*Carbohydrate Research*, 90 (1981) 339–344
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

## Note

# Solubilization of cellulose and other plant structural polysaccharides in 4-methylmorpholine *N*-oxide: an improved method for the study of cell-wall constituents

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(Received June 10th, 1980, accepted for publication in revised form, August 8th, 1980)

The cell walls of plants act as supporting structures and as barriers to pathogens Knowledge of the nature of their macromolecular constituents is, therefore important and although many structural features of the different polysaccharides present in the plant cell-wall are known, their interrelation and mode of attachment remains to be determined with precision[1] A major difficulty in studying the interconnection between the polysaccharides arises from their insolubility In order to study their structures they have to be extracted, and conventional chemical-extraction procedures almost always involve the cleavage of covalent bonds and often alter the structures The problem is especially acute with cellulose, the main constituent of the plant cell-wall, because of its insolubility in most common solvents We have used a new biopolymer solvent for the solubilization of several polysaccharides, including mixtures of polymers of known structure selected as model compounds

The efficacy of the solvent enabled us to solubilize entire cell walls The solvent, 4-methylmorpholine *N*-oxide (MMNO), in it various forms of hydration, has been demonstrated to be a good solvent for cellulose[2,3] Its use in physical studies on cellulose, for crystallization[3] or for n m r spectroscopy[4], shows that it acts as a real solvent and not as a derivatizing reagent For this reason, we used this solvent for the methylation of polysaccharides which, because of their lack of solubility in pure dimethyl sulfoxide ($Me_2SO$), were difficult to methylate[5] In this Note, we report the use of MMNO–$Me_2SO$ as a non-degradative solvent, and its interest for structural studies on mixtures of polysaccharides

Several samples of cellulose from different origins and having various average degrees of polymerization (d p ) were solubilized in MMNO The samples were first dissolved in MMNO monohydrate at 120° and then diluted with $Me_2SO$ (ref 3) to give a final concentration of 0 7% Cellulose samples of increasing d p were selected and dissolved in the same manner Cellulose dissolving-pulp (d p 600) and purified cotton linters (d p 2700) gave the expected solutions[3,4] for cellulose of such

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values. The increase in degree of polymerization had no consequence on the ease of solubilization, as shown by a bacterial cellulose produced by *Acetobacter xylinum* [d p. ≃3000 (ref 6)] or the crude cellulose of untreated, native, cotton hair [d p ≃14 000 (ref 7)] from *Gossypium hirsutum*

During exhaustive dialysis against water, cellulose precipitated in the dialysis bag In each case, the recovery of cellulose after freeze-drying was nearly quantitative (85% for cotton linters, 90% for native cotton hair, and 98% for bacterial cellulose)

The small weight loss observed upon recovery of the polysaccharides after treatment with MMNO demonstrates that the solvent action does not involve degradation from the reducing end by a recurrent stepwise elimination in a "peeling" type of mechanism, which would have caused noticeable loss of material

When the recovered, lyophilized samples were subjected to acid hydrolysis and analyzed by gas–liquid chromatography in the presence of an internal standard for quantitation of the glucose content, yields of ~100% were obtained From this result, it can be deduced that, under our conditions for solubilization of the different cellulose samples, no significant modification by oxidation or degradation of the constituent glucose residues occurred during the time of exposure to the solubilizing agents at high temperature. Similar results were obtained for solubilized samples of bacterial and cotton celluloses that had been kept in the solvent mixture for 6 months at room temperature in a desiccator Further evidence for the stability of the cellulose samples in the presence of MMNO was obtained by comparing the viscosity, measured by use of cupriethylenediamine, of the samples before solubilization and after recovery from the solution. The result showed a decrease of d p of ~50%, which was also confirmed by methylation analysis of the cotton linters As there was no loss of D-glucose, this decrease in chain length does not correspond to significant endwise degradation, but rather to random chain-scission The reason for this scission is unknown.

