In vitro biosynthesis of acetal using electroporated Acetobacter xylinum cells as enzyme preparations

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Acetobacter xylinum strain NRRL B42 and its derivative RCG1 produce a complex exopolysaccharide, acetan, containing glucose, mannose, glucuronic acid and rhamnose in a 4:1:1:1 molar ratio. The in vitro synthesis of acetan, employing electroporated cells as the enzyme system and the respective 14C-labelled sugar nucleotide precursors, is described. The synthesis of the prenyl-linked heptasaccharide repeat unit, already observed in EDTA-treated cells, was confirmed, as well as the formation of other saccharides not related to acetan biosynthesis, including a high molecular mass glucan. The acetan formed was characterized by gel filtration, specific radioactive labelling with each precursor and permethylation analysis. It was also shown that acetan contains acetyl residues and that using [14C]acetyl CoA as donor, radioactivity was detected both at the polysaccharide and at the prenyl-linked oligosaccharide stage.

Introduction

Acetobacter xylinum is a Gram-negative bacterium characterized by its capacity to synthesize cellulose. Work from this laboratory has shown that, in addition, some strains of this organism are also able to produce a complex exopolysaccharide, acetan, that contains glucose (Glc), mannose (Man), glucuronic acid (GlcA) and rhamnose (Rha) in a 4:1:1:1 molar ratio. The in vitro synthesis of acetan, employing electroporated cells as the enzyme system and the respective 14C-labelled sugar nucleotide precursors, is described. Nevertheless the in vitro synthesis of acetan could not be demonstrated with the enzyme preparations employed. A similar situation has been observed with Rhizobium meliloti (Tolmasky et al., 1982), Agrobacterium tumefaciens (Staneloni et al., 1984) and Rhizobium trifolii (Bossio et al., 1986) preparations. In all these systems the synthesis of the respective repeat units was obtained in vitro but the formation of the polysaccharide, i.e. the presumed polymerization process, could not be detected.

This is not the case for xanthan biosynthesis, an exopolysaccharide produced by Xanthomonas campestris that contains glucose, mannose and glucuronic acid in a 2:2:1 molar ratio, and acetate and pyruvate substituents (Jansson et al., 1975). It was possible to demonstrate, using EDTA-treated cells and the appropriate sugar nucleotide donors, the sequential assembly of the respective repeat unit associated with an endogenous lipid, very likely a diphosphate phenol, as well as the polymerization process that follows, leading to the formation of the final polysaccharide (Ielpi et al., 1981a, 1993). The incorporation of acetyl and ketal pyruvate residues at the prenyl phospho-sugar stage, from acetyl CoA and phosphoenol pyruvate, respectively, has been demonstrated also (Ielpi et al., 1981b, 1983).

In this paper the in vitro synthesis of acetan is cumulate lipid-linked heptasaccharide in vitro and the production of acetan in vivo, suggesting a precursor-product relationship (Iñon de Iannino et al., 1988).

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Abbreviations: Glc, glucose; GlcA, glucuronic acid; Man, mannose; Rha, rhamnose.
Acetobacter xylinum described using a novel enzymic system suggested precursor-product relationship between lipid-linked heptasaccharide and acetan is confirmed by means of two-step incubations.

The incorporation of $^{14}$C-acetyl residues into lipid-linked precursors, already detected employing EDTA-treated cells (R. O. Couso and M. A. Dankert, unpublished observations), as well as into acetan, confirmed previous observations (Jansson et al., 1980).

Methods

**Chemicals.** UDP-$[^{14}$C]$\text{Glc}$ (200–300 Ci mol$^{-1}$; 7.4–114 TBq mol$^{-1}$), UDP-$[^{14}$C]$\text{GlcA}$ (200–300 Ci mol$^{-1}$; 7.4–114 TBq mol$^{-1}$), GDP-$[^{14}$C]$\text{Man}$ (250–300 Ci mol$^{-1}$; 9.25–114 TBq mol$^{-1}$) and TDP-$[^{14}$C]$\text{Rha}$ (200–300 Ci mol$^{-1}$; 7.4–114 TBq mol$^{-1}$) were prepared as described previously (Couso et al., 1982). Radiolabelled glucose and $[^{14}$C]acetyl CoA (55 Ci mol$^{-1}$; 2.03 TBq mol$^{-1}$) were purchased from American Radiolabel Chemical Inc. (ARC) and $[^{14}$C]$\text{Man}$ from New England Nuclear. Tetra- and tri-O-methyl glucose derivatives and unlabelled acetan were obtained as described previously (Couso et al., 1987) and $[^{14}$C]$\text{GlcA}$-labelled hexasaccharide (X$_1$) and heptasaccharide (X$_2$), used as standards, were obtained from the respective lipid-linked derivatives, as reported previously (Couso et al., 1982). Acetylated cellobiose and acetylated mannosyl cellobiose, used as standards, were synthesized in incubations performed in the presence of acetyl CoA, either $^{14}$C-labelled or unlabelled and UDP-Glc (also either $^{14}$C-labelled or unlabelled) alone or plus GDP-Man, as reported previously (Garcia et al., 1974; Couso et al., 1980; R. O. Couso and others unpublished observations). The position and number of the acetyl residues is not known. All other chemicals were commercial products.

**Bacterial strains and culture media.** The acetan-forming strain RCGr1, a derivative from strain NRRL B42 (Iñon de Iasnino et al., 1988) was used throughout this work.

