Studies on interaction of cationic dye with bacterial acidic polysaccharide†

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Interaction of acidic capsular polysaccharide isolated from *Klebsiella* K17 with cationic dye pinacyanol chloride has been investigated by spectral measurements, and thermodynamic parameters of the interaction evaluated. The polymer induces metachromasy in the cationic dye and a blue-shift from 600 nm to 500 nm in the visible absorption spectrum of the dye is observed. The spectral changes have been studied during interaction of the dye cations with the polyanions at different polymer/dye molar ratios. The polyanion-dye compound is formed with polymer/dye stoichiometry of 1:1, indicating formation of stacking conformation. The values of thermodynamic constants, enthalpy of complex formation ($\Delta H = -7.02 \text{ kcal mol}^{-1}$), entropy change ($\Delta S = -6.80 \text{ cal mol}^{-1} \text{ deg}^{-1}$) and free energy change ($\Delta G$ at 307 K = $-4.98 \text{ kcal mol}^{-1}$) indicate chromotropic character of the polymer in inducing metachromasy in the cationic dye pinacyanol chloride. Interaction of the polymer with acridine orange dye has also been studied by fluorescence measurements.

Many gram-positive and gram-negative bacteria produce extracellular polysaccharides which surround the bacterium as a capsule. The most interesting and important feature of the capsular polysaccharide is its antigenic character. The highly specific interaction of the antigen with the combining sites on the antibody suggests that the specific antibody conformation dictates conformation of the antigens. Our interest is to understand these conformational characteristics of the antigenic materials in solutions.

Studies on metachromasy of various classes of acidic polysaccharides like polycarboxylates, polysulphates, mucopolysaccharides and different synthetic polyanions with different cationic dyes are available in literature$^1-3$. But such investigations on bacterial polysaccharides are rarely made. The present study forms a part of our programme of investigating chromatropic character of the capsular polysaccharides from different strains of *Klebsiella* and *Esch. coli* inducing metachromasy in cationic dyes$^4-6$.

Materials and Methods

The serological test strain of *Klebsilla* serotype K 17 was obtained from Max-Planck Institute for Immunobiology, Freiburg, West Germany. The strain was checked for agglutination in Difco type-specific antisera. A culture of the strain was grown in nutrient agar medium in big agar plates, harvested, dried, and capsular polysaccharide isolated by phenol-water-Cetavlon method$^7$. The polysaccharide was solubilized in phenol-water and the cell-wall lipopolysaccharide was separated by ultracentrifugation. The acidic capsular polysaccharide was isolated from the supernatant by fractional (0.25-0.06 MNaCl) precipitation with Cetavlon. The final product was dialysed against distilled water and lyophilized.

The polysaccharide was hydrolysed with 0.5 $M$ H$_2$SO$_4$ (20 h, 100°), and the monosaccharides were identified by paper chromatography (Whatman No. 1 paper), using (i) ethyl acetate-pyridine-water (4:1:1) and (ii) ethyl acetate-glacial acetic acid-formic acid-water (18:3:1:4).

Neutral sugars were estimated by GLC of the alditol acetates$^8$, using a Shimadzu gas chromatograph-GC 16A. Uronic acid was determined colorimetrically$^9$ in the unhydrolysed product.

Pinacyanol chloride [1-ethyl-2-[3-[1-ethyl-2[1H]-quinoiyldiene]-propenyl] quinolinium chloride] and acridine orange were purchased from Sigma Chemicals Co., USA. Pinacyanol chloride was used as such. However, acridine orange (double salt as ZnCl$_2$) was purified before use.

A stock solution of the dye was prepared in doubly distilled water and kept in dark at 4°C. The stock solution was always prepared afresh though it could be used for a week.

Absorbance of the solution was measured at 400-700 nm employing a Molton Roy spectrophotome-
Concentrations of the dye and polymer solutions were within the range of $10^{-3}$-$10^{-5} M$. Aqueous solution of pinacyanol chloride (1 ml, $1.003 \times 10^{-5} M$) was added to various volumes of $1.00 \times 10^{-3} M$ aqueous solution of *Klebsiella K17* polysaccharide, and total volume of the mixture was made up to 10 ml by adding the required volume of distilled water in each case.

