PART - III

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DISCUSSION
DISCUSSION

The gram-negative bacterial genus *Klebsiella* belongs to *Enterobacteriaceae* family. *Klebsiella K4-0* is one of the eightyone serologically recognized strains in this genus. The *Klebsiella K4-0* bacteria were grown in nutrient agar-medium from the serological test strain of *Klebsiella K4-0*.

The capsular polysaccharide of *Klebsiella K4-0* as isolated from the dry bacteria by the phenol-water-Cetavlon extraction^{121-125} showed a double peak in the analytical ultracentrifuge (Table-II); the polysaccharide, however, sedimented uniformly with a single peak after mild alkali-treatment^{126}. Appearance of two bands in analytical/centrifuge indicated the typical behaviour in several acidic capsular polysaccharides of *Enterobacteriaceae*^{126}. Two bands indicated different molecular forms and mild alkali-treatment made the polysaccharide smaller in size; physical inhomogeneity disappeared.

The polysaccharide had $\int \alpha \chi D = + 32^\circ$, and its equivalent weight as determined by the conductometric titration of its acidic form was found to be 840. The optical rotations of the sugars, isolated from the hydrolysate, indicated that manrose, galactose, and
glucuronic acid had D configuration and rhamnose L. The K40 glycan contained D-mannose, D-galactose, L-rhamnose, and D-glucuronic acid in molar ratios approaching 1 : 1 : 2 : 1 (Table-III). The alkali-treated material did not show any change in molar composition (Table-III). The sugar analysis of the carboxyl-reduced polysaccharide yielded D-mannose, D-galactose, L-rhamnose, and D-glucose in the approximate molar ratios 1 : 1 : 2 : 1 (Table-III). The appearance of one molar proportion of D-glucose instead of D-glucuronic acid in the carboxyl-reduced product was expected to arise from the D-glucuronic acid present in the original sample. These data suggested that the polysaccharide was composed of pentasaccharide repeating units, and it was in good agreement with the result of equivalent weight titration (Equivalent weight=840).

The \(^1H\)-NMR spectroscopy of the polysaccharide showed the presence of five anomic protons at \(\delta = 5.32, 5.20, 5.05, 4.90\) and 4.48 ppm (Table-X). The ratio of integrals for the signal at \(\delta = 1.32\) ppm indicated the presence of six protons in two methyl groups of the two L-rhamnose units. The absence of signals at
'8 = 1.5 ppm and 2.2 ppm indicated the absence of pyruvate and acetate in the polysaccharide.

The $^{13}$C-NMR spectrum of the K40 polysaccharide (Fig.16) showed five signals for the anomeric carbon atoms appearing at 95.31, 95.71, 99.90, 101.64 and 102.19 ppm. There were also two signals at 17.46 ppm and 17.79 ppm for the CH$_3$ groups of the two L-rhamnose units. The signal at 176.16 was attributed to C-6 of the D-glucuronic residue$^{61,225}$. The values of the chemical shifts for the other carbon atoms C-2, C-3, C-4, C-5 and C-6 as obtained in the $^{13}$C-NMR spectrum are summerised in Table-XII. The spectral data were found to be quite comparable with the published results for the sugars available in the literature$^{214}$. The NMR results as discussed above also indicated that the repeating unit of the Klebsiella K40 capsular polysaccharide was a pentasaccharide.

The polysaccharide was methylated by the method of Hakomori$^{136}$ following the experimental details of Hellerqvist et al$^{137}$. The methylated monomers were analysed by gas-liquid-chromatography (GLC) and mass spectrometry (MS) of the derived alditol acetates.
The methylated polysaccharide yielded (Table-V, column I) 3,4-di-O-methylrhamnitol, 2,3,4,6-tetra-O-methylmannitol, 3-mono-O-methylrhamnitol and 2,4,6-tri-O-methylgalactitol in the approximate molar ratios 1:1:1:1. When a portion of the methylated polysaccharide was carboxyl-reduced-dideuterated before hydrolysis, the derived methylated monomers as alditol acetates yielded one molar proportion of additional 2,3-di-O-methylglucitol (Table-V, column II). This dideuterated product was identified by the appearance of the peak for the dideuterated fragment at m/z = 263 in the mass spectrum (Fig.13), which was observed instead of the normal peak at m/z = 261.

