Carbohydrate Polymers 1 (1981) 129–138

STRUCTURAL STUDIES ON TRIACETATES OF MANNAN AND GLUCOMANNAN

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ABSTRACT

Mannan triacetates prepared from material extracted from ivory nut and Tubera salep *were studied by means of electron and X-ray diffraction. The former is uniquely constituted of acetylated* D*-mannopyranosyl units linked by a (1→4)-β-linkage whereas the latter contains acetylated (1→4)-β-*D*-glucopyranosyl randomly distributed in the backbone with a ratio of mannose to glucose of about 3:1. However, there seems to be no effect on crystallisation due to the presence of the glucosidic units on the conformation of the chain.*

Single crystals of ivory nut triacetate were prepared by slowly cooling a dilute solution of nitromethane and butanol. The crystals were long narrow laths which provide electron diffraction data after annealing at 190°C in a vacuum.

Two different unit cells were derived from the acetylated Tubera salep *X-ray data. A first unit cell with* a = *1·18 nm,* b = *1·54 nm and* c = *1·60 nm contains eight sugar units, whereas the second unit cell with* a = *0.369 nm,* b = *0·96 nm and* c = *1·58 nm would accommodate 16 residues. The latter agrees best with the base-plane parameters derived from electron diffraction of single crystals.*

The X-ray fibre diagram was interpreted in terms of a two-fold helix and an asymmetric unit composed of two triacetyl mannopyranosyl units. This means that two chemically identical mannose units would not be conformationally equivalent along the backbone.

The presence of glucose units in the backbone does not seem to perturb the crystalline conformation. The 'isomorphous replacement' hypothesis was invoked to explain this observation. The helical parameters derived herein for Tubera salep

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Carbohydrate Polymers 0144-8617/81/0001-0129/$02.75 – © Applied Science Publishers Ltd, England, 1981
Printed in Great Britain

mannan triacetate are different from those reported earlier for the same acetylated glucomannan but crystallised using a different technique. This is attributed to the occurrence of polymorphism in this material.

INTRODUCTION

The crystal structure debate concerning native and regenerated cellulose (Gardner & Blackwell, 1974; French, 1978; Stipanovic & Sarko, 1978; Woodcock & Sarko, 1980) has recently been supplemented by electron diffraction studies on lamellar single crystals (Buleon & Chanzy, 1978). In addition, X-ray diffraction studies of the two polymorphs of cellulose triacetate have proceeded with the aid of electron diffraction data on single crystals (Chanzy & Roche, 1974; Roche *et al.*, 1978; Stipanovic & Sarko, 1978). The latter studies showed that the fibre repeat and the basic ribbon-like conformation of the cellulose chain is unchanged as a result of the transformation to cellulose triacetate. Thus a major disruption in intramolecular and intermolecular hydrogen bonds in crystalline cellulose has little effect on the conformational forces which favour the ribbon-like conformation of the cellulose chain. On the other hand, the three-fold helical conformation characteristic of $(1 \rightarrow 4)$-β-D-xylan is converted to a two-fold helix for the crystalline state of the xylan diacetate (Gabbay *et al.*, 1972).

In order to further explore the conformational factors which favour the ribbon-like conformation of β-$(1 \rightarrow 4)$ linked polysaccharides, the report by Bittiger & Marchessault (1971) concerning mannan triacetate has been re-examined. Mannan from plant sources is a $(1 \rightarrow 4)$-β-linked glycan and the most crystalline form, mannan from ivory nut, has been shown to crystallise in a ribbon-like conformation (Nieduszynski & Marchessault, 1972; Preston, 1974; Zugenmaier, 1974). However, the conversion from mannan to mannan triacetate causes a change in the fibre repeat from the familiar 1·04 nm corresponding to the ribbon-shaped molecules with a 2_1 symmetry axis along the chain to a helical conformation with three residues per turn, i.e., a fibre repeat of approximately 1·5 nm. In their study, Bittiger & Marchessault (1971) used a high molecular weight glucomannan from *Tubera salep* which is reported to have a mannose to glucose ratio of 3 : 1. It has been found that on crystallisation from solution this material can lead to crystal lattices corresponding to mannan I and mannan II, both of which have the same fibre repeat of approximately 1·04 nm (Chanzy *et al.*, 1979, 1982).

