2 EXPERIMENTAL

2.1 MATERIALS

2.1.1. Chemicals

Calf thymus DNA, nuclease S1 (Aspergillus oryzae), poly-L-lysine, poly-L-glutamate, bovine serum albumin, agarose, Coomassie Brilliant Blue R 250, xylene cyanole FF, anti-IgG-alkaline phosphatase conjugate, trinitrobenzene sulphonic acid, ethidium bromide, Freund's complete and incomplete adjuvants, standard protein markers and fluorescent dye Hoechst 33258 were purchased from Sigma Chemical Company, U.S.A. Synthetic polynucleotides, micrococcal nuclease, Ox 174 RF DNA Hae III restriction nuclease digest, Sepharose 4B, DEAE Sephadex, DEAE Sephadex A 25, Sephadex G 200, agarose NA, Ficoll 400 were obtained from Pharmacia Fine Chemicals, Sweden. P-nitrophenyl phosphate, Folin-Ciocalteu reagent and Blue Dextran 2000 were purchased from C.S.I.R. Centre for Biochemicals, Delhi.

Polystyrene microtitre flat bottom ELISA plates having 96 wells (7 mm in diameter) were obtained from Dynatech, U.S.A. Tween-20, acrylamide, N, N'-methylenebisacrylamide, ammonium persulphate, N, N, N',N'-tetraethylenediamine, hydroxyapatite were from Bio-Rad Laboratories, U.S.A. Hydrogen peroxide, ninhydrin, sodium dodecyl sulphate and acetic anhydride were purchased from B.D.H. England. All other chemicals were of highest analytical grade available.

2.1.2. Equipment

Bausch and Lomb Spectronic-20, Fraction collector FRAC-100 (Pharmacia, Sweden), Shimadzu spectrophotometer UV-240 equipped with thermoprogrammer and controller unit, Shimadzu spectro-fluorophotometer RF-540, ELISA microplate reader MR-600 (Dynatech, U.S.A.) Beckman
ultracentrifuge model L860 M, Elico pH meter model Ll-10T, gel electrophoresis apparatus GNA-100 (Pharmacia, Sweden), short wavelength ultraviolet lamp (Spectroline R-51, Black Light Eastern Inc. U.S.A.) having maximum emission at 253.7 nm were the major equipment used in this study.

2.2. METHODS

2.2.1. Purification of Calf Thymus DNA

Highly polymerized calf thymus DNA was purified free of proteins, RNA and single stranded regions as described by Ali et al. (1985). The DNA (2 mg/ml) in 0.1 x SSC buffer (0.015 M sodium citrate, 0.15 M sodium chloride, pH 7.3) was extracted with a mixture of chloroform-isoamyl alcohol (24:1) in a stoppered measuring cylinder for one hr. DNA in aqueous layer was separated from organic layer and precipitated with two volumes of cold ethanol and thereafter collected on a glass rod. The extraction process was repeated and DNA was dissolved in 30 mM acetate buffer, pH 5.0 containing 30 mM zinc chloride. The single stranded regions were removed by treatment with nuclease S1 (200 units/mg DNA) at 37°C for 30 min. The reaction was stopped by the addition of one tenth volume of 0.2 M EDTA, pH 8.0. The extraction procedure was repeated as before and final preparation of DNA was dissolved in PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4).

2.2.2. Isolation of DNA Fragments

Purified DNA (2 mg/ml in 100 mM NaCl, 6 mM Tris, 2 mM CaCl₂, pH 8.0) was subjected to controlled digestion with micrococcal nuclease (40 units/mg DNA) at 37°C for 2.5 min to obtain fragments varying approximately from 70 bp to 800 bp. The reaction was stopped by the addition of EDTA, pH 8.0, to a final concentration of 20 mM. The mixture was extracted twice with chloroform-isoamyl
alcohol and DNA fragments were precipitated with ethanol. This was followed by the removal of single stranded regions with nuclease S1 digestion (200 units/mg DNA) at 37°C for 30 min. The digested fragments were extracted as before, dissolved in TBS (10 mM Tris, 150 mM NaCl, pH 8.0) and passed through a 2.06 cm x 57 cm column of Sepharose 4B equilibrated with TBS. Fractions of 4 ml were collected and monitored at 260 nm (Figure 2). DNA fragments were analysed for size by slab gel electrophoresis (inset Figure 2).

2.2.3. Colorimetric Estimation of DNA
Concentration of DNA was determined by the method of Burton (1956) using diphenylamine reagent.

2.2.3.1. Crystalization of diphenylamine
Diphenylamine (2 g) was dissolved in 200 ml of boiling hexane. Approximately 0.5 g of activated animal charcoal was supplemented to the boiling mixture. The solution was filtered while hot and filtrate was kept overnight at 4°C. The crystals were separated by filtration and dried at room temperature.