Two bacterial polysaccharides of known structure were used as model compounds to investigate the possible chemical action of MMNO on acidic polysaccharides Capsular K-antigens from *Klebsiella* serotypes K-41 (ref 8) and K-63 (ref 9) were solubilized under the same conditions as used for cellulose Serotype K-41, which has a repeating unit composed L-rhamnose, D-galactose, D-glucose, and D-glucuronic acid in 1.2.3.1 molar ratio, readily went into solution. Serotype K-63, containing L-fucose, D-galactose, and D-galacturonic acid in 1 1 1 molar proportion was more difficult to solubilize and necessitated longer treatment at 120° in MMNO monohydrate for dissolution It seems that the higher uronic acid content in K-63 is responsible for its lower solubility in the solvent mixture It was noted earlier[9] that K-63 was not soluble in $Me_2SO$ From the recovered yields of polysaccharides and analysis of their sugar constituents, it was established that MMNO has no deleterious effect on the polymers

Because of our interest in plant cell-wall polysaccharides, we evaluated the solvent on a mixture of a hemicellulose and a pectic polymer The hemicellulose, L-arabino-4-*O*-methyl-D-glucurono-D-xylan from wheat straw, and commercial D-

TABLE I

COMPARATIVE DATA[a] FOR ONE-STEP HAKOMORI METHYLATIONS OF VARIOUS CELLULOSE SAMPLES IN $Me_2SO$ AND MMNO–$Me_2SO$

| *Methylated derivative* | *Dissolving pulp d.p. 600* | | *Cotton linters d.p. 2700* | | *Bacterial cellulose* (Acetobacter xylinum) | | *Native cotton* (Gossypium hirsutum) | |
|---|---|---|---|---|---|---|---|---|
| | *$Me_2SO$* | *MMNO–$Me_2SO$* | *$Me_2SO$* | *MMNO–$Me_2SO$* | *$Me_2SO$* | *MMNO–$Me_2SO$* | *$Me_2SO$* | *MMNO–$Me_2SO$* |
| 2,3,4,6 Glc[b] | 0.4 | 1.2 | | 0.1 | | | | |
| 2,3,6 Glc | 90 | 81 | 76 | 81 | 13 | 56 | 3 | 69.5 |
| 2,6 Glc[c] | 2 | 2 | 6 | 2.5 | 6 | 11 | 0.5 | 7 |
| 3,6 Glc[c] | 1.5 | 4.5 | 2.5 | 2 | 5 | 13 | trace | 6 |
| 2,3-Glc[c] | 2.5 | 5.5 | 8 | 8.5 | 13 | 12 | 2 | 9 |
| 2,3-Xyl | 1 | 1.5 | 1 | 2 | | | | |
| Glc[c] | 1.5 | 3.2 | 5.5 | 3 | 63 | 8 | 94.5 | 8.5 |

[a]Results are expressed as percent of glucose derivatives identified. Extraneous peaks were also present. [b]2,3,4,6 Glc = 1,5 di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, etc. [c]The proportion of these derivatives decreased with additional methylation steps.

galacturonan were treated in the same manner as for cellulose The galacturonan did not dissolve completely This insolubility is certainly attributable to the high uronic acid content as observed for antigen K-63 Here again, the recovery was quantitative and the analytical data for the polysaccharides were totally unchanged.