In this study we have attempted to carry forward the experiments of Bittiger & Marchessault (1971) by using more drastic annealing conditions for the oriented glucomannan triacetate films. At the same time, the fibre diffraction data from the glucomannan triacetate oriented films was compared with electron diffraction data on single crystals of mannan triacetate prepared from a completely acetylated mannan from ivory nut. The latter has been shown to be a pure $(1 \rightarrow 4)$-β-D mannan (Timell, 1975).

EXPERIMENTAL

Acetylation of Mannnan and Glucomannan

Ivory nut mannan and glucomannan from *Tubera salep* were peracetylated by a non-degradative method similar to that of Carson & Maclay (1948). A 2-g sample was swollen in 400 ml of formamide for 6 h then a mixture of 200 ml of pyridine and 200 ml of acetic anhydride was added. The mixture was kept for 11 days in the dark until total dissolution had occurred. The acetylated product was recovered by precipitation in a mixture of water and ice.

Crystallisation of Ivory Nut Mannan Triacetate

Two kinds of crystallisation conditions were used giving similar morphology as observed under the electron microscope: (a) Mannan triacetate (5 mg) was dissolved in 190 ml of nitromethane at 80°C, 40 ml of butanol was added to this solution and the mixture was cooled slowly in a water bath to room temperature. (b) Mannan triacetate was dissolved in dibenzyl ether at 220°C, hot tetradecane was then added and the mixture was cooled to 190°C at which point the crystallisation procedure was similar to that of Chanzy & Roche (1974) and Roche *et al.* (1978) for cellulose triacetate.

Preparation of Tubera Salep *Glucomannan Triacetate Films*

A glucomannan triacetate solution (0·42%) in dichloromethane was dried on a teflon plate. The film was placed in a sealed bomb with a free-hanging weight and in the presence of water vapour at 195°C annealing with elongation (10–30%) took place. The water vapour had a plasticising effect at the high temperature and led to well-oriented and moderately crystalline samples.

Electron Microscopy

The crystals of mannan triacetate were washed by repeated centrifugation in methanol. Drops of this suspension were deposited on carbon grids. After shadow-casting with tungsten, specimens were observed using a Philips EM300 electron microscope operating at 100 kV for diffraction experiments and 80 kV for transmission imaging. It was necessary to anneal the crystals at 190°C for 0·5 h in a vacuum oven prior to recording the electron diffractogram.

X-ray Diffraction

X-ray photographs were taken with the beam perpendicular to the film surface in a flat plate camera (Warhus type), using a Philips generator and Ni filtered CuKα radiation. X-ray diagrams were obtained from ivory nut mannan triacetate powders which had been annealed at 190°C for 2 h and from an oriented film of *Tubera salep* glucomannan triacetate. The measured *d* spacings were calibrated against NaF diffraction rings and the unit cell dimensions were refined by a least-squares method.

RESULTS

Mannan Triacetate Single Crystals

Ivory nut triacetate crystallised from nitromethane and butanol is in the form of rectangular laths with a length of 1–5 μm and width of the order of 150 nm (Fig. 1a). Single crystals obtained from a mixture of dibenzyl ether and tetradecane have a more fibrillar and less defined shape (Fig. 1b). Only the crystals shown in Fig. 1a could be characterised by electron diffraction. Six independent reflections were observed and the d spacings were calculated therefrom with a precision of ±0·005 nm (Table 1).

A powder X-ray diagram of the ivory nut mannan triacetate was recorded and the d-spacings were in close agreement as shown in Table 1. The lattice parameters measured from gold calibrations are: $a = 3{\cdot}52$ nm and $b = 0{\cdot}83$ nm. From these data, the d spacings in Table 1 were assigned suitable Miller indexes. Because the crystallinity of the specimen is poor the resolution of spots in the electron diffractogram was poor in spite of the suitable dimensions of the crystals.