2.2.3.2. Preparation of diphenylamine reagent
Recrystallized diphenylamine (750 mg) was dissolved in 50 ml of glacial acetic acid containing 0.75 ml of concentrated sulphuric acid.

2.2.3.3. Procedure
To 1.0 ml of DNA was added 1.0 ml of 1N perchloric acid. The tubes were incubated in a thermostat water bath at 70°C for 15 min. Hundred uL of 5.43 mM acetaldehyde was added to each tube followed by the addition of 2 ml of freshly prepared diphenylamine reagent. The contents were mixed and allowed to stand at room temperature for 16-20 hr. Absorbance was recorded at 600 nm. The concentration of DNA in unknown sample was
Figure 2. Fractionation of calf thymus DNA on Sepharose 4B column digested briefly with micrococcal nuclease. Inset: The results of polyacrylamide gel electrophoresis of fractionated DNA. Lanes (2-8) represent fractions 20-30. Lane 1 represents native DNA and lane 9 contained Hae III digest of φx174 RF DNA.
determined from the standard plot constructed by using 0-100 ug of purified calf thymus DNA (Figure 3).

2.2.4. Determination of Protein Concentration

Protein was estimated colorimetrically by the method of Lowry et al. (1951).

2.2.4.1. Folin-Ciocalteu reagent

The reagent was purchased from C.S.I.R. Centre for Biochemicals, Delhi and diluted 1:4 before use.

2.2.4.2. Alkaline copper reagent

The components of alkaline copper reagent were prepared as follows:
(a) 2% sodium carbonate in 0.1 N NaOH
(b) 0.5% copper sulphate in 1.0% sodium potassium tartrate

The working reagent was prepared fresh just before use by mixing components (a) and (b) in the ratio of 50:1.

2.2.4.3. Procedure

To 1.0 ml of protein sample was added 5.0 ml of alkaline copper reagent. The contents were mixed and allowed to stand at room temperature for 10 min. Working Folin-Ciocalteu reagent (1 ml) was added to each tube, mixed immediately and left at room temperature for 30 min. The absorbance was recorded at 660 nm. The concentration of unknown sample was evaluated from a standard plot constructed by using varying concentrations of bovine serum albumin (Figure 4).

2.2.5. Irradiation of DNA

Aqueous solutions of calf thymus DNA and DNA fragments of 300 bp (0.15 mM) in PBS, pH 7.4 were irradiated under 254 nm light for 30 min at room temperature in the presence of hydrogen peroxide. The
Figure 3. Standard plot for the colorimetric estimation of DNA.
Figure 4. Standard plot for the colorimetric estimation of protein.
temperature during the irradiation period was kept constant by circulating tap water. The concentration of hydrogen peroxide, determined by titration with sodium thiosulphate (Ansari et al., 1985) was 15.1 mM. Excess of hydrogen peroxide after irradiation was removed by exhaustive dialysis against PBS, pH 7.4. Native DNA samples exposed to hydrogen peroxide or UV light alone were used as corresponding controls.

2.2.6. Determination of Melting Temperature
Thermal denaturation analysis of nucleic acids was accomplished in order to ascertain the degree of modification incurred on the nucleic acids by determining midpoint melting temperature ($T_m$).

The $T_m$ of nucleic acid samples was determined by Shimadzu UV 240 spectrophotometer coupled with a temperature programmer and controller assembly (Hasan and Ali, 1990). The samples in PBS, pH 7.4 were heated at the rate of 1°C per min initiating from 30°C up to 95°C. The absorbance was recorded at 260 nm and the percent denaturation evaluated with increase in temperature as follows:

$$\% \text{ denaturation} = \frac{A_T - A_{30}}{A_{\text{max}} - A_{30}} \times 100$$

where, $A_T$ = Absorbance at a temperature $T^\circ C$

$A_{\text{max}}$ = Final maximum absorbance on the completion of denaturation

$A_{30}$ = Initial absorbance at 30°C

2.2.7. Thermodynamic Analysis of Nucleic Acid Denaturation
The thermal transition of nucleic acid from native to denatured state was characterized as single variable, $fD$, 

43
the fraction of nucleic acid in the denatured state. The percent loss in absorbance was used as experimental variable to follow the transition. At any given point along the transition curve, the observed percent change in absorbance was related to the fraction of nucleic acid in the denatured state, by the following expression:

\[
\frac{(A)_{\text{obs}} - (A)_{\text{N}}}{(A)_{\text{D}} - (A)_{\text{N}}}
\]

where \((A)_{\text{obs}}\), \((A)_{\text{N}}\) and \((A)_{\text{D}}\) represent the percent loss in absorbance in any observed, native and fully denatured states respectively. Since each experimental value of \((A)_{\text{obs}}\) will give unique value of \((A)_{\text{D}}\), the later was used to construct transition curves for the thermal denaturation of nucleic acid.

