II EXPERIMENTAL

A. MATERIALS

1. Chemicals
   8-Methoxypsoralen, anti-rabbit, anti-goat and anti-human IgG alkaline phosphatase conjugate, poly(dA-dT), poly(dA-dT), xylene cyanole FF, Coomassie Brilliant Blue R 250 and G 250, bovine serum albumin, methylated bovine serum albumin, calf thymus DNA, poly-D-lysine, poly-L-glutamate, dialysis tubings, Freund’s complete and incomplete adjuvants, nuclease S1, trinitrobenzene sulfonic acid (TNBS), Tris-(hydroxymethyl)-aminomethane, ethidium bromide, standard protein markers and nuclease Bal 31 were purchased from Sigma Chemical Company, U.S.A. Poly(rG).poly(dC), poly(dG).poly(dC), Sepharose 4B, DEAE Sephadex, Sephadex G 200, agarose NA and Ficoll 400 were obtained from Pharmacia Fine Chemicals, Sweden. Tween-20, acrylamide, bisacrylamide, N-N-N’-N’-tetraethylmethylenediamine, ammonium persulphate and hydroxyapatite were the products of Bio-Rad Laboratories, U.S.A. Cyanogen bromide and agarose was from S.R.L., India. Acetaldehyde was from Fluka, Switzerland. Perchloric acid and sodium azide was a product of Ferak-Berlin, Germany. Acetonitrile was from E. Merck, India. Polystyrene flat bottom plates having 96 wells (7 mm in diameter) were obtained from Dynatech U.S.A. A colorigenic substrate p-nitrophenyl phosphate was obtained from C.S.I.R. Centre for Biochemicals, Delhi. Psoralen was obtained from C.D.R.I. Lucknow. All other chemicals were of highest grade available commercially.

2. Equipment
   Bausch and Lomb Spectronic-20, fraction collector
FRAC-100 (Pharmacia, Sweden), Dynatech ELISA microplate reader MR-600, ELICO pH meter model L1-10T, Shimadzu UV-240 spectrophotometer equipped with a thermoprogrammer and controller, Shimadzu spectrophotofluorometer RF-540, gel electrophoresis apparatus GNA-100 (Pharmacia, Sweden), desk top microfuge RM-12C (REMI, India) and ultraviolet lamp having maximum emission at 365 nm (Vilber Lourmat, France) were the major equipment used in this study.

B. METHODS

1. Purification of Calf Thymus DNA

Highly polymerized calf thymus DNA obtained commercially was purified as described by Ali et al. (1985). DNA (2 mg/ml) dissolved in 0.1x SSC buffer (0.015 M sodium citrate, pH 7.3 containing 0.15 M sodium chloride) was mixed with equal volume of chloroform-isooamyl alcohol (24:1) in a sterile stoppered container. The contents were gently shaken for one hr. The aqueous layer containing DNA was separated from the organic layer and extracted again with chloroform-isooamyl alcohol. The deproteinized DNA was precipitated with two volumes of cold 95% ethanol and collected on glass rod by gentle swirling. The DNA thus obtained was air dried to remove the traces of ethanol and was dissolved in 0.03 M acetate buffer, pH 5.0 containing 0.03 M zinc chloride. The sample was digested by incubating with single strand specific enzyme, nuclease S1 (200 units/mg DNA) at 37°C for 30 min. The reaction was terminated by the addition of one-tenth volume of 0.2 M EDTA, pH 8.0. The purified sample was extracted twice with chloroform-isooamyl alcohol. Finally, the DNA was precipitated with two volumes of cold 95% ethanol. The precipitate was air dried and dissolved in 0.01 M sodium phosphate buffer, pH 7.4 containing 0.15 M sodium chloride. The double strandedness of purified DNA was ascertained by sharp
melting of duplex (Fig. 2)

2. Determination of DNA Concentration

The colorimetric method of Burton (1956) using diphenylamine reagent was employed for the determination of DNA concentration.

