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LIGNIN–XYLAN ESTER LINKAGE IN MESTA FIBER (*Hibiscus cannabinus*)

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ABSTRACT

Four successive treatments of mesta fiber with borohydride yielded an off-white fiber, containing ~82% of lignin and ~10% of *O*-acetyl groups originally present in the fiber, that showed in its i.r. spectrum a characteristic absorption band at ~1730 cm^{-1} due to ester groups. Delignification, followed by repeated borohydride treatment of this fiber, resulted in complete reduction of the *O*-acetyl linkages, while only 75% of the 4-*O*-methyl-D-glucuronic acid side-chain residues on the D-xylan component of the fiber were reduced to 4-*O*-methyl-D-glucose. Comparison of the nature of the neutralization curves prepared by titrating (*a*) raw, (*b*) demineralized, and (*c*) cation-free, saponified mesta fibers and a pure D-xylan derived from the untreated fiber suggested that all of the acidic side-chain residues are ester-bonded with lignin in the native state. The last-named fibers also underwent a coupling reaction with a diazonium salt, establishing the existence of free phenolic groups in them. These results indicated that the acidity in the raw and the demineralized fibers arise entirely from free phenolic groups of lignin and that all of the uronic acid units attached to the D-xylan are ester-bonded with lignin in these fibers.

INTRODUCTION

It was shown earlier[1] that ~34% of the 4-*O*-methyl-D-glucuronic acid side-chains on the D-xylan in jute fiber may be reduced to the corresponding neutral sugar by treatment of the fiber with aqueous potassium borohydride, from which it was concluded that about one third of the D-xylan is linked to the lignin component of the fiber through the carboxyl groups of the uronic acid residues *via* ester linkages. A similar study[2] of pineapple fiber revealed that the degree of esterification (d.e.) between the lignin and D-xylan components of this fiber is 28%. However, the nature of the neutralization curves obtained by titration, with dilute alkali, of (*a*) raw, (*b*) demineralized, and (*c*) cation-free, de-esterified jute fibers, and of the

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D-xylan obtained from jute suggested[3] that none of the carboxyl groups of the uronic acids exist in the free state in the raw and the demineralized fibers, implying that the entire xylan is engaged in ester linkage with lignin.

We now report the results of an investigation relating to the possible existence of an ester linkage between these constituents in mesta fiber.

EXPERIMENTAL

Materials and methods. — All reagents and solvents employed in the present investigation were of AR grade, and the latter were distilled before use. Unless stated otherwise, solvents were evaporated under diminished pressure at 40–45°. Preliminary identification of the sugars in polysaccharide hydrolyzates was performed by descending, paper-partition chromatography (hereafter, chromatography), using Whatman No. 1 MM papers. The following solvent mixtures (v/v) were used as irrigants: (*A*) 8:2:1 ethyl acetate–pyridine–water, (*B*) 10:1:2 1-butanol–ethanol–water, (*C*) 5:5:1:3 ethyl acetate–pyridine–acetic acid–water, and (*D*) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water. The chromatograms were stained with (*a*) alkaline silver nitrate, or (*b*) aniline hydrogenoxalate.

Hydrolyzates for chromatographic identification of sugars were prepared as follows. (*a*) The sample of fiber (~100 mg) or water-soluble polysaccharide (~5 mg) was heated in a sealed tube with 0.5M sulfuric acid on a boiling-water bath for 10 h. The acid-soluble portion, separated in the case of fiber samples by filtration of the pulp through a Gooch crucible (porosity G-4), was treated in the usual way, and evaporated to a syrup. (*b*) The insoluble polysaccharides were solubilized by treatment with 72% sulfuric acid for 2 h at room temperature. The resulting solution was diluted 24-fold and then processed as in method (*a*), to accomplish complete hydrolysis of the partially degraded fragments.

The acidic and neutral sugar components in a hydrolyzate were separated[1] from one another by retention of the former sugars on Dowex-1 X-8 (formate) ion-exchange resin (100–200 mesh).

Solutions for spectrophotometric determination of constituent sugars in polysaccharides were prepared by treating the material (~5 mg) with 72% sulfuric acid (~0.5 mL) overnight at 5–10°. The solution was quantitatively transferred to a 25-mL volumetric flask with distilled water (10 vol), the excess of acid neutralized with cold, 2M sodium hydroxide solution, and the volume made to the mark with distilled water.