The molar ratios of the methylated monomers, obtained by hydrolysis of the methylated polysaccharide and by hydrolysis of the carboxyl-reduced product of the methylated polysaccharide, thus indicated that the polysaccharide contained pentasaccharide repeating units. The substitution pattern of the constituents in the polysaccharide could also be established from the results of methylation analysis. These results (Table-V, column I & II) showed that the repeating unit of the Klebsiella K40 capsular polysaccharide contained one residue each of unsubstituted D-mannose,
4-substituted D-glucuronic acid, 3-substituted D-galactose, 2-substituted L-rhamnose, and 2,4-disubstituted L-rhamnose.

The formation of 2,3,4,6-tetra-O-methylmannitol indicated that the unsubstituted D-mannose residue occupied the branch terminal position in the repeating unit. The formation of 3,4-di-O-methylrhamnitol suggested that one of the L-rhamnose residues in the repeating unit was branched. Therefore, it could be deduced that the repeating unit must be branched with a chain L-rhamnosyl residue carrying the branch that terminated in a D-mannosyl unit, and the L-rhamnose was branched at either position 2 or 4.

From the results of quantitative constituent analysis (Table-III), methylation analysis (Table-V) and NMR-spectroscopy (Table-X and XI) it could, therefore, be established that the Klebsiella K40 capsular polysaccharide was comprised of pentasaccharide repeating units, and the repeating unit contained the following constituents.

(1) D-Man \( \rightarrow \) (2) \( \xrightarrow{4} \) D-GlcA \( \rightarrow \)

(3) \( \xrightarrow{3} \) D-Gal \( \rightarrow \) (4) \( \xrightarrow{2} \) L-Rha \( \rightarrow \) and
In order to determine the sequence of the constituent sugars in the repeating unit, the oligosaccharides isolated from the polysaccharide were characterized. By partial acid hydrolysis of the polysaccharide, three acidic oligosaccharides, aldobiouronic acid (H2), aldotriouronic acid (H3), and aldotetraouronic acid (H4) could be isolated.

The aldobiouronic acid (H2) contained D-glucuronic acid and L-rhamnose in the approximate molar ratios 1:1, with L-rhamnose as the reducing end sugar (Table-IV). Methylation of H2 produced only 3,4-di-O-methylrhamnitol (Table-VI, column-I). It was, therefore, evident that H2 had the following structure:

\[
(5) \quad \text{D-GlcA (1→2)-L-Rha}
\]

The aldotriouronic acid (H3) was composed of D-glucuronic acid, L-rhamnose, and D-galactose (1:1:1), and D-galactose was found to be the reducing end sugar (Table-IV). Hydrolysis of the permethylated H3 yielded 3,4-di-O-methylrhamnitol and 2,4,6-tri-O-methylgalactitol in the approximate molar ratios 1:1.
(Table-VI, column II). These results suggested the following structure for H3:

Aldotriouronic acid (H3):

\[ \text{D-GlcA-(1} \rightarrow 2) - \text{L-Rha-(1} \rightarrow 3) - \text{D-Gal} \]

The analysis of the aldotetraouronic acid (H4) indicated the presence of D-glucuronic acid, D-galactose, and L-rhamnose in the molar ratios 1.0 : 1.0 : 1.7, and the reducing end of H4 was identified as L-rhamnose (Table-IV). Methylation analysis of H4 produced 3,4-di-O-methylrhamnitol and 2,4,6-tri-O-methylgalactitol in the molar ratios 1.8 : 1.0 (Table VI, column III). When the permethylated H4 was carboxyl-reduced-dideuterated with calcium borodeuteride before hydrolysis, one molar proportion of additional 2,3,4-tri-O-methyl glucitol was obtained (Table VI, column IV). The dideuterated fragment was identified by the appearance of the peaks at m/z = 191 & 235 in the mass spectra instead of the normal peaks at m/z = 189 and 233 (Fig.14). This methylated monomer was, obviously, derived from the glucuronic acid present in the original H4. These results indicated that H4 had the following structure:
Aldotetrauronic acid ($H_4$):

\[ \text{D-GlcA}-(1 \rightarrow 2)-\text{L-Rha}-(1 \rightarrow 3)-\text{D-Gal}-(1 \rightarrow 2)-\text{L-Rha} \]

