II. EXPERIMENTAL

A. MATERIALS

**Chemicals for immunization.**

Bovine serum albumin and calf thymus DNA were purchased from Sigma Chemical Company, U.S.A. Psoralen was a gift from C.D.R.I., Lucknow. Freund's complete adjuvant was obtained from Difco Chemical Company, U.S.A. Methanol and magnesium turnings were obtained from B.D.H., India and iodine from E. Merck, Germany.

**Chemicals for the determination of anti-DNA antibodies.**

Agarose, 4-methylumbelliferyl phosphate, 2-amino-2 methyl-1-propanol, picrylsulfonic acid, ethidium bromide, alkaline phosphatase (bovine intestine, type VII-S) and goat anti-human IgG and IgM were purchased from Sigma Chemical Company, U.S.A. Calf serum was from Microlab, India. p-nitrophenyl phosphate was obtained from C.S.I.R. Center for Biochemicals, Delhi. Polystyrene flat bottom plates having 96 wells (7 mm diameter) were purchased from Cooke Microtiter Company, U.S.A. Nylon 66 was a product of B.D.H., England. Diphenylamine and diethanolamine were from B.D.H., India. Tween-20 was from Koch-Light Laboratories, England and sodium azide was obtained from Polskie, Poland.
Chemicals for isolation and purification of anti-DNA antibodies.

DEAE-cellulose was obtained from Whatman, England. Sephadex G-200, Blue Dextran-2000 and cyanogen bromide activated Sepharose-4B were purchased from Pharmacia Fine Chemicals, Sweden. Acrylamide, Coomassie Brilliant Blue R and dithiothreitol were from Sigma Chemical Company, U.S.A. Bisacrylamide was from Reanal, Hungary. N,N,N'N'-tetramethylethlenediamine was purchased from B.D.H., England. Glycine was obtained from E. Merck, Germany.

Other chemicals.

RNA (yeast) and orcinol were obtained from Sigma Chemical Company, U.S.A. Hydroxyapatite was obtained from Bio-Rad Laboratories, U.S.A. Glutaraldehyde was purchased from E. Merck, Germany. All other chemicals used were of analytical grade.

Equipment.

Colorimetric reactions were monitored with a Spekol-10 spectrocolorimeter (Veb Carl Zeiss Jena, F.R.G.) and fluorescence measurements were carried out on Aminco-Bowman spectrophotofluorometer. Measurements of absorbance at 260 nm were made by a single beam spectrophotometer (Carl Zeiss Jena VSU 2-P).

Sera.

Normal human sera were obtained from healthy subjects. Sera of patients with various rheumatic diseases were collected.
from outdoor and indoor patients of the Department of Medicine, All India Institute of Medical Sciences, New Delhi. The samples were transported to the laboratory on ice-sodium chloride mixture. Patients with systemic lupus erythematosus, progressive systemic sclerosis, Sjogren's syndrome and rheumatoid arthritis had features meeting preliminary criteria of the American Rheumatism Association for these diseases (1973).

Blood from rabbits and guinea pigs was collected by heart puncture and sera were separated after 4 hours. All sera were stored in small aliquots at -20°C with 0.1 percent sodium azide as preservative.

B. METHODS

(1) Determination of protein concentration.

Protein was estimated by the method of Lowry et al. (1951) using Folin-phenol reagent.

(a) Preparation of Folin-phenol reagent. Folin-phenol reagent was prepared by the procedure given by Folin and Ciocalteu (1927).

100 gm of sodium tungstate, 25 gm sodium molybdate, 700 ml of distilled water, 48.2 ml of 85 percent orthophosphoric acid and 100 ml of hydrochloric acid were refluxed in dark for 10 hours. 128 gm of lithium sulfate, 50 ml of distilled water and few drops of bromine were added to the mixture. Excess bromine
was removed by boiling the mixture for 15 minutes. The solution was cooled, diluted to one liter and filtered. The bright yellow reagent was protected from light by storing in amber colored bottle. The above reagent was diluted 4 times with distilled water before use.

(b) Preparation of alkaline copper reagent. 50 ml of 2 percent sodium carbonate solution prepared in 0.1 N sodium hydroxide was mixed with 1 ml of 0.5 percent copper sulfate in 1 percent sodium potassium tartrate. The reagent was prepared fresh before use.