Tubera Salep *Triacetate Fibre Diagram*

Eighteen independent reflections were measured on the X-ray fibre diagram (Fig. 2). Diffuse central scattering could not be eliminated by annealing at higher temperatures since thermal decomposition occurs in the bomb at temperatures higher than 190°C.

The unit cell parameters obtained by a least-squares method are: $a = 1{\cdot}18 \pm 0{\cdot}01$ nm, $b = 1{\cdot}54 \pm 0{\cdot}015$ nm and $c = 1{\cdot}60 \pm 0{\cdot}02$ nm. A second unit cell with $a = 3{\cdot}69$ nm, $b = 0{\cdot}96$ nm and $c = 1{\cdot}58$ nm was also compatible with the observed d-spacings but the smaller unit cell was chosen since it gives a better agreement with observed data. Nevertheless, the similarity of the base plane of the second unit cell compared with the base plane parameters of the ivory nut mannan triacetate derived from the electron diffractogram is to be noted. A comparison between observed and calculated d spacings is tabulated in Table 2 for the first unit cell.

The measured density (flotation method) of 1·30 g/cm^3 is very similar to that of cellulose triacetate (Dulmage, 1957) (1·29 g/cm^3). The volume of both unit cells is also the same. The calculated number of triacetyl mannose residues per unit cell was deduced to be eight and the theoretical density is 1·32 g/cm^3. It should be noted that the second unit cell would contain 16 triacetyl mannose residues and the calculated density would be 1·37 g/cm^3, this value is only 6% higher than the measured density and therefore does not rule out the possibility of that unit cell.

Although the unit cell parameters were not reported by Bittiger & Marchessault (1971), two well-defined reflections could be measured on their fibre diagram, one with d-spacing corresponding to 0·974 nm on the equator and another with $d = 0{\cdot}50$ nm on the second layer line. Neither of these d-spacings was observed in this study. In addition, as will be discussed later, the three-fold symmetry of the helix proposed by Bittiger & Marchessault (1971) is not in keeping with the clear evidence favouring a two-fold screw axis with fibre repeat of 1·6 nm which is deduced from the