The ionic mobilities of the oligosaccharides in the high voltage electrophoresis ($H_2 = 0.62; \ H_3 = 0.46; \ H_4 = 0.36$) were found to be quite comparable with the published results$^{106,116,120}$. The structures of the oligosaccharides were derived mainly from the methylation data. After deducing the structure of $H_2$, the sequence of the constituent sugars in $H_3$ could be determined easily, and once the structure of $H_3$ was known it was not difficult to establish the sequence of the constituents present in $H_4$.

Having established the structures of $H_2$, $H_3$, & $H_4$, (Fig.28) the sequence of four sugar units in the pentasaccharide repeating unit was known. So it was the next task to locate the position of the fifth sugar residue, D-mannose, in the repeating unit. With the knowledge that D-glucuronic acid was 4-substituted, one of the two L-rhamnose sugars was 2,4-disubstituted (forming branch point), and D-mannose occupied the terminal position (from methylation data), three probable structures for the pentasaccharide repeating
STRUCTURES OF OLIGOSACCHARIDES OBTAINED FROM KLEBSIELLA SEROTYPE K40 CAPSULAR POLYSACCHARIDE

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>Assignment of structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldobiouronic acid</td>
<td>D-GlcA(1→2)-L-RhaOH</td>
</tr>
<tr>
<td>Aldotriouronic acid</td>
<td>D-GlcA(1→2)-L-Rha(1→3)-D-GalOH</td>
</tr>
<tr>
<td>Aldotetrauranic acid</td>
<td>D-GlcA(1→2)-L-Rha(1→3)-D-Gal(1→2)-L-RhaOH</td>
</tr>
</tbody>
</table>

Fig. 28
unit as shown in Fig. 29, could be suggested.

In order to get further information to establish the correct structure, the permethylated polysaccharide was subjected to base degradation reaction\textsuperscript{145,146}. This reaction is particularly useful when there is a 4-substituted uronic acid residue in the heteropolysaccharide. When the permethylated polysaccharide is subjected to base degradation, the 4-substituent on the glycosyluronic acid residue is eliminated along with the uronic acid residue, and if the 4-substituted uronic acid forms a part of the side chain (not terminal), the uronic acid along with the 4-substituent and other attached sugars are eliminated by successive $\beta$-elimination reactions.

In the present studies, the results of the base degradation reaction of the permethylated polysaccharide (Table-VII, column II) showed that $\beta$-elimination caused the loss of 3,4-di-O-methylrhamnitol. This indicated that the parent 2-substituted L-rhamnose residue was linked to C-4 of the D-glucosyluronic acid residue in the repeating unit. As there was neither any loss of 3-O-methylrhamnitol, it was justified to eliminate the structures (a) and (b) leaving only the possible
PROBABLE STRUCTURES FOR KLEBSIELLA SEROTYPE K40 CAPSULAR POLYSACCHARIDE FROM METHYLATION ANALYSIS AND OLIGOSACCHARIDE STRUCTURES.

(a) $\text{D-Man} \xrightarrow{1 \ 4} \text{D-GlcA} \xrightarrow{1 \ 4} \text{L-Rha}(1 \rightarrow 3) \text{D-Gal}(1 \rightarrow 2) \text{L-Rha}(1 \rightarrow 2) \text{D-Man} \xrightarrow{1 \ 4}$

(b) $\rightarrow 4 \ 	ext{D-GlcA}(1 \rightarrow 2) \text{L-Rha}(1 \rightarrow 3) \text{D-Gal}(1 \rightarrow 2) \text{L-Rha}(1 \rightarrow 2) \text{D-Man} \xrightarrow{1 \ 4}$

(c) $\rightarrow 4 \ 	ext{D-GlcA}(1 \rightarrow 2) \text{L-Rha}(1 \rightarrow 3) \text{D-Gal}(1 \rightarrow 2) \text{L-Rha}(1 \rightarrow 2) \text{D-Man} \xrightarrow{1 \ 4}$

Fig. 29
structure (c) for the repeating unit of the polysaccharide.