(c) Procedure. 5 ml of alkaline copper reagent was mixed with 1 ml of the protein sample. The tubes were left for 10 minutes at room temperature and 1 ml of diluted Folin-phenol reagent was added with immediate mixing and allowed to stand at room temperature for 30 minutes. The absorbance was read at 660 nm. Protein content of unknown sample was calculated from a standard plot constructed with bovine serum albumin.

(2) Determination of DNA concentration.

DNA concentration was determined by the method of Burton (1956) using diphenylamine reagent.

(a) Crystallization of diphenylamine. 2 gm of diphenylamine was dissolved in 100 ml of boiling hexane. Approximately 0.5 gm of activated animal charcoal was added to the boiling mixture. The
solution was filtered while hot and filtrate kept at 4°C till the crystallization was complete. The crystals were separated by filtration and air dried at room temperature.

(b) Preparation of diphenylamine reagent. 750 mg of recrystallized diphenylamine was mixed with 50 ml of glacial acetic acid and 0.75 ml of concentrated sulfuric acid. The reagent was prepared immediately before use.

(c) Procedure. One ml of DNA sample was mixed with one ml of 1N perchloric acid. The tubes were incubated at 70°C for 15 minutes in a water bath and 0.1 ml of 54.3 mM acetaldehyde was added followed by 2 ml of diphenylamine reagent. The tubes were mixed and allowed to stand at room temperature for 16-20 hours and absorbance was recorded at 600 nm. The DNA concentration in unknown sample was determined from the standard plot.

(3) Determination of RNA concentration.

RNA was estimated by cupric ion catalysed orcinol reaction of Lin and Schjeide (1969).

(a) Crystallization of orcinol. 5 gm of commercial orcinol was dissolved in 100 ml of boiling benzene and decolorized with 1 gm of activated animal charcoal. The suspension was filtered while hot and kept at room temperature for 1 hour and then at 4°C till the crystallization was complete. The crystals were separated by filtration and dried at room temperature.
(b) **Cupric ion reagent.** 150 mg CuCl$_2$·2H$_2$O was dissolved in 100 ml of concentrated HCl.

(c) **Stock orcinol solution.** 12.5 gm of recrystallized orcinol was dissolved in 95 percent ethyl alcohol and the volume made to 25 ml. The solution was kept in dark.

(d) **Orcinol reagent.** 2 ml of orcinol stock solution was mixed with 100 ml of cupric ion reagent. The reagent was prepared fresh before use.

(e) **Procedure.** 2 ml of RNA solution and 2 ml of orcinol reagent were mixed and kept in boiling water bath for 35 minutes. Tubes were cooled and absorbance recorded at 660 nm. RNA concentration of unknown sample was calculated from a standard plot drawn by using yeast RNA.

(4) **Separation of dsDNA and ssDNA by hydroxyapatite column chromatography.**

Separation was done as described by Bernardi (1971).

(a) **Chromatography of dsDNA.** Native DNA at a concentration of 1 mg/ml in 0.01 M phosphate buffer, pH 7.2 was passed through a 5.5 cm x 1.5 cm column of hydroxyapatite. Stepwise elution was carried out using 0.01M - 0.3M phosphate buffer, pH 7.2. Fractions of 3 ml were collected at a flow rate of 40 ml/hour. DNA in each fraction was estimated by the method of Burton (1956).
Double-stranded DNA was found to be eluted at 0.25 M phosphate buffer, pH 7.2 (Fig. 1). 0.25 M fractions were pooled and used as dsDNA antigen.

(b) Chromatography of ssDNA. Native DNA in 0.01 M phosphate buffer, pH 7.2 was denatured by heating for 15 minutes in boiling water bath. Samples were fast cooled and then passed through hydroxyapatite column. Denatured DNA showed chromatographic behavior quite different from that of native DNA as it was eluted at 0.2 M phosphate buffer, pH 7.2 (Fig. 2). Fractions eluting at 0.2 M phosphate buffer were used as ssDNA antigen.

(5) Preparation of antigens for eliciting antibodies with dsDNA and ssDNA.

(a) Preparation of methylated bovine serum albumin. Methylated bovine serum albumin (MBSA) was prepared by the method of Sueoka and Cheng (1962). One gm of bovine serum albumin was dissolved in 100 ml of absolute methanol and 0.84 ml of 12 N HCl was added. The protein was readily soluble at first but precipitates as it becomes methylated. The reaction mixture was allowed to stand with occasional mixing at room temperature in the dark for at least a day. Thereafter, the mixture was centrifuged and the precipitate washed four times with methanol followed by two washings with anhydrous ether. Residual ether was removed from MBSA by evaporation in air and residual acid by keeping it over KOH in a vacuum desiccator. The dried MBSA was stored in a vacuum desiccator over KOH.
Fig. 1: Chromatography of native calf thymus DNA on hydroxyapatite column.