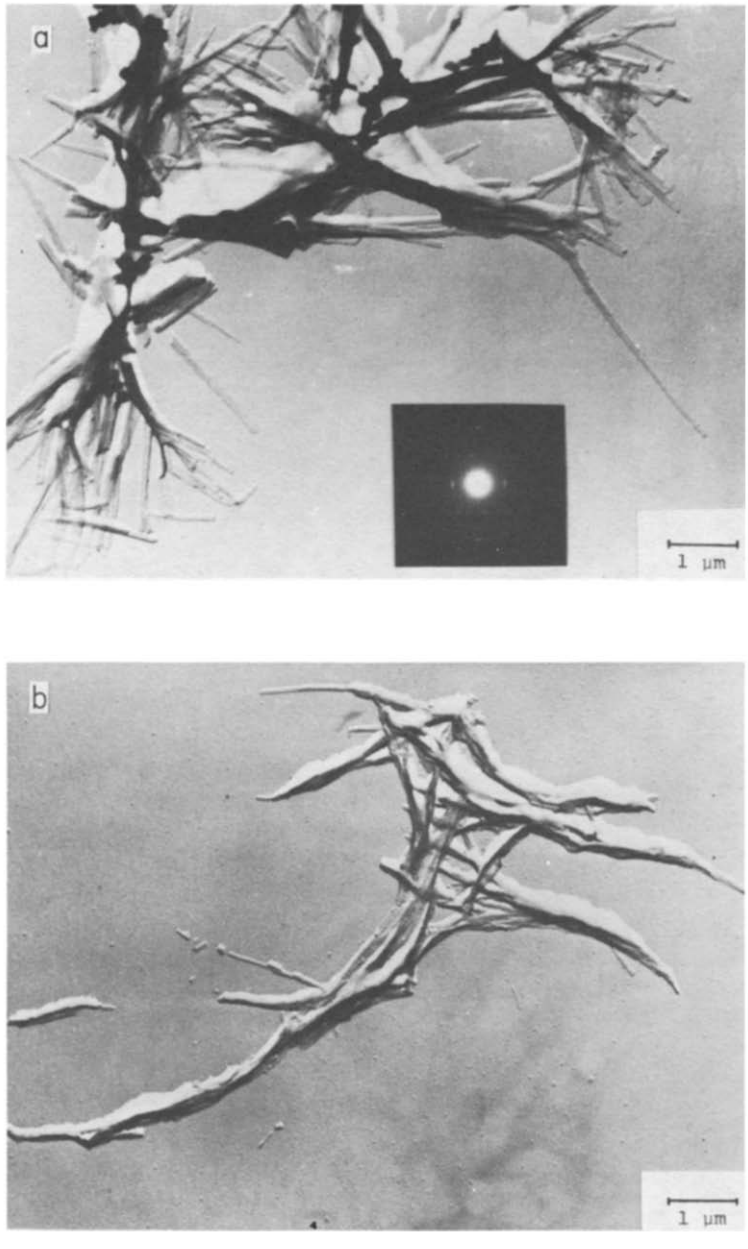

Fig. 1. Electron diffractograms of crystals of ivory nut mannan triacetate. (a) Crystallised by cooling from a nitromethane–butanol mixture at 80°C. The insert corresponds to the electron diffraction pattern. (b) Solution crystallised at 190°C from a mixture of dibenzyl ether and tetradecane.

TABLE 1
Comparison of Interplanar Spacings for Ivory Nut Mannan Triacetate

Miller index	*X-ray powder diffraction (nm)*	*Electron diffraction (nm)*
400	0·89	0·879
210	0·75	0·762
310	0·63	0·658
510	0·55	0·538
020	0·41	0·412
910	0·37	0·360

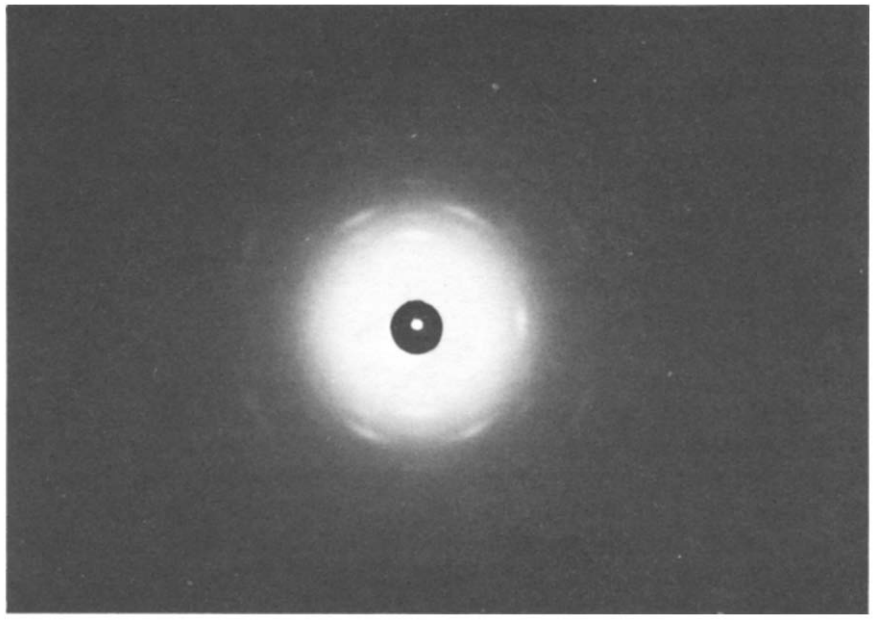

Fig. 2. X-ray fibre diffraction pattern of the glucomannan triacetate fibres recorded with a flat-plate camera. The fibre axis is vertical and the fibre was tilted to the proper angle in order to enhance the meridional reflections.

X-ray diffractogram in Fig. 2 (cf. below). It appears likely that polymorphism in the crystallisation of mannan triacetate is the cause of these differences.

