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UNCERTAINTIES IN THE USE OF PERIODATE OXIDATION FOR DETERMINATION OF DEXTRAN STRUCTURE

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ABSTRACT

A glucan of high molecular weight isolated from stale sugar-cane, and previously shown to have a marked effect on sucrose crystallisation processes[1], is a relatively linear dextran Approximately 96–97% of its D-glucose residues are involved in (1→6)-α-D linkages and constitute the linear backbone of the polymer The remaining 3–4% of D-glucose residues form branch-points by (1→3)-α-D linkages The periodate-oxidation technique, which has been extensively used by other workers to determine dextran structure, gave erroneous results when applied to the dextran from stale sugar-cane

INTRODUCTION

For many years, the presence of elongated sucrose crystal ("needle grain") has been an indicator of trouble in the cane-sugar industry, either in a raw-sugar factory or in a refinery[2,3] Tantaoui[4], among others, has reported the association of needle-shaped crystals, in Egypt, with cane infected by micro-organisms C-axis elongation of crystals from stale cane has been reported also from Mackay[5] and Hawaii[6]

In a preceding paper[1], the major causal agent, after isolation from stale cane and subsequent purification, was identified as glucan B_1 Structural studies of this stale sugar-cane dextran are reported herein

EXPERIMENTAL

Materials and methods — Various dextran fractions, *viz*, Dextran T40 (reported M_w ~40,000), Dextran 110 (M_w ~110,000), Dextran 250 (M_w ~250,000) and Dextran 2000 (M_w ~2×10^6), were obtained from Pharmacia AB, Sweden. Sagavac 2F, an agarose gel (theoretical pore-radius, 440Å, determined upper-limit

of molecular weight of included protein, 181×10^6) used for gel-permeation chromatography (g p c), was obtained from Seravac Laboratories Pty. Ltd Berkshire, England

Carbohydrate assay was performed by the phenol–sulphuric method[7], manually, or automatically[8] (Technicon Auto-Analyser equipment) when continuous monitoring of carbohydrate concentration was required

Neutral sugars, after liberation by acid hydrolysis[9], and partially methylated sugars, after liberation by acid hydrolysis[10], were analysed by gas chromatography (g l c) and paper chromatography (p c)

G l c was performed on a stationary phase of 3% of ECNSS-M on Gas Chrom Q, for alditol acetate derivatives[9], or on 10% of LAC-4R-886 on Gas Chrom P in the case of acetonitrile derivatives[11]

P c. (descending) was carried out on Whatman No. 1 and 3MM paper with (*1*) butanone–water (92 8), (*2*) 1-butanol–ethanol–water (5·1 4, upper phase), (*3*) benzene–ethanol–water–ammonia (200 47 15 1, upper phase) Detection was effected with alkaline silver nitrate[12] and *p*-anisidine hydrochloride[13] D-Glucose oxidase (Sigma Chemical Co , Type 11 from *Aspergillus niger*), horseradish peroxidase (Sigma Chemical Co , U S A), and *o*-dianisidine were used in the procedure for enzymic determination of D-glucose[14]

Homogeneity of dextran B_1 — A solution (~1%) of dextran B_1 was examined in 0 05M borate buffer (pH 9 6) by free-boundary electrophoresis, using a Perkin–Elmer model 38 apparatus During the electrophoresis in a standard 15-mm Tiselius-type cell at 3° for 150 min, several Schlieren diagrams were obtained. In all cases, a single peak was obtained

Each of three samples (~4 mg in each case) of dextran B_1, after solubilisation in three different ways, was subjected to gel chromatography on Sagavac 2F The column (1 5 × 28 cm) was eluted with water at 0 1 ml/min (controlled by the Auto-Analyser pump), and the eluate was continuously monitored for carbohydrate The area under each of the two peaks obtained on each chromatogram (typical examples are shown in Fig 1) was measured by a planimeter. Sample (*a*) was solubilised by trituration in water (2 ml) at ambient temperature, subsequently heated at 75° for ~2 h, and then heated in a boiling-water bath for 10 min before cooling to room temperature Sample (*b*) was solubilised by trituration with 0 5M sodium hydroxide (0 7 ml), followed by standing at room temperature for 2–3 h before neutralisation (with acetic acid) and dilution to 2 ml Sample (*c*), after trituration with 0 15 ml of methyl sulphoxide, was allowed to stand overnight before dilution to 2 ml with water.

