J. Indian Chem. Soc.,
Vol. LXIII, January 1986, pp. 138-143

Characterisation of the Tal Fruit (*Borassus flabellifer*) Polysaccharide. Its Induction of Metachromasia and Circular Dichroism with Pinacyanol†

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Aqueous extract of the mesocarp of ripe palmyra palm (*Borassus flabellifer*, local name ; *Tal* fruit) furnished a polysaccharide material (BF-A) which on saponification gave polysaccharide material (BF-B) containing GalA (53), Glc (25) Gal (3.16), Ara (2.66), Xyl (2.24), and Rha (1.5%). The results of pectinase treatment and periodate oxidation indicate that BF-B is essentially a polygalacturonan strongly associated with a glucan which is different from starch. The unsaponified BF-A has an equivalent weight (average weight containing one anionic group) of 825 (as K-salt) and does not induce metachromatic spectral shift of toluidine blue (TB). The corresponding saponified product BF-B has an equivalent weight of 338 (as K-salt), and induces metachromatic spectral shift of the dyes TB and pinacyanol chloride (PCYN), thereby indicating that many of the carboxylic groups in BF-A are esterified. The stoichiometry of the compound of BF-B and TB shows that TB binds surprisingly to alternate carboxyl groups of BF-B, the other carboxyl groups of the polygalacturonan chain appear to be masked in being associated with the glucan portion. From the strong CD (in the visible region) of the compound of the dye PCYN and BF-B, it is postulated that the polygalacturonan portion of BF-B has an extended helical structure encasing or associating the glucan component.

BORASSUS flabellifer is one of the four species of the family *palmae* and belongs to the genus *Borassus*. It is a native of tropical Africa and is found in India, Burma and Ceylone.

The black trunk of the plam tree attains a height of 12-18 m (even more) and girth of 1-2 m. It bears terminal crown of large fan like leaves 1-1.5 m in width. The spathes begin to appear in November-December. Male and female flowers are borne on different trees. The fruits, when ripe, are large having brownish black skin and weigh 1-2.5 kg a piece. Just below the thin skin, the body of the ripe fruit is covered with fibres which hold a soft yellow edible viscous mass (mesocarp) encasing 1-3 hard seeds or nuts[1]. The fruit has several medicinal uses[2]. The green fruits have large tender nuts which contain a colourless soft sweet and essentially carbohydrate material that is much relished in the summer. A galactomannan[3] and a mannan[4] have been characterised from this source.

In this communication, we report the average carbohydrate composition, polyelectrolytic and chromotropic properties of the saponified polysaccharide material obtained by water extraction of the yellow pulp.

Experimental

Descending paper chromatography (pc) was performed on Whatman No. 1 MM papers using the solvent systems (v/v) : (A) ethyl acetate-pyridine-water (8 : 2 : 1), (B) 1-butanol-ethanol-water (4 : 1 : 1) and (C) 1-butanol-acetic acid-water (3 : 1 : 1). Staining reagents were (*a*) alkaline silver nitrate and (*b*) benzidine-periodate.

Hexuronic acids were estimated by the carbazole method[5] and neutral sugars by the phenol sulphuric acid method[6]. Absorbances were measured with a Beckman DB spectrophotometer ; metachromatic spectra were read with a Toshniwal CL 10-A_3 vis-spectrophotometer or Cary 17D spectrophotometer. Circular dichroism (CD) were recorded with a JASCO J-500C spectropolarimeter using 1.00 cm circular cuvettes. Conductimetric titrations were carried out with a Toshniwal CLO 1.02 A conductivity bridge. All evaporations were carried out below 40° under reduced pressure.

Glc was performed with a glass column (6 mm × 1.83 m) containing 3% of ECNSS-M on Gas Chrom Q(100-120 mesh) at 190° for alditol acetates of neutral sugars using the same instrument as reported[7]. Pectinase (polygalacturonase ; poly-

† Dedicated to Professor Sadhan Basu on the occasion of his 65th birthday.

α-1,4-galacturonide glycuronohydrolase ; E.C.No. 3.2.1.15 ; from *Aspergillus niger*, Sigma) was used in this investigation. Ir spectrum was taken as KBr pellets using a Perkin-Elmer 297 instrument. Specific rotation values were recorded with a Jasco DIP-181 digital polarimeter.

