Bacteriophage Receptor Development and Synthesis of O-Specific Side Chains After Addition of d-Galactose to the Uridine Diphosphate-Galactose-4-Epimeraseless Mutant Salmonella typhimurium LT2-M1

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The formation of complete cell wall core lipopolysaccharide (LPS) and O-antigenic side chains after addition of d-galactose to the uridine diphosphate-galactose-4-epimeraseless mutant, Salmonella typhimurium LT2-M1, has been studied by (i) determination of adsorption rates of smooth and rough specific bacteriophages, (ii) passive hemagglutination inhibition, and (iii) qualitative and quantitative determination of the polysaccharide composition and structure. A rapid synthesis of the complete core LPS and O side chains occurred in bacteria in the log phase and the early stationary phase. Phage C21, which attaches to unsubstituted Rc structures, was adsorbed by the bacteria for only 10 min after the addition of d-galactose. Unsubstituted Rc structures, however, could still be detected after 160 min by immunological and chemical assays. Attachment of the P22 phage, which requires O-specific side chains with more than one repeating unit for adsorption, was demonstrated 10 min after the addition of d-galactose. Attachment of the Felix O-1 phage, which requires a complete core, was observed between 20 and 80 min after the addition of d-galactose. The rough specific phases 6SR and Br2 did not adsorb to the bacteria at any time after the addition of d-galactose. By passive hemagglutination inhibition, the presence of O-specific structures could be demonstrated after 10 min. No antigenic activity of the Ra and Rb structures was observed in the LPS preparations isolated at any time after the addition of d-galactose. Methylation analysis of LPS preparations isolated at 10 and 160 min after the addition of d-galactose showed that the O-specific side chains contained an average of 11 and 15 repeating units, respectively. In the 10-min sample, every 25th “Rc structure” carried a side chain, compared to every 3rd residue in the 160-min sample.

Mutants of Salmonella typhimurium lacking uridine diphosphate (UDP)-galactose (Gal)-4-epimerase are unable to synthesize d-galactose. When grown on d-galactose-free media, the polysaccharide part of the cell wall lipopolysaccharide (LPS) contains only 2-keto-3-deoxyoctonate (KDO), a heptose, and d-glucose. This structure corresponds to chemotype Rc (Fig. 1). Besides being a constituent of the core polysaccharide, d-galactose is also part of the tetrasaccharide which forms the repeating unit of the O-specific side chain in S. typhimurium (O-antigen 4.12). Therefore, the UDP-Gal-4-epimeraseless mutant was also unable to synthesize the O-specific hapten. Addition of d-galactose to the epimeraseless mutant yields a smooth phenotype with concomitant appearance of the receptors for the smooth specific P22 phage (4, 5, 15).

The LPS of Salmonella bacteria has been found to contain receptor structures for bacteriophages (10, 12; R. G. Wilkinson, Ph.D. Thesis, University of London, 1966). The P22 phage attaches to bacteria with the serological specificity 4.12 or the closely related 2.12 and 9.12 (23). The Salmonella phage Felix O-1
(FO) is dependent on the presence of the terminal \(N\)-acetyl-D-glucosamine of the core polysaccharide for attachment \(10\). Linking of O side chains to the core makes the FO-receptor structures less accessible, which is reflected in a reduced adsorption rate of the phage to the bacteria \(11\). The rough specific bacteriophages 6SR and Br2 have their receptors in the core region. They are even more susceptible to interference by O side chains than is the FO phage \(\text{(A. A. Lindberg, M. Sarvas, and P. H. Mäkelä, unpublished data).}\)

Phage C21 attaches only to mutants of chemotypes Rc and Rd \(\text{(R. G. Wilkinson, Ph.D. Thesis, University of London, 1966).}\)

The core polysaccharide is synthesized by sequential addition of individual sugar residues, whereas the O-specific side chain is obtained by assembly and polymerization of tetrasaccharide repeating units from a lipid-bound intermediate \(17, 18, 23\). The O side chain “bapten” is then linked to the core. The completed side chain thus consists of two different polymers covalently linked after having been synthesized by different mechanisms.

The aim of the present investigation was to study the development of phage receptors in the galactose-epimerase-less mutant \(S. \text{typhimurium}\) LT2-M1, after the addition of \(D\)-galactose. The results were correlated with a qualitative and quantitative chemical analysis of the polysaccharide structure. The polysaccharide preparations were assayed further by passive hemagglutination inhibition.