The X-ray pattern shown in Fig. 2 shows discrete layer lines with a spacing of 0·40 nm and meridional reflections occur on layer lines 2 and 4. This is consistent with the presence of a two-fold screw axis. The fibre repeat being 1·60 nm (advance per crystallographic repeat is 0·8 nm), a disaccharide must be considered as the asymmetric unit since the virtual bond length of a (1 → 4)-β-mannopyranoside ring in

TABLE 2
Comparison of Predicted and Observed D-Spacings for *Tubera Salep* Glucomannan Triacetate Fibres Using $a = 1{\cdot}18$ nm, $b = 1{\cdot}54$ nm, $c = 1{\cdot}60$ nm

h k l	*Predicted (nm)*	*Observed (nm)*
1 1 0	0·936	0·935
0 2 0	0·770	0·765
2 0 0	0·589	0·591
3 1 0	0·380	0·381
0 2 1	0·695	0·693
1 1 1	0·806	0·794
2 2 1	0·449	0·448
2 2 2	0·404	0·404
1 1 2	0·609	0·614
1 1 3	0·464	0·466
1 2 3	0·411	0·415
0 3 3	0·370	0·371
2 2 3	0·352	0·352
2 2 3	0·314	0·313
0 2 4	0·355	0·356
1 0 5	0·310	0·311
0 0 2	0·801	0·801
0 0 4	0·400	0·394

the $^4C_1$ chair form is ~5·45 nm. Accordingly an asymmetric unit consisting of two triacetylated pyranoside units is proposed.

DISCUSSION

The interpretation of the data obtained from the X-ray fibre diffractogram of glucomannan triacetate is not straightforward even in the light of the studies published recently on crystallisation of mannans (Chanzy *et al.*, 1979, 1982). One model stands out among others with very interesting implications. The helical parameters and meridional reflections of the glucomannan triacetate fibre diagram imply a disaccharide of asymmetric units. Although both glucose and mannose units are in the backbone of the chain it is unlikely that the asymmetric unit is an acetylated glucosyl mannose since the ratio of mannose to glucose units is more than 3 : 1. Nothing is known about the distribution of the glucose units along the backbone but glucomannans are generally random copolymers (Timell, 1975; Shimahara *et al.*, 1975). The X-ray fibre diagram of Fig. 2 is from a sample which is only moderately crystalline. However, the diffraction arcs are well defined. It is improbable that the crystalline part of the chain was constituted solely of the rarely occurring sequences of acetylated glucosyl-mannose units.

The preferred model for describing the chain conformation of this glucomannan triacetate is based on the conformational equivalence of β-D-mannose and β-D-glucose

in the backbone, as reported by Chanzy *et al.* (1982). Glucose and mannose differ only at C-2 where hydroxyls are equatorial and axial respectively. As a result of this difference, only a small part of the conformational space around the glycosidic linkage will be different for mannobiose versus glucosyl mannose. Comparison of the Φ, ψ space on the energy contour maps of mannan and glucan show that very little difference is observed (Sundararajan, 1969).

In their recent study, Chanzy *et al.* (1982) found that *Tubera salep* glucomannan and four other sources of glucomannan with different molecular weights and different mannose to glucose ratios can yield mannan I and mannan II on crystallisation. The factors influencing the crystallisation are the temperature of crystallisation, the polarity of the solvent and the molecular weight of the crystallising sample. The mannose to glucose ratio does not play any role, and only random variations of the unit cell parameters of glucomannan as a function of the glucose content were observed. This behaviour, isomorphous replacement, which seems to occur in crystalline glucomannan is probably operative in the triacetate also.

A recent chain statistics study on glucomannans (Hallman & Whittington, 1973) supports the conformational equivalence concept. It was shown that introduction of glucose into a mannan chain has little or no influence on the calculated radius of gyration. It is possible that different orientation of two neighbouring C-6 acetyls are responsible for the non-equivalent mannose units in the disaccharide repeating unit. These different orientations would have to be regularly alternated along the chain. Such an explanation was recently used in the case of V-amylose (Zugenmaier & Sarko, 1976).

