (Fig.42 lanes 2-7). After 1 hr the linear form is further degraded to lower fragments seen as a smear in the gel (Fig.42 lane 8).

pBR322 relaxed DNA was incubated in the nicking assay buffer with increasing concentrations of DBNP-B at two different temperatures 37°C and 65°C and the reaction products were electrophoresed. As can be seen from Fig.43A and 43B there is no difference in the activity of DBNP-B at both the temperatures. Thus this activity is not dependent on temperature. At 65^0C also the DNA is converted to linear form.

SV40 DNA was incubated with increasing concentrations of DBNP-B at 37°C in the nicking assay buffer. In the case of SV40 DNA, the linear form progressively increased with increasing DBNP-B concentration. The supercoiled form progressively decreased with increasing concentrations of DBNP-B. At high DBNP-B concentration (>30 protein to DNA ratio) most of the supercoiled DNA is converted to relaxed or linear forms with faint smear in the gel (Fig.44A lanes 9, 10).

pUC19 DNA containing supercoiled and relaxed forms was incubated with increasing concentrations of DBNP-B in the nicking assay buffer at 37°C for 1hr. The reaction products were electrophoresed after deproteinising with 1% SDS. As can be seen from Fig.44B (lanes 6-11) a smear is observed between the relaxed and supercoiled forms of DNA indicating nonspecific nicking by DBNP-B. Thus the DNA does not seem to be intact and the relaxed form is degraded into lower forms.

Fig. 41: Influence of pH on the nicking activity of DBNP-B:

pBR322 relaxed DNA (300 ng) was incubated with DBNP-B at a DNA:protein concentration of 1:8 at 37^0C for 1 hr in 50 mM Tris-Cl or 50 mM Na acetate in 50 mM KCl, 0.1 mM EDTA and 0.5 mM DTT containing buffers at different pH. The reaction products were deproteinised with 1% SDS and electrophoresed on a 1% agarose gel.

Lane 1: DNA without DBNP-B in 50 mM Tris-Cl (pH 7.5) (control).
Lane 2-5: DNA incubated with DBNP-B in 50 mM Na acetate (pH 5.0, 5.5, 6.0 and 6.5 respectively).
Lane 6-9: DNA incubated with DBNP-B in 50 mM Tris-Cl (pH 7.0, 7.5, 8.0 and 8.5 respectively).
Lane 10: DNA incubated with DBNP-B in 50 mM Tris-Cl (pH 7.5) for 1 hr, deproteinised and mixed with pBR322 *Hind* III digest containing supercoiled, relaxed and linear forms before loading onto the gel.

Fig. 42: pBR322 DNA (300ng) was incubated with 2400 ng of DBNP-B in 50 mM Tris-Cl (pH 7.5), 50 mM KCl, 0.1 mM EDTA, 0.5 mM DTT and 10 mM $MgCl_2$ at 37^0C for different time intervals. The reaction products were deproteinised with 1% SDS and electrophoresed on a 1% agarose gel.

Lane 1: DNA incubated without DBNP-B (control).
Lane 2-8: DNA incubated with DBNP-B for 2 mins, 5 mins, 10 mins, 15 mins, 30 mins, 60 mins and 120 mins respectively.
Lane 10: pBR322 *Hind* III digest containing relaxed, linear and supercoiled forms.

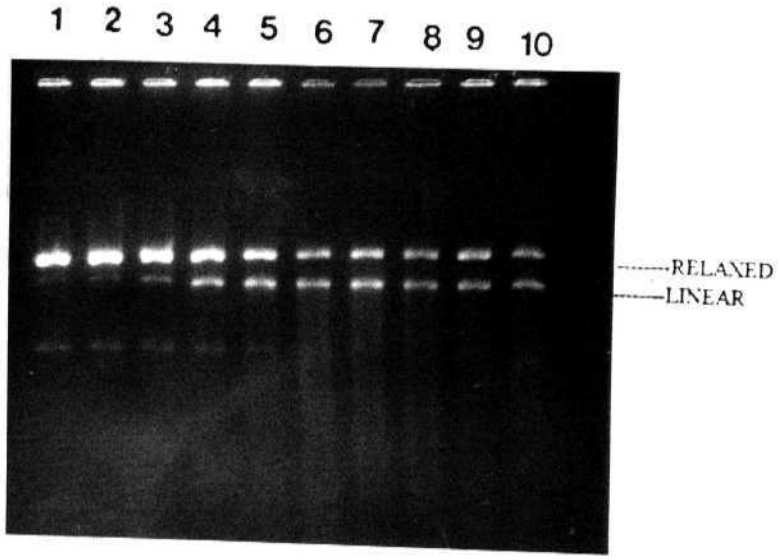

Figure 41

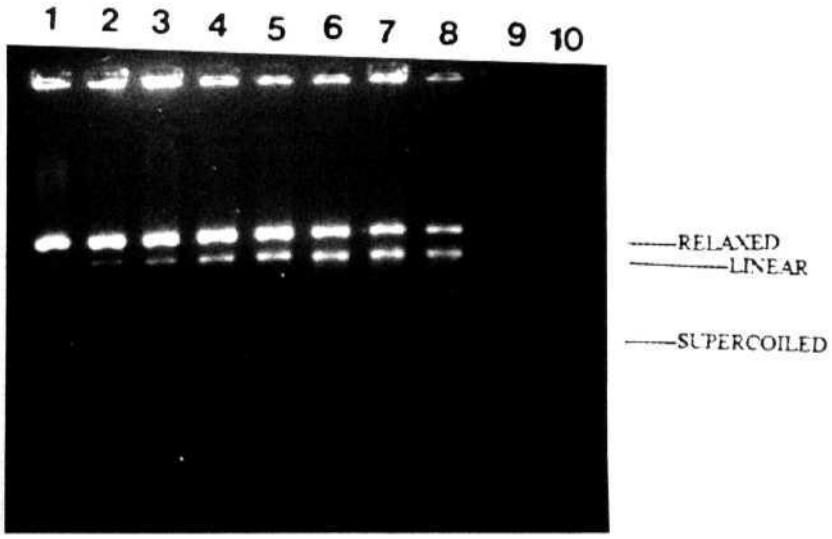

Figure 42

Fig. 43: pBR322 relaxed DNA (300 ng) was incubated with increasing concentrations of DBNP-B in 50 mM Tris-Cl (pH 7.5), 50 mM KCl, 0.1 mM EDTA, 0.5 mM DTT and 10 mM $MgCl_2$. The reaction products were deproteinised with 1% SDS and electrophoresed on a 1% agarose gel.

Fig. 43A: Assay at 37^0C for 1 hr.

Lane 1: DNA without DBNP-B (control).
Lane 2-11: DNA incubated with 0.15µg, 0.3µg, 0.6µg, 0.9µg, 1.2µg, 2.4µg, 3µg, 6µg , 9µg and 12 µg DBNP-B respectively.

Fig. 43B: Assay at 65^0C for 1 hr.