From the results of base degradation it could, therefore, be concluded that the 4-substituted D-glucuronic acid was linked to the L-rhamnose unit in the chain which was branched with the D-mannose residue occupying the terminal position. After locating the position of D-mannose, the sequence of all the five constituent sugar residues present in the pentasaccharide repeating unit of the polysaccharide was thus established.

The polysaccharide was then subjected to periodate oxidation for obtaining further structural information. On oxidation of the polysaccharide with sodium metaperiodate, followed by the treatment with sodium borohydride, a polyol was obtained. The complete hydrolysis of the polyol yielded rhamnose and galactose in the molar ratios of 0.9 : 1.0 as determined by GLC as alditol acetates. The presence of rhamnose was expected, because the branched rhamnosyl residue was 2,4-disubstituted, and galactose must have arisen from the 3-substituted galactosyl residue. As expected, the other sugar residues in the repeating unit did not survive during the periodate oxidation due to their
substitution pattern. The 4-substituted glucuronic acid, the 2-substituted rhamnose, and the unsubstituted mannose (terminal sugar) were completely degraded by periodate oxidation. These results corroborated the findings of the methylation analysis of the polysaccharide.

The anomeric configurations of all the glycosidic linkages were assigned from the results of NMR spectral analysis. The $^1$H-NMR spectrum of the polymer showed the presence of five signals in the anomeric region of which three ($\delta = 5.32, 5.20$ and 5.05 ppm) were derived from $\alpha$-linked and two ($\delta = 4.80$ and 4.48 ppm) from $\beta$-linked sugars (Table-X). From the signal at $\delta = 4.64$ ppm ($J = 7.5$ Hz) in the spectrum of H$_2$ (Table-XI), $\beta$-linkage was assigned to D-glucuronic acid. In the spectrum of H$_3$, the anomeric signals at $\delta = 5.22$ and 5.15 ppm were integrated together for 1.5 integrals of protons, and hence it was obvious that the L-rhamnose in H$_3$ was $\alpha$-linked; the signal at $\delta = 4.60$ ppm ($J = 7.0$ Hz) was assigned to the $\beta$-D-glucuronic acid. In the spectrum of H$_4$, the signal at $\delta = 4.85$ ppm ($J = 7.5$ Hz) was assigned to the $\beta$-D-galactose. From the signals at $\delta = 5.32$ ppm ($J = 3.0$ Hz) and 5.05 ppm ($J = 2.5$ Hz) in the spectrum of the polysaccharide...
(Table-X), the D-mannose and the second L-rhamnose (2-substituted) of the repeating unit were assigned to be $\alpha$-linked.

The $^{13}$C-NMR spectrum of the polymer (Fig.16) also clearly showed five signals in the anomeric region, of which three (95.31, 95.71 and 99.90 ppm) were assigned for $\alpha$-linkages and two (101.64 and 102.19 ppm) for $\beta$-linkages. The signals for the anomeric carbon atoms were assigned in accordance with the assignment of anomeric configurations by the $^1$H-NMR spectral data.

From the above discussion on the $^1$H-NMR spectral data of the polysaccharide (Table-X) and its derived oligosaccharides (Table-XI), and the $^{13}$C-NMR spectral data of the polysaccharide (Table-XII), the anomeric configurations of all the glycosidic linkages in the repeating unit could be clearly determined.