In addition, a preparative-scale fractionation of dextran B_1 (1 75 g solubilised in 160 ml of 0 02M aqueous ammonium acetate) was achieved on a column (5 5 × 90 cm) of Sagavac 2F by upward-flow elution with 0 02M aqueous ammonium acetate Fractions (5 ml) were collected and grouped, after monitoring for carbohydrate concentration, into Fraction 1 and Fraction 2 Polymer was isolated from the above fractions by lyophilisation.

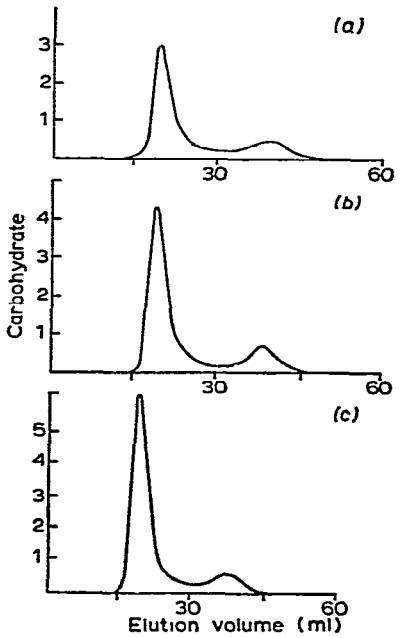

Fig 1 Gel-permeation chromatography of dextran B_1 on Sagavac 2F Solubilised in (*a*) hot water, (*b*) 0 5M sodium hydroxide, (*c*) methyl sulphoxide

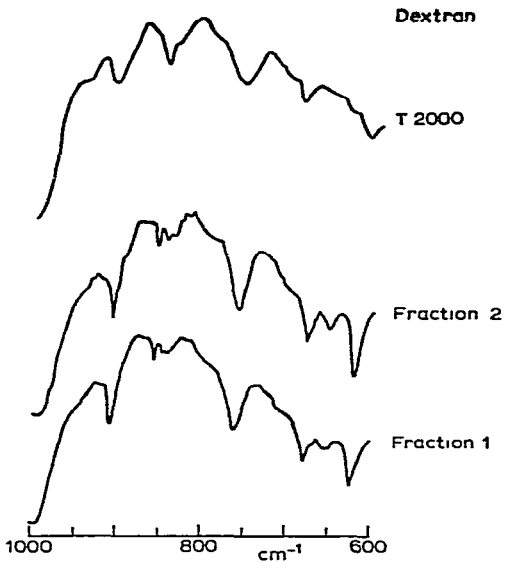

Fig 2 Infrared spectra of dextrans

Preliminary analysis — Carbohydrate assay of dextran B_1 (16 9 mg/500 ml), by the phenol–sulphuric method, corresponded to 97% of glucan In addition, the same polymer (~ 17 mg in triplicate samples) was acid-hydrolysed and then derivatised to yield glucitol hexa-acetate as the only product (100 1%, as glucose equivalents) after g.l.c. (galactitol hexa-acetate as internal standard) analysis.

Fraction 1 and Fraction 2, obtained from dextran B_1, together with Pharmacia Dextran 2000 and the original dextran B_1, were examined in KBr discs (1 mg of polymer/100 mg of KBr) by i r spectroscopy (Fig 2)