The *O*-acetyl and Klasson-lignin contents of the fiber were respectively determined according to the methods of Sen Gupta[4,5] and co-workers.

Holocellulose from native, or reduced, fibers was prepared[6] by heating a suspension of the fiber in sodium acetate–acetic acid buffer (0.1M, pH 4.0; fiber to liquid ratio, 1:50) containing 0.7% of active sodium chlorite, on a thermostatically controlled water-bath for 5 h at 75 ±1°.

Saponification and demineralization of fibers were accomplished by proce-

dures described earlier[3]. The pH–neutralization curves (n.c.'s) for these fibers were obtained, and their coupling reactions with *p*-nitrobenzenediazonium hydrogensulfate were conducted, by methods developed earlier[3]. The starting material for titration of polysaccharides was prepared by decationization, followed by dialysis and lyophilization of the decationized solution. The n.c. in this case also was obtained as previously[3].

Uronic acid was estimated spectrophotometrically[7], employing appropriate corrections[1] for interference due to hexoses and pentoses, which were separately estimated by L-cysteine–H_2SO_4 methods[8,9].

Pellets for recording infrared spectra of fibers were prepared from an intimate mixture of pulverized fiber and spectral-grade potassium bromide (~2 mg per 100 mg).

The specific rotation of sugars was determined at equilibrium with a JASCO digital polarimeter, model DIP 181. All measurements of pH were made with an EC pH-meter, model PH 5651.

Spectral studies in the u.v. and visible regions were conducted with a Hitachi Spectrophotometer, model 200-20. Infrared spectra were recorded with a Perkin–Elmer IR Spectrophotometer, model 399B. G.l.c. analyses of derived alditol acetates of neutral sugars were conducted at 195° with a Hewlett–Packard Gas Chromatograph, model 5730A, using glass columns (1.83 m × 3.2 mm) containing (*1*) 3% of ECNSS-M on Gas Chrom Q (80–100 mesh) and (*2*) 1% of OV-225 supported on SIL.RUB (80–100 mesh). Nitrogen was used as the carrier gas. The per cent areas of individual sugars appearing in the g.l.c. profile were calculated with the help of an integrator, model 3380A.

Preparation of fiber. — The fiber strands (~2.7 m long) were collected from commercially available mesta fiber (*Hibiscus cannabinus*, grade[10] M-2, as per specifications stipulated by ISI). The root (~100 cm) and top (~70 cm) ends were discarded, and the middle portion was combed, to get rid of adhering dirt and clay matter. The clean fiber was cut into short lengths (~2–3 cm), pulverized in a Wiley mill, and then dewaxed by extensive extraction with 1:2 (v/v) ethanol–benzene in a Soxhlet apparatus, to afford the starting material (hereafter, the control fiber).

Preparation of fully reduced fiber. — The control fiber (~12 g) was reduced by the procedure described earlier[1], using 0.2M potassium borohydride. Four successive borohydride treatments of the control fiber afforded an off-white fiber containing 0.38% [on oven-dry (o.d.) weight of treated fiber] of *O*-acetyl groups. This fiber (~2 g) was delignified with aqueous sodium chlorite, to give a snow-white fiber, the *O*-acetyl content of which was found to be 0.31%. The delignified fiber was successively treated twice with M potassium borohydride solution, to afford fully reduced fiber, free from *O*-acetyl linkages.

Isolation and purification of D-*xylan from control and fully reduced fibers.* — The D-xylan was isolated by extraction of the holocellulose (containing 3.8% of lignin and 2.9% of *O*-acetyl), prepared from the control fiber in ~88% yield, with 9.5% (w/w) aqueous sodium hydroxide (fiber to liquid ratio 1:50) for 2 h at room