Fluorescence of acridine orange dye solution ($1.00 \times 10^{-5} M$) was measured using a Shimadzu-RF 5000 spectrofluorophotometer. The solutions were excited at 430 nm and fluorescence intensity was measured at 522 nm. Required volume of polymer solution was thoroughly mixed with the dye solution to measure fluorescence spectra at different polymer/dye molar ratios.

**Determination of stoichiometry**

Stoichiometry of the dye-polymer compound was determined by isolating the metachromatic compound according to the method of MacIntosh. To aliquots of dye solution (10 ml each, $1.003 \times 10^{-5} M$), increasing amounts of the polymer solution (0.2-2.0 ml, $1.00 \times 10^{-4} M$) were added, and the contents were thoroughly mixed. An aliquot (10 ml) of the mixture was shaken with petroleum ether (5 ml) for 10 min, the coloured aqueous layer was separated and the colourless organic layer discarded. The process was repeated thrice. The metachromatic dye-polymer compound was thus removed; uncomplexed dye was insoluble in the organic layer. Concentration of free dye was estimated colorimetrically in the aqueous layer. Concentrations of complexed dye were plotted against the moles of polymer added, and stoichiometry of polymer and dye in the metachromatic dye-polymer compound was determined from the point of intersection of the linear curves.

Stoichiometry was also determined by centrifugation method. When the metachromatic solution was centrifuged at 23,000g (11,000 rpm) for 30 min, the dye-polymer compound was sedimented. The supernatant was analysed colorimetrically for the free dye and stoichiometry was then graphically determined.

**Reversal of metachromasy**

The reversal of metachromasy was investigated by measuring absorbance of metachromatic solution (polymer/dye molar ratio, 5.0) at the metachromatic band ($\mu$-band, 500 nm) of the spectra and also at the monomeric band ($\alpha$-band, 600 nm) of the pure dye solution upon addition of different cosolvents like 1-propanol, ethanol, methanol and urea in increasing amounts (alcohols, $0 \sim 70\%$; urea, $0 \sim 8 M$).

**Spectrophotometric titration**

For carrying out spectrophotometric titration, increasing amounts of polymer solution ($0.1 \sim 1.0$ ml, $1.00 \times 10^{-4} M$) were added to a fixed volume of the dye solution ($0.5$ ml, $1.003 \times 10^{-4} M$) and total volume of the mixture was made up to 10 ml by adding water. Absorbance of the mixtures was measured at the monomeric band ($\alpha$-band, 600 nm) of the pinacyanol chloride dye solution after thorough mixing. Absorbance versus polymer solution added was plotted, and from the point of intersection of the two linear lines, volume of the polymer solution required for equivalence of the anionic groups of the polymer and the dye cations was measured.

**Spectrofluorometric titration**

Spectrofluorometric titrations were carried out by adding polymer solution in increasing quantities to a fixed volume of acridine orange solution ($1.00 \times 10^{-5} M$). The solutions were first excited at 430 nm, and fluorescence intensity measured at 522 nm upon addition of polymer. End point of titration was determined graphically as was done in spectrophotometric titration.

**Results and Discussion**

*Klebsiella K17* capsular polysaccharide is a heteroglycan consisting of repeating units that contain $\beta$-glucose, $\alpha$-glucuronic acid and $\alpha$-rhamnose in approximate molar ratio of 1:1:3. The pentasaccharide repeating unit is represented by structure (I):

\[
\beta-D-GlcP-(1 \rightarrow 2)-\alpha-L-Rhap-(1 \rightarrow 4)-\alpha-D-GlcAP-(1 \rightarrow 3)-\beta-L-Rhap(1 \rightarrow 4)-\beta-D-GlcP-(1 \rightarrow 2)-\alpha-L-Rhap
\]

The cationic dye pinacyanol chloride (2) belongs to cyanine group of dyes.