The assignment of anomeric configurations was also supported by the results of exoglycosidase treatment and chromium trioxide oxidation. In agreement with the $^1$H-NMR spectral data of $\text{H}_2$, glucuronic acid was liberated when the aldobiouronic acid was treated with
$\beta$-D-glucuronidase. When the acetylated polysaccharide is oxidised with chromium trioxide, the equatorially linked sugar residues are oxidised, and the sugar residues having axial orientations survive. The results of chromium trioxide oxidation of the polysaccharide (Table-VIII) indicated that the proportion of D-galactose decreased considerably and it suggested that D-galactose had $\beta$-glycosidic linkage. The results also indicated that D-mannosyl and L-rhamnosyl residues were $\alpha$-linked. Similar results were observed when the carboxyl-reduced polysaccharide was subjected to chromium trioxide oxidation. In this case the D-glucose originated from the D-glucuronic acid also considerably decreased, indicating that the D-glucuronic acid of the native polysaccharide was $\beta$-linked.

From the above discussion on the results obtained in the present investigation it could be conclusively established that the Klebsiella 940 capsular polysaccharide was composed to pentasaccharide repeating units having the primary structure as shown in Fig. 30.

This part of the work showing the results on the primary structure of the polysaccharide has been published in the Eur. J. Biochem. 226.
Primary structure of *Klebsiella K40* capsular polysaccharide.

Fig. 30
This *Klebsiella* K40 belongs to one of the twenty chemotypes which contains three serologically different K-types having the same qualitative composition (glucuronic acid, mannose, galactose and rhamnose). The other two K-types in this chemotype are K53 and K80, and the structures of the capsular polysaccharides from them have already been reported. The K53 polysaccharide has a hexasaccharide repeating unit and K80 polysaccharide has the pentasaccharide repeating unit, and both of them have the branched structure. Only K80 polysaccharide has also pyruvic acid substitution in its repeating unit.

In the present investigation, viscometric properties of the *Klebsiella* K40 capsular polysaccharide were studied. The reduced viscosity ($\eta_{sp}/C$) of the aqueous solution of the native polysaccharide showed the characteristic behaviour of polyelectrolytes in which the reduced viscosity increased sharply at low concentration. The value of $\eta_{sp}/C$ normally rises with increasing concentration. But the anomalous shape was explained on the reason that with the decrease in polyelectrolyte concentration, the degree of ionization increased. In the case of polysalts (sodium salt of the polysaccharide) the
genginous (Na\(^+\) ions in this case) formed an atmosphere around the chains of the polyelectrolyte macroions. In the absence of added salt, in very dilute solutions the diameter of the ion atmosphere was greater than the diameter of the coiled molecule. The carboxylate ions, \(\text{COO}^-\), of the polysaccharide repelled each other, increasing the chain rigidity and expanding the polymer coil with consequent increase in the viscosity. Another contributing factor might be that because the polyelectrolyte ions were so greatly expanded, they could be expected to interfere with each other so that there might be an influence of concentration on configuration of the polymer molecule. The effect of addition of salt (upto 10\(^{-2}\) M NaCl) did not, however, change the results significantly (Fig. 17, Table-XIII to XVI). The intrinsic viscosity of the aqueous solution of the polysaccharide was obtained by plotting the curve of \(C/\eta_{sp}\) vs \(1/\sqrt{C}\) (Fig. 18) according to Fuoss and Strauss\(^{220}\). The results are given in Table-XVII. The intrinsic viscosity, obtained by extrapolation of the linear plot to \(1/\sqrt{C} \rightarrow 0\), was found to be 400 dl/g.

The interaction of the Klebsiella K40 polysaccharide with the cationic dye was investigated by spectral measurements in the visible range. Methylene blue,
acridine orange, neutral red, and pinacyanol chloride are the four different dyes which were preliminarily used for the interaction with the polysaccharide. The absorption spectra of the pure dyes and the dye-polymer solutions at polymer/dye ratio (P/D) of 10.0 are shown in Fig.19. From the spectral data (Table-XVIII) only pinacyanol chloride was chosen for further studies as significant spectral shift was observed with this dye; no appreciable induction of metachromasy in other three dyes by the polysaccharide was observed.