Lane 1: DNA without DBNP-B (control).
Lane 2-10: DNA incubated with 0.15µg, 0.3µg, 0.6µg, 0.9µg, 1.2µg, 2.4µg, 3µg, 6µg and 9µg DBNP-B respectively.
Lane 11: pBR322 *Hind* III digest containing supercoiled, relaxed and linear forms.

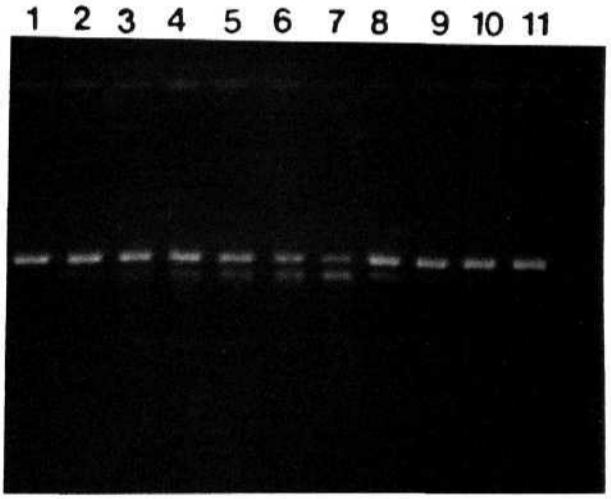

Figure 43A

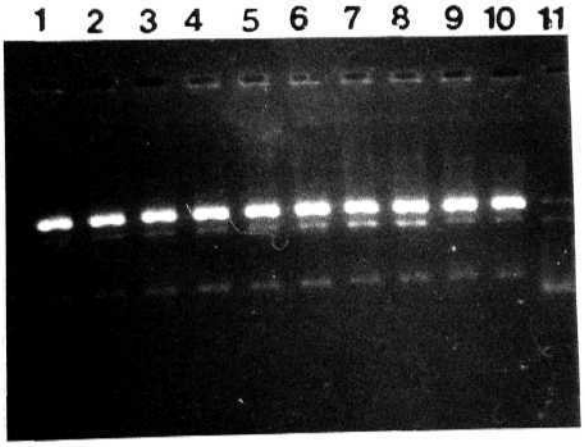

Figure 43B

Fig. 44: Nicking activity of DBNP-B on different DNA:

DNA was incubated with increasing concentrations of DBNP-B in 50 mM Tris-Cl (pH 7.5), 50 mM KCl, 0.1 mM EDTA, 0.5 mM DTT and 10 mM $MgCl_2$ at 37^0C for 1 hr. The reaction products were deproteinised with 1% SDS and electrophoresed on a 1% agarose gel.

Fig. 44A: SV40 DN A (1200 ng)

Lane 1: DNA incubated without DBNP-B (control).
Lane 2-10: DNA incubated with 0.15µg, 0.3µg, 0.6µg, 0.9µg, 1.2µg, 2.4µg, 3µg, 6µg and 9µg DBNP-B respectively.

Fig. 44B: pUC19DNA (300 ng)

Lane 1: DNA incubated without DBNP-B (control).
Lane 2-11: DNA incubated with 0.15µg, 0.3µg, 0.6µg, 0.9µg, 1.2µg, 2.4µg, 3µg, 6µg , 9µg and 12µg DBNP-B respectively.

Fig. 44C: *X* DNA (300 ng)

Lane 1: DNA incubated without DBNP-B (control).
Lane 2-10: DNA incubated with 0.15µg, 0.3µg, 0.6µg, 0.9µg, 1.2µg, 2.4µg, 3µg, 6µg and 9µg DBNP-B respectively.

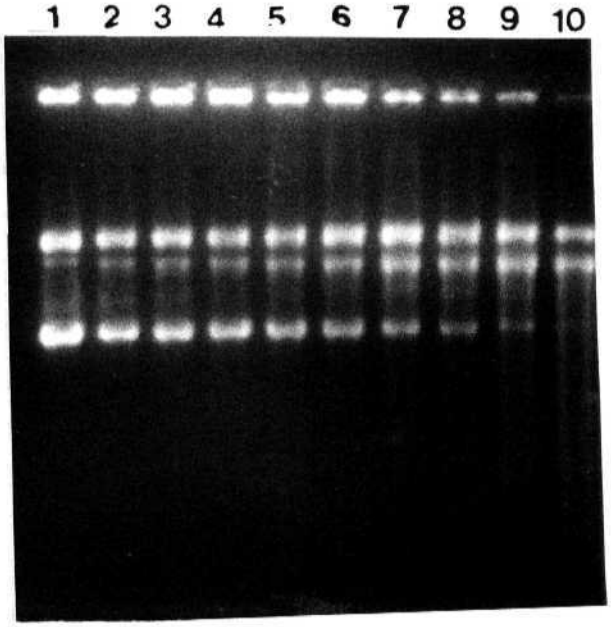

Figure 44A

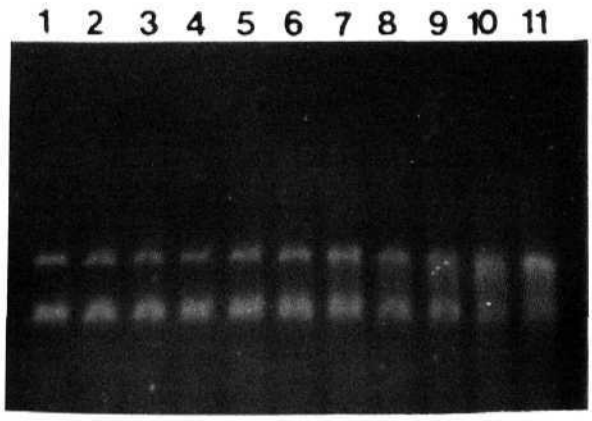

Figure 44B

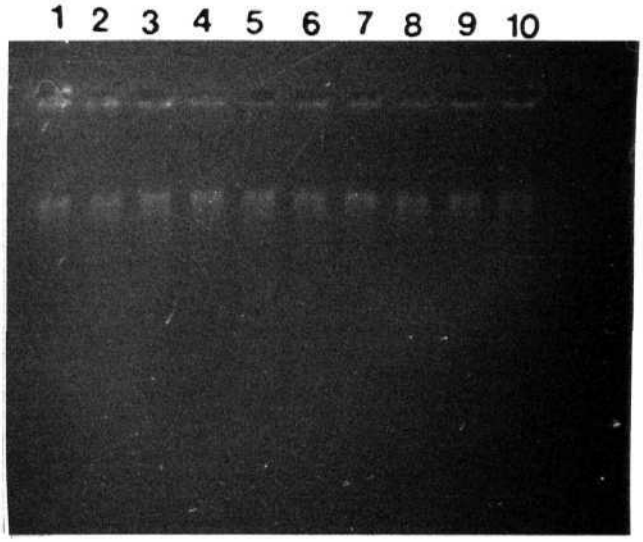

Figure 44C

Nicking activity of DBNP-B was tested on λ DNA by incubating the DNA with increasing concentrations of the protein under similar conditions at 37°C for 1 hr (Fig.44C). The DNA looks intact and nicking activity on *X* DNA could not be detected.