By assuming that unfolding of the strands of nucleic acid is a two state process due to the presence of A-T and G-C regions respectively, the initial and final states of thermal denaturation of nucleic acids were related to apparent equilibrium constant, defined as:

\[
K_{\text{app}} = \frac{(A)_{\text{obs}} - (A)_{\text{N}}}{(A)_{\text{D}} - (A)_{\text{obs}}}
\]

The free energy change, \(\Delta G\), for the thermal reaction (native double stranded conformation to single stranded conformation) was calculated by using the following relationship:

\[
\Delta G^O = -RT \ln K_{\text{app}}
\]

where, \(K_{\text{app}}\) is the apparent equilibrium constant
2.2.8. Hydroxyapatite Column Chromatography

Hydroxyapatite column chromatography was employed to discriminate nucleic acids endowed with different secondary structures. Modified and unmodified DNA samples were chromatographed on hydroxyapatite column before and after heat denaturation (Dardalhon and Averbeck, 1988). Three ml of DNA sample (100 ug/ml) in 10 mM sodium phosphate buffer pH 6.8 was loaded onto a hydroxyapatite column (1.5 cm x 14 cm) previously washed and equilibrated with 10 mM sodium phosphate buffer pH 6.8. The bound material was eluted with 0.125 M and 0.25 M potassium phosphate buffer, pH 6.8. Fractions of 3.0 ml were collected at a flow rate of 10 ml/hr and monitored at 260 nm. DNA samples were denatured by heating for 15 min at 100°C, at a concentration of 100 ug/ml in 10 mM sodium phosphate buffer and fast cooled.

2.2.9. Detection of Single Strand Breaks

The damage to DNA mediated by hydroxyl radical was ascertained by sucrose density gradient centrifugation and polyacrylamide gel electrophoresis.

2.2.9.1. Alkaline density gradient centrifugation

Nucleic acid samples (0.15 ml) were treated by the addition of an equal volume of 0.2 N NaOH. After a ten min delay to permit denaturation of DNA, 0.2 ml of each sample was layered on top of a 3.8 ml alkaline sucrose gradient (5-20% sucrose in 0.1 N NaOH and 0.01 M EDTA) (Ali and Sauerbier, 1978). The gradients were centrifuged at 37,000 rpm for 10.5 hr at room temperature in the swinging bucket rotor of Spinco E, Beckman ultracentrifuge. Six drop fractions were collected and diluted to about 0.5 ml by the addition of 0.3 ml of double distilled water and their absorbance recorded at 260 nm.
2.2.9.2. Polyacrylamide slab gel electrophoresis

Electrophoresis of nucleic acid samples was performed with 7.5% acrylamide gels under denaturing and non-denaturing conditions according to the method of Sealey and Southern (1985).

2.2.9.2.1. Electrophoresis under non-denaturing conditions

(i) Following stock solutions were prepared

A - 40% acrylamide in distilled water
B - 2% bisacrylamide in distilled water
C - Resolving gel buffer: 0.9 M Tris-borate, 25 mM EDTA (pH 8.3)
D - TEMED
E - 1.5% ammonium persulphate (freshly prepared)

(ii) Recipe for the preparation of non-denaturing polyacrylamide gels

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Amount in ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>7.5</td>
</tr>
<tr>
<td>B</td>
<td>7.5</td>
</tr>
<tr>
<td>C</td>
<td>4.0</td>
</tr>
<tr>
<td>D</td>
<td>0.025</td>
</tr>
<tr>
<td>E</td>
<td>2.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>19.0</td>
</tr>
</tbody>
</table>

in a total volume of 40 ml and final polyacrylamide gel concentration of 7.5%

(iii) Procedure

The gel plates (19 cm x 16 cm) were cleaned by soaking them in chromic acid overnight, rinsed with water
and then with ethanol. The bottom of the gel mould was sealed with 1% agarose. The gel mixture was prepared by adding the correct volumes of all components, gently mixed and poured between the glass plates within 0.5 cm of the top. Immediately, a comb was inserted between the glass plates and the assembly left undisturbed for the gel to polymerise (30-50 min). DNA samples in buffer of low ionic strength and at concentration of 1 ug/uL were loaded directly onto the gels by adding one tenth volume of the ‘stop mix’ (30% Ficoll, 0.025% xylene cyanole FF, 0.5 M EDTA, 10 times concentrated electrophoresis buffer). The gels (19 cm x 16 cm x 0.2 cm) were run for 6-8 hr at 70 volts. After electrophoresis, the gels were stained directly by immersion in electrophoresis buffer containing 1 ug/ml ethidium bromide for 30-60 min and were viewed by illumination with UV light.