Bacteria were grown and freed from cellulosi as described by Hestrin & Schramm (1954). The cell suspension was centrifuged and the supernatant saved for isolation of acetan. The cell pellet was used either to obtain EDTA-treated cells as described previously by Garcia et al. (1974) or to prepare cells for electroporation. In the latter case they were washed twice with 10% (v/v) glycerol and resuspended in the same solution (0.65 ml for an original culture volume of 200 ml, to a final concentration of 8–10 mg protein ml$^{-1}$).

Starved cells were obtained by resuspending the cell precipitate in 10 mM-sodium phosphate buffer, pH 7.0 (one half, 100 ml, of the original culture volume). They were incubated at 28 °C for 30 min, with shaking and then centrifuged, washed once with water and resuspended in water (0.65 ml for an original culture volume of 200 ml).

**Electroporation of cells and standard incubation.** The following amounts (nmol) of the different sugar nucleotide precursors were added to freshly prepared Acetobacter cells, either starved or not (about 1 mg protein), in a 0.4 ml electroporation cell: UDP-Glc, 24; UDP-GlcA, 24; GDP-Man, 30; TDP-Rha, 9.6.

As indicated in each case, one of the precursors was replaced by the following amounts (pmol) of the corresponding labelled nucleotide: UDP-$[^{14}$C]$\text{Glc}$, 0.69 (10$^6$ c.p.m.); UDP-$[^{14}$C]$\text{GlcA}$, 0.41 (10$^6$ c.p.m.); GDP-$[^{14}$C]$\text{Man}$, 0.42 (0.6$^6$ c.p.m. and TDP-$[^{14}$C]$\text{Rha}$, 0.41 (0.6$^6$ c.p.m.). Where indicated, $[^{14}$C]$\text{acetyl}$ CoA (27 pmol; 0.76–10$^6$ c.p.m.) was also added. In each case the final volume was brought to 0.15 ml with water and kept at 0 °C. Electroporation was performed at 1500 V, 400 μF and 25 μF with a Gene Pulser Transfection Apparatus (Bio-Rad). Usually the resulting time constant was 7.0–8.2.

The solution was then transferred quickly to incubation vials containing Tris/HCl, pH 8.2 and MgCl$_2$ to reach final concentrations of 70 and 10 mm, respectively, in a total volume of 0.20 ml. Incubations were at 30 °C for 60 min. They were ended by adding 0.005 ml 250 mM EDTA adjusted to pH 8.0 by the addition of 2 M-Tris to chelate the Mg$^{2+}$ essential for activity of the sugar transfer enzymes (Garcia et al., 1974).

**Two-step incubations.** The first incubation was performed as described for the standard incubations, but at 10 °C, for 30 min. The reaction was stopped by adding 250 mm-EDTA–Tris, pH 8.0 (0.005 ml), and the cell resuspension was centrifuged in a tabletop Eppendorf centrifuge for 2 min at 10000 r.p.m. The cell pellet was then washed three times with 70 mM-Tris/HCl buffer, pH 8.2, and resuspended in

![Structure proposed for the repeat unit of acetan (Couso et al., 1987; Jansson et al., 1993).](image-url)
Acetobacter xylinum biosynthesis in Acetobacter xylinum

**Results**

**Analysis of the incorporation products**

Previous studies have shown that in incubations performed at 30 °C with EDTA-treated *Acetobacter xylinum* cells, UDP-[\(^{14}\)C]Glca and the other unlabelled sugar nucleotides, the radioactive products in 1203 extracts consisted of prenyldiphosphate heptasaccharide and a lipid-bound glucuronide (Couso et al., 1982, 1986).

Similar experiments have now been performed, but using electroporated cells as enzyme source, and good incorporation of radioactivity into the 1203 extracts (fraction 2) has been obtained also. Analysis by DEAE-cellulose column chromatography of these extracts showed the formation of both compounds mentioned above: lipid-bound glucuronic acid (compound I, eluted at 0.7 M-ammonium acetate) and the prenyl diphosphate oligosaccharide (compound II, eluted at 1.1 M-ammonium acetate) (Fig. 2).

These results prompted a systematic study of the incubation conditions to detect polymer formation. To simplify the analysis of the 1203 extracts, they were not chromatographed on DEAE-cellulose columns but submitted to paper electrophoresis either untreated or after hydrolysis at pH 2.

Preliminary incubations were performed in the presence of UDP-\([^{14}\text{C}]\)Glca, and the other donors unlabelled, at different temperatures (0–30 °C). Good incorporations into the 1203 extracts (fraction 2), that increased with

**Fractionation of the incorporation products.** For each incubation vial, five fractions were obtained, i.e. ten fractions for two-step incubations. The reactions were ended by adding 250 mM-EDTA-Tris, pH 8.0 (0.005 ml to each incubation vial). The cells were spun down and washed three times with 70 mM-Tris/HCl buffer (0.2 ml each). The washings were combined with the incubation supernatants (fraction 1, acetan). In the standard incubations and in the first step of the two-step incubations, this fraction contains the excess of sugar nucleotides and water-soluble products liberated during the incubation period, such as exopolysaccharide (EPS). This fraction (1) was freed from the excess of nucleotides as follows: it was brought to pH 2 with HCl, heated at 100 °C for 10 min and then dialysed against 20 mM-Tris/HCl buffer (pH 7.4) for 4 h at 4 °C, changing to fresh buffer every hour. Then it was dialysed against deionized water at 4 °C for 36-48 h, with two changes for fresh water. The dialysed material was analysed by filtering through a Bio-Gel Agarose A 5 m column (only acetan was detected). In the second step of the two-step incubations this fraction contains the water-soluble products liberated during the second incubation. It was directly analysed by filtering through a Bio-Gel A 5 m column (fraction 6, acetan and other saccharides).