temperature under a nitrogen atmosphere. The pulp thus obtained was squeezed through Nylon cloth, and the liquid was clarified by filtration through a Gooch crucible (porosity G-2). The filtrate was acidified in the cold with ice-cold, 6M acetic acid, whereupon a flocculent precipitate separated out. This was centrifuged off (12,000 r.p.m., 40 min), and the clear, supernatant liquor was extensively dialyzed against distilled water. The dialyzed solution was concentrated, and then lyophilized, to afford crude D-xylan (polysaccharide A_1; yield, 469.4 mg; λ_{max}^{water} 280 nm, $E_{1\%}^{1\,cm}$ 19.8). To a dispersion of A_1 (~350 mg) in acetate buffer (12.5 mL; 0.2M, pH 4.0) were added aqueous sodium chlorite (3.6%; 4.9 mL) and distilled water (7.6 mL), to make a final chlorite concentration of 0.7%. The mixture was heated for 5 h at 75 ±1°, cooled to room temperature, dechlorited by dropwise addition of sodium metabisulfite solution (2%), made neutral with dilute alkali, dialyzed, and the dialyzed solution lyophilized, to afford polysaccharide A_2 (yield 227.7 mg; λ_{max}^{water} 280 nm, $E_{1\%}^{1\,cm}$ 10.92). A further chlorite treatment of A_2 furnished a pure D-xylan (fraction A_3; λ_{max}^{water} 280 nm, $E_{1\%}^{1\,cm}$ 4.41). Crude D-xylan (polysaccharide A_4) from fully reduced fiber was isolated as just described, and then purified through the barium complex[11], to furnish polysaccharide fraction A_5.

RESULTS AND DISCUSSION

Extractive-free, pulverized fibrils (control fiber) obtained from the middle portion of mesta-fiber strands (*Hibiscus cannabinus*), grade M-2, served as the starting material for the present investigation. This fiber contained lignin 10.21%, *O*-acetyl 3.47%, and holocellulose 88.5%, and its i.r. spectrum showed, at ~1730 cm^{-1}, a characteristic absorption band due to the stretchng vibrations of ester carbonyl groups.

It had been observed[1,2] in the case of at least two ligno-cellulosic fibers that substantial proportions of the 4-*O*-methyl-D-glucurono-D-xylan components are attached to the respective lignin components by ester linkages through the carboxyl groups of the uronic acid side-chains. Most of these fibers also contain a considerable percentage of *O*-acetyl groups, which are presumed to originate from esters of acetic acid and secondary hydroxyl groups of the carbohydrate constituents (possibly the D-xylan) of these fibers. It is also known that the framework of the D-xylan present in mesta fiber[12] resembles those of jute[13] and other lignocellulosic fibers[14–17]. Because the biosynthetic route for the synthesis of chemical constituents in all these fibers is likely to be similar, there is a strong possibility that an ester linkage between lignin and xylan would also exist in mesta fiber. In consideration of this aspect, control fiber was successively treated four times with aqueous potassium borohydride (0.2M), to reduce the expected ester linkages just mentioned. The off-white fiber thus obtained still retained ~10% of *O*-acetyl groups, and its lignin content was decreased by ~18% (see Table I). The acetyl content could not, however, be further diminished by repeating the borohydride treatment. Perhaps, the reason is that the reaction sites are not accessible to the reducing

TABLE I

CHEMICAL ANALYSIS OF BOROHYDRIDE-TREATED FIBERS AND UNTREATED FIBERS

Fiber sample	*Sugars detected*[a]	*Composition (%)*[b]		
		Lignin	O-*Acetyl*	*Holocellulose*
Control fiber	Uronic acid Galactose Glucose Mannose Arbinose Xylose Rhamnose	10.21	3.47	88.5
Holocellulose prepared from control fiber	Uronic acid Galactose Glucose Mannose Arabinose Xylose Rhamnose	3.84	2.93	—
Four-times borohydride-treated fiber	Uronic acid Galactose Glucose Mannose Arabinose Xylose 4-*O*-Methylglucose[c] Rhamnose	8.39	0.35	82.7
Fully reduced fiber	Uronic acid Galactose Glucose Mannose Arbinose Xylose 4-*O*-Methylglucose[c] Rhamnose	—	0.00	—

[a]By chromatography; sugars arranged in order of increasing chromatographic mobility (solvent *A* and *B*); uronic acids detected using solvents *C* and *D*. [b]On o.d. weight of control fiber. [c]Identified also by g.l.c. (see text).