Isolation of the Tal polysaccharide (BFA) : The outer skin of the Tal fruit (2 kg) was carefully removed and the edible gummy yellow pulp (the mesocarp) was extracted by pressed-sieving. It (750 g) was suspended in a double-walled nylon-cloth bag. A yellowish viscous juice (100 ml) gradually oozed out and collected in a container below. It was diluted with water (100 ml) and filtered under suction. To the filtrate KOAc (7 g) was added. From the resulting solution the polysaccharide material was precipitated by gradual addition of ethanol. The first turbidity (a gray precipitate) appearing on addition of ethanol (125 ml) was centrifuged (7000 r.p.m., 45 min, 25°) out. To the supernatant liquor more ethanol (275 ml) was added and the mixture allowed to stand overnight. The precipitate (BF-A, 3.1 g, yellowish) was collected by centrifugation and dried by solvent (ethanol, acetone) exchange.

Saponification of BF-A : BF-A (2.6 g) dissolved in KOH (0.1 *M*) was flushed with nitrogen and left overnight under nitrogen at room temperature (25°). The solution was centrifuged and the supernatant liquor neutralised (*p*H ~8) with cold AcOH and dialysed (24 h) against water. The bag content was centrifuged (7000 r.p.m., 30 min, 25°) and a small precipitate appearing was rejected. To the supernatant liquor, KOAc (7 g) was added and the polysaccharide precipitated by gradual addition of ethanol (2.5 vol). The precipitate (BF-B ; 1.5 g, yellowish) was collected in the usual way.

Identification of the monosaccharide components of BF-B : BF-B (12.92 mg) was hydrolysed with sulphuric acid (0.5 *M*, 4 ml) in a sealed tube at 100° for 16 h. The hydrolysate was neutralised with prewashed $BaCO_3$, centrifuged and filtered. The filtrate was decationised (Dowex-50, H^+) and concentrated to syrup. Pc of the syrup (solvents A, B and C, staining reagent *a*) gave two prominent spots corresponding to Glc and GalA. There were trace amounts of Gal, Ara, Xyl and Rha.

Another weight of BF-B (4.58 mg) was mixed with myo-inositol (1032 μg) and hydrolysed with sulphuric acid (0.5 *M*, 3 ml) as described earlier. The hydrolysate was neutralised ($BaCO_3$), decationised (Dowex-50, H^+) and Dowex-1 (formate) and the neutral monosaccharides were converted[8] into the alditol acetate mixture. The glc of the product furnished Glc, Gal, Xyl, Ara and Rha in the molar ratios of ~15 : 2 : 2 : 2 : 1.

Enzymic hydrolysis of BF-B : To a solution of BF-B (18.95 mg) in NaOAc buffer (5 ml, *p*H 4.0), pectinase (2.93 mg) was added and incubated at 33.5° under a drop of toluene for 16 h. The reaction mixture was then heated to 100° for 20 min, cooled, centrifuged and filtered. The filtrate was decationised (Dowex-50, H^+) and the solution was concentrated (~2 ml) and examined by pc (solvent A, stain *a*). This furnished a single spot corresponding to GalA.

The above solution was passed through a Dowex-1 (formate) column (1 × 30 cm) and the mixture of neutral monosaccharides (monitored by Molisch[9] test) was eluted with water (200 ml). It was concentrated to syrup and subjected to pc (solvent A, stains *a* and *b*). It revealed the presence of Gal, Ara, Xyl and Rha ; along with, there were some carbohydrate materials staying at the base line. The syrup was diluted (~2 ml) and extensively dialysed. The bag content was concentrated and the polysaccharide (~1.2 mg) precipitated with ethanol (2 vol). The precipitate (0.8 mg) was hydrolysed (0.5 *M* H_2SO_4, 1 ml, 100°, 16 h), and the hydrolysate after neutralisation and usual work up was examined by pc. It furnished a single spot corresponding to Glc.