The washed cell pellets were then extracted three times (0.2 ml each) with chloroform/methanol/water (1:2:0.3, by vol.; 1203 solvent) and the extracts combined (1203 extract; fractions 2 and 7). These fractions contain all the lipid-linked sugars formed, that may differ according to the labelled precursor used. It may also contain part of the glucan formed. The 1203-extracted pellets were then washed with water three times (0.2 ml each) and the washings combined (water washings, fractions 3 and 8). These fractions contain water-soluble compounds liberated after the disruption of the cell membranes produced by the 1203 solvent extraction. They contain glucan and lipid-linked oligosaccharide degradation products. The 1203-extracted and water-washed cell precipitates were then resuspended in 1% (v/v) NaOH (0.1 ml) and heated at 100 °C for 5 min, a classical alkaline treatment to isolate cellulose as an insoluble material, since other polysaccharides are soluble (Elbein et al., 1966). After spinning down a supernatant (fractions 4 and 9) and a pellet (cellulose, fractions 5 and 10) were obtained. Samples of each fraction were counted for radioactivity.

Radioactivity was counted in Bray's solution (Bray, 1960) with a 2124 Rackbeta Wallac Liquid Scintillation Counter. 

**Acid hydrolysis.** Sugar-phosphate linkages were hydrolysed by heating at pH 2 (0.01 M-HCl), 100 °C for 10 min as reported previously (García et al., 1974), and total acid hydrolysis of oligosaccharides and polysaccharides was performed in 10 M-HCl, at 100 °C for 20–24 h in a sealed tube.

Borohydride reduction was carried out as described by Couso et al. (1987).

**α-Amylase treatment.** The incubations were performed in 100 mM-Tris/HCl buffer, pH 7.4, containing 50 mM-CaCl\(_2\), 0.22 units of enzyme [2 mg ml\(^{-1}\), 2124 units mg\(^{-1}\) kindly provided by Dr C. Krisman (this Institute)] and substrate in a total volume of 0.1 ml at 37 °C for 48 h in a toluene atmosphere.

**Chromatography and electrophoresis.** Paper chromatography and electrophoresis were done using Whatman No. 1 paper as described previously (García et al., 1974). The following solvents were used: A, chloroform:acetic acid/water (27:4:9, by vol.); B, butan-1-ol/pyridine/water (6:4:3, by vol.); C, 12 M-pyridinium acetate buffer, pH 6.5; D, 0.1 M-sodium molybdate buffer, pH 5.0.

Reducing substances were located with alkaline silver nitrate (Trevelyan et al., 1950) and radioactive areas with a radiochromatogram scanner (Packard, model 7201). UV-absorbing compounds were detected with a Miniray UV lamp.

**TLC** was performed on silica gel G plates (250 μm thick, Merck) in solvent E, benzene/acetone/water/NH\(_4\)OH (sp. gr. 0.91) (50:200:1:0:1:35, by vol.).

The non-radioactive samples were located by spraying with 5% (v/v) H\(_2\)SO\(_4\) in ethanol and heating at 120 °C for 10 min (Li et al., 1978). Labelled compounds were located by radioimaging as above, or by autoradiography using Kodak X-Omat A-R film (X-AR-5).
Fig. 2. DEAE-cellulose column chromatography of a $[^{14}\text{C}]\text{GlcA}$-labelled 1203 extract. The 1203 extract (300000 c.p.m.) was prepared in a four-fold standard incubation at 30 °C. The column was run as described in Methods, collecting 3 ml fractions. After fraction 33, the 0–2 M-ammonium acetate gradient was connected. Samples (0.3 ml) were counted for radioactivity. The numbers on top of the peaks indicate the elution concentration. Compound I: lipid-bound glucuronic acid; compound II: prenyl diphosphate oligosaccharide.

Table 1. Effect of the incubation temperature on the incorporation of $[^{14}\text{C}]\text{GlcA}$

The incubations were performed as for the standard assay but with unlabelled UDP-Glc, GDP-Man and TDP-Rha (16.5 nmol each) and UDP-$[^{14}\text{C}]\text{GlcA}$ (0.19 pmol; 0.28 x $10^6$ c.p.m.) in a final volume of 0.20 ml, at the indicated temperatures, for 60 min. To isolate the polymer fraction, the incubation supernatants (Fraction 1) were processed as indicated in Methods. The Bio-Gel A 5 m filtrates eluting in the position of the acetan carrier, were pooled and counted for radioactivity (Polymer). See Fig. 4.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Polymer* (c.p.m.)</th>
<th>1203 extract* (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt; 30</td>
<td>17060</td>
</tr>
<tr>
<td>10</td>
<td>&lt; 30</td>
<td>26100</td>
</tr>
<tr>
<td>20</td>
<td>1000</td>
<td>33630</td>
</tr>
<tr>
<td>30</td>
<td>3600</td>
<td>55000</td>
</tr>
<tr>
<td>30†</td>
<td>&lt; 30</td>
<td>66240</td>
</tr>
</tbody>
</table>

*The numbers indicate either c.p.m. or pmol GlcA (mg protein)$^{-1}$, on the basis of the specific activity of the $[^{14}\text{C}]\text{GlcA}$.
† In this case EDTA-treated cells were used as enzyme preparation.