a) Preparation of diphenylamine reagent

750 mg of diphenylamine was added to 50 ml of glacial acetic acid followed by the addition of 0.75 ml of concentrated sulphuric acid. The reagent was prepared immediately before use.

b) Procedure

One ml of purified DNA solution was mixed with 1.0 ml of 1 N perchloric acid. The contents were mixed thoroughly and incubated for 15 min in a water bath maintained at 70°C. Hundred uL of 5.43 mM acetaldehyde was added to each assay tube. Two ml of freshly prepared diphenylamine reagent was then added. The contents of each tube were thoroughly mixed and allowed to stand at room temperature for 16-20 hr and the absorbance was recorded at 600 nm. The DNA concentration in unknown sample was determined from the standard plot constructed by using 0-100 ug of purified calf thymus DNA (Fig. 3).

3. Determination of Protein Concentration

Protein was estimated by the method of Lowry et al. (1951) and Bradford (1976).

(i) Estimation of protein by Folin-phenol reagent

The protein estimation by this method involves the complexing of protein with Cu²⁺ in alkaline solution. In addition, the copper appears to catalyze the reduction, by the tyrosine and tryptophan residues of the phosphomolybdate/phosphotungstate anions in the Folin-phenol reagent, added subsequently. This later reaction leads to
Fig. 2. Melting profile of calf thymus DNA in PBS.

$T_m = 87.5^\circ C$
Fig. 3. Standard plot for the estimation of DNA by diphenylamine reagent.
a blue colour, which can be measured at 660 nm.

a) Folin-Ciocalteu reagent

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

b) Alkaline copper reagent

The components of alkaline copper reagent were:

i) 2% sodium carbonate in 0.1 N NaOH

ii) 1% copper sulphate in 2% sodium potassium tartrate

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

c) Procedure

One ml of protein sample was mixed with freshly prepared 5.0 ml of alkaline copper reagent. The tubes were allowed to stand at room temperature for 10 min in order to complete the reaction. One ml of 1:4 times diluted Folin-Ciocalteu reagent was added with immediate mixing. After the lapse of 30 min, absorbance was recorded at 660 nm. The concentration of unknown protein sample was computed from standard plot constructed with bovine serum albumin. (Fig. 4).

(ii) Estimation of protein by dye binding

The methodology is based on the change in colour that occurs when Coomassie Brilliant Blue G 250 binds to protein in acidic solution. The protonated form of Coomassie Brilliant Blue dye is pale orange-red in colour. The dye binds strongly to proteins, interacting both hydrophobically and at positively charged (basic) groups on the protein. In the environment of these positively charged groups protonation is suppressed, resulting in the formation of blue colour.
Fig. 4. Standard plot for the estimation of protein by Folin-Ciocalteu reagent.
a) Preparation of dye

One hundred mg of Coomassie Brilliant Blue G 250 dye was dissolved in 50 ml of 95% ethanol and filtered to remove the undissolved residues. The dissolved dye was brought to acidic medium by adding 100 ml of 85% (v/v) orthophosphoric acid and was diluted to a final volume of 1 litre with double distilled water.

b) Assay procedure

Appropriate dilutions of protein samples were pipetted in test tubes and the final volume was adjusted to 0.1 ml with buffer. Five ml of dye reagent was added and the contents of each tube were thoroughly vortexed. The reaction was allowed to proceed at room temperature for 15 min and the absorbance was recorded at 595 nm against a reagent blank. The colour is stable for 30 min, after which the precipitation of dye-protein complex may occur. The concentration of protein in an unknown sample was determined from the plot constructed with bovine serum albumin (0-100 ug).

4. Photomodification of Nucleic Acids with 8-Methoxypsoralen

The bifunctional skin photosensitizer, 8-methoxypsoralen was covalently photolinked with calf thymus DNA as well as synthetic polymer, poly(dA-dT).poly(dA-dT) in a controlled two step process.