**MATERIALS AND METHODS**

**Bacterial strains.** The UDP-Gal-4-epimeraseless mutant \(S. \text{typhimurium}\) LT2-M1 and its parent strain \(S. \text{typhimurium}\) LT2 were used.

**Preparation of cell wall LPS.** The bacteria were grown in a medium of the following composition (g/liter): Tryptone (Difco), 10; Yeast Extract (Difco), 5; \(D\)-glucose, 10; \(\text{NaH}_{2}\text{PO}_4\), 2.5; \(\text{KH}_{2}\text{PO}_4\), 15; \(\text{Na}_{2}\text{SO}_4\), 40; \(\text{MgSO}_4\cdot 7\text{H}_2\text{O}\), 0.5; \(\text{MgCl}_2\cdot 7\text{H}_2\text{O}\), 0.2. No galactose could be demonstrated by gas-liquid chromatographic (GLC) analyses of peracetylated samples of the Tryptone or Yeast Extract. \(D\)-Galactose was then added to the bacterial cultures to a final concentration of 2 g/liter. The cultures were grown as described earlier \(9\). Samples were cooled to 4°C within 3 min and centrifuged for 20 min at 3,900 \(\times g\) at 4°C in an International PR-2 centrifuge. The bacteria were disintegrated in a cell homogenizer (W. Braun, Apparatenbau, Melsungen, Germany). The preparation of cell walls and the extraction of LPS were performed as described earlier \(7\).

**Phages.** The smooth specific phage P22, the smooth and rough specific phage FO, and the rough specific phages 6SR, Br2, and C21 were used \(11\). The experimental procedures for determining phage adsorption rate constants (ARC) by measuring either free phage in the supernatant fraction or the number of phage-infected bacteria have been described earlier \(13\).
Passive hemagglutination-inhibition tests. The experimental procedure for the hemagglutination-inhibition tests was the same as described by Lindberg and Holme (11). The hemagglutination systems were chosen to represent Rc (S. typhimurium 395 MR9), Rb (S. typhimurium 395 MR6 and TV 160), and Ra (S. typhimurium TV 163) chemotypes as well as the wild-type strain (S. typhimurium 395 MS).

Chemical analyses of the cell wall LPS. (i) Sugar analyses. The LPS was hydrolyzed by 0.25 μ H2SO4 at 100°C for 12 hr; the resulting sugars were reduced with NaBH4 and acetylated with acetic acid anhydride: pyridine (1:1). The mixtures of alditol acetates were analyzed by GLC (20).

(ii) Methylation analyses. The LPS was methylated by treatment with 2 m methyl-sulphyniolsodium-methyliodide as described by Hakomori (6). The methylated LPS was hydrolyzed with 90% formic acid for 3 hr at 100°C and then by 0.13 m H2SO4 at 100°C for 12 hr. The hydrolysate was converted into alditol acetates and analyzed by GLC-mass spectrometry (2, 3).

RESULTS

Development of phage receptors. S. typhimurium LT2-M1 was grown in the Tryptone-Yeast Extract medium to about 2 × 10⁸ bacteria/ml (total count). Then D-galactose was added to a final concentration of 2 g/liter. Logarithmic growth continued for approximately 90 min after the addition. At various times (10, 20, 40, and 80 min), samples were taken, and potassium cyanide was added immediately to give a final concentration of 0.001 m. The bacteria were diluted in broth to about 2 × 10⁸ bacteria/ml (total count) before the phages were added.

The ARC for phage C21 before the addition of D-galactose to LT2-M1 was 22 × 10⁻¹¹ ml/min (Fig. 2). Ten minutes after the addition, attachment of phage C21 could no longer be demonstrated.

The FO phage did not attach to LT2-M1 bacteria since the mutant did not contain the receptor structure for this phage. After the addition of D-galactose, attachment was noted after 20 min. The maximum adsorption occurred after 40 min, at which time the ARC was 27 × 10⁻¹¹ ml/min. After 80 min, the ARC was <5 × 10⁻¹¹ ml/min.

The smooth specific phage P22 did not attach to LT2-M1 bacteria. By 10 min after the addition of D-galactose, sufficient receptor material for attachment had been synthesized and the ARC was 96 × 10⁻¹¹ ml/min. After 40 min, the observed ARC was 327 × 10⁻¹¹ ml/min, i.e., the same adsorption rate as with the parent strain S. typhimurium LT2.

No attachment of the rough specific 6SR and Br2 phages was observed at any time.