The incorporation temperature, were obtained (Table 1). Analysis of the extract obtained at 30 °C showed that, as expected, the radioactivity was associated to lipids since upon paper electrophoresis it remained at the origin of the run (Fig. 3a). Mild acid hydrolysis (pH 2, 100 °C, 10 min) of the 1203 extract released all the radioactivity in two water-soluble compounds that, upon paper electrophoresis, behaved as the heptasaccharide repeat unit ($R_{\text{GlcA}} = 0.5$) and as free $[^{14}\text{C}]\text{GlcA}$, as reported previously (Couso et al., 1982) (Fig. 3b). Gel filtration of the former compound indicated that in addition to the heptasaccharide some hexasaccharide (the repeat unit without the terminal rhamnose) was also present (about 30%) (Fig. 4). The identity of the $[^{14}\text{C}]\text{GlcA}$ was confirmed by paper electrophoresis with buffer D.

Similar results were observed by analysing the 1203 extracts obtained at the other temperatures indicated in Table 1 (not shown).

The incubation supernatants (fraction 1) from the different incubation temperatures were processed as indicated in Methods. At 30 °C, incorporation of radioactivity into a polymer that coeluted with carrier aceten was evident (Fig. 5a). Polymer formation was not
Acetan biosynthesis in *Acetobacter xylinurn*

**Fig. 4.** Bio-Gel P2 gel filtration. The compound of $R_{UMF} = 0.5$ from Fig. 3(b) (about 15000 c.p.m.) was eluted from the electrophorogram and filtered through a Bio-Gel P2 column as indicated in Methods. The arrows indicate the elution position of glucose (Glc), sucrose (Suc), raffinose (Raf) and stachiose (Sta) added as standards of mono-, di-, tri- and tetrascarharides, respectively, detected by the phenol-H$_2$SO$_4$ method (Smith & Montgomery, 1956); $X_6$ and $X_7$, hexa- and heptascarharide repeat units from *A. xylinurn*. BD, Blue Dextran and CoCl$_2$ were added as indicators of total exclusion and inclusion volumes, respectively.

These results are summarized in Table 1, that, in addition, shows a control experiment performed at 30 °C under the same conditions but using EDTA-treated cells. Although a slightly higher incorporation of radioactivity into 1203 extract was observed, no polymer formation was detected, as reported in previous publications (Couso et al., 1982; Ifon de Iannino et al., 1988). Mild acid hydrolysis of this 1203 extract, upon paper electrophoresis, produced a similar radioactive profile as in Fig. 3(b), as expected.

**Incorporation of the other components of acetan**

To confirm that the polymer formed was acetan, incorporation studies similar to the above described were performed using all the different sugars precursors, labelled one at a time. The results are summarized in Table 2. The profiles obtained upon paper electrophoresis of the mild-acid-treated 1203 extracts (fraction 2) confirm previous results obtained with EDTA-treated cells; for each label the main compound detected was the heptascarharide repeat unit ($R_{UMF} = 0.5$) (Fig. 3c, d, e). Bio-Gel A 5 m gel filtration of the incubation supernatants showed that all the sugars were incorporated only into the acetan fraction although in different apparent amounts (Table 2, fraction 1 and Fig. 5a, b). The differences observed are possibly due to the presence of endogenous donors and acceptors in the enzyme preparations used. From experience with EDTA-treated cells it is known that they contain endogenous donors and acceptors. The donors can be partially removed by dialysis, but not the acceptors, that very likely are closely associated to the cell membrane (Ielpi et al., 1993). With electroporated cells the situation should be similar.

To reduce the amount of donors and acceptors, the washed cells were incubated for different periods of time in a buffer without nutrients but with aeration, to allow them to consume their reserves (starved cells).

Cells electroporated after 30 min starvation showed increased incorporation of radioactivity into most fractions (Table 2). With shorter or longer times of starvation, the increment of radioactivity incorporated into the polymer was smaller. The increase in incor-
Table 2. Incorporation of the acetan components into the different fractions analysed

Standard incubations were performed in the presence of the indicated \( ^{14} \text{C} \)-labelled precursor and the incorporation products were fractionated, as indicated in Methods. Incorporation is expressed as c.p.m. (mg protein\(^{-1}\)). Some of the compounds included in the ‘other’ columns were identified as indicated below. Abbreviation: Hepta, prenyl diphosphate hexa-heptasaccharide.

<table>
<thead>
<tr>
<th>Labelled donors</th>
<th>Fraction 1 (supernatant)</th>
<th>Glucan</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP-GlcA</td>
<td>3600</td>
<td>33810</td>
<td>15190*</td>
</tr>
<tr>
<td>GDP-Man</td>
<td>2500</td>
<td>26564</td>
<td>6310†</td>
</tr>
<tr>
<td>TDR-Rha</td>
<td>1100</td>
<td>6910</td>
<td>540‡</td>
</tr>
<tr>
<td>TDR-Rha (starved cells)</td>
<td>2750</td>
<td>14380</td>
<td>–</td>
</tr>
<tr>
<td>UDP-Glc</td>
<td>3330</td>
<td>2560</td>
<td>30400§</td>
</tr>
<tr>
<td>UDP-Glc (starved cells)</td>
<td>6130</td>
<td>118000</td>
<td>618000∥</td>
</tr>
<tr>
<td>Acetyl CoA</td>
<td>4600</td>
<td>8200</td>
<td>46120§</td>
</tr>
<tr>
<td>Acetyl CoA††</td>
<td>8710</td>
<td>10100</td>
<td>49100‡</td>
</tr>
</tbody>
</table>