In order to apply this solubilization procedure to the structural investigation of polysaccharides from bacterial and plant sources, it was important for the solvent to be compatible with the derivatization of polysaccharides and, in particular, with permethylation, which is of basic interest for this study All of the aforementioned samples were subjected to methylation by the Hakomori procedure[10] in a single step, in $Me_2SO$ only, or in solution in the MMNO-$Me_2SO$ system The efficiency of the methylation conditions was estimated from the yields of the expected 2 3,6-tri-*O*-methylglucose derivative obtained after hydrolysis and g l c analysis The results are compared in Table I, and show that, for the samples known to be insoluble under the Hakomori conditions (as with the cellulosic materials) the efficiency of the methylation in a single step. as judged by the proportion of 2,3,6-tri-*O*-methylglucose, was considerably enhanced for the cellulose of higher d p Remarkably, such high d p values correspond also to higher degrees of crystallinity[11] It is clear that the MMNO–$Me_2SO$ solvent completely breaks down the crystalline texture of cellulose, thereby increasing accessibility towards the methylating reagents It may also be observed that MMNO does not impede the methylation reaction at all Other derivatizations of polysaccharides may be achieved in this solvent, as shown by carboxyl reduction of D-galacturonan by the carboimide procedure of Taylor and Conrad[15], which could be performed in one step on the foregoing mixture of xylan and galacturonan When performed in water, the complete reduction of D-galacturonan necessitated three successive treatments Various derivatization reactions of cotton linters have been reported previously. such as acetylation or propionylation, in the presence of MMNO[2]

MMNO–$Me_2SO$ thus seems to be a useful solvent-mixture for biological carbohydrate polymers, especially those whose high degree of crystallinity renders them insoluble in conventional solvents Under the conditions described in this paper, we could solubilize the entire cell-wall of suspension-cultured cells of *Rosa glauca*[13] From the solution, very mild separation and purification of homogeneous polysaccharide fractions could be effected for structural studies[13] We consider that this solvent system has utility for the study of cell-wall components, as the problems associated with solubilization of cell-wall polymers is considered a major impediment to progress in this field

## EXPERIMENTAL

*Polysaccharide samples* — Cell walls of suspension-cultured cells of *Rosa glauca* were prepared as previously described[12] Cellulose from *Acetobacter xylinum* was prepared as in ref 14. Three cellulose samples having various d p values were used· cellulose dissolving-pulp (d p = 600), linters powder d p 2700 (Schleicher and

Schull) and native cotton-hair from *Gossypium hirsutum* The K-antigens from *Klebsiella* have been described[8,9]

*Preparation of solutions* — The polysaccharides (0 1 g) in MMNO monohydrate[3] (2 5 g) were heated for 30 min at 120° in a tightly stoppered tube under nitrogen After complete solubilization of the samples, $Me_2SO$ (10 mL) was added and the mixtures were cooled to room temperature All samples were dissolved in the same manner One-half of each polysaccharide solution was directly subjected to methylation, and the analytical results were compared with those obtained for the same sample methylated in $Me_2SO$ (Table I) The other half of each fraction was dialyzed against water, and recovered the yield of the polysaccharides their sugar constituents were determined

*Hydrolysis* — A portion of each solution was exhaustively dialyzed against distilled water, and lyophilized To a known amount of the material, *myo*-inositol was added as an internal standard The mixture was hydrolyzed in 72% sulfuric acid for 30 min at room temperature and then diluted to 0 5M and heated for 6 h at 100° (ref. 12) Sugar analysis was performed by g l c of the alditol acetate derivatives as in ref 12

*Methylation* — All of the polysaccharides were methylated by the Hakomori procedure[10] in two different media firstly in $Me_2SO$ (ref 10) and secondly in MMNO–$Me_2SO$ with the reagents of ref 10, in a single step The methylated products were recovered by dialysis and freeze-drying, and were hydrolyzed Hydrolyses were performed as described in ref 12 for the cellulosic polymers and in 90% formic acid and 2M trifluoroacetic acid for polysaccharides K-41 and K-63 (refs 8 and 9) The products were analyzed as in ref 8

*Carboxyl reduction* — Hemicellulose (40 mg) and D-galacturonan (40 mg) were dissolved as before in MMNO–$Me_2SO$ solution (12 mL) Water (12 mL) was added, and reduction was effected directly by the procedure of Taylor and Conrad[15] After dialysis and freeze-drying of the mixture, analytical data for the polysaccharides were obtained

ACKNOWLEDGMENTS

The authors are grateful to Dr Henri Chanzy for helpful discussions and suggestions, and to Dr A Peguy for advice on the preparation of the solutions

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