2.2.9.2.2. Electrophoresis under denaturing conditions

Electrophoretic fractionation of nucleic acids was carried out under denaturing conditions using urea as a denaturing agent. The composition of the stock solutions were same as for non-denaturing gels. The gels were prepared as non-denaturing gels except that the volume of distilled water was reduced to allow 16.8 g of urea to be added to give a final volume of 38.0 ml. Thereafter, 0.025 ml of solution D and 2.0 ml of solution E was added immediately before pouring the gel. The final gel mixture contained 7 M urea. Samples were prepared by adding urea (upto 7 M) and 0.025% of tracking dye xylene cyanole FF. To destroy any aggregates, the preparations were heated for 10 min at 90°C before their application onto the gel. The reservoir buffer contained 7 M urea. The gels were run for 10-12 hr at 70 volts. Rest of the procedure was same as above.

2.2.9.3. Isolation of DNA by diffusion

After locating the bands, the gel was cut into
lanes. Each lane was sliced into 5 mm pieces and DNA extracted by diffusion. Elution by diffusion was carried out by placing intact piece of thin gel slice into a 0.5 ml buffer consisting of 0.15 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. The gel was gently macerated using a glass rod (Maxam and Gilbert, 1980). After overnight incubation (more than 12 hr) at 37°C, the mixture was centrifuged at 12,000 g for 10 min in a microfuge. The supernatant containing DNA was filtered through Millipore filter (0.45 μ).

2.2.9.4. Fluorometric assay for DNA with ethidium bromide

The fluorescence of ethidium bromide is enhanced on binding to DNA (Karsten and Wallenberger, 1972). Enhancement involves intercalation of molecules of the dye between the nucleic acid bases.

2.2.9.4.1. Reagents
(a) Phosphate-buffered saline (PBS) (170 mM NaCl, 3.3 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4).
(b) 100 μg/ml of ethidium bromide in PBS.

2.2.9.4.2. Method
To 0.2 ml of extracted DNA was added 2.2 ml of PBS, pH 7.4. The sample was treated with 0.1 ml of ethidium bromide solution and fluorescence intensity of ethidium-DNA complex (λ<sub>ex</sub> = 360 nm, λ<sub>emit</sub> = 580 nm) was recorded with respect to free ethidium bromide of the same concentration.

2.2.10. Nuclease S1 Sensitivity of Modified DNA Samples
Twenty μL of sample containing approximately 2 μg DNA in acetate buffer (30 mM each of sodium acetate and zinc chloride, pH 5.0) was treated with 40 units of nuclease S1 (Matsuo and Ross, 1987). After 30 min incubation at 37°C, the reaction was stopped by the addition of one tenth volume of 0.2 M EDTA, pH 8.0. The
digested sample was subjected to agarose gel electrophoresis.

(a) Gel preparation

Agarose (0.18 g) in 30 ml of TAE (40 mM Tris-acetate, pH 8.0 containing 2 mM EDTA) was dissolved by heating in boiling water bath. The solution was cooled to between 50°C and 60°C. The solution was poured into the gel tray and left at room temperature for 30 min for complete solidification.

(b) Sample preparation and loading

The nuclease S1 treated nucleic acid samples were prepared for loading onto the gels by adding one fifth volume of concentrated loading buffer (0.125% bromophenol blue, 30% Ficoll 400, 0.5 M EDTA and 10x electrophoresis buffer). The samples were loaded in the wells of the submerged gel (0.4 cm thick) and electrophoresed for 2-3 hr at 40 V. Ethidium bromide was incorporated both in the gel during its preparation and in the running buffer (0.5 ug/ml).

2.2.11. Separation of Modified Bases

The separation and quantification of modified bases in nucleic acid samples was carried out according to Hasan and Ali (1990).

2.2.11.1. Acid hydrolysis of nucleic acid samples

Modified DNA samples were precipitated with two volumes of cold ethanol, dessicated and dissolved in perchloric acid (70%). The samples were heated at 100°C for 1 hr to release the bases. The solution was neutralized and chromatographed on DEAE Sephadex A 25 column.

2.2.11.2. DEAE Sephadex A 25 column chromatography

The swollen ion exchanger was mixed with
starting buffer (1 mM Tris-HCl, pH 7.6) to form a fairly thick slurry. The ion exchanger suspension was poured into the column and gel was allowed to settle. In order to allow the system to reach equilibration and to stabilize the bed, about ten bed volumes of buffer were run through the ion exchanger bed. Three ml of sample was loaded onto the column (2.4 cm x 20 cm) preequilibrated with 1 mM Tris-HCl buffer and was eluted with Tris-HCl buffer using a linear gradient (1-20 mM) at a flow rate of 40 ml/hr. Fractions of 2.5 ml were collected and their absorbance recorded at 260 nm. The control experiments were carried out with equimolar mixture of individual bases and hydrolysed native DNA in order to locate the pattern of unmodified bases. The individual modified/unmodified bases were identified by their characteristic UV absorption profile.