1.5 mg of DNA in 1.5 ml of 0.01 M phosphate buffer, pH 7.2 was loaded on a column of hydroxyapatite (5.5 cm x 1.5 cm). The column was washed with 0.01 M, 0.1 M and 0.2 M phosphate buffer and dsDNA eluted at 0.25 M phosphate buffer, pH 7.2.
Fig. 2: Chromatography of denatured DNA on hydroxyapatite column.

Native DNA (1 mg/ml in 0.01M phosphate buffer, pH 7.2) was denatured by heating and applied onto a column of hydroxyapatite (5.5 cm x 1.5 cm). Stepwise elution was made from 0.01M-0.3M phosphate buffer, pH 7.2. Fractions of 3 ml were collected and monitored for DNA. ssDNA was eluted at 0.2M phosphate buffer, pH 7.2.
(b) **Formation of DNA-MBSA complex.** Suitably immunogenic protein conjugates were prepared by mixing equal volumes of DNA (1 mg/ml) and MBSA (0.5 mg/ml). The reaction was carried out at room temperature with gentle mixing.

(c) **Immunization schedule.** Rabbits (nine to twelve months old, weighing 1 to 1.5 kg) were injected intramuscularly for 3 weeks with freshly prepared antigen-MBSA complex containing 350 μg of hapten in a total volume of 1.4 ml. A single rabbit received a total of 1.05 mg of hapten in the course of three injections. Antisera were separated from blood obtained by cardiac puncture 7 to 10 days following the last injection.

(6) **Isolation of IgG.**

Serum IgG was isolated by DEAE-cellulose adsorption method of Stanworth (1960). IgG was also isolated by DEAE-cellulose chromatography of the crude immunoglobulin precipitate obtained by 40% ammonium sulfate saturation of serum.

(a) **Isolation of IgG by DEAE-cellulose adsorption method.** Approximately 17 gm of moist DEAE-cellulose previously washed and equilibrated with 0.01 M sodium phosphate buffer containing 0.015 M NaCl, pH 7.5 was mixed with 2.5 ml of cold phosphate buffer, 2.5 ml of serum (previously dialyzed for 16 hours at 4°C against phosphate buffer) was added dropwise to the exchanger with continuous mixing. The mixture was kept for 5 hours at 4°C.
Thereafter 5 ml of cold buffer was added with stirring. The resulting slurry was centrifuged in cold for 15-20 minutes. The supernatant was removed and cleared of cellulose, if any, by recentrifugation. The purity of the isolated IgG was checked on Sephadex G-200 column (54.6 cm x 1.8 cm) using 0.1 M phosphate buffer, pH 6.5 as eluent and by polyacrylamide gel electrophoresis using 10 percent gel. The protein was found to be essentially homogeneous.

(b) Preparation of crude immunoglobulins. 4 ml of 100 percent saturated ammonium sulfate solution was slowly added to 6 ml of serum in cold and the mixture was left at 4°C for 1 hour for complete precipitation of the immunoglobulins. The precipitate was recovered by centrifugation and washed thrice with 40 percent saturated ammonium sulfate solution. The washed precipitate was dissolved in and dialyzed against 10 mM phosphate buffer, pH 8.0.

(c) DEAE-cellulose chromatography. Dialyzed crude immunoglobulins were loaded onto a column of DEAE-cellulose (20 cm x 2 cm) previously equilibrated with the starting buffer (10 mM phosphate buffer, pH 8.0). The column was eluted at room temperature with linear ionic strength gradient of 10mM - 300mM phosphate buffer, pH 8.0. 3 ml fractions were collected and monitored for protein (Fig.3). First peak of the chromatogram was pooled and used as purified IgG. The material was homogeneous and gave a single band in polyacrylamide gel electrophoresis.
Fig. 3: Fractionation of serum immunoglobulins by DEAE-cellulose chromatography.