Effect of DBNP-B on the relaxation **activity of topoisomerase I:**

DBNP-B-pBR322 supercoiled DNA complexes were formed in the topoisomerase I assay buffer at 37°C for 15 minutes with increasing concentrations of DBNP-B and then topoisomerase I was added and incubated at 37°C. Results presented in Fig.45 (lanes 3-10) show that DBNP-B neither inhibits nor stimulates the relaxation activity of wheat germ topoisomerase I. The extent of relaxation of supercoiled form to relaxed form by topoisomerase I in the absence of DBNP-B (control Fig.45 lane 2) and in the presence of different concentrations of DBNP-B (Fig.45 lanes 3-10) was the same. DBNP-B does not inhibit topoisomerase I even at high concentrations (1:25 of DNA:DBNP-B). Under these conditions, the nicking activity of DBNP-B on pBR322 DNA could not be detected.

Effect of DBNP-B on topoisomerase II activity:

DBNP-B-pBR322 DNA complexes were formed in the assay buffer at 37°C for 15 minutes with increasing concentrations of the protein and then rat testis topoisomerase II was added and incubated at 37°C for 30 minutes. The reaction products after deproteinisation with 1% SDS were subjected to proteinase K digestion and electrophoresed on a 1% agarose gel. DBNP-B does not inhibit the activity of topoisomerase II. All of the supercoiled DNA is relaxed both in control (Fig 46 lane 2) *i.e* DNA incubated with topoisomerase II in the absence of DBNP-B and in the reaction mixtures incubated with different concentrations of DBNP-B (Fig 46 lanes 3-9). DBNP-B does not inhibit topoisomerase II activity even at high concentrations as 1:20 or 1:25 of DNA:DBNP-B.

Fig. 45: Effect of DBNP-B on wheat germ topoisomerase I:

Wheat germ topoisomerase I assay was performed as described in materials and methods with 500 ng of pBR322 supercoiled DNA in the presence of increasing amounts of DBNP-B. The reaction products after deproteinising with 1% SDS were subjected to proteinase K digestion and electrophoresed on a 1% agarose gel.

Lane 1:	DNA alone incubated in the absence of DBNP-B and topoisomerase I.
Lane 2:	DNA (control) incubated in the presence of topoisomerase I.
Lane 3-10:	DNA incubated with 0.25µg, 0.5µg, 1.5µg, 2.5µg, 4µg, 5µg, 10µg and 12.5µg of DBNP-B respectively for 15 mins and then subjected to topoisomerase I addition.

Fig. 46: Effect of DBNP-B on the relaxation activity of rat testis topoisomerase 11:

Rat testis topoisomerase II relaxation assay was carried out as described in materials and methods with 500 ng of pBR322 supercoiled DNA in the presence of increasing amounts of DBNP-B. The reaction products after deproteinisation with 1% SDS were subjected to proteinase K digestion and electrophoresed on a 1% agarose gel.

Lane 1:	DNA alone incubated in the absence of topoisomerase II and DBNP-B.
Lane 2:	DNA incubated with topoisomerase II (control).
Lane 3-9:	DNA incubated for 15 mins with 0.5 µg, 1.5µg, 2.5µg, 4µg, 5µg, 10µg, and 12.5µg DBNP-B respectively prior to the addition of topoisomerase II.

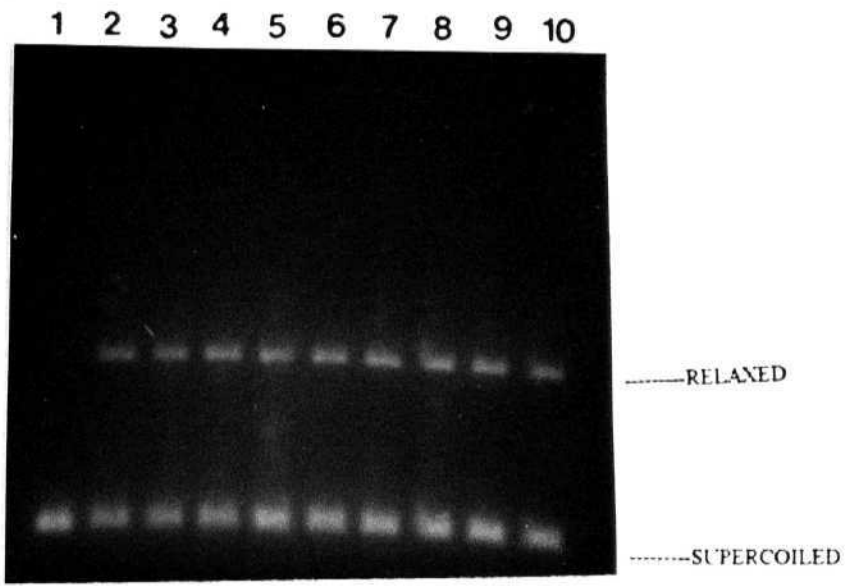

Figure 45

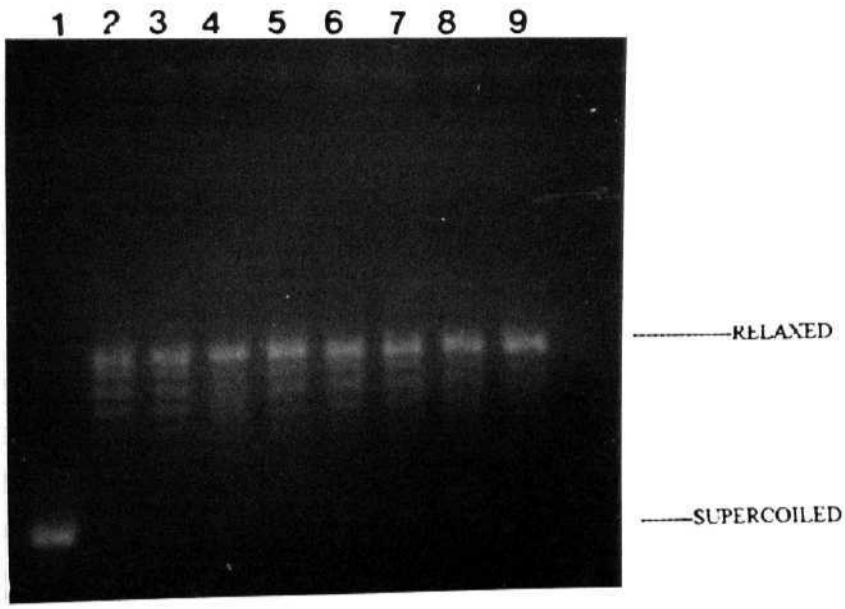

Figure 46

Effect of DBNP-B on *E. coli* DNA polymerase **I activity:**

DNA-DBNP-B complexes were formed in the DNA polymerase I assay buffer with increasing concentrations of the protein and incubated at 37°C for 15 minutes and then *E. coli* DNA polymerase I was added and incubated for DNA synthesis as described in methods section. In the control the DNA was incubated with *E. coli* DNA polymerase I in the absence of DBNP-B. Upto 1:3 ratio of DNA:DBNP-B there is an enhancement in the activity of DNA polymerase I compared to the control. At 1:5 ratio of DNA:DBNP-B, the level of DNA polymerase I activity is almost same as that of the control. Beyond this concentration there is a complete inhibition in the activity of DNA polymerase 1. After 1:5 concentration of DNA:DBNP-B the DNA is saturated with the protein thus blocking the acess of DNA by DNA polymerase I. These results indicate that DBNP-B forms different kinds of complexes with DNA at different protein concentrations (Fig.47).