2.2.12. Immunization Schedule

Antibodies against modified DNA were elicited in goat complexed with methylated BSA (MBSA, 0.5 ml each) and emulsified with 1.0 ml of Freund’s complete adjuvant. Intramuscular injections were given for five weeks at weekly intervals with incomplete adjuvant (Ishaq and Ali, 1984). The amount of antigen was 100 ug/injection. Booster dose was administered after the lapse of one month from the last injection. Blood was collected through jugular vein and serum separated. The separated serum was decomplemented by heating at 56°C for 30 min and stored in small aliquots at -20°C with 0.1% sodium azide as preservative. Preimmune sera were collected before immunization of the animals.

Antibodies against ROS-DNA fragments of 300 bp were raised in rabbits after intramuscular injection with 50 ug of antigen complexed with MBSA and emulsified with complete Freund’s adjuvant. The rabbits received six similar injections but with incomplete Freund’s adjuvant at weekly intervals. Animals were bled by cardiac
puncture and serum separated, preserved in sodium azide (0.1%) and stored at 4°C.

2.2.13. Isolation and Purification of IgG

2.2.13.1. Precipitation of immunoglobulins

To 6.5 ml of serum was added 3.5 ml of saturated ammonium sulphate gradually under continuous stirring to avoid local high concentrations leading to precipitation of contaminants. The solution was stirred for one hr at 4°C and centrifuged at low speed (15 min, 4,000 g). The pellet was washed twice with 40% saturated ammonium sulphate, redissolved in buffer of same volume and reprecipitated. The precipitate was collected by centrifugation, dissolved in sodium phosphate buffer, pH 8.0 and excess of salts were removed by dialysis.

2.2.13.2. Purification of IgG on DEAE Sephacel

Ion exchange chromatography was performed using DEAE Sephacel (Ali et al., 1991). The resin was swollen in distilled water, treated with 0.1 N NaOH for 1 hr at room temperature and washed with distilled water till pH became neutral. It was then treated with 0.1 N HCl for an hr. The regenerated resin was washed extensively with distilled water and packed in a column (1.5 cm x 28 cm) and equilibrated with the operating buffer (0.01 M sodium phosphate, pH 8.0). The immunoglobulins were applied to the column and eluted with phosphate buffer using a linear gradient of 0.01 M-0.3 M. Fractions of 4 ml were collected and absorbance was monitored at 280 nm. The homogeneity of the isolated IgG was ascertained by SDS-PAGE.

2.2.13.3. Purification of IgG by gel filtration

Gel filtration was performed on a Sephadex G 200 column. About 10 g of gel was allowed to swell in distilled water for 5 hr in a boiling water bath. The
column (2.4 cm x 50 cm) was filled with the operating buffer upto 3 cm. An extension column was connected to the column and then degassed gel slurry was carefully poured into the column. The gel was left overnight at room temperature to settle under gravity. As the gel settled down, the flow rate was increased gradually to a value slightly higher than that was to be employed for elution. The column was equilibrated with operating buffer by passing a volume equal to 3 times of the total bed volume. Uniform packing of the column was checked by passing 0.2% w/v solution of Blue Dextran.

Three ml of the sample containing 30 mg protein, was applied onto the column with the help of an applicator. The column outlet was opened slowly and the sample was allowed to percolate through the upper surface of the gel. The flow rate of the column was then adjusted to 20 ml/hr and the column was connected to a reservoir containing operating buffer. After rejection of discarded volume, fractions of 4.0 ml were collected and absorbance monitored at 280 nm.

2.2.13.4. Polyacrylamide gel electrophoresis

The homogeneity of the IgG was probed by polyacrylamide slab gel electrophoresis according to the method of Laemmli (1970).

2.2.13.4.1. Stock solutions
(i) Acrylamide-bisacrylamide (30:0.8): prepared by dissolving 30 g of acrylamide and 0.8 g bisacrylamide in a total volume of 100 ml water. The solution was stored at 4°C in a dark bottle.
(ii) TEMED: used as supplied.
(iii) Ammonium persulphate (1.5%, w/v): 0.15 g of ammonium persulphate was dissolved in 10 ml water. The solution was made fresh just before use.
(iv) SDS (10% w/v): prepared by dissolving 10 g SDS in water to 100 ml.
(v) Resolving gel buffer (3 M Tris-HCl, pH 8.8): prepared by dissolving 36 g Tris in 48.0 ml of 1 N HCl. The contents were mixed thoroughly, pH adjusted to 8.8 and the final volume brought to 100 ml.

(vi) Stacking gel buffer (0.5 M Tris-HCl, pH 6.8): Tris (6.05 g) was dissolved in 40 ml distilled water, titrated to pH 6.8 with 1 N HCl (around 48 ml) and the volume made up to 100 ml with water.

(vii) Reservoir buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3): 3.03 g Tris, 14.4 g glycine and 1.0 g SDS were dissolved in and made to one litre with water.

(viii) Sample buffer

(A) - Tris (6.0 g) was dissolved in 80.0 ml of water and pH adjusted to 6.8 with o-phosphoric acid. The volume was made to 100 ml with water.