Calf thymus DNA and poly(dA-dT).poly(dA-dT) in TNE buffer (0.01 M Tris, 0.0004 M EDTA, 0.05 M NaCl) pH 7.6 were mixed separately with 8-MOP in the ratio of 3.2:1 (w/w) in a total volume of 1.0 ml. The mixture was kept in dark for 4 hr at room temperature with constant stirring. The intercalated complexes were irradiated for 40 min using illuminating wavelength of 365 nm. The covalent photoadducts thus formed were extensively dialyzed against 0.01 M Na-Pi buffer (pH 6.8) to remove
unbound drug. Unirradiated samples of DNA, poly(dA-dT).poly(dA-dT) and 8-MOP served as corresponding controls. The occurrence of photoreaction and the formation of photoadduct was studied by ultraviolet and fluorescence spectroscopy.

5. Determination of Melting Temperature

Thermal denaturation of calf thymus DNA, DNA-8MOP photoadduct (in TNE buffer, pH 7.6), poly(dA-dT).poly(dA-dT) and poly(dA-dT)-8MOP photoconjugate (in 0.01 M Na-Pi, pH 6.8) was induced by a temperature scan from 30°C to 95°C at a rate of 1°C/min on Shimadzu UV-240 spectrophotometer equipped with temperature programmer and controller (Hasan and Ali, 1990). Melting curves were recorded at a fixed wavelength of 260 nm. Since Tm is dependent upon the ionic strength of the solvent and the nature of counterions, a single solvent was chosen for test and control samples. The percent denaturation was evaluated by the equation:

\[
\text{Percent denaturation} = \frac{A_T - A_{30}}{A_F - A_{30}} \times 100
\]

Where,

- \( A_T \) is the sample absorbance at various temperatures
- \( A_F \) is the final absorbance at 95°C
- \( A_{30} \) is the absorbance prior to heating

The change in melting temperature (\( \Delta Tm \)) was calculated using the formula:

\[
\Delta Tm = Tm^c - Tm^s
\]

where \( Tm^c \) and \( Tm^s \) are the melting temperatures of native and modified sample respectively.
6. Detection of Photoadduct by Nuclease S1 Sensitivity Assay

Nucleic acid-8MOP photoadducts were also characterized by nuclease S1 treatment (Matsuo and Ross, 1987) followed by agarose gel electrophoresis.

a) Preparation of gel

0.3 gm of agarose in 30 ml of TAE buffer, (0.04 M Tris acetate, pH 8.0 containing 0.002 M EDTA) was brought to molten state by boiling. The solution was allowed to cool to 50-60°C and was poured onto a horizontal tray of GNA-100 electrophoresis apparatus (Pharmacia, Sweden). The poured solution was left at room temperature for 1 hr for complete solidification. The casted gel was 4 mm thick with wells 3 mm deep and 3 mm wide. The capacity of each well was 9 uL and the electrode buffer was TAE.

b) Digestion of photoadduct with nuclease S1

Two ug each of modified and unmodified nucleic acids were incubated with nuclease S1 (20 units/ug of nucleic acid) in acetate buffer, pH 5.0 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.

c) Sample preparation

The loading buffer consist of 0.025% xylene cyanole, 30% Ficoll 400, 0.5 M EDTA in 10 x electrophoresis buffer. The digested and undigested samples contained one-tenth volume of above solution.

d) Running condition

40 volts for 2 hr.

e) Staining

The DNA bands were visualized by staining with
ethidium bromide (1 ug/ml).

7. Hydroxyapatite Column Chromatography

Hydroxyapatite possesses a unique property of distinguishing between single stranded and double stranded conformations of nucleic acids and thus it was employed to ascertain the mono- or diadduct nature of photomodified nucleic acid polymers.

