EXPERIMENTAL

(A) Materials

Staphylococcus aureus was kindly supplied by Dr. S.C. Santra, Reader in the Department of Ecology, Kalyani University, W.B. and the culture was regularly maintained by B.N. Chakraborty in the Department of Botany of Kalyani University.

Acridine orange (AO, as $ZnCl_2$ double salt, E. Merck, Germany) was made zinc free and purified by the method of Armstrong et al [143].

Methylene blue (MB, E. Merck, Germany) was purified by recrystallization from hot dilute HCl solution in water according to the method of Pal and Schubert [152].

The cationic dyes pinacyanol chloride (PCYN), Sigma Chemical Co.); N,N'-diethylpseudoisocyanine (PIC, Serva Feinbiochemica, Heidelberg); 1,9-dimethylmethylene blue (DMMB, Serva Feinbiochemica, Heidelberg) and 1,1'-diethyl-4,4'-carbocyanine iodide (CCI, Aldrich Chemical Co., USA) were pure samples as checked from molar absorbances of 10^{-5}M aquecus solutions of these dyes, and used as such. Calf thymus DNA, daunomycin (DMC), actinomycin D (AMD) gramicidin D (GRD), neomycin sulfate (NMC) all of Sigma chemical Co., USA); tryptone, yeast extract (Oxoid Limited, England) and poly (styrene sulfonate) (PSS) (Serva Feinbiochemica, Heidelberg) were used as such. Ion exchange resin Dowex-1 (Cl^-), was of Sigma Chemical Co., USA. It was treated by two successive treatments with 1.00M NaCl followed by washing with distilled water (Seven times).

Alcohols were used after redistillation. Other chemicals like sodium chloride, magnesium chloride, calcium chloride, ammonium molybdate, dextrose anhydrous etc. were of GR/AR qualities and used as such. Ordinary distilled water was redistilled with alkaline permanganate using all -glass distillation set.

(B) Methods

Purification of dyes

5.00 g of MB was dissolved in 100 ml of 0.03M HCl solution at 50°C, filtered hot, and to the filtrate concentrated HCl (10 ml) was slowly added while the filtrate was chilled at 5°C. The crystals were separated by centrifugation, washed with chilled ethanol and ether, and dried in vacuum desiccator, yield 3.5 g.

AO (3.00 g) was dissolved in 50 ml of water and filtered. To the filtrate liquor NH_3 was added in slight excess after the precipitation of AO base and $Zn(OH)_2$ was complete. AO base, thus precipitated along with $Zn(OH)_2$, was extracted with 50 ml of chloroform. The chloroform layer was separated, washed repeatedly with 0.01M aqueous NH_4EDTA solution and evaporated at room temperature. The residue containing AO base was dissolved in 50 ml ethanol and slight excess of dilute HCl solution was added. AO-HCl thus formed was precipitated by adding ether and filtered, washed with ether and dried in vacuum desiccator, yield 2.00 g.

The molar absorbances of 10^{-5}M solution of AO and MB are checked to be sure of their purification.

Extraction and purification of *Staphylococcus aureus* wall teichoic acid (TA)

Teichoic acids can be extracted from isolated cell walls as well as from the whole bacteria. There are several methods for the extraction and purification of teichoic acids [1,142,144-147]. Among them we have judiciously selected the method of Zenek et al [142] with little modification as detailed below.

The fresh overnight culture of *S. aureus* cell was grown in the liquid culture medium containing 1% tryptone, 0.50% yeast extract, 1.0% glucose, 0.3% dipotassium hydrogen orthophosphate in 4 litre de-ionized water; the medium was previously sterilised by autoclaving for 15 minutes at a pressure of 15 lbs. The pH of sterile medium usually comes between

7.3-7.5. To have a sufficient growth the incubated medium was kept in a mechanical shaker at 37°C with aeration for 24 hours.