| Unidentified \( ^{14} \text{C} \)-Rha-containing compounds. |
| Glucan (8300 c.p.m.) (Garcia et al., 1974), prenyl monophosphate galactose (16200 c.p.m.) (Romero et al., 1977a) and an unidentified product (6080 c.p.m.). |
| Glucan (29000 c.p.m.) (Garcia et al., 1974), prenyl monophosphate galactose (15800 c.p.m.) (Romero et al., 1977a) and an unidentified product (17000 c.p.m.). |
| Prenyl diphosphate acetyl cellobiose, prenyl diphosphate acetyl mannosyl cellobiose and their degradation products, the cyclic phosphate esters of acetyl cellobiose and of acetyl mannosyl cellobiose (Garcia et al., 1974; Couso et al., 1980; R. O. Couso and M. A. Dankert, unpublished observations). |
| **Cyclic phosphate esters of acetylated cellobiose and mannosyl cellobiose, as above.** |
| †† Incubation in the presence of unlabelled sugar nucleotide donors. |

* Lipid-bound glucoronic acid (Couso et al., 1986).
† Prenyl monophosphate mannose (Couso et al., 1980).
‡ Unidentified \( ^{14} \text{C} \)-Rha-containing compounds.
§ Glucan (8300 c.p.m.) (Garcia et al., 1974), prenyl monophosphate galactose (16200 c.p.m.) (Romero et al., 1977a) and an unidentified product (6080 c.p.m.).
∥ Prenyl diphosphate acetyl cellobiose, prenyl diphosphate acetyl mannosyl cellobiose and their degradation products, the cyclic phosphate esters of acetyl cellobiose and of acetyl mannosyl cellobiose (Garcia et al., 1974; Couso et al., 1980; R. O. Couso and M. A. Dankert, unpublished observations).

Incorporated radioactivity observed was possibly due in part to the decrease in endogenous donors that isotopically diluted the labelled sugar nucleotide donor. Since the specific activity of the incorporation products was not determined, the increase in sugar incorporation observed may be more apparent than real (Table 2).

In contrast, recent studies have shown that acetan also contains about 4-0% acetyl ester residues (R. O. Couso & M. A. Dankert, unpublished results; Jansson et al., 1993). The incorporation of labelled acetate into the different fractions under study was then investigated using \( ^{14} \text{C} \)acetate as donor. Even in the absence of unlabelled sugar nucleotide donors, \( ^{14} \text{C} \)acetate was detected in the polymer fraction and this incorporation was almost doubled in the presence of the unlabelled sugar donors (Table 2, Fraction 1 and Fig. 5b). As expected \( ^{14} \text{C} \)acetate was also detected in the 1203 extracts (Table 2, Fraction 2). The prenyl diphosphate hexa-heptasaccharide was clearly labelled, but most of the radioactive substances were degraded (fraction 2, other). This subfraction ‘other’ contained neutral and negatively charged components. Analysed by Bio-Gel P2 gel filtration and paper chromatography with solvent A, the neutral components (27500 c.p.m.) were characterized as a mixture of acetylated cellobiose and mannosyl cellobiose. In a similar way the negatively charged fraction (21600 c.p.m.), as well as the water-soluble
Fig. 6. TLC analysis of the permethylation products. Samples of \[^{14}C\]Glc acetan (31000 c.p.m.) (a) and of \[^{14}C\]Glc oligosaccharide repeat unit (20000 c.p.m.) isolated by paper electrophoresis (as in Fig. 2c (b) were permethylated and hydrolysed (see Methods) and the products analysed by TLC with solvent E. The structures of an internal (a) and of a free repeat unit (b) show the origin of the different permethylated glucoses (solid lines), the numbers indicating the position of the methyl groups and the arrows, the respective mobilities. The dashed line corresponds to the terminal glucose originated when the rhamnose is absent and the asterisk (*) indicates the position of the branching sugar. R, rest of the acetan molecule; Or, origin; F, solvent front.

from incubations carried out in the presence of all the sugar donors, TDP-Rha among them, a fair amount of 2,3,4,6-tetra-O-methyl-glucose was consistently detected (Fig. 6b), confirming the presence of hexasaccharide observed by gel filtration (Fig. 4). As expected, in this case instead of 2,6-di-O-methyl-glucose, the 2,4,6-tri-O-methyl derivative was observed, since at this stage the repeat unit is still linear.

The presence of incomplete repeat units both at the acetan- and prenol-linked levels might simply reflect minor damage to the last transglycosylase enzyme produced during the \textit{in vitro} manipulation. In contrast, it demonstrates the possibility of obtaining truncated polysaccharides. This opens the way to look for mutants that might produce new polysaccharides with, perhaps, interesting properties.