(B) - To 12.5 ml of 'A' was added 1.0 mg bromophenol blue and 12.5 ml glycerol.

(C) - One part of B and four parts of sample were mixed and heated in a boiling water bath for 5 min just prior to electrophoresis.

Recipe for 7.5% SDS-PAGE

(Total volume 30.0 ml)

<table>
<thead>
<tr>
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<th>Volume</th>
</tr>
</thead>
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<tr>
<td>Acrylamide-bisacrylamide</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>3.75 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>1.5% Ammonium persulphate</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>16.95 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>15.0 ul</td>
</tr>
</tbody>
</table>
Recipe for stacking gel  
(Total volume 10.0 ml)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-bisacrylamide</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>1.5% Ammonium persulphate</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>5.65 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>7.5 uL</td>
</tr>
</tbody>
</table>

2.2.13.4.2. Procedure  
The resolving gel mixture was prepared by mixing the components in the volumes listed above and poured into the space between the glass plates leaving sufficient space (about 3.5 cm) at the top for a stacking gel to be polymerized later and sample wells formed. Samples were carefully loaded onto the gel surface using a microsyringe. The gels were electrophoresed at 60 V for 6-8 hr and were stained with Coomassie Brilliant Blue R 250.

2.2.14. Detection and Quantitation of Antibodies  

2.2.14.1. Enzyme linked immunosorbent assay (ELISA)  
The anti-ROS DNA antibodies were detected and quantitated by ELISA on flat bottom 96 well microtitre plates according to the method of Aotsuka et al. (1979) with slight modification.

2.2.14.1.1. Buffers and reagents  
(a) Tris buffer saline (TBS), pH 7.4  
10 mM Tris, 150 mM NaCl, pH 7.4 containing 0.02% sodium azide as preservative.

(b) Tris buffer saline Tween-20 (TBS-T), pH 7.4  
20 mM Tris, 144 mM NaCl, 2.68 mM KCl and 500 uL
Tween-20
(c) Bicarbonate buffer, pH 9.6
15 mM sodium carbonate, 35 mM sodium bicarbonate and
2 mM magnesium chloride, pH 9.6 containing 0.02% sodium azide as preservative.
(d) Substrate
Five hundred ug/ml p-nitrophenyl phosphate in bicarbonate buffer, pH 9.6.

2.2.14.1.2. Procedure
Polystyrene plates were preincubated with 100 uL poly-D-lysine (50 ug/ml in water) for 30 min at room
temperature. After washing three times with TBS, pH 7.4, the wells were coated with 100 uL of modified DNA
antigens (2.5 ug/ml TBS) for 2 hr at room temperature and overnight at 4°C. The wells were emptied and washed three
times with TBS-T, pH 7.4. Hundred uL of poly-L-glutamate (50 ug/ml TBS) was added to antigen coated wells for 2
hr. The plates were washed three times with TBS-T and unoccupied sites were blocked with 150 uL of 2% BSA (in
TBS) for 6 hr at room temperature. Antibodies (100 uL/well) to be tested (dilution in TBS) were adsorbed for
2 hr at room temperature and overnight at 4°C. The unbound material was washed four times with TBS-T and two
times with distilled water. An appropriate anti-immunoglobulin alkaline phosphatase conjugate was added
to each well. The substrate was added and absorbance recorded at 410 nm on an automatic microplate reader
after proper color development. Each sample was run in duplicate. The control wells were treated similarly but
were devoid of antigen. Results were expressed as a mean of $A_{test} - A_{control}$.

2.2.14.2. Competition ELISA
The specificity of the induced antibodies was ascertained by competition ELISA employing modified
polymers and various synthetic polynucleotides as
inhibitors. Anti-ROS DNA antibodies (constant amount) were incubated with increasing amounts (0-10 ug) of modified polymers and synthetic polynucleotides. Following incubation for 2 hr at room temperature and overnight at 4°C, immune complexes were added to ROS-DNA coated plates and the direct binding ELISA was performed. Each inhibitor dilution was run in duplicate and corrected for nonspecific reactivity in control wells. Percent inhibition was calculated using the formula:

\[
\% \text{ inhibition} = \left[ 1 - \frac{A_{410 \text{ inhibited}}}{A_{410 \text{ uninhibited}}} \right] \times 100
\]

2.2.14.3. Quantitative precipitin titration

The antigen-antibody interaction was investigated by performing precipitin analysis under equilibrium conditions. Increasing amounts of antigen (ROS-DNA) (0-50 ug) were added to a series of Eppendorf microfuge tubes containing equal amounts (100 ug) of immune IgG. The mixture was incubated at 37°C for two hr and then transferred to 4°C for a day. This was followed by the addition of anti-goat IgG to the assay mixture. The tubes were incubated at 37°C for 2 hr and overnight at 4°C. The precipitates thus formed were separated from the supernatant by centrifugation at maximum speed for 2 min in a microfuge and both (precipitate and supernatant) were saved. The precipitate was washed twice with cold PBS, pH 7.4 and redissolved in a fixed volume of 1 N NaCl. The amount of bound and unbound antigen in the dissociated immune complex precipitate and supernatant respectively were determined colorimetrically using diphenylamine reagent (Burton, 1956).