The uronic acid portion retained by the Dowex-1 (formate) column was eluted with formic acid (0.3 *M*, 60 ml) and the effluent evaporated to dryness. Pc of the residue (~4 mg) gave a single spot corresponding to 'GalA'. The residue was dried over P_2O_5, and the acid converted[10] into methyl ester methyl glycoside by refluxing with dry methanolic HCl (1%, 5 ml, 10 h). The solvent was evaporated and the residue treated with Dowex-1 (formate) resin in cold water and filtered. The filtrate was concentrated (~2 ml) by lyophilisation. To it KBH_4 (50 mg) was added and left for 20 h at 10°. The excess of KBH_4 was decomposed with Dowex-50 (H^+) resin and filtered. The filtrate was evaporated to dryness and boric acid was removed as methyl borate. The residue was hydrolysed with sulphuric acid (0.5 *M*, 3 ml, 100°, 16 h). The hydrolysate was neutralised ($BaCO_3$), centrifuged, filtered and the filtrate decationised (Dowex-50, H^+) and lyophilised. The residue was converted into the alditol acetate and subjected to glc when only one peak corresponding to Gal was obtained.

Oxidation of BF-B with periodic acid : Solutions of BF-B (21.7 mg) in water (10 ml) and periodic acid (0.1 *M*, 10 ml) were separately cooled (4°) and mixed together. The mixture was kept in the dark under stirring condition. Aliquotes (0.1 ml) were taken out at intervals and titrated for formic acid with standard NaOH (0.01 *M*) using methyl red indicator. In 72 h, the liberation of formic acid became constant. The reaction mixture was then treated with $Ba(OH)_2$ (0.1 *M*) and the precipitate was removed by centrifugation. Excess $Ba(OH)_2$ was precipitated by bubbling CO_2 and the precipitate centrifuged out. The resulting solution was concentrated (2 ml) and a small amount of precipitate appearing at this stage was filtered off. The filtrate was reduced with KBH_4 (~50 mg) for overnight at room temperature. Excess borohydride was decomposed by acidifying (cold AcOH,

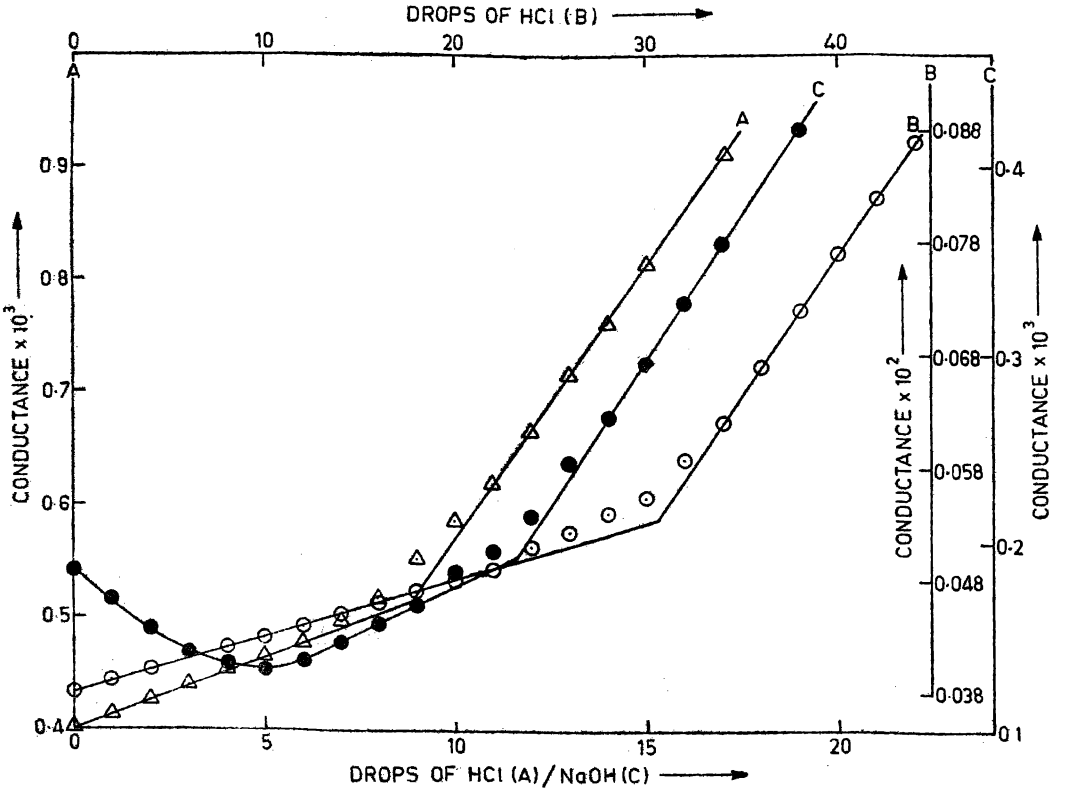

Fig. 1. Conductimetric titration of (A) BF-A (26.3 mg) by 0.084 *N* HCl, (B) BF-B (36.9 mg) by 0.084 *N* HCl and (C) BF-B (17.0 mg) in the acid form by 0.103 *N* NaOH.