The commercial sample of hydroxyapatite was washed several times with 0.01 M sodium phosphate (Na-Pi) buffer, pH 6.8 to remove the fine particles and thereafter packed in a glass column (1.5 cm x 6.0 cm). After equilibration with 0.01 M Na-Pi buffer, pH 6.8, heat denatured photoadducts (DNA-8M0P and poly(dA-dT)-8M0P) in equilibrating buffer were adsorbed onto the column. Control samples of native and heat denatured nucleic acid polymers (DNA and poly(dA-dT)) were also subjected to hydroxyapatite column chromatography under identical conditions. Stepwise elution was carried out with 0.125 M and 0.25 M Na-K-phosphate buffer, pH 6.8 (Dardalhon and Averbeck, 1988). Fractions of 3.0 ml were collected at a flow rate of 14 ml/hr. Single stranded DNA was eluted with 0.125 M Na-K-phosphate buffer, pH 6.8. Heat denatured photoadducts and double stranded DNA were found to be eluted with 0.25 M Na-K-phosphate buffer, pH 6.8. All samples were denatured (80 ug/ml) by heating in a boiling water bath for 15 min and fast cooled in ice-NaCl bath. The fractions eluted as diadduct were pooled and used as antigen.

8. Bal 31 Digestion of Photoadducts

The study was based on the digestion of double stranded DNA by the exonuclease Bal 31, which was found to be arrested at psoralen-DNA interstrand crosslinked sites (Zhen et al., 1986). DNA samples were
electrophoresed by the procedure of Sealey and Southern (1985). The following stock solutions were prepared:

i) 40% acrylamide in distilled water
ii) 2% bisacrylamide in distilled water
iii) Gel buffer: 0.9 M Tris borate, pH 8.3 containing 0.025 M EDTA.
iv) Electrode buffer: 0.09 M Tris borate, pH 8.3 containing 0.0025 M EDTA.

Recipe for 6% resolving gel
(Total volume 40.0 ml)

- 40% acrylamide: 6.0 ml
- 2% bisacrylamide: 6.0 ml
- Gel buffer: 4.0 ml
- 1.5% ammonium persulphate: 2.0 ml
- Distilled water: 22.0 ml
- TEMED: 0.025 ml

Photoadduct, hydroxyapatite purified photoadduct and calf thymus DNA (1.0 ug each) in Bal 31 buffer (0.6 M NaCl, 0.02 M Tris, 0.012 M MgCl₂, 0.012 M CaCl₂, 0.001 M EDTA) pH 8.0 were treated with 0.5 units of Bal 31 for 0, 5, 10 and 20 min at 30°C. The reaction was stopped by the addition of one-tenth volume of 0.2 M EDTA, pH 8.0. The DNA samples were run for 6 hr at 70 volts after the addition of 3.0 uL of "stop mix" (30% Ficoll 400, 0.025% xylene cyanole FF, 0.5 M EDTA in 10 x electrophoresis buffer). On completion of electrophoresis, the gel was stained with ethidium bromide (1 ug/ml) and DNA bands were visualized under UV illumination.

9. Separation and Quantitation of Photomodified Bases

The separation, quantitation and identification of the drug (8-MOP) photoconjugated to bases was
accomplished as described earlier (Hasan and Ali, 1990).

a) Acid hydrolysis of DNA-8MOP photoadduct

The photoadduct was precipitated with two volumes of cold ethanol and was collected after centrifugation at 5000 rpm for 10 min. The precipitate was dessicated in order to remove the traces of ethanol and dissolved in perchloric acid (60%). The sample was treated in a boiling water bath for 1 hr to release the bases. The solution was neutralized and concentrated.

b) DEAE Sephadex A 50 column chromatography

The separation of bases was performed by ion exchange chromatography on DEAE Sephadex A 50 column. The supplied gel was swelled by keeping at 90°C for 1 hr in distilled water. The gel was packed in a column (1.6 cm x 30 cm) and left overnight for gravity packing. The column was equilibrated with copious volume of 0.02 M Tris buffer, pH 7.2. Sample (2.5 ml) was applied onto the column with the aid of an applicator and eluted with same buffer at a flow rate of 40 ml/hr. Two hundred fractions of 2.5 ml each were collected and their absorbance recorded at 260 nm. The control experiment was carried out with hydrolyzed calf thymus DNA. The individual bases were identified by their characteristic UV absorption profile.