Methylation analysis of dextran B_1 — The initial methylation of a sample (2 164 g) of dextran B_1 was by the Haworth procedure[15] This procedure was repeated twice before recovery of the partially methylated glucan (1 92 g) by dialysis and lyophilisation. Further methylation by the Kuhn procedure was attempted, using essentially the experimental conditions described by Hirst and Percival[15], and subsequently by repetition of a modified[16] Purdie methylation procedure When no further change occurred in the i r. spectrum of the product (1 2 g, fractionally precipitated from chloroform solution with light petroleum in the usual manner), a sample (88 mg) was hydrolysed with formic acid (5 ml of 90%) in a sealed tube at 100° for 1 h Further hydrolysis with 0 25M sulphuric acid (5 ml) at 100° for 14 h, with subsequent removal of sulphate ions with Amberlite IRA-400 (acetate) resin and evaporation [38° (bath), 2–3 cmHg], produced a syrup of partially methylated sugars The molar ratios of these sugars, after conversion into acetonitrile derivatives, were determined by g l c

Subsequently, a sample (0 25 g) of dextran B_1 in methyl sulphoxide (12 ml) was methylated by use of methylsulphinyl carbanion[16–18]. Without isolation of the product, this methylation procedure was applied three times A colourless film of methylated polymer (0 24 g) was isolated by dialysis and chloroform extraction in the usual manner Fractional precipitation of this product from a chloroform solution produced a major fraction (203 mg) after addition of ~4 volumes of light petroleum (b p 60–80°) An i r. spectrum of a polymer film of this major fraction, cast on a KBr disc, showed zero hydroxyl absorption Analysis of the methylated fragments, after acid hydrolysis and work-up in the usual manner, was made by g l c (of acetonitrile and alditol acetate derivatives) (Table I), p c , and paper electrophoresis Preliminary fractionation was achieved by descending paper chromatography ~2 h on 3MM Whatman paper with solvent (*1*) After location, each fraction was eluted from the respective strip of the chromatogram. Each of the three fractions was subsequently analysed by co-chromatography with various reference compounds on Whatman No 1 paper (Table II) In addition, the di-*O*-methyl fraction was analysed electrophoretically (400 V, 30 mA) in 0 05M borax for 3 h on Whatman 3MM paper (11 × 34 cm) against reference sugars

Periodate oxidation — (*a*) The dextran B_1 (62 mg/100 ml) was oxidised in the dark at 35° with an aqueous solution of sodium metaperiodate (1 5 mmoles/100 ml) Periodate reduced[19,20] (Fig 3) and formic acid liberated[21] were measured Pharmacia Dextran T2000 was treated in the same way for comparison

TABLE I

G L C IDENTIFICATION OF METHYLATED FRAGMENTS DERIVED FROM DEXTRAN B_1

Methylated sugar	T_G[a] *of alditol acetate derivative*	T_G[a] *of acetonitrile derivative*	*Molar ratio of acetonitrile derivatives*
Tetra-*O*-methyl fraction[b]	1 0	1 0	3 5
2,3,4,6-Tetra-*O*-methylglucose[c]	1 0	1 0	
Tri-*O*-methyl fraction[b]	2 57	2 46	100 0
2,3,4-Tri-*O*-methylglucose[c]	2 57	2 46	
2,3,6-Tri-*O*-methylglucose[c]	2 64	2 57	
2,4,6-Tri-*O*-methylglucose[c]	1 84	1 60	
Di-*O*-methyl fraction[b]	5 33	4 32	3 5
2,3-Di-*O*-methylglucose[c]	5 88	6 75	
2,4-Di-*O*-methylglucose[c]	5 33	4 32	

[a]T_G = retention time relative to that of 2,3,4,6-tetra-*O*-methylglucose (as alditol acetate or acetonitrile derivative) [b]Methylated fragments derived from dextran B_1 [c]Authentic samples

TABLE II

PAPER-CHROMATOGRAPHIC IDENTIFICATION OF METHYLATED FRAGMENTS DERIVED FROM DEXTRAN B_1

Methylated sugars	R_G[a] *(Solvent 1)*	R_G[a] *(Solvent 2)*	*Migration distance (Solvent 3)*
Band III[b]	47	5 4	Coincident
2,3,4,6-Tetra-*O*-methylglucose[c]	47	5 4	
Band II[b]	31	4 5	22 1 cm
2,3,4-Tri-*O*-methylglucose[c]	31	4 5	22 1 cm
2,3,6-Tri-*O*-methylglucose[c]	29	4 3	18 6 cm
2,4,6-Tri-*O*-methylglucose[c]	26		17 8 cm
Band I[b]	13	3 1	3 0 cm
2,3-Di-*O*-methylglucose[c]	16	3 4	3 8 cm
2,4-Di-*O*-methylglucose[c]	13	3 1	3 0 cm