Predictions of the effect of the acetate substituents on the conformation of polysaccharides have been studied by Marchessault & Sundararajan (1975) who predicted a significant effect on the torsional angles of acetyl oligomers compared with the free sugar oligomers. These differences are then responsible for the variations in crystalline chain symmetry going from the free to the acetylated polysaccharides. Cellulose is an exception since the free and acetylated chains of (1 → 4)-β-D-glucan have been reported to have a similar conformation (Gardner & Blackwell, 1964; Stipanovic & Sarko, 1978). In the case of acetyl mannobiose, two theoretical conformations of minimum energy can be proposed based on the energy maps of the disaccharides. They correspond to two different orientations of the acetate groups. Using $\chi(5)$ and $\chi'(5)$ to define the torsion angle O(5)-C(5)-C(6)-OAc (Fig. 3) in the first and second residue respectively, the first minimum where $\chi(5) = -60^\circ$ and $\chi'(5) = +60^\circ$ corresponds to a Φ, ψ angles set of $\Phi = 65^\circ$ and $\psi = 10^\circ$. The second minimum with $\chi(5) = \chi'(5) = +60^\circ$ is located at $\Phi = 35^\circ$ and $\psi = 20^\circ$.

Examining the helical parameters of those minima one finds that $(\Phi, \psi) = (65^\circ, 10^\circ)$ corresponds to a helix with 3·6 mannose units per turn and a fibre repeat of 1·92 nm. The other minimum corresponds to the structure proposed by Bittiger & Marchessault (1971), i.e., a three-fold helix with a fibre repeat of 1·5 nm. These two different minima of about the same energy and leading to two different conformations support the hypothesis that polymorphism occurs in the crystallisation of mannan triacetate.

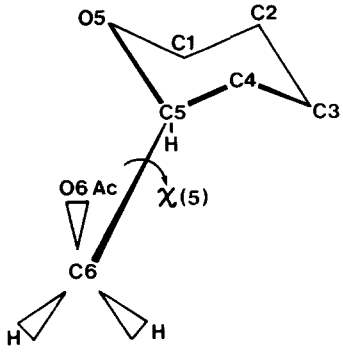

Fig. 3. Schematic diagram showing the definitions of the dihedral angle $\chi(5)$ for β-D-mannose.

The effect of more drastic conditions (high pressure in a sealed bomb with water vapour at 195°C) compared to 210°C in the absence of water vapour led to different crystal forms of glucomannan.

Interestingly, the conformation predicted by those calculations (Marchessault & Sundararajan, 1975) for a cellulose triacetate chain would also correspond to a three-fold helix, whereas the experiments have shown that a two-fold helix is observed in both the cellulose triacetate I and cellulose triacetate II. This indicates that caution should be exercised in the choice of a chain conformation based on energy map calculations when the maps show several minima.

CONCLUSION

A detailed conformational analysis of an acetylated mannan chain based on an acetylated mannobiose unit is needed before any final model is proposed. The same analysis could be done using glucosyl mannose as asymmetric units in order to verify the conformational equivalence of acetylated glucose and mannose in the case of crystalline glucomannan triacetate. This study is underway.

The concept of conformational equivalence or isomorphism has not been examined extensively in polysaccharide crystallography. If one considers the proteins and polynucleotides and their classical helical structures, it is clear that a wide range of copolymer compositions fits into a given helical arrangement. For example, the Pauling helix, the triple helix of collagen and the DNA double helix all accommodate a wide range of monomer compositions with the same helical parameters. Since the same 'grammar' of structural molecular biology applies to polysaccharides, one may ask: what is the potential for isomorphous replacement of one sugar by another? This question is crucial in the case of bacterial polysaccharides when complex and specific sequences of carbohydrates translate into important biological information in terms of conformation.

The influence of acetate substitution on helical parameters of mannan triacetate compared to pure mannan is significant. On the other hand, the cellulose crystalline conformation is relatively unchanged by acetylation.

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