40% saturated ammonium sulphate precipitated serum fraction was dissolved and dialyzed against 10 mM phosphate buffer, pH 8.0 and loaded on a column of DEAE-cellulose (20 cm x 2 cm). The elution was performed by applying linear ionic strength gradient of 10 mM - 300 mM phosphate buffer, pH 8.0. Fractions of 3 ml were collected and monitored for proteins.
Sheared DNA was used as antigen in immunodiffusion, counterimmunoelectrophoresis and passive hemagglutination techniques for the measurement of anti-DNA antibodies. It was prepared by passing 1 mg/ml dsDNA in PBS (0.01 M phosphate buffer in 0.15M NaCl, pH 7.2) or McIlvaine buffer (0.05 M citric acid, 0.1M Na₂HPO₄, pH 4.9) three times through a sterilized 21-gauge needle. Single-stranded DNA was prepared by heat denaturation.

(a) Immunodiffusion. The precipitin reactions were carried out by Ouchterlony double diffusion using glass petri dishes as described by Seligmann (1957) and Tan et al. (1966). 6 ml of molten, filtered, 0.4 percent agarose in PBS containing 0.1 percent sodium azide was poured in 5 cm x 1.5 cm glass petri dishes and allowed to harden at room temperature. The petri dishes were stored at 4°C. All the sera tested were decomplemented by heating at 56°C for 30 minutes. 50 μl of each serum and antigen was placed in the wells and the reaction was allowed to proceed for 2-4 days in a moist chamber at room temperature. Petri dishes were washed with 5 percent sodium citrate for 4 hours to remove nonspecific precipitin lines. The precipitin lines were analysed visually and the results recorded.

(b) Counterimmunoelectrophoresis. Counterimmunoelectrophoresis (CIE) was performed as described by Davis and Winfield (1974).
3 ml of 0.6 percent molten agarose prepared in 25 mM barbital buffer, pH 8.4 containing 0.1 percent sodium azide, was pipetted onto alcohol cleaned microscopic slides (2.5 cm x 7.5 cm) and allowed to solidify at room temperature. The slides were stored in a moist chamber at 4°C. 4 mm wells were cut on the slide at a distance of 5 mm between the two opposite wells. Antigen was placed in the cathodal well and decomplemented serum in the anodal well (25 μl in each well). Electrophoresis was performed in 50 mM barbital buffer, pH 8.4 with a current of 3-4 mA per slide. Electrophoresis was performed for 30-50 minutes. Nonspecific precipitin lines were abolished by washing the slides with 5 percent sodium citrate for 2-4 hours. Finally the slides were examined for precipitin lines and the results recorded.

(c) Passive hemagglutination technique. Microhemagglutination test of Inami et al. (1973) with slight modifications was used for the detection of anti-DNA antibodies in the sera of patients with various rheumatic diseases and animals immunized with dsDNA, ssDNA and DNA-psoralen adduct. Sheared DNA was used as the source of antigen and formalinized sheep erythrocytes were employed as their passive carriers. Sheep erythrocytes were formalinized according to the procedure of Inami and associates (1973).

To 0.3 ml of packed cells, 4.2 ml of 0.005 percent tannic acid in PBS was added. The resulting suspension was incubated
at 37°C for 45 minutes with occasional shaking. The cells so treated were washed 4 times with 20 volumes of PBS. The fifth and final wash was carried out with McIlvaine buffer (citric acid 0.05M, Na₂HPO₄ 0.1M, pH 4.9). Formalinized tanned cells were coated with dsDNA, ssDNA and DNA-psoralen adduct. DNA (50 μg/ml) in McIlvaine buffer was added to the packed cells to give 4 percent suspension. Mixture was incubated at 37°C for 1 hour with frequent shaking. The cells were packed and washed 4 times with PBS and resuspended in PBS containing 0.07 percent BSA to a final concentration of 2.5%. The antigen coated cells were stored at 4°C and then used within a week.

All decomponented sera tested were adsorbed for 30 minutes at room temperature with equal volumes of fresh packed sheep erythrocytes that had been washed 4 times with PBS. This was done to get rid of heterophilic antibodies that would otherwise nonspecifically agglutinate the antigen coated erythrocytes.

(i) Check of specificity by inhibition studies. The specificity of hemagglutination reaction was checked by inhibition experiments. In a parallel row of wells, 50 μl of ds- or ssDNA (100 μg/ml in PBS containing 0.2 percent bovine serum albumin) was placed in all but the first well. To the first well were added 50 μl of ds- or ssDNA at a concentration of 200 μg/ml in PBS and 50 μl of test serum, the solutions were mixed and serially diluted in subsequent wells containing constant level of antigen. The plates
were shaken and incubated at 37°C for one hour and at 4°C for 16 hours. 25 μl of ds- or ssDNA coated cells were added to each well, mixed and read after 2 hours. Decrease in the hemagglutination titer compared to test without added DNA confirmed the presence of anti-DNA antibodies in the test serum.