The affinity of the interaction was probed by Langmuir isotherm plot. A plot of 1/r versus 1/[Ag] yielded a straight line. The results were analysed by least square fit method. Affinity constant was computed
from the slope of the plot.

2.2.14.4. Gel retardation assay

For the visual detection of antigen-antibody binding and formation of immune complexes, electrophoresis was performed with 1% agarose gels (Alam and Ali, 1992).

(a) Electrode buffer
40 mM Tris-acetate (pH 8.0) containing 2 mM EDTA.

(b) Preparation of gel
The gel tray was cleaned with water and a mild detergent, rinsed well with distilled water and dried. The ends of the gel tray were sealed with a tape and gel comb was placed in position. Agarose NA (0.3 g) in 30 ml of electrophoresis buffer was dissolved by heating in a boiling water bath. The solution was cooled (50°C-60°C) and poured into the gel tray. The gel was left at room temperature for complete solidification. After the gel was completely set, the comb and tape was carefully removed and gel mounted in the electrophoresis tank.

(c) Sample preparation and loading
Samples were prepared by incubating constant amounts of antigen and antibody in PBS, pH 7.4 for 2 hr at 37°C and overnight at 4°C. One fifth volume of concentrated loading buffer (6x) containing 0.25% xylene cyanole and 15% Ficoll 400 were added to samples prior to loading them onto the gel.

(d) Electrophoresis conditions
The gels were run with 40 mM Tris-acetate buffer, pH 8.0 at 30 V.

(e) Staining
The gels were stained with fluorescent dye ethidium bromide (1 ug/ml) for 15-30 min. After staining, gels
were viewed by illumination with UV light.

2.2.15. Serum Specimens

Normal human sera were obtained from healthy laboratory personnel. Sera of patients with systemic lupus erythematosus were obtained from outdoor and indoor patients of Department of Medicine, All India Institute of Medical Sciences, New Delhi and J.N. Medical College Hospital, A.M.U., Aligarh. The samples were transported to the laboratory in ice sodium chloride mixture. Patients with this disease satisfied the American Rheumatism Association Criteria for the diagnosis of SLE (Tan et al., 1982). All sera were stored at -20°C with 0.1 percent sodium azide as preservative until examined. Serum samples were heated at 56°C for 30 min before use to inactivate complement.

2.2.16. Immunological Techniques

2.2.16.1. Direct binding ELISA

Antibodies in SLE sera were detected and quantitated by ELISA as described earlier (Aotsuka et al., 1979). Decomplemented sera were serially diluted in TBS-BSA and were added to antigen coated plates for 2 hr at room temperature and overnight at 4°C. Bound antibodies were assayed with anti-human IgG alkaline phosphatase conjugate (1:1500 dilution in TBS) using p-nitrophenyl phosphate as substrate. The absorbance of each well was monitored at 410 nm on an automatic microplate reader.

2.2.16.2. Inhibition ELISA

Antibody specificity was evaluated by competitive binding assay (Hasan et al., 1991). Serum samples at a dilution of 1:100 were incubated with increasing amounts (0-10 ug) of competitors for 2 hr at room temperature and subsequently overnight at 4°C.
Duplicate aliquots of immune complexes were added to native DNA coated plates and direct binding ELISA was performed. Inhibition was expressed as the amount of inhibitor resulting in a 50% decrease in antibody binding to the solid phase antigen.

2.2.17. Data Analysis

Absorbance values obtained with sera of 20 normal healthy individuals were used for determining the cut off level for distinguishing antibody positive and negative samples. The mean and standard deviation (S.D.) of the absorbance values was calculated and a value of 2 S.D's above the mean 0.17 was chosen as a cut off value. A test sample was positive if the mean absorbance of duplicate measurements was greater than 0.17 and negative if the mean absorbance was lower.

2.2.18. Isolation of IgG from SLE Serum

IgG was isolated from SLE serum by the method of Ali et al. (1991). IgG was precipitated as 40% of the total Y-globulin fraction by 35% ammonium sulphate fractionation. After dissolving the precipitate in minimal volume of 0.01 M sodium phosphate buffer, pH 8.0, the excess of salt was removed by dialysis. The sample was loaded on DEAE Sephacel column (1.5 cm x 28 cm) previously equilibrated with 0.01 M sodium phosphate buffer, pH 8.0. IgG was eluted with phosphate buffer, using a linear gradient (10-300 mM). Fractions of 4.0 ml at a flow rate of 18 ml/hr were collected and monitored at 280 nm. Three peaks emerged, the first, at about 20 mM phosphate, contained pure IgG with a yield of about 80%.