agent owing to steric hindrance, the hydrophobicity of lignin, and the heterogenity of the reaction mixture. In view of this, the fiber that had been treated four times with borohydride was delignified, in order to minimize these inhibitory factors, and then successively treated twice with M borohydride solution, affording a snow-white fiber (hereinafter termed fully reduced fiber) which was devoid of *O*-acetyl groups. The extent of reduction achieved through successive treatments with borohydride was monitored by determining the relative intensity of the band at 1730 cm^{-1} in the i.r. spectrum of the fiber at each step. The spectral data suggested that the proportion of ester linkages progressively decreased with each borohydride treatment (see Fig. 1). However, a broad, small shoulder at 1730 cm^{-1} persisted in

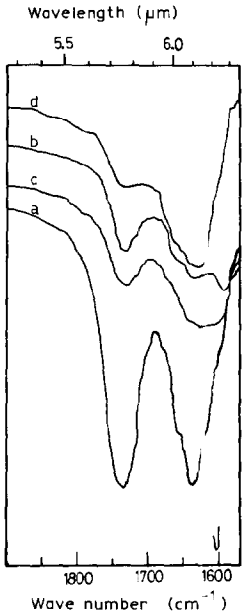

Fig. 1. Infrared spectra of (a) control fiber, (b) four-times borohydride-treated fiber, (c) holocellulose prepared from four-times borohydride-treated fiber, and (d) fully reduced fiber.

the i.r. spectrum of fully reduced fiber, and this could not be eliminated by further treatment with borohydride.

The effect of borohydride treatment was examined by comparing the chromatographic behavior of the hydrolyzates prepared from control, four-times borohydride-treated, and fully reduced fibers. Both of the treated fibers showed the presence of one sugar component in excess of those present in the hydrolyzate of the control fiber (see Table I). The additional sugar travelled with a chromatographic mobility (solvents *A* and *B*) comparable to that of 4-*O*-methyl-D-glucose, and produced a yellow coloration with staining reagent *b*, which is a characteristic property of the latter sugar. The partially identified sugar was separated from the associated sugar mixture by repeated chromatography (solvent *B*), on a preparative scale, of the hydrolyzate of the four-times borohydride-treated fiber. This was eventually identified as 4-*O*-methyl-D-glucose by comparing its specific rotation value ($[\alpha]_{589}$ +60.8°, *c* 0.8 in water; lit.[1] +61.3°), and the g.l.c. profile of the alditol acetate derived from it, with those of an authentic specimen of the sugar*. The retention times, relative to D-glucitol hexaacetate as unity, were found to be 0.83 and 0.84**, respectively, in columns *1* and *2*.

Potassium borohydride being a specific reductant for esters (and not for free

*Prepared during earlier studies.

**The authors regret the erroneous reporting[1] of this value, which escaped their notice during preparation of that manuscript.

carboxylic acids or their salts), the identification, in the borohydride-treated fibers, of 4-*O*-methyl-D-glucose, which must have been introduced into these fibers by virtue of reduction of 4-*O*-methyl-D-glucosyluronic acid side-chains on D-xylan, establishes that at least some of these side-chains were ester-bonded in the native fiber. It may be calculated, by comparing the lignin contents of control fibers and four-times borohydride-treated fibers, that ~18% of the original lignin passes into the aqueous phase as a consequence of splitting of the ester bonds, indicating that the alcohol parts of these bonds are contributed by lignin. It is evident that the number of glycosyluronic acid residues that are ester-bonded in the control fiber would give rise to the same number of 4-*O*-methylglucosyl groups in the fully reduced fiber, assuming that quantitative reduction is achieved during the borohydride treatments. The d.e. may, therefore, be calculated either by monitoring the increase in the ratio of xylose to uronic acid in the latter fiber, or by comparing the same ratio in control fiber with that of xylose to 4-*O*-methylglucose in the fully reduced fiber. It would be desirable to conduct the estimation of these sugars on the respective D-xylans after their isolation and subsequent purification, in order to avoid unnecessary complications and misinterpretation of the results.