4 *N*) to *p*H ~5 and boric acid was removed as methyl borate. The resulting solution was decationised (Dowex-50, H^+), concentrated (1 ml) and examined by pc (solvent A, staining reagents *a* and *b*). In both the cases there was an immobile white patch at the base line and a spot corresponding to GalA.

The reaction mixture was slowly passed through a Dowex-1 (formate) column (1×15 cm). The column was washed with water and the effluent concentrated. Pc (solvent A, stains *a* and *b*) indicated the same white patch at the base line and there was no monosaccharide spot. The solution was evaporated and a part of the solid (~2.8 mg) was hydrolysed with H_2SO_4 as described earlier. The hydrolysate was worked up in the usual way and examined by pc (solvents A and B, stain *a*) when a single spot corresponding to Glc was obtained. The Dowex-1 (formate) column was then eluted with formic acid (0.3 *M*, 10 ml) and the effluent evaporated. The residue was hydrolysed with trifluoroacetic acid (1 ml, 50% solution) for 12 h at 100°. The acid was evaporated and the residue examined by pc (solvent A, stain *a*), which revealed the presence of GalA, glycerol and threic acid. There was no Glc spot.

Equivalent weight of BF-A and BF-B : Equivalent weight (average weight containing one anionic group) of BF-A (26.3 mg) was determined by direct conductimetric titration of its solution with standard HCl (0.084 *N*) and found to be 827. A similar titration of BF-B (36.9 mg) gave an equivalent weight 338. Equivalent weight of BF-B was also determined by converting it (17.0 mg) to the acid form by treatment with Dowex-50 (H^+) followed by conductimetric titration[11] against standard NaOH (0.103 *N*) and found to be 335 (Fig. 1).

Stoichiometry of the compound formed between toluidine Blue (T.B) and BF-B : Portions (10 ml) of 1.64×10^{-4}*M* TB in 1.00×10^{-3}*M* NaCl containing different amounts of BF-B in a series of stoppered test tubes were shaken with petroleum ether (b.p. 60-80°) (2 ml). The compound between TB and BF-B was thus removed from the aqueous phase[12,13]. The excess dye remaining in the aqueous phase in each case was estimated from Beer's law and the dye removed as compound with BF-B was calculated by difference. From the plot of μ-mol of dye removed *vs* μ-mol of BF-B added, the stoichiometry of TB—BF-B came to be 1 : 2.

Results and Discussion

The polysaccharide material present in the juice of ripe Tal fruit was isolated by precipitation with ethanol in presence of KOAc. The product BF-A being considerably esterified as evidenced[14] by ν_{max} at 1 735 and 1 260 cm^{-1}, was saponified with

KOH into a polyanion of higher charge density (hence low equivalent weight); the saponified product BF-B, as well as the unsaponified product BF-A were isolated as their potassium salts. Conductimetric titration results (Fig. 1) gave equivalent weights (average weight containing one anionic group) 825 ± 3 and 338 ± 3, respectively for BF-A and BF-B. Hydrolysis of BF-B furnished mainly GalA (53%) and Glc (25%) along with minor components, like Gal (3.16%), Ara (2.66%), Xyl (2.24%) and Rha (1.53%). This composition suggested that BF-B (or the original product BF-A) was likely to be pectic substance. The presence of considerable amount of glucose was interesting as it indicated the possibility of an associated starch like component. But neither BF-A nor BF-B gave any colour with iodine.