(8) Spectrophotometric assay for the detection of anti-DNA antibodies.

A spectrophotometric procedure utilizing purified calf thymus DNA as antigen was developed and carried out as follows.

(a) Procedure. 50 μg of ds- or ssDNA (purified by hydroxyapatite column) in 0.9 ml of 0.15 M borate sodium chloride buffer, pH 8.0 was mixed with 0.1 ml of decomplemented serum. The mixture was incubated at 37°C for 1 hour followed by 16-24 hours incubation at 4°C. One ml of saturated ammonium sulfate solution was added and the contents mixed thoroughly. The mixture was kept for 1 hour at 4°C for the complete precipitation of immune complexes. The contents were centrifuged and supernatant separated. Absorbance of the supernatant was recorded at 260 nm. Each test serum was run along with a control which contained all reagents except the antigen. The results were expressed in terms of percent DNA bound to anti-DNA antibodies, calculated according to the formula:

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\text{Percent DNA bound} = 100 - \left[ \frac{A_{\text{test}} - A_{\text{control}}}{A \text{ 50 μg of DNA}} \times 100 \right]
\]
(b) **Specificity of antigen-antibody reaction in spectrophotometric assay.** The specificity of anti-DNA antibody measurement by spectrophotometric assay was studied by an inhibition experiment. Varying concentrations of DNA were mixed with 0.1 ml of anti-DNA antibody positive SLE serum to a final concentration of 2 to 50 μg DNA/ml and incubated at 37°C for one hour and at 4°C for 16 hours. Incubated contents were used in the assay instead of serum.

(9) **Fluorometric assay for anti-DNA antibodies using ethidium bromide as intercalating dye.**

(a) **Fluorescence measurement.** The excitation and emission spectra of ethidium bromide (EB), DNA and EB-DNA complexes were obtained by plotting fluorescence intensity (FI) versus wavelength. The excitation and emission wavelengths were 300 nm and 590 nm respectively.

(b) **Principle of the fluorometric assay.** EB forms a fluorescent complex with double-stranded DNA which shows strikingly enhanced fluorescence intensity as compared to free DNA and EB. The increase in fluorescence is a function of DNA concentration (Fig. 4). The method described measures the amount of DNA bound in the immune complexes by the enhanced fluorescence due to dye intercalation. The fluorescence intensity is proportional to the amount of DNA which in turn is dependent on the concentration of the anti-DNA antibodies.
Fig. 4: Emission spectra of EB, DNA and EB-DNA complex.

Inset: Fluorescence intensity of EB-DNA complex as a function of DNA concentrations.

Emission spectra were recorded with excitation wavelength of 300 nm. Concentration of DNA in EB-DNA complex: 2 µg (●), 4 µg (○), 6 µg (▲), 8 µg (■), 10 µg (▲), free EB (●), 10 µg of free DNA (▲). The concentration of EB was kept constant (5 µg/ml).
(c) Procedure of the fluorometric assay. 10 µg of hydroxyapatite purified dsDNA in 0.5 ml of borate buffer (0.15 M borate-sodium chloride buffer, pH 8.0) was mixed with 0.5 ml of decomplemented serum in siliconized plastic tubes. All the sera were tested at a dilution of 1:5. The mixture was incubated at 37°C for one hour and at 4°C for 20 hours. One ml of saturated ammonium sulfate solution was added and the resulting fifty percent saturated mixture was held at 4°C for one hour for complete precipitation of immune complexes. The precipitated immune complexes were separated by centrifugation (12,000 rpm for 15 minutes at 4°C) and dissolved in 4.5 ml of borate buffer at 4°C. 25 µg of EB in 0.5 ml of borate buffer was added and fluorescence intensity recorded. Each test serum was run along with a control which contained all reagents except antigen. The fluorescence reading of the control was subtracted from the test and the results were expressed in terms of percent DNA bound in the immune complexes. The amount of DNA bound to the immune complexes was calculated from a standard plot of fluorescence intensity of EB-DNA complexes as a function of DNA concentration.

(d) Inhibition experiment. The specificity of the assay was checked by performing an inhibition experiment as described previously in the spectrophotometric assay.
(10) Enzyme-linked immunosorbent assay (ELISA) for anti-DNA antibodies on polystyrene plates.