Absorption spectra of the pure dye solution and the mixtures containing *Klebsiella K17* polymer at different polymer/dye molar ratios ($P/D = 0 \sim 80$)
are shown in Fig. 1. An aqueous solution of the dye (1.003 \times 10^{-5} M) showed two peaks at 600 nm (\(\alpha\)-band) and 545 nm (\(\beta\)-band) corresponding to monomeric and dimeric forms of the dye molecule respectively. Upon addition of polymer, intensities of both \(\alpha\) and \(\beta\)-bands decreased and a new band (\(\mu\)-band) appeared at shorter wavelength, indicating metachromasy. At P/D = 10, a distinct \(\mu\)-band appeared at 500 nm. On further increase of P/D value, the spectral behaviour changed significantly, and the \(\mu\)-band became stronger with complete disappearance of \(\beta\)-band at 545 nm and \(\alpha\)-band at 600 nm. The metachromatic blue shift of 100 nm indicated induction of strong metachromasy in the cationic dye by the polymer.

Stoichiometry of the polymer-dye compound was determined by the isolation method\(^\text{10}\) and also by centrifugation method\(^3\). The results are presented in Fig. 2. Both the methods yielded identical results. It was observed that the metachromatic compound was formed at polyanion: dye cation of 1:1. The results were in good agreement with the reported values\(^{16,17}\) for interaction of dyes like pinacyanol chloride and acridine orange with synthetic polyanions. The 1:1 stoichiometry of the metachromatic compound indicated that every potential anionic site of the polymer was associated with the dye cation resulting in stacking conformation\(^3\). It has been suggested that the dye cations are held sufficiently close to the surface of the polyanions to allow them to interact and form aggregates similar to those that were supposedly responsible for the metachromatic behaviour in concentrated dye solutions\(^{18}\). Aggregation of such rigid and planar dyes like methylene blue, acridine orange, pinacyanol chloride, etc. on anionic polymers is expected to lead to the formation of a card pack stacking\(^9\) of the individual dye monomers on the surface of the polyanion so that the allowed transition gives rise to a blue-shifted metachromasy.

Figure 3 represents the results of metachromatic titration. Aqueous solution of the cationic dye pinacyanol chloride was titrated spectrophotometrically by adding Klebsiella K17 polymer solution. It was observed that intensity of absorbance of the \(\alpha\)-band decreased linearly when increasing amount of the polymer solution was added to a fixed volume of the dye solution until it became constant. The point of intersection of the linear curves indicated neutralisation point. The stoichiometry of the polyanion-dye compound at the neutralisation point was found to
be 1.02:1.00 which was almost identical to that obtained by isolation and centrifugation methods (Fig. 2). This technique of spectrophotometric titration can be conveniently used to determine either concentration of the polymer sample of known molecular weight or anionic group content of the polyanionic sample of unknown structure in a relatively dilute solution. The equivalent weight of *Klebsiella* K 17 polysaccharide calculated from the results of metachromatic titration was found to be 778 and this was very close to the value 794 obtained from the known structure of the repeating unit.

Results of reversal of metachromasy are shown in Figs 4 and 5. Absorbances at 600 nm (α-band) and 500 nm (μ-band) were measured upon addition of different co-solvents like 1-propanol, ethanol, methanol, and urea to the pure dye solution and also to the dye-polymer mixture (P/D = 5). Absorbance of the metachromatic solution (at 600 nm) increased with increase in alcohol or urea concentration and finally reached a constant value corresponding to that of pure dye solution, indicating complete destruction of the metachromatic compound. Minimum concentration of the co-solvent required for complete reversal of metachromasy was different for different co-solvents, viz., 40%, 30% and 20% respectively for methanol, ethanol and 1-propanol. About 7M urea solution was sufficient for complete reversal of metachromasy. Lower curves of Fig. 4 indicated that absorbance at 500 nm decreased gradually and reached constant value at the same corresponding co-solvent concentration. Absorption spectra of metachromatic solution and reversal of metachromasy by 40% methanol are shown in Fig. 5. At this methanol concentration, the pure dye solution gave
a spectrum showing an intense peak at 600 nm. The dimeric μ-band of the aqueous dye solution almost completely disappeared. It also appeared that the metachromatic band (μ-band) at 500 nm of the dye-polymer mixture (P/D = 5) disappeared and the spectrum became identical to that of pure dye solution. Progressive destruction of metachromatic compound by alcohols and urea might be attributed to the cleavage of hydrophobic bonds by alcohols and urea in the induction process of metachromasy, leading to the dimerization of the dye as well as metachromatic compound formation. The efficiencies of various alcohols in disrupting metachromasy followed the order: methanol < ethanol < 1-propanol, indicating that reversal became quicker with increasing hydrophobic character of alcohol.