10. Preparation of Antigen and Immunization Schedule

a) Antibodies against poly(dA-dT)-8MOP photoadduct

Poly(dA-dT)-8MOP-MBSA conjugate was formed by mixing equal amounts of both poly(dA-dT)-8MOP and MBSA by weight of the solutes. One ml of poly(dA-dT)-8MOP photoadduct (100 ug) was complexed with 100 uL of MBSA (100 ug). The complex was emulsified with equal volume of Freund’s complete adjuvant for the first injection. All subsequent
injections were in incomplete adjuvant. Goat was injected intramuscularly in hind limbs weekly for five weeks (Ishaq and Ali, 1984). A single animal received a total of 500 ug of photoadduct in the course of five injections. Ten days after the last dose, animal was bled through jugular vein. The serum samples were heated at 56°C for 30 min to inactivate complement which inhibits the formation of and also dissolves preformed antigen-antibody complexes (Miller and Nussenzweig, 1975; Schifferli et al., 1980). The decomplemented samples were stored at -20°C with 0.02% sodium azide as preservative. Animal was also bled prior to immunization to obtain preimmune serum.

b) Antibodies against DNA-8MOP photoadduct

Rabbits (8-12 months old, weighing 1.0 to 1.5 kg) were injected intramuscularly in hind limbs with freshly prepared solution of DNA-8MOP photoadduct (50 ug) and MBSA (50 ug) in Freund’s complete adjuvant in a total volume of 1.0 ml. Blood was collected by cardiac puncture prior to immunization (Ali and Ali, 1983). Subsequent injections were given weekly for four weeks with the same amount of antigen but in Freund’s incomplete adjuvant. Each animal received a total of 250 ug of antigen in the course of five injections. A week after the last injection blood was drawn by cardiac puncture and serum separated. The Clq complement component was heat inactivated by keeping serum samples at 56°C for 30 min. Serum was aliquoted in small volumes and kept at -20°C with 0.02% sodium azide as preservative.

c) Sera

Normal sera were obtained from healthy subjects. SLE sera were obtained from patients showing high titer anti-DNA antibodies and fulfilled the American Rheumatism Association Criteria for this disease (Tan et al., 1982). SLE negative and positive controls were obtained from
Sigma, U.S.A. All sera were heat inactivated (56°C, 30 min) and stored at -20°C with 0.02% sodium azide as preservative. Specific monoclonal anti-ZDNA antibody Z22 (IgG) was a generous gift from Dr. B.D. Stollar (U.S.A.).

11. Isolation of IgG

The preimmune as well as immune IgG were isolated from their respective serum by DEAE Sephacel column chromatography of 35% saturated ammonium sulphate precipitated immunoglobulin fractions (Ishaq and Ali, 1987).

a) Preparation of crude IgG

Saturated ammonium sulphate was added dropwise to 6.5 ml serum, allowing each drop to disperse before the next was added. Most of the immunoglobulins got precipitated by 35-40% of saturation. The mixture was allowed to stand at 4°C for 1 hr in order to attain complete precipitation. The suspension was stirred for 15-30 min and then centrifuged at 10,000 rpm for 15 min. The pellet thus obtained was washed three times with 40% saturated ammonium sulphate. The washed precipitate was dissolved and dialyzed against 500-1000 volumes of 0.01 M sodium phosphate buffer, pH 8.0. The dialysis fluid was changed several times at intervals of few hr.

b) DEAE Sephacel chromatography

The crude immunoglobulins were loaded onto a DEAE Sephacel column (1.5 cm x 28 cm) preequilibrated with 0.01 M sodium phosphate buffer, pH 8.0. The column was washed with 0.01 M sodium phosphate buffer, pH 8.0 until the absorbance at 280 nm reached base line. Fractions of 3.0 ml were collected with a linear gradient of 0.01 M - 0.3 M sodium phosphate buffer, pH 8.0. Absorbance of each fraction was monitored at 280 nm. First peak of the chromatogram was pooled and used as IgG.
12. Purification of Isolated IgG

Pure preparations of immune IgG was obtained by performing a preparative gel chromatography on Sephadex G 200 column (2 cm x 75 cm). The column was packed according to the instructions provided by the manufacturer. Fifty mg of DEAE Sephacel isolated IgG in a total volume of 3.0 ml was loaded onto the pre-equilibrated column. Fractions of 4.0 ml were collected with 0.01 M sodium phosphate buffer, pH 7.4 containing 0.15 M sodium chloride at a flow rate of 20 ml/hr. The protein content of each fraction was monitored at 280 nm. The fractions showing A_{278}/A_{251} ratio of 2.5 and single band in SDS-PAGE were pooled, dialyzed against appropriate buffer and used as purified IgG. The pooled IgG was stored at -20°C in small aliquots with 0.02% sodium azide as preservative.