(a) Buffers and other reagents.

(i) Bicarbonate buffer. 15 mM sodium carbonate, 35 mM sodium bicarbonate, pH adjusted to 9.6. Sodium azide (0.02%) was added as preservative.

(ii) Phosphate buffered saline-Tween (PBS-T). 136 mM sodium chloride, 2 mM potassium dihydrogen orthophosphate, 8 mM disodium hydrogen phosphate, 0.5 ml Tween-20/lit, pH adjusted to 7.4.

(iii) Diethanolamine buffer. 1 M diethanolamine containing 100 mg/lit MgCl$_2$.6H$_2$O, pH adjusted to 9.8 with hydrochloric acid. 0.02 percent sodium azide was added as preservative.

(iv) 2-amino-2-methyl-1-propanol (AMP) buffer. 150mM AMP, 3mM MgCl$_2$, 0.1 ml Triton x-100/lit, pH adjusted to 10.3. The solution was filtered before use.

(v) Substrates. 5 x 10$^{-3}$M p-nitrophenyl phosphate in diethanolamine buffer and 1 x 10$^{-3}$M 4-methylumbelliferyl phosphate (4MU-P) in AMP buffer.

(b) Preparation of rabbit anti-human-IgG alkaline phosphatase conjugate. Rabbits were immunized with four, weekly intramuscular injections of purified normal human IgG in PBS with equal volume of Freund's complete adjuvant. Each rabbit received 4 mg
IgG per injection. The animals were bled a week after 4th injection and IgG was isolated by DEAE-cellulose chromatography from the pooled sera.

2 mg of isolated rabbit anti-human IgG in 0.1 ml of PBS was mixed with 5 mg of alkaline phosphatase. The mixture was dialyzed for 20 hours against PBS at 4°C with 3 changes of buffer. 25% glutaraldehyde was added to a final concentration of 0.2%. The resulting mixture was kept at room temperature for 2 hours and then dialysed at 4°C for 16 hours against PBS with 3 changes of buffer. Dialysis sack containing the conjugate was transferred to 0.05M Tris buffer pH 8.0 and dialyzed for 16 hours at 4°C. Finally the dialysis sack contents (conjugate) were diluted to 4 ml with Tris buffer containing 1% BSA and 0.02% sodium azide.

The conjugate was partially purified by gel filtration on 58 cm x 1.9 cm column of Sephadex G-200 in 0.1M phosphate buffer, pH 7.2 and the active fractions were pooled and stored at 4°C.

(c) ELISA procedure. Technique of Voller et al. (1976) with slight modification was used for the detection and quantitation of anti-DNA antibodies. DNA in coating buffer (12.5 μg/0.25 ml in 0.05 M bicarbonate buffer, pH 9.6) was added to the wells of the microtiter plate which was then incubated at 4°C for 16 hours. The wells were washed three times with PBS-T and unoccupied sites
were saturated with fetal calf serum (1% v/v in PBS-T) by incubation at 37°C for 1 hour. After washing the plate with PBS-T, 0.25 ml of serum (1:100) or IgG (1:100 at 12 mg/ml) was added and the plate was incubated at 37°C for one hour. 0.25 ml of rabbit antihuman IgG alkaline phosphatase conjugate was added, after washing the excess antibody. The plate was once again incubated at 37°C for 2 hours. At the end of incubation period, the plate was emptied and washed. 0.20 ml of substrate (PNP-P, 5x10^{-3} M in diethanolamine buffer, pH 9.8 or 4MU-P, 1x10^{-3} M in AMP buffer, containing 1.5x10^{-3} M Mg^{++} pH 10.3) was pipetted into each well. The plate was incubated at 37°C for one hour and the reaction was stopped by the addition of 0.05 ml of 3N NaOH. The contents of each well were either read at 400 nm or fluorometric measurements were made, depending upon the substrate. Excitation and emission wavelengths were 365 nm and 455 nm respectively.

(11) ELISA with nylon as solid phase.

A highly sensitive enzyme-linked immunosorbent assay was developed for the detection and quantitation of anti-DNA antibodies in the sera of patients with various rheumatic diseases and animals immunized with DNA-psoralen adduct, using nylon as solid support for antigen immobilization.

(a) Activation of nylon. Nylon beads (3mm x 2mm) were treated with 3.5N HCl for 36 hours at 37°C with occasional shaking and
thereafter washed thoroughly with distilled water till free of acid. The exposed amino groups were detected by the treatment of activated beads with picrylsulfonic acid (Synder and Soborinski, 1975).