Pc of the pectinase hydrolysate of BF-B detected considerable amount of GalA and the neutral fraction obtained from the hydrolysate gave faint spots of Gal, Ara, Xyl and Rha. The presence of these four minor components was also corroborated by glc. It was significant to note that Glc constituting 25% of BF-B was not detectable in the above hydrolysate even in the concentrated state. It has been found[15] before that the crude pectinase used in the present investigation is a non-specific enzyme capable of complete depolymerisation of pectic substances (irrespective of their having insertions[15] or substitutions of neutral monosaccharide residues) as well as the accompanying galactans and arabinans. It has also been found that any starch like component alone or associated with such pectic substances was also depolymerised. But in the present case, the glucan portion remains unaffected by the pectinase and stayed as a white patch at the base line in the pc of the pectinase hydrolysate.

The above hydrolysate was dialysed when the bag content retained the glucan portion and the dialysate contained the rest of the monosaccharides as evidenced by pc and glc. No Glc could be detected even in the concentrated dialysate. This finding clearly indicated that BF-B was essentially a polygalacturonan (pectic substance) associated with the glucan component. The absence of any aldobiuronic acid in the acid hydrolysate of BF-B was also consistent with this conclusion.

Since there is no reliable existing method for the separation of such a polymer mixture, further evidence for such a model was sought from the results of Smith degradation. BF-B was oxidised with periodic acid[16] in dark at 4°, the liberation of formic acid became constant in 72 h. The oxidised product was reduced and the inorganic ions were removed. Pc of the resulting product showed an immobile patch at the base line and some GalA. Apparently some GalA residues survived the oxidation and were released during the subsequent operations. The acidic components (oxidised or unoxidised) were held on a Dowex-1 (formate) column and the effluent was worked up to solid. Hydrolysis of the solid followed by pc and glc, identified it to be the pure glucan. Thus, isolation of the pure glucan portion once again substantiated the earlier contention that the glucan was a separate entity and the other (very minor quantity) monosaccharides were some how attached with the pectic acid portion.

The acidic portion was eluted from the Dowex-1 (formate) column and hydrolysed. Pc of the hydrolysate furnished GalA, glycerol and threic acid. As complete oxidation of pectic acid is rather difficult, therefore, occurrence of some GalA was quite expected[17]. The formation of threic acid, the results of enzymic reaction and the high specific rotation value of BF-B, $[\alpha]589.5^{+}$ 165° (*c* 0.275, H_2O) confirmed the structure of the pectic acid as of the conventional type, *i.e.* essentially a (1→4)-α-D-galacturonan. Failure of the accompanying glucan to develop any colour with iodine and its resistance to periodate indicated that in all probability the glucan was not a starch like polymer.

An attempt was made to determine the relative mode of distribution of the major sugar residues (GalA and Glc) in BF-B from some spectral measurements. Broad metachromatic spectra of BF-B-toluidine blue system (Fig. 2) showed that BF-B was rather a weak chromotrope. This would be a bit unbecoming of a polygalacturonan having an anionic site on every monosaccharide residue[18,19]. In the present case, this requires either (i) GalA residues to be interpersed by the glucosyl residues in the same chain or (ii) there should be blocking of the alternate anionic sites of

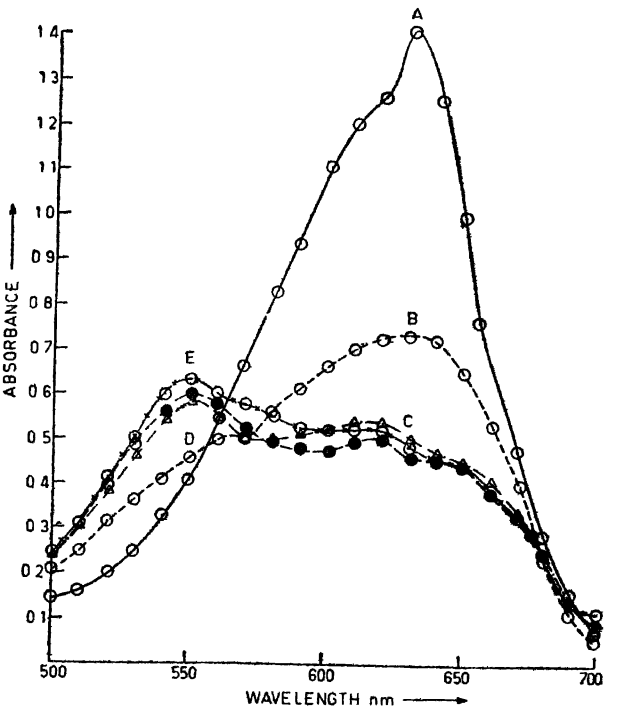

Fig. 2. Absorption spectra of 5.2×10^{-5} *M* toluidine blue in water (A), in presence of BF-B in the polymer-dye mol ratio of (B) 1.1, (C) 3.3, (D) 5.5, and (E) 11.0.