In view of these considerations, the control fiber was delignified, and the resulting holocellulose was extracted with aqueous alkali. Acidification of the alkaline extract with 6M acetic acid afforded a flocculent precipitate which was centrifuged off and discarded. A crude D-xylan (polysaccharide A_1) was isolated from the supernatant liquor by dialysis, followed by lyophilization of the dialyzed solution, which absorbed strongly at 280 nm ($E^{1\%}_{1\,cm}$ 19.8), indicating that the polysaccharide is associated with a substantial proportion of lignin. Chromatography, and g.l.c. analysis, of the hydrolyzate of A_1 revealed the presence of significant proportions of contaminating sugars (*viz.*, galactose, glucose, mannose, arabinose, and rhamnose). The lignin content of A_1 was diminished by repeated treatment of its aqueous solution with chlorite. The polysaccharide was isolated after each delignification treatment by removing free chlorine with aqueous sodium metabisulfite, neutralizing the acid with dilute alkali, dialyzing, and lyophilizing the dialyzed solution. The absorbance at 280 nm of the polysaccharide (A_3), obtained after two successive chlorite treatments of A_1, decreased substantially ($E^{1\%}_{1\,cm}$ 4.41), and A_3 was found to contain uronic acids (13.7%), xylose (78.4%), and rhamnose (traces) as the only constituent sugars. It may be noted that the hexosans in A_1 disappeared upon delignification of the polysaccharide. The probable reason may be that the hexosans of very low molecular weight are attached to a lignin core of high molecular weight; as a result, the delignification treatment produces low-molecular-weight lignin–hexan complexes (or simply hexans) which escape during the dialysis step. Another explanation could be that hexans are degraded into small fragments that escape during dialysis.

The crude D-xylan (polysaccharide A_4) obtained by extraction of the holocellulose, prepared from fully reduced fiber, also contained significant proportions of hexans as impurities that were removed by repeated treatment of an aqueous solu-

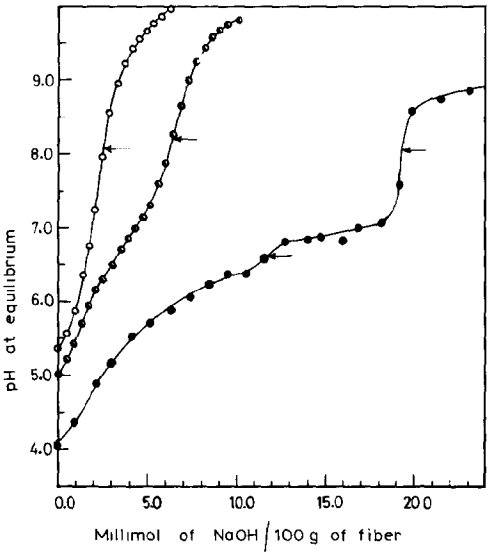

Fig. 2. Neutralization curves furnished by chemically treated and untreated mesta fibers. [Key: —○—○—, control fiber; —◐—◐—, DM fiber; and —●—●—, cation-free, de-esterified mesta fiber. The arrow marks indicate the equivalence points.]

TABLE II

ACIDIC PROPERTIES OF CONTROL, DEMINERALIZED, AND CATION-FREE, DE-ESTERIFIED MESTA FIBERS

Fiber sample	*Neutralization equivalent (mmol/100 g)*	*pH at equivalence point*	*Diazonium salt uptake (mmol/100 g)*
Control fiber	2.6	8.1	5.2
Demineralized (DM) fiber	6.4	8.2	5.6
Cation-free, de-esterified	11.8	6.6	5.6
(DD) fiber	19.4	8.1	
Decationized polysaccharide A_3	74.0	6.7	—

tion of A_4 with baryta. The purified D-xylan (polysaccharide A_5) isolated from the supernatant liquor of the Ba complex was found to contain xylose and 4-*O*-methylglucose in the molar ratio of 100:9.4 (estimated by g.l.c.). This estimate does not, however, account for the xylose which escaped as D-xylo-oligosaccharide during preparation of the alditol acetate derivatives of the neutral sugar components in polysaccharide A_5. It was assumed that the xylose unaccounted for would not alter this ratio to an appreciable extent, since the loss would be expected to be negligibly small. The xylose and uronic acid contents of polysaccharide A_3 being 78.4% (spectrophotometric estimation) and 74mM% (titrimetric estimation; see later), it may be derived that this material contains 12.5 glycosyluronic acid groups per 100 xylosyl residues. Consequently, the d.e. between lignin and xylan compo-

nents of the fiber (control fiber) in the native state may be shown to be 75.2%. The rest of the glucosyluronic acid groups possibly remain as free acid or as salt.