(b) Procedure. Activated nylon beads were treated with DNA (100 μg/ml) in McIlvaine buffer (citric acid 0.05M, Na₂HPO₄ 0.1M, pH 5) for 24 hours at 4°C with occasional shaking. The antigen coated beads were washed 4 times with ST-20 (0.15M NaCl, 0.05% Tween-20) to remove the unreacted DNA. Unoccupied sites were saturated with 1% BSA in ST-20 by keeping the beads immersed in the BSA solution for 12 hours at 4°C. The beads were washed 4 times with ST-20, dried on Whatman filter paper and stored at -20°C until use. Activated beads treated with BSA, without prior antigen treatment, were used as control. Six beads were used for ELISA. All the sera were tested at a dilution of 1:100. Beads (test and control) in siliconized glass tubes were incubated with 0.5 ml of serum for 1 hour at 37°C with occasional shaking. The beads were washed 4 times with ST-20 and incubated with 0.5 ml of conjugate (1:1000 dilution or 11 μg/ml protein) for 2 hours at 37°C. Unreacted conjugate was washed four times with ST-20. The final washing was done by distilled water. The beads were dried on Whatman filter paper and transferred to a clean tube containing 1 ml of substrate (PNP-P or 4MU-P). The tubes were incubated at 37°C, for 30 minutes with occasional shaking. The
reaction was stopped with 1 ml of 3N NaOH for PNP-P and 1 ml of 1M K$_2$HPO$_4$-KOH buffer, pH 10.4 for 4MU-P. The absorbance (A) was monitored at 420 nm. Excitation and emission wavelengths were 365 nm and 455 nm respectively. Results were expressed as $\frac{A/FI_{\text{test}}}{A/FI_{\text{control}}} - 1$.

(c) Inhibition ELISA. The specificity of antigen-antibody reaction in ELISA was checked by inhibition experiments. Varying concentrations of DNA were mixed with an anti-DNA positive SLE serum to a final concentration of 2 to 20 µg DNA/ml. The mixture was incubated at 37°C for 1 hour and then at 4°C for 20 hours and used in ELISA instead of serum.

(12) Production of anti-DNA antibodies by immunization with DNA-psoralen adduct.

(a) Preparation of DNA-psoralen adduct. The formation of inter-strand cross-linkages by photoreaction of psoralen with dsDNA was studied by column chromatography on hydroxyapatite. The renaturation capacity shown by drug intercalated DNA after heat denaturation was the basis of adduct formation (Lawley and Brookes, 1967).

1 mg of hydroxyapatite purified dsDNA in TNE (0.001M Tris, 0.015M NaCl, 2x10^{-5}M EDTA, pH 7.5) buffer was thoroughly mixed with 100 µg of psoralen in the same buffer at 25°C for one hour.
The mixture was irradiated at a dose rate of 2x10^6 ergs cm^{-2} min^{-1} for 30 minutes using illuminating wavelength of 365 nm. Samples of DNA and psoralen without irradiation were the experimental control. Both samples were dialyzed against PBS to remove unbound psoralen. Dialyzed contents were heated for 10 minutes in a boiling water bath and then immersed in ice-sodium chloride mixture for 15 minutes. Each sample was chromatographed on a column (5.5 cm x 1.5 cm) of hydroxyapatite. Stepwise elution was carried out using 0.01M - 0.3M phosphate buffer, pH 7.4. Fractions of 3 ml were collected at a flow rate of 40 ml/hour. DNA in each fraction was estimated with diphenylamine reagent. Fractions eluting at 0.25M phosphate buffer (cross-linked) were pooled, dialyzed against PBS and used as DNA-psoralen antigen for eliciting antibodies in guinea pigs.

(b) Equilibrium dialysis. The amount of psoralen intercalated with DNA was evaluated by equilibrium dialysis measurements as described by Romeu et al. (1976).

Dialysis bags with 4 ml of DNA-psoralen adduct were immersed in 60 ml of TNE buffer in stoppered round bottom flask. Equilibrium was allowed to attain by shaking the flask for 30 hours at 4°C. The fluorescence intensity of the dialysate was recorded at 450 nm (320 nm excitation). The concentration of psoralen dialyzed out was quantitated with the help of a standard plot obtained by drawing fluorescence intensity of irradiated
psoralen as a function of its concentration. The amount of psoralen thus intercalated with DNA was determined.