2.2.19. Formation and Quantitation of Immune Complexes

Antigen-antibody interaction was performed by precipitin analysis under equilibrium conditions in Eppendorf microfuge tubes. Increasing amounts of antigen (0-50 ug) were added to a constant amount of antibody
(100 ug SLE IgG) in a total volume of 250 uL. The mixture was incubated for 2 hr at room temperature and overnight at 4°C. The immune complexes were pelleted at maximum speed for 2 min, washed twice with cold PBS, pH 7.4 and dissolved in 250 uL of 1 N NaCl. The concentration of protein and DNA in each sample was determined by the colorimetric assay of Lowry (1951) and Burton (1956) respectively.

2.2.20. Calculation of Antibody Affinity

The data from immune precipitation reaction were used to estimate the quantity of bound antigen in an equilibrium mixture of antigen and antibody for the calculation of apparent antibody affinity by the method of Langmuir (1918). The results were analysed by least square fit method. Affinity constant was computed from the slope of the plot.

2.2.21. Chemical Modification of Amino Groups of Anti-ROS DNA IgG

2.2.21.1. Acetylation of IgG

Acetylation of IgG to modify lysine residues was performed according to the method of Riordan and Vallee (1972) using acetic anhydride as an acetylating agent. A solution of IgG of known concentration in 0.06 M sodium phosphate buffer was taken and to this was added the required amount of acetic anhydride with continuous stirring. The reaction was performed at 4°C and the pH of the reaction mixture was maintained between 7.4 to 7.6 by adding 1 N NaOH. The reaction was allowed to proceed for 40 min. The modified protein was then dialysed against 0.06 M sodium phosphate buffer, pH 7.0. Keeping all the reaction conditions as above, the molar excess of acetic anhydride over protein was changed from 50 to 1000 to effect different degrees of modification.
2.2.22. Quantification of Modification

The extent of lysine residue modification of anti-ROS DNA IgG was determined by treatment with trinitrobenzene sulphonic acid (TNBS) as described by Habeeb (1966) and by ninhydrin reaction (Moore and Stein, 1954).

2.2.22.1. Reaction with TNBS

To 1.0 ml of protein solution (modified and native IgG) was added 0.1 ml of TNBS solution in water. The mixture was incubated at 40°C for 2 hr at room temperature and 1.0 ml of 10% (w/v) SDS was added to solubilize the protein and to prevent its precipitation on addition of 0.5 ml of 1 N hydrochloric acid. The color intensity was recorded after 10 min at 335 nm against a blank treated in the same way except that it contained 1 ml of water instead of protein solution. The extent of modification was calculated from the straight line plots between amount of protein (in mg) and absorbance at 335 nm, for modified and unmodified preparations by using the following equation:

\[
\text{Percent modification} = 100 \left( 1 - \frac{m'}{m} \right) \quad (i)
\]

where \( m' \) and \( m \) represent the slopes of the straight lines for modified and unmodified preparations respectively.

2.2.22.2. Ninhydrin reaction

Estimation of free amino groups was accomplished by the method described by Moore and Stein (1954).

(a) Preparation of hydridantin

Hydridantin was prepared by reducing ninhydrin with ascorbic acid (Moore and Stein, 1954). One gram of ninhydrin was dissolved in 25 ml of water at 90°C. Five ml of 20% (w/v) ascorbic acid was added slowly
to ninhydrin solution with continuous stirring. The reaction was allowed to proceed for 30 min and the contents were cooled under tap water. The hydrindantin crystals, thus formed were filtered and dried in a dessicator, under reduced pressure. Dried crystals were stored in the dark.

(b) Preparation of ninhydrin reagent
Three hundred mg of ninhydrin and 100 mg of hydrindantin were dissolved in 76 ml of methyl cellosolve. To this was added 24 ml of 4 M sodium acetate buffer, pH 5.5. The contents were mixed thoroughly and filtered through Whatman filter paper. The reagent thus obtained was stored at 4°C in an amber colored bottle.

(c) Method
Increasing amounts of protein (0.1 to 1 mg) were taken in a series of tubes and volume made upto 1 ml with water. One ml of 4 M sodium acetate buffer, pH 5.5 was added to each tube followed by the addition of 1 ml ninhydrin reagent. The tubes were placed in a boiling water bath for exactly 20 min and then cooled under tap water. Five ml of 50% (v/v) ethanol was added to each tube. The contents were mixed and filtered through a Whatman filter paper. The absorbance was recorded at 570 nm against an appropriate blank. Absorbance values at 570 nm were plotted against protein concentration (in mg) and data analysed by least square analysis. The extent of modification was determined by using equation (i).

2.2.23. Effect of Modification on the Reactivity of Anti-ROS DNA IgG
The reactivity of modified IgG was determined by direct binding ELISA on microtitre polystyrene plates. The unmodified anti-ROS DNA was used as control.