During these studies it was observed that with material obtained from unstarved cells the first two glucoses, at the reducing end of the repeat unit, were very difficult to label, indicating the presence of partially built lipid-linked repeat units working as endogenous acceptors (not shown). This difficulty was partially overcome when starved cells were used as enzyme source. For instance, only with these preparations a 1:6 sorbitol:glucose radioactivity ratio was obtained (instead of the theoretical 1:3 ratio) after total acid hydrolysis of borohydride-reduced hexa-heptasaccharide, showing that the reducing glucose had a specific activity smaller than the other glucoses. With material from unstarved cells, the sorbitol:glucose radioactivity ratio was higher than 1:14, indicating a still greater isotopic dilution of the reducing glucose. These results indicate that starvation also reduces the amount of partially built repeat units, working as acceptors.

\textit{Prenyl-linked oligosaccharides as intermediates in the synthesis of acetan}

To show that the 1203-solvent-soluble lipid-linked oligosaccharides are intermediates in the synthesis of acetan, a two-step incubation assay was carried out.

In the first step, in the presence of the four sugar nucleotide donors, one of them labelled, a standard incubation was performed, but at 10 °C, because at this temperature the polymerization process is highly diminished and the lipid-linked sugars accumulate (Table 1). The excess of sugar nucleotides was then removed by centrifugation and the washed cells were reincubated, this time at 30 °C, to allow polymerization.

The results are summarized in Table 3. For instance, using UDP-[\(^{14}C\)]GlcA as labelled donor, from the prenyl diphosphate hexa-heptasaccharide mixture accumulated at the end of the first step (21200 c.p.m., fraction 2, hepta), about half of it remained as such (10100 c.p.m., fraction 7, hepta) after the second incubation, but a significant amount (3000 c.p.m., fraction 6, acetan) was polymerized into acetan. In addition, free hexa-heptasaccharides (6000 c.p.m., fraction 6, hepta) were also detected in this water phase. Furthermore, most of the lipid-bound GlcA released the sugar into this water phase (fraction 6, other).

Using UDP-[\(^{14}C\)]Glc as marker, the distribution of radioactivity was more complex, but even so, easy to interpret. Again, the prenyl diphosphate hexa-
A two-step incubation was performed and processed as described in Methods, at the temperatures and for the times indicated, in the presence of the four sugar nucleotide donors, one of them labelled. Incorporation is expressed as c.p.m. (mg protein)^{-1}. Abbreviations: Hepta, prenyl diphasphate hexa-heptasaccharide; Free hepta, free hexa- and heptasaccharides.  

<table>
<thead>
<tr>
<th>Fraction</th>
<th>1st incubation: 10 °C, 30 min</th>
<th>2nd incubation: 30 °C, 120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 1 (1203 extract)</td>
<td>Heptasaccharide (5160 c.p.m., fraction 7, hepta)</td>
<td>Lipid-bound glucuronic acid with unusual properties (500000 c.p.m., fraction 5, total)</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>free [14C]Man (18000 c.p.m., fraction 2, hepta)</td>
<td>Lipidated glucuronic acid (4600 c.p.m., fraction 5, acetan)</td>
</tr>
<tr>
<td>Fraction 3 (1203 extract)</td>
<td>free [14C]GlcA (18000 c.p.m., fraction 2, hepta)</td>
<td>Lipidated glucuronic acid (22000 c.p.m., fraction 5, hepta)</td>
</tr>
<tr>
<td>Fraction 4</td>
<td>Other glucan (3100 c.p.m.)</td>
<td>Other glucan (4600 c.p.m., fraction 5, acetan)</td>
</tr>
<tr>
<td>Fraction 5</td>
<td>Other glucosulphate (17000 c.p.m., fraction 2, hepta)</td>
<td>Other glucosulphate (31531 c.p.m., fraction 5, acetan)</td>
</tr>
<tr>
<td>Fraction 6 (supernatant)</td>
<td>Hepta (3000 c.p.m.)</td>
<td>Hepta (3000 c.p.m., fraction 5, acetan)</td>
</tr>
<tr>
<td>Fraction 7 (supernatant)</td>
<td>Other glucosulphate (6000 c.p.m.)</td>
<td>Other glucosulphate (6000 c.p.m., fraction 5, acetan)</td>
</tr>
<tr>
<td>Fraction 8 (alkali insoluble)</td>
<td>Hepta (5160 c.p.m.)</td>
<td>Hepta (5160 c.p.m., fraction 6, hepta)</td>
</tr>
<tr>
<td>Fraction 9 (alkali soluble)</td>
<td>Hepta (55565 c.p.m.)</td>
<td>Hepta (55565 c.p.m., fraction 6, acetan)</td>
</tr>
<tr>
<td>Fraction 10 (water (alkali insoluble))</td>
<td>Hepta (35565 c.p.m.)</td>
<td>Hepta (35565 c.p.m., fraction 6, acetan)</td>
</tr>
</tbody>
</table>

Heptasaccharide accumulated during the first incubation (18000 c.p.m., fraction 2, hepta) followed three ways: about one-third of it remained as such (5160 c.p.m., fraction 7, hepta); another third was polymerized (4600 c.p.m., fraction 6, acetan) and the rest was liberated into the water layer as free hexa-heptasaccharide (6225 c.p.m., fraction 6, hepta).

A similar correlation was observed with the Man-labelled precursor, although in this particular case, the amount of polymer formed was low. It should be stressed that in all these experiments, for each label, the radioactivity recovered at the end of the first and second incubations (Table 3, 1st and 2nd totals) were in reasonable agreement, especially considering the number of operations involved. The positive and negative differences obtained are within the experimental error.

Finally, with each label, the compounds not related to acetan biosynthesis were also detected in the respective fractions, as expected (Table 3).