Effect of DBNP-B on *E. coli* RNA polymerase activity:

DNA-DBNP-B complexes were formed in the RNA polymerase assay buffer with increasing concentrations of DBNP-B and incubated at 37^0C for 15 minutes and then RNA polymerase was added and incubated at 32°C for 30 minutes. In the control, the DNA was incubated with RNA polymerase in the absence of DBNP-B. RNA synthesis was measured as described in methods section. As before, upto 1:3 concentrations of DNA:DBNP-B there is a stimulation in the activity of RNA polymerase. At 1:3 ratio of DNA:DBNP-B the level of RNA polymerase activity is same as that of the control. Beyond this concentration there is a progressive inhibition in the activity of RNA polymerase (Fig.48).

Joint molecule formation:

DNA renaturation and aggregation are considered as important intermediate states in DNA recombination. Most of the proteins known to catalyse strand exchange are capable of DNA aggregation and renaturation. Joint molecules (triple stranded structures) between complementary DNA molecules were formed

Fig. 47: Influence of DBNP-B on *E. coli* DNA polymerase I:

DNA polymerase assay was performed as described in materials and methods at different DNA:DBNP-B concentrations. In the control this assay was performed in the absence of DBNP-B. The efficiency of DNA polymerase I was studied by measuring the number of pico moles of dTTP incorporated at various DBNP-B:DNA concentrations.

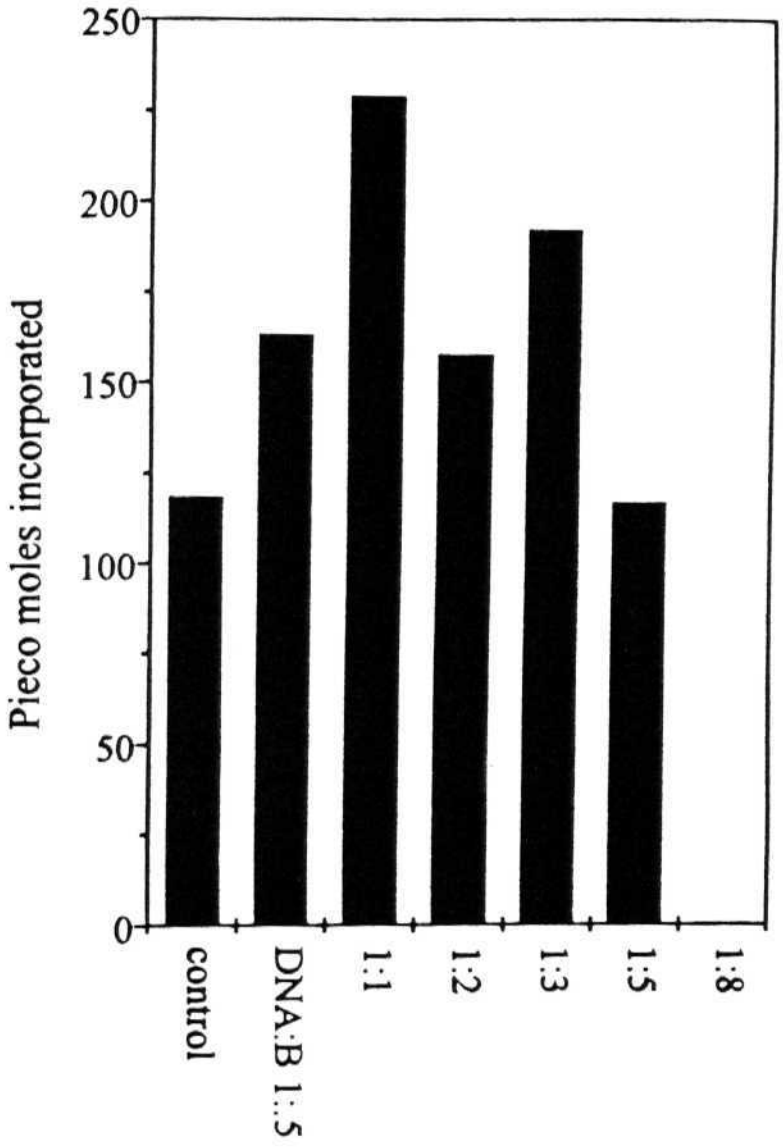
Pieco moles incorporated
250
200
150
100
50
0
control
DNA:B 1:.5
1:1
1:2
1:3
1:5
1:8

Figure 47

Fig. 48: Influence of DBNP-B on *E. coli* RNA polymerase:

RNA polymerase assay was performed as described in materials and methods at different DNA:DBNP-B concentrations. In the control this assay was performed in the absence of DBNP-B. The efficiency of RNA polymerase was studied by measuring the number of pico moles of UTP incorporated at various DBNP-B:DNA concentrations.