the pectic acid because of its having a special type of helicity due to its association with the glucan. While the first possibility is untenable because of the hydrolysis products of BF-B, the second one receives the strong support from the study of stoichiometry of TB–BF-B compound by the 'shaking with immiscible organic solvent' method[12]. Fig. 3 gives the stoichiometry of TB–BF-B compound to be 1 : 2 indicating that the dye cations are bound at alternate anionic sites of polygalacturonate in BF-B and every alternate COO^- group is engaged in forming strong association with the glucan component and hence, is not available for the dye binding.

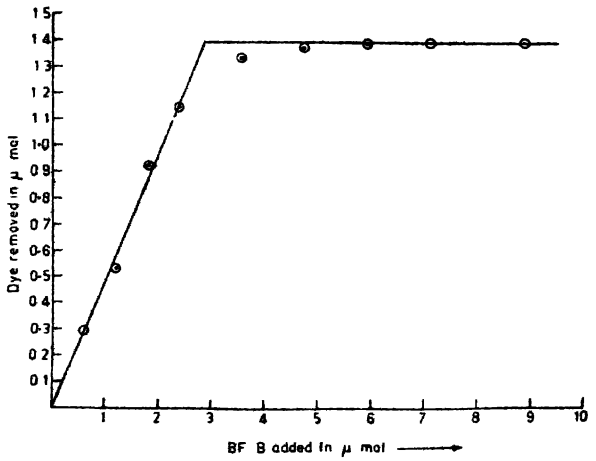

Fig. 3. Stoichiometry of BF-B—toluidine blue compound. Removal of BF-B — toluidine blue (TB) compound from aqueous phase by shaking with petroleum ether. 10 ml portions of 1.64×10^{-4} *M* TB containing various amounts of BF-B as indicated were shaken with petroleum ether and the dye left in the aqueous layer estimated.

The possibility of a helical structure in BF-B might be inferred from a parallel study of its metachromasia and circular dichroism (CD) of the BF-B–pinacyanol (PCYN) system in the visible range as depicted in Figs. 4 and 5, respectively. PCYN is a cyanine dye, and the shape of the metachromatic spectrum of this dye strongly depends on the nature of the polyanions inducing the spectral change[20,21]. Apparently BF-B induced sharp but multiple banded spectral change in PCYN (Fig. 4) having metachromatic bands at 485 and 590 nm with a minor band around 635 nm. The shape of the metachromatic spectrum was rather insensitive to polymer/dye ratio at least upto 33. Fig. 5 depicts the strong CD exhibited by BF-B–PCYN system in the visible region. PCYN or its aggregate does not have any chirality and metachromatic compound of PCYN and polyanion like Na-polyacrylate does not exhibit CD[22]. However, compound of PCYN with poly (L-glutamic acid) at both acidic and neutral *p*H exhibits dichroism in the visible absorption range of the dye, the shapes of the metachromatic and CD spectra characteristically