[a]R_G value = migration of methylated sugar relative to D-glucose [b]Chromatographic bands of methylated sugars derived from dextran B_1 after fractionation on 3MM paper [c]Authentic samples

(*b*) In a separate experiment, dextran B_1 (126 mg/200 ml) was oxidised with periodate over a longer period, and the proportion of unoxidised D-glucose (by D-glucose oxidase assay) was measured after reduction with sodium borohydride and acid hydrolysis[22,23] (Table III)

RESULTS AND DISCUSSION

Although the stale-cane dextran B_1 produced a sharp carbohydrate peak[1] on Sepharose 2B or 4B, this was not necessarily indicative of homogeneity because the peak occurred near the exclusion limit of the gel A large molecular size (appar-

ent molecular weight $>20\times10^6$) or, alternatively, the occurrence of molecular aggregates was thus indicated. In fact, rechromatography on a more porous gel, Sagavac 2F, gave two carbohydrate peaks However, the ratio of peak areas changed according to experimental conditions (Fig 1)

Sample	*Solvent*	*Area ratio (peak 1/peak 2)*	*Sum of areas (peak 1+peak 2)*
(*a*)	Cold water	3 8 1	3 0
(*b*)	Hot water	3 8 1	3 0
(*c*)	Alkali	4 3 1	4 1
(*d*)	Me_2SO	9 4 1	5 6

It is unlikely that the changes in relative amounts of the species of different molecular weight have resulted from any change in covalent bonding (*e g*, between water and Me_2SO solvation) The two peaks, therefore, do not represent structurally different polysaccharides The i r. spectra of Fraction 1 and Fraction 2 are identical, the small shoulder at $\sim790\ cm^{-1}$ in each case indicated the same low percentage (probably[24] less than 5%) of (1→3)-linkages (Fig 2) In addition, free-boundary electrophoresis of dextran B_1 in borate solution indicated a single molecular species

It is possible that the two fractions (peaks 1 and 2) represent aggregates, rather than chemical heterogeneity, because the ratio of peak areas changes according to the method of solubilisation From these results, it appears that the sample solubilised by the more effective hydrogen bond-breaker (Me_2SO) produced the highest proportion of species of higher molecular weight, but this would be surprising if peak 1 represents aggregates produced from peak 2 However, the mass of polymer taken for each chromatographic run was approximately the same, and the sum of the peak areas shows that more polysaccharide was recovered from the column after treatment with the more effective solubilising agents It seems more likely, therefore, that, in dextran B_1, a proportion of the material is present as aggregated and relatively insoluble forms, perhaps produced by intermolecular hydrogen bonding during previous drying or storage of the sample Subsequent treatment of the solid with various solvents may then dissolve a variable percentage of the relatively insoluble species, depending on the effectiveness of the solvent as a hydrogen bond-breaker In such circumstances, it is envisaged that the incompletely dispersed material was mechanically held on the column

Comparison of i r spectra of Fractions 1 and 2 with a spectrum of Pharmacia Dextran 2000 indicated structures possessing characteristic absorption bands for (1→6)-glucosidic linkages (917 ±2 and 768 $\pm1\ cm^{-1}$) and for α-glucosidic linkages[25 26] (844 $\pm8\ cm^{-1}$) (Fig 2)

Complete methylation was not achieved by a commonly used sequence of methods, *i e.*, Haworth, Kuhn, and Purdie methods, even though several repetitive Purdie-type methylations were used The final Purdie methylation, although the

polymer was soluble in methyl iodide, produced no change in the i r spectrum, and appreciable hydroxyl absorption was still evident Hydrolysis of the methylated polymer at this stage showed an imbalance of "branch points" to "end groups", *i e*, the ratio of di-*O*-methyl to tetra-*O*-methyl derivatives was ~2 5 1, instead of the anticipated 1.1, as shown below.