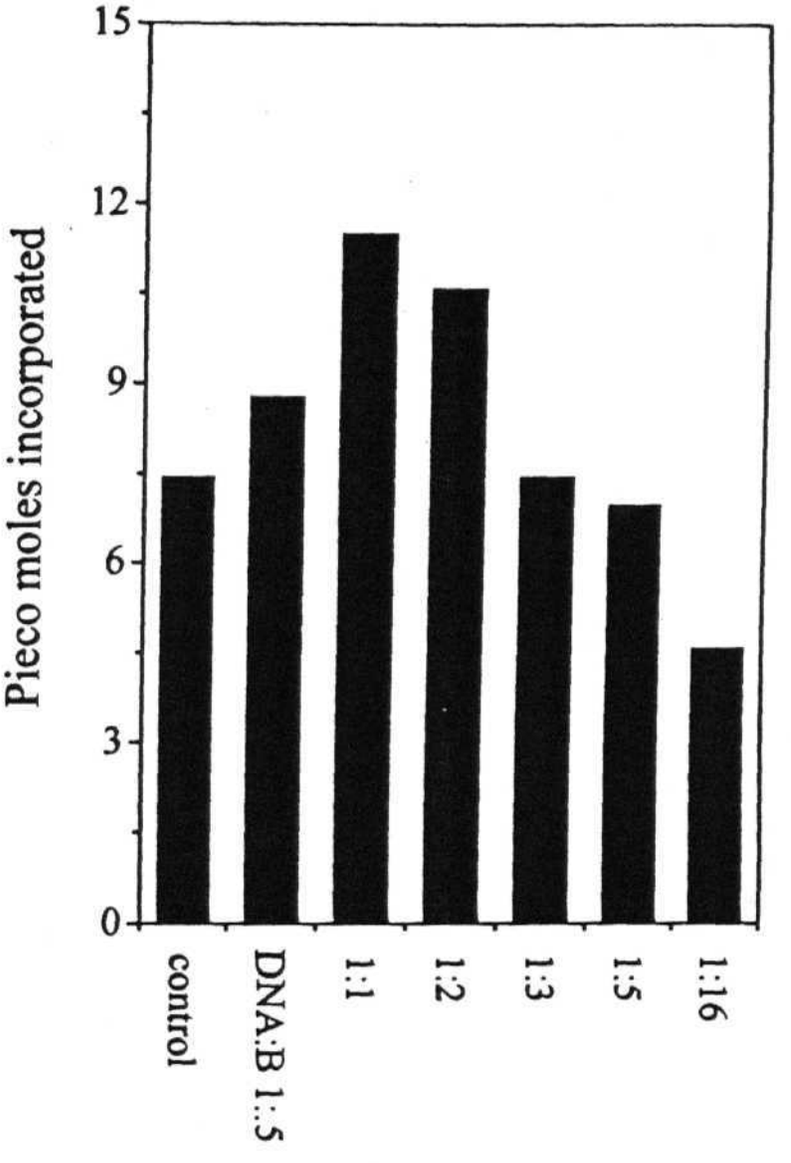
Pieco moles incorporated
15
12
9
6
3
0
control
DNA:B 1:.5
1:1
1:2
1:3
1:5
1:16

Figure 48

during these strand exchange reactions and their formation can be visualised on agarose gels. The ability of DBNP-B to promote strand exchange reaction between M13 ss DNA and linearised M13 ds DNA was tested according to Menetski *et al,* 1990. Results presented in Fig.49 show that DBNP-B failed to promote the strand exchange reaction at different concentrations of DBNP-B (Fig.49 lanes 2-6). The reactants incubated with DBNP-B show the same electrophoretic mobility as the reaction mixtures incubated without DBNP-B (Fig.49 lane 1). No joint molecules were seen even at high concentrations of DBNP-B (1:25 or 1:50 of DNA: DBNP-B).

Tetranitromethane modification of DBNP-B:

Since DBNP-B exhibited interesting fluorescence properties under different solution conditions and the fluorescence intensity was quenched upon nucleic acid binding, the importance of tyrosyl residues in nucleic acid binding was assessed by chemical modification.

Nitration of tyrosine residues of DBNP-B with tetranitromethane (TNM) at pH 8.0 was carried out as described in methods section. TNM irreversibly and specifically nitrates tyrosine to 3-nitrotyrosine. Intermolecular covalent crosslinking of protein in the presence of TNM is found in some proteins (Williams and Lowe, 1971). The TNM modified DBNP-B was analysed on a 15% SDS-PAGE for intermolecular crosslinking. As seen in Fig.50A lane 4 treatment with TNM did not result in any crosslinking. The protein fluorescence of DBNP-B is characteristic of tyrosine with an emission maxima at 304 nm and an excitation maxima at 274 nm. The fluorescence emission spectrum of TNM modified and native DBNP-B as seen in Fig.50B in 10 mM Tris-Cl (pH 7.6), 30 mM NaCl shows that the intrinsic fluorescence of the protein due to tyrosine has been quenched about 50% upon TNM modification compared to the unmodified protein.

The effect of tyrosine modification of DBNP-B on protein-nucleic acid interaction was studied by affinity chromatography on a single stranded DNA-cellulose column as described in methods section to identify whether tyrosine is involved in the interaction of nucleic acids. DBNP-B binds strongly to ss DNA-cellulose even at 125 mM concentration and is eluted only at 500 mM KC1

Fig. 49: Joint molecule formation by DBNP-B:

Formation of joint molecules between M13 ssDNA and linearised M13 dsDNA by DBNP-B was tested at different protein concentrations. M13 ssDNA (200 ng) was incubated with DBNP-B for 10 minutes at 37°C in 1 mM $MgCl_2$ in 25 mM Tris-Cl (pH 7.4) and 1 mM DTT buffer before the addition of M13 lineansed dsDNA (400 ng). Then the $MgCl_2$ concentration was increased to 12 mM after the addition of dsDNA and the incubation was continued for another 30 minutes. The reaction products were deproteinised with 1% SDS and 25 mM EDTA and electrophoresed on a 0.8% agarose gel.

Lane 1: M13 ssDNA and linearised dsDNA mixture in the absence of DBNP-B.
Lane 2-6: Reaction mixtures incubated in the presence of 0.6μg, 3μg, 6μg, 15μg and 30μg of DBNP-B respectively.

Fig. 50A: SDS-PAGE analysis of tetranitromethane modified DBNP-B:

DBNP-B was modified with 5 mM TNM as described in materials and methods, dialysed exhaustively against 10 mM Tris-Cl (pH 7.5), 30 mM NaCl and 7 mM β−mercaptoethanol and electrophoresed on a 15% SDS-polyacrylamide gel followed by silver staining.

Lane 2: DBNP-B control (4 μg).
Lane 4: TNM modified DBNP-B (4 μg).
Lane 8: Molecular weight markers (66 kDa- BSA, 45 kDa -Ovalbumin, 25 kDa-Chymotrypsinogen, 20.1 kDa -Trypsin inhibitor and 12.3 kDa-Cytochrome-C).

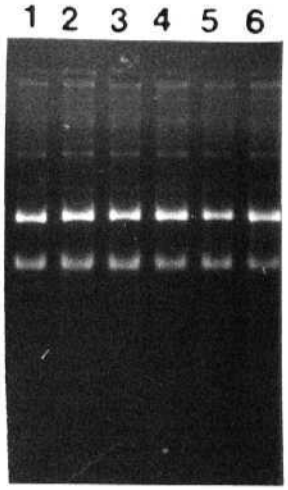

Figure 49

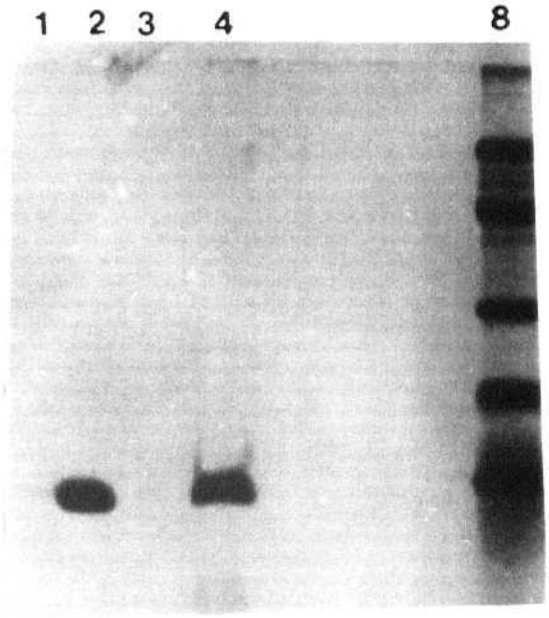

Figure 50A

Fig. 50B: Fluorescence emission spectrum of tetranitromethane modified DBNP-B:

Fluorescence emission spectrum of DBNP-B, normal and TNM modified (10 μg) in 1 ml of 10 mM Tris-Cl (pH 7.6), 30 mM NaCl buffer was recorded between 280-360 nm by exciting at 274 nm. The fluorescence intensity measured is in arbitrary units.