The bacteria was collected from the medium by centrifuging (7000 rpm, 10 minutes, 4°C) and washed repeatedly (4 times) with 0.9% aqueous NaCl solution to remove the fat containing materials of the cells. The wet weight of the collected defatted bacteria comes to be around 8.30 g. It was then treated with 170 ml of 0.01M aqueous NaOH solution stirred with a magnetic stirrer for 8 hours and kept overnight at (4°C), when teichoic acid was removed from the bacterial cell walls and get dissolved in the NaOH solution. The bacteria from the solution was separated by centrifuging (7000 rpm, 15 minutes, 4°C) and the pH of the supernatant containing teichoic acid was adjusted to 7.6 from 8.1 by adding 0.2M HCl. The solution was passed through an anion exchanger column (Dowex-1 $(Cl)^-$) and desorbed with 100 ml of 0.2M HCl. 300 ml of ethanol was then added to the resulting solution and kept over night at 4°C to precipitate the teichoic acid. 2.0 g of potassium acetate was added to the liquid to facilitate the precipitation. The purified TA thus obtained was washed several times with ethanol and finally with acetone. It was vacuum desiccated to make dry; the dry weight of the purified TA obtained finally comes to be around 23 mg; moisture content of the sample was insignificant.

To check the purity of our sample of TA its phosphorus content was estimated colorimetrically following the standard procedure [148]. At first a standard curve was made for which the following reagents are prepared.

(i) 0.438% (w/v) of potassium dihydrogen phosphate, KH_2PO_4 was prepared in water; 1.0 ml of this solution was diluted to 100 ml by trichloroacetic acid (TCA) (50 g/l) and this solution in TCA was used as standard phosphate.

(ii) Copper acetate buffer, pH 4.0 was prepared by dissolving 250 mg copper sulphate ($CaSO_4$, $5H_2O$) and 4.6 g of sodium acetate (CH_3COONa, $3H_2O$) in 100 ml of 2 mol/litre acetic acid.

(iii) A 5% (w/v) ammonium molybdate ($(NH_4)_6MO_7O_{24},4H_2O$) was prepared in water.

(iv) The reducing agent was prepared by dissolving 2.0 g of p-methylaminophenol sulphate $((CH_3.NH.C_6H_4.OH)_2.H_2SO_4)$ in 100 ml of 10.0% (w/v) sodium sulphite $(Na_2SO_3.7H_2O)$ solution in water, and stored in dark place until required.

In a series of test tubes increasing volume (0.1 to 1.0 ml) of standard phosphate in TCA was pipetted, followed by pipetting of 3.0 ml of copper acetate buffer, 0.5 ml of ammonium molybdate solution, 0.5 ml of the reducing agent, and the final volume was made up to 5.0 ml by water. The solution in test tube was mixed up thoroughly and allowed to stand for 10 minutes at laboratory temperature.

Absorbance of the solutions were measured at 880 nm using matched cuvette of 1.0 cm path length against a blank prepared by adding 1.0 ml of 5% TCA in place of standard phosphorus to the respective same volumes of the reagents as in the experimental solutions. The plot of OD at 880 nm vs. cencentration of phosphorus (mg/ml) as represented in **Fig.** 4.1) was used as the standard curve.

To estimate phosphorus content of TA 2.0 ml solution of the sample (0.3 mg/ml) was pipetted in 5.0 ml volumetric flask, followed by pipetting 0.5 ml of 60% perchloroacetic acid. The mixture was digested over a low flame for 1 hour. After cooling the volume was made upto 5.0 ml by adding water. 0.2, 0.4 and 0.8 ml of this digested TA solution was pipetted to three different test tubes containing the same reagents as in the preparation for the standard curve and the volume made upto 5.0 ml by pipetting water in each case. OD at 880 nm was measured after waiting for 10 minutes. Phosphorus content of TA was calculated by compairing with the standard curve and taking the average of the three values. The sample of TA showing phosphorus content higher or less than 6.0 ± 0.4% was rejected.