13. SDS-Polyacrylamide Gel Electrophoresis

The homogeneity of purified IgG was ascertained by polyacrylamide slab gel electrophoresis as described by Laemmli (1970) under denaturing conditions.

a) Acrylamide-bisacrylamide (30:0.8)

The stock solution was prepared by dissolving 30 gm of acrylamide and 0.8 gm of bisacrylamide in a total volume of 100 ml. The solution was stored at 4°C in light protective bottles.

b) Resolving gel buffer

Stock buffer solution was prepared by dissolving 36.3 gm Tris in 48.0 ml of 1 N HCl (3.0 M). The contents were mixed properly, pH adjusted to 8.8 and final volume brought to 100 ml with distilled water.

c) Stacking gel buffer

6.05 gm Tris was dissolved in 40.0 ml distilled
water, titrated to pH 6.8 with 1 N HCl (around 48 ml) and
the volume adjusted to 100 ml with distilled water
(0.5 M).

d) Electrode buffer

0.025 M Tris and 0.192 M glycine, pH 8.3 containing
0.1 percent SDS.

e) Sample buffer

i) 6.0 gm Tris was dissolved in 80 ml distilled water and
ph pH adjusted to 6.8 with phosphoric acid. The volume
was made to 100 ml with distilled water.

ii) To 12.5 ml of the above sample buffer was added 1 mg
bromophenol blue and 12.5 ml glycerol.

One part of (ii) and four part of the sample were
mixed prior to electrophoresis and heated in a boiling
water bath for 3 min. This ensures denaturation of
protein. Samples were electrophoresed at 80 V at room
temperature for 8-10 hr.

Recipe for 7.5% SDS-PAGE
(Total volume 30.0 ml)

<table>
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<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
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<td>Acrylamide-bisacrylamide</td>
<td>7.5 ml</td>
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<tr>
<td>Resolving gel buffer</td>
<td>3.75 ml</td>
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<tr>
<td>10% SDS</td>
<td>0.30 ml</td>
</tr>
<tr>
<td>1.5% ammonium persulfate</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>16.95 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.015 ml</td>
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</table>

Recipe for stacking gel buffer
(Total volume 10 ml)

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<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-bisacrylamide</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>2.50 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.10 ml</td>
</tr>
</tbody>
</table>
1.5% ammonium persulfate 0.50 ml
Distilled water 5.65 ml
TEMED 0.075 ml

The resolving gel buffer was mixed and poured between the glass plates separated by 1.5 mm thick spacer. The gel was allowed to polymerize at room temperature. The stacking gel was added thereafter. The samples were loaded, electrophoresed and stained with Coomassie Brilliant Blue R 250 in 25% isopropyl alcohol and 10% glacial acetic acid. Destaining was carried out in a mixture of 10% acetic acid and 10% methanol.

14. Immunoaffinity Purification of Antibodies

Epitope specific paratopes in the heterogeneous immune sera were obtained by affinity purification of induced antibodies on polylysyl-Sepharose 4B coupled with the immunogen (Hasan et al., 1991).