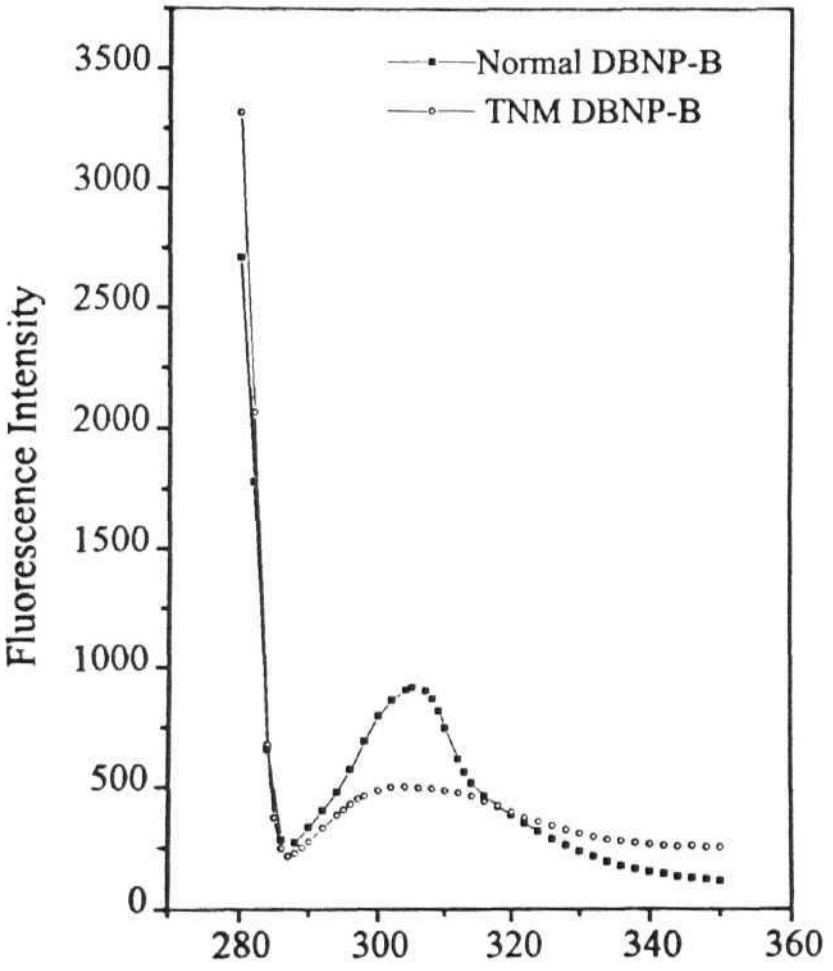
Normal DBNP-B
TNM DBNP-B
Fluorescence Intensity
3500
3000
2500
2000
1500
1000
500
0
280
300
320
340
360
Wavelength(nm)

Figure 50B

concentration. TNM modified DBNP-B was passed through the ss DNA-cellulose column and the column was eluted with 300 mM and 500 mM **KCI containing** buffer. SDS-PAGE analysis of the fractions show that TNM modified DBNP-B eluted in the 300 mM KCl fractions. Treatment with TNM resulted in decreased affinity of DBNP-B to single stranded DNA, indicating that tyrosine residues of DBNP-B probably play an important role in the strong binding of the protein to ss DNA. The effect of nitration of tyrosine on DNA aggregation, renaturation and nicking activity is worth investigating.

CHAPTER 4

DISCUSSION

In this thesis, results are presented on the structure of archaeal nucleoid and on interactions of archaeal nucleoid associated DNA binding protein DBNP-B. Although extensive work has been carried out on the organisation of eukaryotic chromatin, no clear picture has emerged regarding either bacterial chromatin (nucleoid) or archaeal chromatin (nucleoid). In an effort to understand the organisation of archaeal chromatin, methods were developed to isolate nucleoid under different conditions from *Sulfolobus acidocaldarius*.

S. acidocaldarius nucleoid can be isolated as a rapidly sedimenting nucleoprotein complex. In order to ascertain which of the proteins are truly components of the nucleoid, the nucleoid was purified by gel filtration chromatography and DEAE-cellulose chromatography. To facilitate gel filtration chromatography the viscous nucleoid was sonicated. Chromatography on sephacryl S-1000 columns resulted in the elution of the nucleoid material in two distinct regions. Nucleoid isolated from cells grown upto early and midlogarthmic phases showed similar characteristics on gel filtration chromatography. Analysis of protein composition by SDS-PAGE showed that peak-I fraction consists of predominantly high molecular weight proteins along with very high molecular weight protein(s) which remained at the top of the gels. Peak-II fraction contained both high molecular weight and low molecular weight proteins, the latter in abundance. To identify DNA binding proteins other than low molecular weight histone like proteins, the nucleoid peak-II fraction was digested with DNase I and chromatographed on a sephadex G-50 column. The protein fraction depleted of histone like proteins was chromatographed on DNA-cellulose column. The DNA bound fraction as analysed by SDS-PAGE, contained proteins in molecular weight range of 45 kDa, 25 kDa and 15 kDa apart from HSNP-A, DBNP-B and HSNP-C'.

Similar analysis of peak-I nucleoid fraction on DNA-cellulose also identified a 15 kDa protein and a few proteins with Mr. of 20-30 kDa. These results suggest that both peak-I and peak-II nucleoid fraction have essentially similar DNA binding proteins including the low molecular weight proteins. These results indicate that the high molecular weight material which stayed at the top of

the SDS-gels could be an aggregate of the DNA binding proteins and low molecular weight proteins. Nucleoid purified by DEAE-cellulose showed very similar protein composition as the DNA-cellulose bound protein fraction. At this moment, the result suggest that purified nucleoid contains apart from the four DNA binding acid soluble proteins (HSNP-A, DBNP-B, HSNP-C and HSNP-C'), a 15 kDa protein and atleast two to three 25-27 kDa proteins. The interactions among these proteins and with DNA needs to be worked out.

Micrococcal nuclease digestion of both purified and crude nucleoid resulted in the protection of DNA of approximately 600 bp length. Mild to exhaustive micrococcal nuclease digestion did not detect any high molecular weight DNA intermediates other than 600 bp and 1200 bp. These preliminary results suggest that the overall condensation of DNA and its Organisation by the nucleoid proteins in archaea may be different from the organisation of eukaryotic DNA.