The evidence so far obtained suggests that the control fiber contains an acidic component that may be titrated with alkali, especially if the counter-cations are removed by pretreatment with a mineral acid. This was experimentally verified by preparing neutralization curves for the control and the demineralized (DM) mesta fibers. The titratable acidities were found to be of the order of 2.6 and 6.4 mmol per 100 g of the respective fiber. It may be noted that these n.c.'s contain only one inflection (see Fig. 2), suggesting that there is only one kind of acid present in these fibers. In order to ascertain whether this acidity is due to the unesterified glycosyluronic acid groups on the D-xylan, the nature of these n.c.'s was compared with that of decationized polysaccharide A_3. Surprisingly, the n.c.'s furnished by the fibers were remarkably different, in that the initial and end-point pH values (see Table II and Fig. 2) were considerably higher than those observed in the case of D-xylan (polysaccharide A_3; see Fig. 3), suggesting that the acidic component manifested in the former curves is different from, and weaker than, the acidic D-xylan, and this is likely to be due to the phenolic groups present in the lignin.

This concept was fully corroborated in subsequent experiments wherein the control and DM fibers were found to undergo a coupling reaction with *p*-nitrobenzenediazonium hydrogensulfate, to afford non-leachable, deep-yellow-colored fibers. This property provided positive evidence demonstrating that both of these fibers contain a phenolic acid component (originating from lignin), as the coupling reaction is characteristic of phenolic compounds in which the *ortho* or *para* posi-

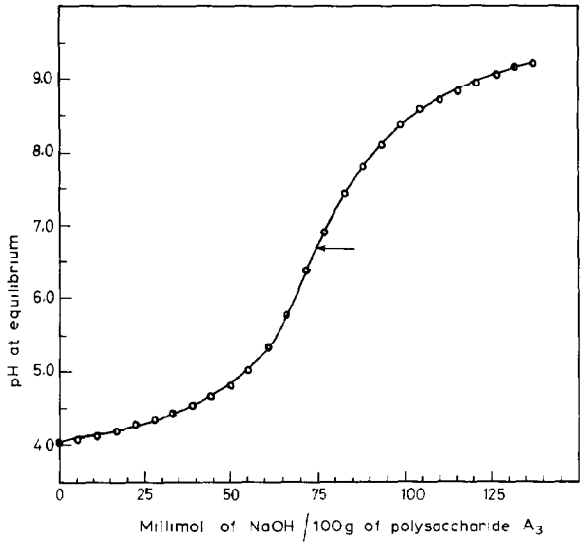

Fig. 3. Neutralization curve furnished by decationized D-xylan (polysaccharide A_3) isolated from control fiber. [The arrow mark indicates the equivalence point.]

tions, or both, with respect to the phenolic function, are available for reaction. The diazonium salt uptakes were estimated to be 5.2 and 5.6 mmol per 100 g of control and DM fibers, respectively. The parity in these figures is understandable, because the coupling reaction is conducted under slightly alkaline conditions in order to enable the formation of phenolate ions (a prime requirement for the coupling reaction), which are obviously formed in comparable proportions in each fiber. Interestingly, the uptake of diazonium salt of the control and the DM fibers is in good agreement with the free acidity of the latter fiber. This may, however, be regarded as a mere coincidence, as each molecule of a phenolic compound may theoretically react with three molecules of the coupling reagent. In the present instance, there is an average of one substitution by the diazonium salt for each phenolic group.

The aforementioned titration and coupling-reaction data convincingly suggest that the entire acidities appearing in the control and DM fibers arise from phenolic groups, indirectly implying that unesterified glycosyluronic acid groups are absent from these fibers. This contention was further substantiated by examining the n.c. furnished by cation-free, de-esterified mesta fiber (see Fig. 3). This n.c. contained two inflections; the first was due to neutralization of uronic acids, newly generated in the fiber during the saponification step, and the second was due to that of phenolic groups. The difference between the first and second neutralization equivalents (7.6 mmol/100 g), which gives a measure of the acidity contributed by the latter component, is in fair agreement with the acidity of the DM fiber.

It may be noted that the d.e. obtained from the reduction experiments is lower than that to be expected from the titration data. This may be attributed to incomplete reduction of the ester linkages arising out of inaccessibility of the reaction sites to the reducing agent. It may be mentioned that such incomplete reduction was also observed[18] in the case of jute fiber, where only 72% of the ester linkages could be reduced under similar conditions.

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