a) Activation of matrix

Fifteen ml of Sepharose 4B slurry obtained from Pharmacia was suspended in distilled water, filtered and washed with 300 ml of double distilled water on a sintered glass funnel (porosity G 2). Ten gm of moist gel, mixed with 10 ml of 2 M sodium carbonate was kept in an ice-NaCl bath placed on a magnetic stirrer. A total of 0.8 ml acetonitrile having 1.0 gm CNBr was added dropwise to the gel and contents were allowed to interact for 12 min in cold. The reaction mixture was filtered through sintered glass funnel and washed with 400 ml of cold 0.1 M sodium bicarbonate (coupling buffer) and resuspended in same buffer. The unreacted CNBr (drained effluent) was reacted with ferrous sulphate to convert it into harmless ferrocyanide. All steps were carried out in fume-hood chamber (Ali, 1984).
b) Coupling of poly-L-lysine with activated Sepharose 4B

The method described by Wilchek (1973) was followed. The polycationic homopolymer polylysine (100 mg) was brought into solution form by dissolving in 10.0 ml of 0.1 M sodium bicarbonate and was added to the gel immediately after CNBr activation. The mixture was kept at 4°C for 12 hr with constant but slow stirring. The buffer was drained out and the gel was washed successively with 100 ml each of cold (i) distilled water (ii) 0.1 N HCl (iii) 0.1 M sodium bicarbonate and (iv) distilled water till neutral. Finally the gel was resuspended in 40 ml of 0.15 M acetate buffer, pH 4.5. The extent of poly-L-lysine depletion due to covalent coupling with activated Sepharose 4B was quantified by treatment of drained effluent with TNBS as described by Habeeb (1966).

c) Affinity purification of anti-photoadduct antibodies

DNA-8MOP-[polylysyl-Sepharose 4B] column was prepared as described by Nicotra et al. (1982) with slight modification. Twenty ml of polylysyl-Sepharose 4B was packed in a glass minicolumn (1.6 cm x 5.0 cm) and equilibrated with acetate buffer. DNA-8MOP photoadduct (12.5 ml of 100 ug/ml in acetate buffer) was applied onto the preequilibrated column and subjected to recycling for a couple of times in order to ensure maximum electrostatic binding of the photoadduct to the matrix. The unbound material was washed with 50 ml of PBS, pH 7.4. IgG isolated from anti-photoadduct serum, dialyzed previously against PBS, was loaded onto the affinity column. The unbound antibodies were removed by washing the column with 40 ml of PBS. The bound antibodies were eluted with 0.3 M NaCl in PBS, pH 7.4. Fractions of 3.0 ml were collected at a flow rate of 18 ml/hr. The contents of each tube were monitored at 251 nm, 260 nm, 278 nm and 280 nm.
d) Regeneration of affinity column

For further use, the column was regenerated by washing successively with 50 ml each of the following:

i) Cold distilled water
ii) Cold 0.1 N HCl
iii) Cold 0.1 M NaHCO₃ and
iv) Cold distilled water till neutral

15. Immunological Techniques

a) Immunodiffusion

The method described by Tan et al. (1966) was followed. The solid phase was 0.4% agarose in PBS, pH 7.4.

i) Preparation of agarose petri dishes

Six ml of 0.4% molten agarose in PBS containing 0.1% sodium azide was poured into a 5 cm x 1.5 cm glass petri dishes and allowed to solidify at room temperature. Wells, each 5 mm in diameter, were made with the help of a gel puncture and were separated from each other by a distance of 8 mm. The agarose layered petri dishes were stored at 4°C.

ii) Assay procedure

Twenty five uL of serially diluted decomplemented serum and antigen were placed in wells. The petri dishes were allowed to stand in a moist chamber at room temperature for 48-72 hr. The gels were washed with 5% sodium citrate to eliminate non specific precipitin lines. The precipitin lines were analyzed visually and the results recorded.

b) Quantitative precipitin titration

An attempt was made to investigate the immunointeraction of the induced antibodies with polyvalent immunogen having distinct conformation by
means of quantitative precipitin titration. Antigen-antibody interaction was carried out in sterilized Eppendorf microfuge tubes.