Detailed studies were carried out on DBNP-B, one of the abundant nucleoid associated DNA binding proteins. This protein showed novel and interesting properties with respect to oligomerisation, temperature and metal ion induced conformational changes, DNA aggregation, renaturation and DNA nicking activity.

One of the interesting aspects of DBNP-B is its oligomeric aggregation. The protein seems to exist in a very high molecular weight aggregated state as indicated by increase in aggregation pattern at 80°C (the optimum temperature for growth of *S. acidocaldarius*). The largest multimeric state that is clearly identifiable is octameric aggregate with a Mr of about 70 kDa (at pH 7.6 in the presence of Mg^{++} ions). However, under most of the crosslinking conditions dimeric, tetrameric and hexameric species are dominant over odd numbered aggregates (trimer, pentamer *etc)* indicating that the stable aggregated form of DBNP-B could be a tetramer or a hexamer and that the other high molecular weight and very high molecular weight aggregates could be artefactual. Crosslinking with dimethyl suberimidate could only detect dimeric species, and no higher molecular weight species were detected. These results may suggest that the self aggregation of DBNP-B may involve closely packed interactions and that the necessary groups (e NH_2 of lysine) involved in bifunctional crosslinking are not available. This is also supported by the observation that DBNP-B is totally

resistant to cleavage by trypsin (which cleaves at arginine or lysine). Gel filtration chromatography of native DBNP-B also detected tetrameric species. However this experiment could not be performed at high temperature (> 65^{0}C).

Another interesting physical property of DBNP-B is variability in the environment of aromatic amino acids as a function of the solution conditions. The protein contains two tyTOsines and one phenylalanine as calculated from amino acid composition (Reddy and Suryanarayana, 1989). The protein emission fluorescence is enhanced dramatically in the presence of divalent metal ion Mg^{++} and is stimulated by moderate concentration of Na^{+}. These results indicate clear conformational changes in the protein and the aromatic amino acids are highly exposed in the presence of these salt ions. Results on the nucleic acid binding, aggregation, renaturation *etc.* which occur optimally in the presence of Mg^{++} and the observed protein fluorescence enhancement (or exposure of aromatic amino acids) suggest a functional involvement of these amino acids in the interactions of this protein (self interactions and binding to nucleic acids).

The protein is remarkably stable to digestion by proteolytic enzymes suggesting highly compact 3-dimensional structure.

Nucleic acid binding of DBNP-B by mobility shift assays suggest that the protein binds all types of nucleic acids, single stranded and double stranded RNAs and DNAs, circular or linear DNAs with varying affinities. As already reported, the protein forms different types of novel complexes with ss DNA or ds DNA at different protein:DNA ratios. At subsaturating level the protein forms two types of complexes. At very low protein to DNA (upto 2:1), it forms DBNP-B.2ds DNA structure with circular duplex DNA containing double strand loops and at moderate concentrations (> 5:1, protein/DNA), the protein forms Aggregated condensed complex (Sreenivas *et al.*, 1998). The mobilities of the DNA:DBNP-B complexes at different protein concentrations also reflect this. The retardation of circular DNA by DBNP-B was low upto a ratio of 5-6 (protein/DNA) which increased dramatically beyond this ratio.

The coaggregation complexes formed by DNA:DBNP-B was studied by light scattering which confirms the results obtained by other methods (Sreenivas *et*

al., 1998) that the aggregation of DNA by DBNP-B is stimulated by Mg^{++} ions with an optimum concentration of 10 mM.

DNA renaturation reaction promoted by DBNP-B as studied earlier (Sreenivas *et al.,* 1998) was analysed in more detail using DNA of differing sizes. Results presented in the present study indicate that DBNP-B promotes renaturation of complementary single stranded DNA not only to net work like structures but also to form linear double stranded DNA molecules as observed in the case of DNA fragments (50 bp - 1 kb). Renaturation promoted by DBNP-B could be an independent function of this protein or it could be also a generalised effect of DBNP-B such that the topology or structure of DNA in DNA:DBNP-B complexes at a particular protein concentration may be suitable for pairing and action by other enzymes and proteins acting on DNA.

Results presented on the effect of DBNP-B on enzymes acting on DNA (RNA polymerase and DNA polymerase and topoisomerases) show that DNA in DNA:DBNP-B complexes is not only available for action by these enzymes but it is in a more favourable conformation as indicated by stimulation of DNA and RNA polymerase activities.

The novel and interesting activity detected during the course of this study is the nicking activity of DBNP-B. As already mentioned in the results section, it was observed during mobility shift experiments that some of the supercoiled pBR322 DNA is slowly converted to relaxed form. This led to detailed characterisation of the nicking activity with respect to substrate specificity and optimum conditions. The activity is optimal at a ratio of 4-8:1 (protein /DNA) beyond which there was inhibition. Electron microscopy pictures of DNA:DBNP-B complexes formed at very high protein to DNA ratio ($\geq$ 10) show that the protein is uniformly coated all along the ds DNA and the DNA is in relaxed circular form. Lack of nicking activity at very high protein concentration may be due to this mode of binding. The nicking reaction is optimal in broad pH range (7 to 8.5) and is absolutely dependent on Mg^{++} ions.

These results clearly indicate nicking by DBNP-B is a specific enzymatic reaction. Use of different DNA as substrate indicate lack of nicking activity with linear DNA *(X* DNA), a small but non specific nicking on smaller circular

DNA (pUC19 DNA, 2686 bp) and specific and enhanced nicking of moderately longer circular DNA (pBR322, 4363 bp and SV40 DNA, 5243 bp). Sequence specific nicking by DBNP-B can be ruled out because a larger linear DNA like λ DNA (48,502 bp) is unlikely not to have a target sequence that would be present in small DNAs. The preferential and specific nicking of circular DNA of size 4-5 kb may suggest formation of specific DNA structure in DNA.DBNP-B complex that is amenable to cleavage by the protein which is not formed with linear DNA or short circular duplexes. Further work is necessary to analyse the mode of action, site of cleavage and the DNA structure in the DNA.DBNP-B complex that is amenable for nicking by DBNP-B.

Past results from our laboratory and the results presented in this thesis suggest that DBNP-B may have a multifunctional role in *S. acidocaldarius* facilitating several reactions connected with DNA metabolism such as DNA replication, transcription and recombination. This may be related to the ability of the protein to form distinctly different types of complexes with DNA at different protein concentrations.

CHAPTER 5

SUMMARY

Chapter 1 (Introduction):

A brief review of archaea and their molecular biological aspects is given in this chapter. It was followed by a review of histone like DNA binding proteins from eubacteria and archaea. A brief survey on proteins which promote renaturation and aggregation from eubacteria and eukarya is also presented. This was followed by objectives and scope of the present investigation.