**Sugar incorporation into compounds not related to acetan**

The *in vivo* formation of several lipid-linked sugars not related to acetan biosynthesis, like prenyl monophosphate galactose, prenyl monophosphate mannose and a lipid-bound glucuronic acid with unusual properties have been described in previous publications (Romero et al., 1977; Couso et al., 1980, 1986). All have been detected in the present study (Table 2, fraction 2). Paper electrophoresis of the pH 2-treated 1203[^14C]GlcA-labelled extracts showed, in addition to the hexa-heptasaccharide (*R*$_{MP}$ = 0.5), free[^14C]GlcA, as mentioned above (Fig. 3b) (Couso et al., 1986) and DEAE-cellulose column chromatography of the untreated extracts clearly separated the intact prenyl diphasphate hexa-heptasaccharides (compound II) from the lipid-bound glucuronide (compound I) (Fig. 2).

In a similar way,[^14C]Man and[^14C]Glc-labelled pH 2-treated 1203 extracts produced significant radioactive neutral peaks (Fig. 3c, d). The[^14C]Man neutral compound was confirmed to be free[^14C]Man derived from the hydrolysis of prenylmonophosphate mannose (Couso et al., 1980), but the composition of the[^14C]Glc neutral peak was more complex. Filtered through a Biogel A 5 m column, the profile obtained showed two main components. One was eluted at the position corresponding to a 500000 Da molecular mass glucan. The other one corresponded to the total inclusion volume; it consisted of galactose originated in the hydrolysis of prenylmonophosphate galactose (Romero et al., 1977a) and of an unidentified disaccharide (Table 2, fraction 2, other). The glucan was not retained by filtration through a mixed bed ion-exchange Amberlite MB3 column. It...
was soluble in 5% (w/v) TCA and not degraded by α-amylase treatment.

Furthermore, especially when working with UDP-[14C]Glc as labelled precursor, a series of labelled compounds have been detected in other fractions (Table 2). A water-soluble glucan (about 120000 c.p.m.), already observed in previous studies (García et al., 1974) was by far the most important component. Filtered through a Bio-Gel A 5 m column, this material produced a single peak of radioactivity with an apparent molecular mass of 500000 Da (Fig. 7a), but when chromatographed through an Amberlite MB3 column it was seen to contain at least two components. One was neutral (80450 c.p.m.), since it was not retained by the column, and the other was eluted with 0.8 M-NaCl, indicating the possession of some kind of charge (38700 c.p.m.) (Table 2, fraction 3). This latter material was insoluble in 5% TCA, suggesting that it was associated with a protein. The neutral fraction was not precipitated by 5% TCA and the other was eluted with (80450 c.p.m.), since it was not retained by the column, because it was retained by filtration through an Amberlite MB3 column and more than 80% of the radioactivity was precipitated with 5% TCA. Fraction 4 was insensitive to α-amylase degradation.

Partial acid hydrolysis (0.1 M-HCl, 100 °C, 1 h) of all these glucan fractions produced free glucose and a series of oligosaccharides of decreasing size, as judged by paper chromatography with solvent D. The smallest one was identified as glucosyl(1→2)-β-glucose, sophorose, upon borohydrate reduction and paper electrophoresis with buffer D (not shown).

In the two-step incubation experiment using UDP-[14C]Glc as marker (Table 3) a new interesting point was observed: the shift of radioactive glucan from fraction 4 in the 1st incubation to fraction 8 after the second incubation and the corresponding decrease in fraction 9, suggesting a precursor–product relationship. Since in this particular experiment the different glucan fractions were not analysed in detail, with the present data this result has to be considered as a working hypothesis.

Discussion

The in vitro sequential synthesis of the prenyl-diphosphate-linked heptasaccharide repeat unit of acetal, using EDTA-treated cells as enzymic preparation and the respective sugar nucleotide donors, has been described in previous publications (García et al., 1974; Couso et al., 1980, 1982). Nevertheless, with this enzymic preparation the polymerization product, acetal, was very difficult to detect (L. Ielpi & M. A. Dankert, unpublished observations), although an indirect approach suggested a precursor–product relationship (Ibón de Ihammad et al., 1988). The use of electroporated cells as enzyme source has now allowed the synthesis of polymer in substantial amounts (Tables 1 and 2, Fig. 5).

It looks as if this technique, already widely applied to introduce large molecules into bacterial cells (Poter, 1988) causes less damage to the cell envelope than EDTA treatment, known to release a series of compounds from the interior of the cell, polysaccharides, proteins and nucleic acids among them (Leive, 1974). With this new electroporation technique, the polymerizing enzyme(s) remains active. It is interesting to note that the in vitro synthesis of lipid-linked repeat units using EDTA-treated cells, has been reported for several systems (Tolmasky et
al., 1982; Staneloni et al., 1984; Bossio et al., 1986) but only in the case of xanthan, has a clear in vitro polymerization process been reported (Ielpi et al., 1981, 1993). A detailed study of the electroporation technique, as well as its application to a series of exopolysaccharide-synthesizing systems, will be described elsewhere (C. E. Semino & M. A. Dankert, unpublished results).

The identification of the polymer formed with acetal was based not only on its size but also on the incorporation of the different constituents, glucose, mannose, glucuronic acid, rhamnose and acetate (Fig. 5), and in the permethylation analysis of the [14C]Glc-labelled acetal produced. The branching glucose was clearly detected in the polymer (Fig. 6a), and was replaced by the respective mono-substituted derivative in the heptasaccharide precursor (Fig. 6b). As in the case of xanthan biosynthesis, part of the linear repeat unit becomes a branch in the final product as a consequence of the polymerization process (Ielpi et al., 1981, 1993).