In order to determine thermodynamic parameters of the dye-polymer interaction, absorbance of the pure dye solution (A₀) and that of dye-polymer mixture (A) at μ-band (500 nm) were measured at five different temperatures in the range 31°C to 49°C ± 0.1°C for different sets of solutions containing varying amounts of polymer (Cₛ) in a fixed volume of dye solution (C₇). The values of C₇.Cₛ/(A-A₀) were plotted against Cₛ when linear relationship was obtained at all the five different temperatures. The results are shown in Fig. 6. From the slope and intercept of the linear relationship, equilibrium constant or interaction constant (Kₑ) was calculated at each temperature using the following equation:

\[
K_e = \frac{C_7.C_s}{(A - A_0)}
\]

By plotting log Kₑ against 1/T, change in enthalpy of complex formation (ΔH) was calculated from the slope of the linear plot obtained according to van't Hoff equation. Free energy change (ΔG) was obtained from the expression \( \Delta G = -RT \ln K_e \) at all the four temperatures. Finally, entropy change (ΔS) was obtained by plotting ΔG values against T according to thermodynamic expression, \( \Delta G = \Delta H - T \Delta S \). The slope of the linear plot yielded ΔS. The results are given in Table 1. The value of Kₑ decreased with increase in temperature and ΔH value of -7.02 kcal mol⁻¹ was quite reasonable for such interaction. The negative value of ΔG (-4.98 kcal mol⁻¹) at 307 K was also within the range of a reversible biological process. The negative entropy change, ΔS = -6.80 cal mol⁻¹deg⁻¹ was also not unreasonable as it indicated more ordered state of the ions due to aggregation. Such negative entropy changes were also reported earlier in the induction of metachromasy in cationic dye, Azure A, by heparin in solution. All these thermodynamic parameters, evaluated on the assumption of simple equilibrium: D + S = DS(Kₑ = [DS]/[D][S]) suggested that there was interaction between anionic sites of the polyanion and the dye counterions, resulting in induction of metachromasy.

Fluorescence spectral studies were carried out with the fluorescent dye acridine orange which is also a cationic dye. Emission spectra of the dye in the presence and absence of Klebsiella K17 polymer are shown in Fig. 7. When the dye solution was excited at 430 nm and emission spectral values were
Fig. 7—Emission spectra of acridine orange in presence and absence of *Klebsiella* K17 polymer at different polymer/dye molar ratios: dye conc., \(1 \times 10^{-5} M\) \(\lambda_{em} = 522\ nm\) and \(\lambda_{ex} = 430\ nm\). Recorded, the maximum emission peak was observed at 522 nm (\(\lambda_{em}\)). Upon addition of polymer solution to the dye solution, quenching of fluorescence was observed in the dye-polymer mixture (Fig. 7).

The results of fluorescence were also treated with Stero-Volmer equation\(^2\) to study the interaction phenomenon between the dye and polymer molecules in solution. In Stern-Volmer equation, \(\frac{\phi_i}{\phi} = 1 + K_{SV}[Q]\), \(\phi_i\) is the fluorescence intensity of dye solution and \(\phi\) is that of dye-polymer mixture, and \([Q]\) is the concentration of the quencher. In our studies \([Q]\) indicates the molar concentration of the polymer; \(K_{SV}\) is known as Stern-Volmer constant. The linear plot of \(\frac{\phi_i}{\phi}\) versus polymer concentration indicated that Stern-Volmer equation was satisfied.

The results of spectrofluorometric titration show that when increasing amounts of polymer solution (0-2 ml, \(1 \times 10^{-5} M\)) were added to a dilute solution of dye (1 ml, \(1 \times 10^{-5} M\)), fluorescence intensity decreased gradually, showing quenching of dye due to interaction with the added polymer. The intensity progressively dropped till the amounts of the dye cations and the polyanion were equivalent. After the equivalence point, no significant change in the intensity was observed. Equivalent weight of the polymer was calculated at the equivalence point and it was found to be 794. This method can also be used to estimate the amount of an anionic polymer of known structure in a very dilute solution.

From the results as discussed above, chromotropic character of *Klebsiella* K17 polysaccharide in inducing blue-shifted metachromasy in cationic dye pinacyanol chloride and quenching of fluorescence in acridine orange, was established.

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References