Chapter 2 (Materials and methods):

This chapter lists the materials used in the study and gives a brief description of the methods that were followed in the investigation. A purification procedure for nucleoid from *S. acidocaldarius* was presented. A purification protocol for DBNP-B is presented. The protein chemical properties were studied by using various cleaving agents like V8 protease, trypsin, chymotrypsin and cyanogen bromide. The solution state of DBNP-B was studied by employing formaldehyde and dimethyl suberimidate crosslinking. The nucleic flcid binding properties of the 11 kDa DBNP-B were studied by employing a variety of techniques like fluorimetry, gel mobility shift assays, renaturation assays, nicking assays and aggregation assays. The functional properties of DBNP-B were also studied. The effect of DBNP-B on various enzymes like topoisomerase I, topoisomerase 11, DNA polymerase and RNA polymerase are presented.

Chapter 3 (Results):

This chapter deals with the results that were obtained in the present investigation and contain the following sections.

3.1: Characterisation *of S. acidocaldarius* nucleoid:

Nucleoid from *S. acidocaldarius* was purified and passed through a sephacryl S-1000 column for further purification which resulted in the separation

of nucleoid into two peaks, peak-I and peak-II which differ in quality. Peak-I nucleoid shows high molecular weight proteins in abundance along with low amounts of low molecular weight histone like proteins. The DNA looks like a high molecular weight sharp band. Peak-11 nucleoid shows low molecular weight histone like proteins in abundance along with high molecular weight proteins. The DNA is resolved as a broad smear on an agarose gel. This separation of nucleoid into two peaks is observed in freshly harvested early logarthmic phase cells and midlogarthmic phase cells and is thus not growth phase dependent. The nucleoid proteins in both the peaks which bind to DNA were characterised by passing through a DNA cellulose column. The tight binding proteins in the nucleoid were also characterised. Micrococcal nuclease digestion of nucleoid resulted in ~ 600 bp to 1500 bp fragments.

3.2: Purification and properties of **DBNP-B:**

DBNP-B was purified from cell extracts by a single CM-cellulose chromatographic step. The solution state of DBNP-B was studied by formaldehyde crosslinking at three different temperatures 37°C, 65°C and 80°C. Crosslinking results indicate that DBNP-B predominantly exists in a dimeric state in pH 5.0 containing buffer at 37^0C and 65°C and it probably exists as oligomeric forms in the presence of $MgCl_2$ at 80°C. DBNP-B when passed tlirough a gel filtration column, sephadex G-50 eluted as aggregates corresponding to dimer predominantly along with some tetrameric protein at room temperature. DBNP-B was cleaved chemically with cyanogen bromide and proteolytically with V8 protease, trypsin and chymotrypsin. DBNP-B seems resistant to trypsin and chymotrypsin.

3.3: Nucleic acid binding properties of DBNP-B:

The interaction between DBNP-B and DNA as well as RNA was studied using mobility shift assays, fluorescence assays, DNA renaturation and other assays. The binding interaction between DBNP-B and poly (U) or poly (A) was characterised and the site size of DBNP-B was calculated from the fluorescence titrations with poly (U) and poly (A). DBNP-B aggregated double stranded DNA. The aggregation was dependent on protein concentration and increases with increase in DBNP-B concentration. Complementary' ss DNA was renatured into

high molecular weight network by DBNP-B. This DNA renaturation requires Mg^{++} and showed an optimum of pH 5.0. Renaturation was dependent on protein concentration and high concentrations of protein reduced renaturation. pUC19 supercoiled DNA showed increased sensitivity to DNase I at intermediate concentrations of DBNP-B and at high concentrations, a protection from DNase 1 digestion was observed. A non specific nicking activity of DNA by DBNP-B was observed which is Mg^{++} dependent and protein concentration dependent.

DBNP-B neither inhibits nor enhances the activity of DNA topoisomerase 1 and DNA topoisomerase II. DBNP-B enhances the activity of DNA polymerase I and RNA polymerase at low concentrations and inhibits their activity at high concentrations. Chemical modification of tyrosine residues in the protein by tetranitromethane resulted in the decreased affinity of DBNP-B to DNA-cellulose.

Chapter 4 (Discussion):

This chapter deals with the implications of the results obtained in the present investigation.

S. acidocaldarius nucleoid can be isolated as a rapidly sedimenting nucleoprotein complex. In order to ascertain which of the proteins are truly components of the nucleoid, the nucleoid was purified by gel filtration chromatography and DEAE-cellulose chromatography, Nucleoid isolated from cells grown upto early and midlogarthmic phases showed similar characteristics on gel filtration chromatography. Both peak-I and peak-II nucleoid fraction have essentially similar DNA binding proteins including the low molecular weight proteins. The purified nucleoid contained apart from the four DNA binding acid soluble proteins (HSNP-A, DBNP-B, HSNP-C and HSNP-C') a 15 kDa protein and atleast two to three 25-27 kDa proteins. Micrococcal nuclease digestion results of nucleoid suggest that the overall condensation of DNA and its organisation by the nucleoid proteins in archaea may be different from the organisation of eukaryotic DNA.

DBNP-B seems to exist in a very high molecular weight aggregated state. The largest multimeric state that is clearly identifiable is an octameric aggregate.

However under most of the crosslinking conditions, dimeric, tetrameric and hexameric species are dominant indicating that stable aggregated form of DBNP-B could be a tetramer or a hexamer. The protein is remarkably stable to digestion by proteolytic enzymes suggesting highly compact 3 dimensional structure. Results on nucleic acid binding, aggregation, renaturation *etc* which occur optimally in the presence of Mg^{++} and the observed fluorescence enhancement suggest a functional involvement of aromatic amino acids in the interaction of DBNP-B. Nucleic acid binding of DBNP-B by mobility shift assays suggest that the protein binds all types of DNA ss or ds or circular or linear with varying affinities. The coaggregation complexes formed by DNA:DBNP-B show that the aggregation of DNA by DBNP-B is stimulated by Mg^{++} ions with an optimum concentration of 10 mM.

DBNP-B promotes renaturation of complementary ss DNA not only to network like structures but also to form linear ds molecules. Renaturation promoted by DBNP-B could be an independent function of this protein or it could be also a generalised effect of DBNP-B such that the topology or structure of DNA in the DNA:DBNP-B complexes at a particular protein concentration may be suitable for the action of other enzymes and proteins acting on DNA.

Results presented on the effect of DBNP-B on enzymes acting on DNA show that DNA in DNA:DBNP-B complexes is not only available for action by these enzymes but is in a more favourable conformation. The novel and interesting activity detected during the course of this study is the nicking activity of DBNP-B which is optimal in a broad pH range of 7 to 8.5 and is absolutely dependent on Mg^{++} ions. The preferential specific nicking activity of circular DNA of size 4-5 kb may suggest formation of specific DNA.protein complex that is amenable to cleavage by the protein which is not possible with linear DNA or short circular duplexes. DBNP-B may have a multifunctional role in *S. acidocaldarius* facilitating several reactions connected with DNA metabolism such as DNA replication, transcription and recombination.

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