Finally, the two-step incubation experiment showed that at least part of the lipid-linked repeat units are transferred to the polymer fraction confirming the role of intermediates originally assigned (Table 3) (Couso et al., 1982). One point for which there was no clear answer was the presence of free repeat units in the water-soluble supernatant of this experiment (Table 3, fraction 6, hepta). This result may reflect the 'physiological' liberation of repeat units (Amemura et al., 1983) or simply indicate that the enzyme(s) involved in the export process is not size-specific, and less damaged than that involved in the polymerization reaction.

The analysis of the products of permethylated acetal, as mentioned in Results, showed the formation of small amounts of 2,3,4,6-tetra-O-methyl glucose (Fig. 6a), originated in a terminal glucose. Its presence can be explained assuming that in the polymer, some of the repeat units (about 20%) lack the terminal rhamnose. The relative amount of this tetra-methyl derivative is higher in the lipid-linked repeat unit (30-35% of hexasaccharide; Fig. 4 and Fig. 6b), suggesting that the polymerizing mechanism preferentially selects complete heptasaccharide precursors. The presence of truncated repeat units in the polymer opens the possibility, through the isolation of appropriate mutants, to obtain new exopolysaccharides, as described for the xanthan system (Betlach et al., 1987; A. A. Vojnov & M. A. Dankert, unpublished observations). In fact, whilst this paper was in preparation the isolation of Acetobacter xylinum cellulose-minus mutants, one of them called CR 1/4, producing a 'truncated acetal' polymer with a repeat unit of only four sugars, was reported (MacCormick et al., 1993). It is interesting to note that the yield of this 'truncated acetal' was less than that of the wild-type acetal, suggesting that the polymerizing system works better with the wild-type prenyl heptasaccharide precursor, as observed in this study.

Acetal is acetylated in some, for the moment, unknown position. Substantial amounts of [14C]acetate were incorporated both in the acetal and in the hexa-heptasaccharide fractions (Table 2, fractions 1 and 2, hexa-heptasaccharide). But, surprisingly, larger amounts of radioactive were incorporated into the neutral and charged components of the 1203 extracts and of the water washings (Table 2, fraction 2, other; and fraction 3, other). They were identified as a mixture of lipid-linked and free acetylated di- and trisaccharides (cellobiose and mannosyl cellobiose) and their corresponding cyclic phosphate esters, respectively. Lipid diphosphate sugars readily decompose, especially in mild alkaline media, producing the respective 1,2-cyclic phosphate esters, provided the hydroxyl group at C-2 is free and in cis configuration with the phosphate bridge (Couso et al., 1982). It looks clear that [14C]acetate labels only acetal or acetal precursors, but it is not so clear why the different amounts of the acetylated tri- and disaccharides accumulate. It is tempting to think that once the lipid-linked oligosaccharide is acetylated the following sugar has some difficulties in being incorporated, as if the acetylation process should follow the sugar transfer. In contrast, these results show that the first two or three hexoses next to the reducing end of the repeat unit carry one or more acetyl groups.

The enzyme preparation used not only led to the production of acetal but also to all the other compounds already described working with EDTA-treated cells: prenyl monophosphate β-galactose (Romero et al., 1977a), prenyl monophosphate β-mannose (Couso et al., 1980), and a lipid-bound GlcA (Couso et al., 1986).

The synthesis of a glucan, mentioned in early work (Garcia et al., 1974) has been confirmed. Its presence and structure were also reported by other laboratories (Sandermann & Dekker, 1979; Amemura et al., 1985). The glucan formed has now been fractionated into at least three groups: (a) a neutral glucan of apparent molecular mass 500 000 Da, partially extracted with 1203 solvent (Table 2, fraction 2, other) and with water (Table 2, fraction 3, neutral, and Fig. 7a), (b) a protein-associated glucan (charged and TCA-insoluble), also of apparent molecular mass 500 000 Da (Table 2, fraction 3, charged, and Fig. 7a); and (c) a series of smaller molecular mass glucans partially associated with protein (Table 2, fraction 4, glucan alkali soluble, and Fig. 7b). Results from the two-step experiment suggest that this fraction (Table 3, fraction 9) could be a precursor of fraction 8. The biosynthesis of β-1,2-cyclosphorans has been carefully studied in Rhizobium and Agrobacterium systems (Zorreguieta et al., 1990). It looks as if this linear glucan (Amemura et al., 1985) follows a similar bio-
polymerization process. For instance, it is not clear if the newly formed repeat units are incorporated at the non-reducing end (Ielpi et al., 1993) as demonstrated for the O-antigen chains of Salmonella lipopolysaccharide (Robbins et al., 1967).

Reactions 10 and 11 refer to the formation of the prenyl phosphate acceptor, either by removal of a phosphate through a specific phosphatase (Romero, 1977) or by phosphorylation of the free phenol by means of a specific kinase (Romero et al., 1977b).

Reactions 12, 13 and 14 refer to the formation of the galactose, mannose and glucuronic acid derivatives already mentioned, and reaction 15 simply indicates that both cellulose (Ross et al., 1991) and glucans follow independent pathways of biosynthesis. A more detailed study of the latter process is under way.

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