Methylated fragment	*Molar ratio*
Tetra-*O*-methyl sugar	6
Tri-*O*-methyl sugar	89
Di-*O*-methyl sugar	14
Mono-*O*-methyl sugar	4

In this case, the mono-*O*-methyl derivative(s) was only tentatively identified by paper chromatography and could indicate incomplete[27] methylation The unusually high resistance to methylation may be related to the unexpectedly low result in the periodate oxidation Hawes[28] also observed that a dextran isolated from sugar-cane juice which had been allowed to undergo microbial deterioration resisted complete methylation by conventional procedures. The incomplete methylation of HO-3 was shown to be responsible for the production of an excess of 2,4-di-*O*-methyl-D-glucose after fragmentation of this methylated dextran

Exhaustive methylation was only achieved by four applications of the Hakomori procedure; the product then exhibited no significant i r. absorption for hydroxyl Almost complete recovery of the polymer was obtained The component sugars, after acid hydrolysis and subsequent g l c analysis, are shown in Table I The molar ratios (3 5 100 3 5) of tetra-, tri-, and di-*O*-methylglucoses indicated that a branch point occurred every 28 or 29 glucose residues The complete absence of mono-*O*-methylglucoses and the good correlation of tetra- with di-*O*-methylglucoses indicate the completeness of methylation and the reliability of the results

The identification of the major methylated fragment, as 2,3,4-tri-*O*-methyl-D-glucose, was made after paper-chromatographic analysis (Table II) and g l c It was interesting to note that, whereas 2,3,4-tri-*O*-methyl-D-glucose was not well-separated from the 2,3,6 derivative but easily resolved from the 2,4,6 derivative by g l c , the 2,3,4 derivative was easily separated from the 2,3,6 derivative, but not the 2,4,6 derivative, by paper chromatography Such an identification indicated that the major part of this glucan consisted of regions of (1→6)-linked, linear chains of D-glucopyranose residues

Similarly, the two minor fragments were identified by p c , g l c , and paper electrophoresis as 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,4-di-*O*-methyl-D-glucose (electrophoretic mobility, M_G 0 09; *cf* 0 21 for 2,3-di-*O*-methyl-D-glucose This indicated that non-reducing end-groups (some 3–4% of all D-glucose residues) are D-glucopyranose residues, while branching of the molecule (some 3–4% of all D-glucose residues) is entirely by (1→3)-linkages

The results of the methylation study are therefore consistent with the IR spectrum of glucan B_1.

Periodate oxidation of dextran B_1, by the procedure which has been very extensively used in structural analysis of dextrans (*e g*, Jeanes *et al* [29]), revealed unexpected results Approximately 1 53 millimoles of periodate per millimole of "anhydroglucose" unit (AGU) (by extrapolation) were consumed with concomitant liberation of 0 75 mequiv of formic acid The periodate uptake formed a plateau after 10–15 h (Fig 3), without significant increase in level subsequently up to 55 h