Photoadducts of varying concentration (0-50 ug/0-25 ug) were allowed to interact electrostatically in Eppendorf tube with a constant amount of immune IgG in an assay volume of 0.2 ml. The interaction was allowed to attain its minimum Van der Waal’s radii by incubating the mixture at 37°C for 2 hr followed by overnight incubation at 4°C. The assay tubes were spun in microfuge at 8,000 rpm for 2.5 min. The supernatant was carefully drained out and precipitated immune complex was washed thrice with cold PBS, pH 7.4. The precipitate was dissolved in PBS, pH 7.4 containing 1 M NaCl. The photoadduct and antibody in the dissociated immune complex was estimated by diphenylamine and dye binding assay respectively. The binding data were analyzed and antibody affinity was calculated by Scatchard (1949) as well as Langmuir (1918) isotherm plot.

c) Enzyme linked immunosorbent assay

Preparation of buffers and substrate

i) Tris buffer saline (TBS): 0.01 M Tris, 0.15 M NaCl, pH 7.4.

ii) Tris buffer saline - Tween-20 (TBS-T): 0.02 M Tris, 0.144 M NaCl, 0.00268 M KCl, 500 uL Tween-20, pH 7.4.

iii) Bicarbonate buffer: 0.015 M sodium carbonate, 0.035 M sodium bicarbonate and 0.002 M magnesium chloride, pH 9.6 containing 0.02% sodium azide as preservative

iv) Substrate: 500 ug p-nitrophenyl phosphate/ml in bicarbonate buffer.
Assay procedure

Polystyrene flat bottom microtiter plates were precoated with 100 uL of poly-D-lysine (50 ug/ml in distilled water) for 30 min at room temperature to increase antigen immobilization. The poly-D-lysine coated wells were washed thrice with TBS, pH 7.4. One hundred uL nucleic acid antigens (2.5 ug/ml in TBS) were coated for 2 hr at room temperature followed by overnight incubation at 4°C. The plates were washed thrice with TBS-T and coated with 100 uL/well of poly-L-glutamate (50 ug/ml in TBS) for 2 hr at room temperature to neutralize the positive charges of unreacted poly-D-lysine. The plates were again washed and finally the unoccupied sites, both in control and antigen coated wells, were saturated with 100 uL of 1% bovine serum albumin (in TBS) for 5 hr at room temperature. The plates were washed once with TBS-T and antibodies (diluted in 1% BSA-TBS) were added both in antigen and control wells. The plates were incubated for 2 hr at room temperature and overnight at 4°C. The unbound material on the plates was eliminated by washing thrice with TBS-T, pH 7.4 and appropriate anti-IgG-alkaline phosphatase conjugate (1:1500 dilution in TBS) was coated for 2 hr at room temperature. A colorigenic substrate, p-nitrophenyl phosphate was added and the absorbance of each well was recorded at 410 nm on an automatic microplate reader. The results were expressed as a mean of \( A_{\text{Test}} - A_{\text{Control}} \).

d) Competition-inhibition ELISA

The specificity of antigen-antibody interaction was checked by inhibition ELISA (Ali and Ali, 1986). Varying amounts of soluble competitors were incubated with constant amounts of antibody (serum or IgG). The preparation was incubated for 2 hr at 37°C and overnight at 4°C before they were coated onto the test plates. Rest of the steps were same as in simple ELISA. The inhibition was expressed according to the formula:
(OD inhibited) - (Background)
Inhibition (%) = 1 - \[\frac{\text{(OD uninhibited) - (Background)}}{(OD inhibited) - (Background)}\] X 100

16. Gel Retardation Assay
Varying amounts (0-28 ug) of antibodies were incubated with photoadducts (DNA-8MOP or poly(dA-dT)-8MOP) in a total volume of 15 uL for 2 hr at 37°C (Alam and Ali, 1992). The antigen-antibody interaction was stabilized by incubating the mixture overnight at 4°C. After completion of incubation period, 1.0 uL of "stopmix" was added in each assay tube. The immune complex thus formed was electrophoresed on 1% agarose using 0.04 M Tris acetate and 0.002 M EDTA pH 8.0. Control experiments were carried out in presence or absence of preimmune goat IgG. On completion of electrophoresis, the gel was stained with ethidium bromide (1 ug/ml) and photographed under UV illumination.