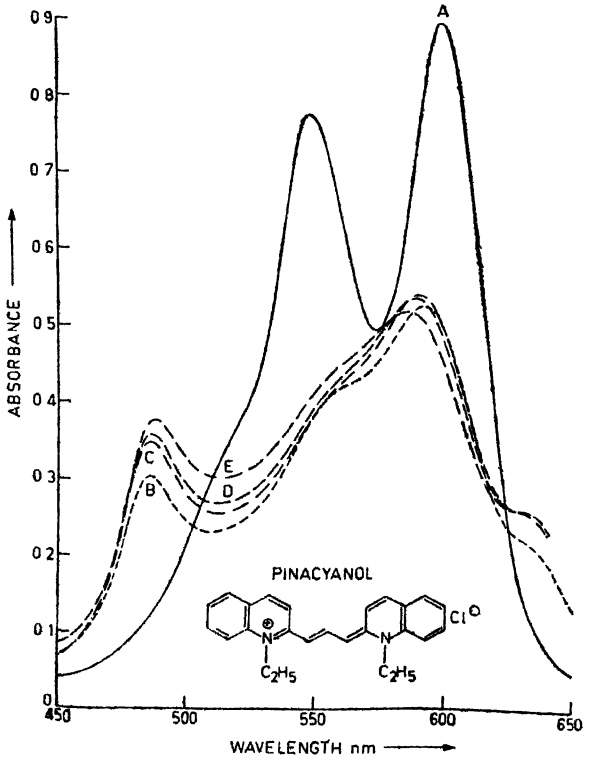

Fig. 4. Absorption spectra of 1.16×10^{-5} *M* pinacyanol in water (A); in the presence of BF-B at polymer-dye mol ratio of (B) 1.52, (C) 3.79, (D) 11.38, and (E) 33.37.

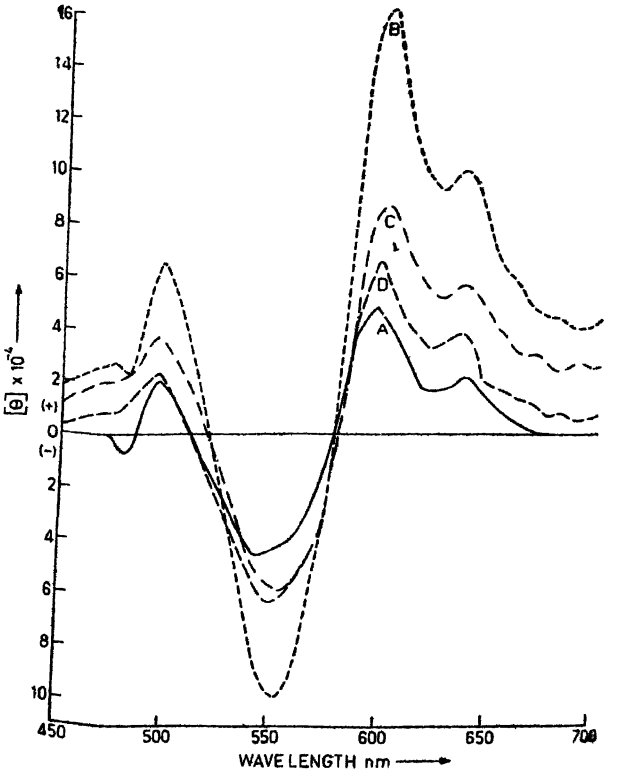

Fig. 5. CD spectra of 1.16×10^{-5} *M* pinacyanol in the presence of BF-B in water at polymer-dye mol ratio of (A) 1.52, (B) 3.79, (C) 11.38, and (D) 33.37.

depend on the conformation of the polyglutamic acid[28]. The importance of chromophore-chromophore interaction in metachromasia and extrinsic dichroism of dye-polyanion complexes has been pointed out[24-26]. The extrinsic optical activity of a symmetric chromophore bound on the side chains of a helical polyanion could be[27] (i) strong chromophore-chromophore interaction, the polyanion serving only to fix the relative geometry with transition dipoles of the chromophores coupled in a chiral arrangement ; (ii) local interaction between the chromophore and the dissymmetric field of the helix ; and (iii) perturbation of the chromophore by the dissymmetric field of the chiral atom in the polyanion. The exhibition of CD by BF-B – PCYN system indicated strong chromophore-chromophore interaction[28], and high [θ] values observed for the system suggested that mechanism (i) played the predominant role ; lower [θ] values would be expected for other two mechanisms[29]. Considering the similarities between the CD spectra of PCYN induced by BF-B (Fig. 5) and by Na-poly (L-glutamate)[28], it could reasonably be suggested that poly GalA in BF-B, like Na-polyglutamate[30], had an extended helical conformation around the glucan component ; the alternate possibility of a double helix involving both glucan and galacturonan could not be ruled out on the basis of the present data, though the proportions of GalA and Glc (molar ratio 2 : 1) supported the former model, PCYN cations were, therefore, bound around the BF-B extended helix with their transition moments coupled in a chiral arrangement, either helical or with systematic constraints, to exhibit circular dichroism.

Acknowledgement

The authors are thankful to Prof. M. Chaudhuri, I.A.C.S., Calcutta, for putting the spectropolarimeter at their disposal and to (Miss) Rina Ghosh, research scholar of the Chemistry Department, Jadavpur University, for various assistance.

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