The purity of TA was also checked by recording the UV absorption spectra in the range 200-300 nm. TA does not have any peak in this region. Any sample of TA showing a peak at around 260 nm was rejected, since such a peak indicates the contamination with DNA. We like to point out that occasionally we found samples of TA contaminated with DNA, and

such samples were not used.

The purity of the samples were also checked from the determination of its equivalent weight which is defined as the average weight containing one anionic phosphate group. The equivalent weight was determined by spectrophotometric and conductometric titrations using dyes and drugs, as described under "**Results and Discussion**". The equivalent weight came to 520 ± 5 which is in parity with the phosphorus content of the sample.

Preparation of solutions of ligands (dyes and drugs)

Stock solutions of dyes ($\sim$ x 10^{-3}M) were prepared in water and stored in the dark when not in use. Stock solutions of dyes, except PCYN and CCI, were kept for a month; PCYN and CCI solutions were made fresh once in a week. Absorbance was checked before use. Stock solutions of daunomycin, actinomycin D, neomycin sulfate were prepared in water and that of the gramicidin D was prepared in 40% ethyl alcohol.

Preparation of solutions of nucleic acids and teichoic acids

Stock solutions of DNA and TA ($\sim$x10^{-3}M) were prepared in water. The pH of the stock solution of TA was adjusted to 7.6 by adding 0.01M NaOH. The stock solutions were kept in refrigerator at 4°C.

Other details

Normal order of pipetting is polyanion-water-dye unless otherwise mentioned in the text. Spectra were recorded after waiting for at least half an hour of mixing the reagents. All experiments were done at room temperature (22°). All temperatures recorded were °C.

Ditermination of equivalent weight of polyanion and/or Stoichiometry of polyanion-dye system or polyanion-drug system by,

(i) Spectrophotometric titration

Increasing volume of aqueous solution of the polyanions of known concentrations were pipetted in a series of test tubes each containing a fixed amount of dye (usually AO). The final volume was made upto 5.0 ml with water so that the final concentration of dye in each was 1.20 x

10^{-5}M. The tubes were kept for 30 minutes at dark and the optical density (OD) of each solution at the λ_{max} of the dye was measured with respect to water. A plot of OD vs. volume of polyanion was drawn; from the break in the curve the stoichiometry of dye binding or equivalent weight (average weight containing one anionic site) of the polyanion was determined.

(ii) Conductometric titration

In a known concentration of dye or drug (usually 20.0 ml of 1.00 x 10^{-4}M) the electrode of the conductometer was immersed and the concuctance was recorded after gradual dropwise addition of the polyanion solution from a microburrette. A time gap of at least 5 minute was maintained between the addition of each drop. From the plot of concuctance vs. amount of polyanion added, the equivalent weight was calculated. In some cases the reverse order was maintained i.e. by immersing the electrode of the conductometer in the polymer solution, the conductance was recorded after gradual dropwise addition of the ligands solution from a microburrette.

Spectroscopic studies

Recording of absorption spectra

Absorption spectra as well as absorbance at particular wavelengh were recorded with a Shimadzu Spectrophotometer UV-150-02 using 1.00 cm quartz cuvettes at laboratory temperature (22°C). The instrument was calibrated with 6.00 mg $K_2Cr_2O_7$ per 100 ml 0.01 N H_2SO_4. This solution has peaks at 257 and 350 nm with molar absorbances of 145.6 and 107.0 respectively and minima at 235 and 313 nm with molar absorbances 125.02 and 48.9.

Recording of CD spectra :

CD spectra were recorded with Jasco-500-C spectropolarimeter. The instrument was routinely calibrated using d-10 camphor sulphonic acid in water and/or L-tartaric acid [149] in the UV region and 1,9-dimethylmethylene blue-hyaluronate metachromatic complex [150] in the visible region. Circular cuvettes with 1.0 cm pathlength were used, and

amplifications varying from 1.0 m°/cm to 2.0 m°/cm were used depending on the ellipticity of the samples; base lines also recorded with the same amplification as used in the relevant solutions. Spectra were recorded at the laboratory temperature (22°C).