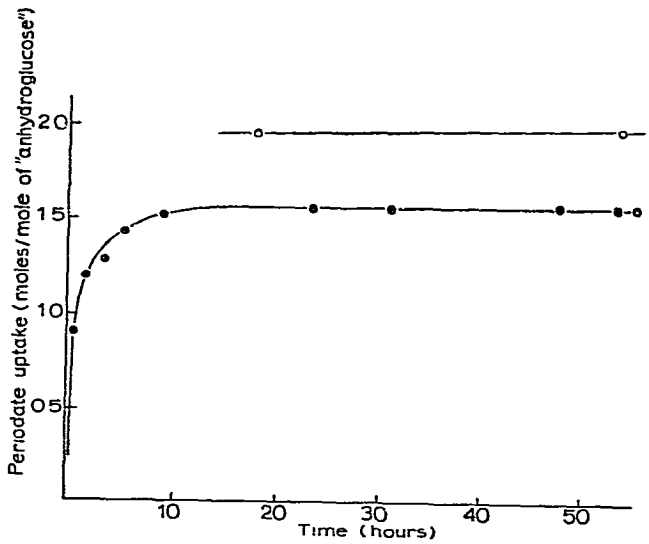

Fig 3 Periodate oxidation of dextrans —O—O—, Dextran T2000, —●—●—, Dextran B_1

Considering the majority of linkages as (1→6), with some possible branch-points at (1→2), (1→3), or (1→4), these results would indicate that 75% are (1→6)-linkages. The moles of periodate reduced minus 2 × moles of formic acid produced [claimed[26] to be a measure of (1→2)- and/or (1→4)-linkages] is 0 03 or ~3% of (1→4)- and/or (1→2)-linkages, while 22% appear to be (1→3)-like linkages Thus indication of D-glucose residues involved in (1→3)-linkages, or alternatively resistant to oxidation, was confirmed when 23% of D-glucose was recovered from the Smith-degradation products of dextran B_1 (Table III) Such results are contradictory to those obtained from the methylation analysis

Resistance to periodate attack of some "hindered" C-3 hydroxyl groups, perhaps weakly bonded[30,31] with aldehyde groups already formed by periodate oxidation, seemed likely. Therefore, dextran B_1 was subjected to periodate oxidation over a period of 38 days to detect any further periodate attack at position 3, in anticipation of a corresponding decrease in the glucose isolated after Smith degradation Further, slow uptake of periodate occurred and subsequent analysis for glucose,

TABLE III

RESULTS OF A 38-DAY PERIODATE OXIDATION OF DEXTRAN B_1

Reaction time (days)	*Periodate uptake (mole/mole of AGU)*	*Glucose (%) recovered after Smith degradation*	*Glucose (%) theoretically expected from periodate uptake*
4	1 53	23	24
11	1 78	8 5	11
18	1 88	4 1	6
38		<1	3–4[a]

[a]3–4% of glucose is expected from the methylation study

recovered after Smith degradation at various time-intervals, revealed a decrease in glucose directly related to the increase in periodate reduced Thus, if one assumes that this dextran contains only (1→6)- and (1→3)-linkages, the expected recovery of glucose (calculated from the periodate reduced) is in reasonable agreement with the glucose actually recovered (Table III)

If the slow, "secondary" oxidation phase represented the further oxidation of previously oxidised glucose residues, the glucose liberated by Smith degradation would have been expected to remain approximately the same, while periodate uptake increased The observed periodate-uptake after 18 days, *viz* , 1 88 moles of periodate/mole of AGU, indicates approximately 6% of (1→3)-linkages, in reasonable agreement with the methylation results However, further slow consumption of periodate was observed for periods up to 35 days, both for dextrans B_1 and T2000

A comparison of the behaviour of dextran B_1 with Pharmacia dextrans, during simultaneous periodate oxidation for 38 days, indicated that the periodate-oxidation method gave the anticipated results with the latter dextrans, *i e* , 1 9 moles uptake after 1 day followed by slow "over-oxidation" up to 2 4 moles after 30 days. Significant differences therefore do exist between the behaviour of this dextran and the Pharmacia dextrans, despite the fact that they have similar numbers of (1→3)-branch points as shown by methylation. Although prolonged periods of oxidation of dextran B_1 may lead to more reasonable results, the problem of differentiation between specific and non-specific oxidation becomes very difficult.

The foregoing results clearly indicate that the periodate-oxidation technique, at least when applied to various stale sugar-cane dextrans, can give very misleading results

We have implied, in the above, that the "hindrance" to complete periodate oxidation may be associated with bonding of hydroxyl groups (*e g* , on C-3) to aldehyde groups formed during early stages of the oxidation (*cf* ref 31), and no doubt this is a partial explanation of the effect However, it seems quite probable that the resistance to complete periodate oxidation may also be related to the abnormal resistance to complete methylation through some form of temporary protection

(*e g*, non-covalent) of HO-3 which is not, as yet, understood It is possible that such effects may also be related to the variable macromolecular association indicated in Fig 1, and that with some dextrans, polymer aggregates may persist in solution during methylation or oxidation, most likely as relatively highly ordered regions If this is the case, then our results indicate that HO-3 is prominently involved in such interpolymer bonding

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