(c) Immunization. DNA-psoralen-MBSA complex was prepared as described previously for DNA-MBSA. Guinea pigs (6 to 8 months old, weighing 400 to 500 gm) were injected intramuscularly in the hind limbs with the complex (2 ml) weekly for seven weeks. A single animal received a total of about 1.5 mg intercalated DNA in the course of seven injections. Serum was separated from blood obtained by cardiac puncture 7 days after the last injection.

(d) Reaction of DNA-psoralen immunized sera with various nucleic acids by ELISA with nylon as solid phase. Preimmunized and DNA-psoralen immunized guinea pig sera at different dilutions were tested by ELISA for the reaction against adduct, dsDNA, ssDNA and RNA using goat anti-guinea pig IgG alkaline phosphatase conjugate (prepared as for rabbit anti-human IgG alkaline phosphatase conjugate).

Activated nylon beads were treated with DNA-psoralen adduct, dsDNA, ssDNA and RNA (100 μg/ml) in McIlvaine buffer, pH 5 for 24 hours at 4°C with occasional shaking. Remaining steps were as described earlier. Sera were tested at a dilution of 1:100 in siliconized glass tubes with antigen sensitized and control beads. Immune complex attached to nylon beads was
detected by incubating the beads in 0.5 ml of goat anti-guinea pig IgG alkaline phosphatase conjugate (1:500 dilution or 28 µg/ml protein).

(13) Isolation and purification of anti-DNA antibodies.

(a) Preparation of DNA-Sepharose column. DNA was coupled with CNBr activated Sepharose-4B according to the method for Arndt-Jovin et al. (1975).

1 gm of CNBr activated Sepharose-4B was swollen in 20 ml of 1mM HCl for 15 minutes. The gel was transferred onto sintered glass filter and washed with 1mM HCl, 10mM potassium phosphate buffer, pH 8.0. The contents were transferred to a stoppered cylinder containing hydroxyapatite purified double-stranded DNA (500 µg/ml) in 50 ml of 10 mM potassium phosphate buffer, pH 8.0 (coupling buffer). The mixture was rotated end-over-end for 2 hours at room temperature and then kept at 4°C for 12 hours. The DNA-Sepharose product was washed on a sintered funnel with 10 mM potassium phosphate, pH 8.0 followed by 1M potassium phosphate, pH 8.0 and 1M KCl. The gel was then washed with 0.2 M glycine, pH 8.0 and transferred to a stoppered cylinder containing 20 ml of 0.2 M glycine. The contents were mixed end-over-end for 2 hours at room temperature and filtered. The gel was then washed with coupling buffer and finally equilibrated with TEDG buffer.
(50mM Tris, 0.1mM EDTA, 0.1mM dithiothreitol and 5% glycerol, pH 7.9). The gel was stored at 4°C.

(b) **Purification of anti-DNA antibodies by affinity chromatography.**

IgG was isolated from an anti-DNA positive SLE serum by DEAE-cellulose adsorption method and dialyzed against equilibrating buffer at 4°C for 24 hours with 3 changes of buffer. The dialyzed contents were applied to affinity column (7 cm x 2 cm). The column was washed with equilibrating buffer followed by 50mM Tris, 0.1mM EDTA, 0.1mM dithiothreitol, 5% glycerol and 0.1M NaCl buffer, pH 7.9. Bound protein was eluted with 1M NaCl in TEDG buffer. The eluate was dialyzed at room temperature against 0.01M phosphate buffer containing 0.015M NaCl, pH 7.5 and concentrated.

(c) **Reaction of affinity purified material with different nucleic acids by CIE and ELISA.** The specificity and crossreactivity of the affinity purified material was checked by CIE and ELISA.

ELISA was carried out at various antibody dilutions (120 μg/ml to 0.06 μg/ml) employing DNA-psoralen adduct, dsDNA, ssDNA and RNA sensitized nylon beads. Rest of the steps were as described previously.

(d) **Demonstration of IgG/IgM response.** Crude immunoglobulins were isolated from an anti-DNA positive SLE serum by 40 percent satu-
rated ammonium sulfate solution. The precipitate was dissolved in and dialyzed against equilibrating buffer.

Dialyzed contents were applied on affinity column and bound protein was eluted as described previously. IgG/IgM response in purified material was checked by ELISA using goat anti-human IgG alkaline phosphatase (1:1000 dilution) and goat anti-human IgM alkaline phosphatase (1:1000 dilution) conjugate employing dsDNA coated beads.