Pinacyanol chloride (1-ethyl-2-$\text{\text{-}}$3-(1-ethyl-2(1H)-quinolylidene) propenyl$\text{/}$ quinolinium chloride) is the cationic dye belonging to cyanine group of dyes and its structure is shown in Fig.31. The absorption spectra of the aqueous solutions of the dye of different concentrations ($4.8 \times 10^{-7}$ M to $1.91 \times 10^{-5}$ M) measured at 450 - 650 nm are shown in Fig. 20. Some of the results are given in Table-XIX. At comparatively higher concentrations ($0.953 \times 10^{-5}$ M), the dye showed two prominent peaks one at 600 nm ($\alpha$-band) for the monomeric band, and the other at 550 nm ($\beta$-band) corresponding to the dimeric band. Further experiments were carried out with the dye concentration of about $10^{-5}$ M.
1-Ethyl-2-\([3-(1\text{-ethyl}-2(1H)-quinoly lidene)\text{propenyl}]\) quinolinium Chloride

**STRUCTURE OF PINACYANOL CHLORIDE**

![Chemical Structure of 1-Ethyl-2-(3-(1-ethyl-2(1H)-quinolylidene)propenyl) quinolinium Chloride](image-url)
When the polysaccharide was added to the dye solution, the intensity of both α and β bands decreased and a new absorption band (μ-band) at about 500 nm appeared. The blue-shifted metachromatic spectral changes observed at different polymer/dye ratios are shown in Fig. 21. From spectral data of the metachromatic solutions (Table-XX), it appeared that at P/D = 5.0 the polymer induced a distinct metachromatic blue-shifted band at 500 nm. At higher P/D values the spectral character did not change significantly. The blue-shift value of about 100 nm exhibited induction of strong metachromasy in the pinacyanol chloride dye by the Klebsiella K40 polymer.

A stoichiometry of about 1:1 for the polymer/dye was found to exist in the polyanion-dye compound. The results are shown in Table-XXI & XXII and Fig. 22, 23.

The reversal of metachromasy was studied by measuring the absorbances of the metachromatic solution after addition of increasing amounts of ethanol (Fig. 24 & 25). The spectral data are shown in Table-XXIII & XXIV. As observed in Fig. 25, the metachromatic solution (P/D = 5.0) gave a single banded spectrum with the peak only at 607 nm with disappearance of β and μ bands.
upon addition of about 40% ethanol, and the spectral behaviour was found to be identical in nature with that of pure dye. The absorption values at 600 nm increased and those at 500 nm decreased with ethanol concentration, and finally reached to constant values corresponding to those of pure dye at about 40% ethanol concentration (Fig. 24).

The effect of urea on the reversal of metachromasy was also similarly studied. It was observed (Fig. 26 & 27) that urea had similar effect like ethanol on complete reversal of metachromasy.

The results of metachromatic titration (Table-XXI) were obtained by measuring the absorbances of the metachromatic solutions at 600 nm after gradual addition of the polymer to the dye solution at lower P/D values. It was observed that intensity of the absorbance at 600 nm decreased linearly until it became constant (Fig. 22). The volume of polymer solution required for equivalence of dye cations and polyanions could be calculated. The results indicated dye/polyanion stoichiometry of 1:1 in the dye-polyanion compound. This technique of metachromatic titration can be satisfactorily used to determine the equivalent weight...
of the polymer, and can be conveniently utilized to determine stoichiometry of the polymer/dye in the metachromatic compound provided the structure of the polymer is known.

From the results of dye-polymer interaction as discussed above the chromotropic character of the acidic capsular polysaccharide of *Klebsiella* K40 was established and it was indicated that the polymer could induce strong metachromasy in the cationic dye pinacyanol chloride. The polymer/dye stoichiometric result was found to be in good agreement with the values available in the literature for interaction of the dyes like acridine orange and pinacyanol chloride with different synthetic polyanions. The stoichiometry of the polymer/dye (1:1) also suggested that every potential anionic site of the repeating unit of the polymer (here COO−) was associated with the aggregation of dye cations as a result of which they were expected to form stacking conformation.

Some of the results discussed above were presented at the 21st Annual Convention of Chemists (India), 72nd Sessions of Indian Science Congress Association, and 73rd Sessions of Indian Science Congress Association.
and XIII th International Carbohydrate Symposium (Cornell Univ., USA)\textsuperscript{233}.