Measurement of free phage in the supernatant fraction is not a sensitive method when the fraction of phages that has adsorbed is small. Therefore, the ARC for the FO, 6SR, Br2, and C21 phages were determined also by counting the number of phage-infected bacteria. Table 1

![FIG. 2. Adsorption rate constants (ARC) for the smooth specific P22 (▲), the smooth and rough specific FO (■), and the rough specific C21 (●) bacteriophages to Salmonella typhimurium LT2-M1 at different times after the addition of galactose to a culture in the logarithmic growth phase. The ARC was estimated by determining the number of unadsorbed bacteriophages in the supernatant fraction.](http://jb.asm.org/)

<table>
<thead>
<tr>
<th>Time after the addition of D-galactose</th>
<th>ARC</th>
<th>FO</th>
<th>6SR</th>
<th>Br2</th>
<th>C21</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>ml/min</td>
<td>ml/min</td>
<td>ml/min</td>
<td>ml/min</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>17.0 × 10⁻¹¹</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.7 × 10⁻¹¹</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>2.7 × 10⁻¹¹</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>5.6 × 10⁻¹¹</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>10.2 × 10⁻¹¹</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*ARC for the rough and smooth specific FO bacteriophage and the rough specific 6SR, Br2, and C21 bacteriophages to Salmonella typhimurium LT2-M1 bacteria taken at different times after the addition of D-galactose to a culture in the logarithmic growth phase. The ARC was estimated by counting the number of phage-infected bacteria.

TABLE 1. Adsorption rate constant (ARC) for the FO, 6SR, Br2, and C21 bacteriophages*
shows that attachment of the FO phage was not demonstrable until 20 min after the addition of galactose. The maximum ARC value, \(10.2 \times 10^{-11}\) ml/min, was reached after 80 min. Not even with this method could any attachment of the 6SR and Br2 phages be demonstrated, which means that their receptor structures were not accessible on the LT2-M1 cells supplemented with \(\beta\)-galactose. Attachment of phage C21 to the LT2-M1 cells before the \(\beta\)-galactose addition was maximal (ARC \(17.0 \times 10^{-11}\) ml/min). Thereafter, measurable adsorption was observed only in the 10-min sample.

**Passive hemagglutination inhibition.** After addition of \(\beta\)-galactose to bacteria in the early stationary phase, samples were taken at various times for the preparation of cell wall LPS. The extracts were tested for their inhibiting capacity in a number of passive hemagglutination systems (Fig. 3).

A rapid synthesis of the O side chains was indicated, since, by 10 min after the addition of \(\beta\)-galactose, inhibition of the LPS S. typhimurium 395 MS (4, 5, 12a) anti-395 MS serum systems was observed; the concentration was 2 \(\mu\)g of LPS/ml compared to >256 \(\mu\)g of LPS/ml at zero time. All extracts made at later times inhibited the 395 MS/anti-395 MS system by using concentrations <2 \(\mu\)g of LPS/ml.

The LPS S. typhimurium 395 MR9/anti-S. typhimurium 395 MR9 system (chemotype Rc) was inhibited by 2 \(\mu\)g/ml of the zero-time LPS. The extracts up to 60 min after the addition of galactose showed inhibition of this system in concentrations <16 \(\mu\)g/ml. The LPS from LT2-M1 cells grown in the presence of \(\beta\)-galactose overnight, as well as the LPS from the parent strain S. typhimurium LT2 (4, 5, 12a) and from strain 395 MS, did not show any inhibition of the 395 MR9 system in the concentrations tested.

None of the LPS preparations inhibited the S. typhimurium 395 MR6 (chemotype Rb), TV 160 (chemotype Rb), and TV 163 (chemotype Ra) LPS/antiserum systems.

**Sugar analyses of cell wall polysaccharide.** Sugar analyses were performed on LPS preparations from two cultures of S. typhimurium LT2-M1, one to which \(\beta\)-galactose was added during the logarithmic growth phase and the other to which it was added during the early stationary phase. The bacterial densities at the time when \(\beta\)-galactose was added were 0.4 and 2.0 mg/ml (dry weight), respectively.

When \(\beta\)-galactose was added to the culture in the early stationary phase, synthesis and polymerization of the core and the O side chains occurred within 5 min after the addition (Table 2 and Fig. 4). The quantities of the individual

![FIG. 3. Passive hemagglutination inhibition with cell wall lipopolysaccharides (LPS) extracted from Salmonella typhimurium LT2-M1 at different times after the addition of \(\beta\)-galactose to a culture in the early stationary phase. Hemagglutination systems: homologous alkali-treated LPS and serum. ▲, Salmonella typhimurium 395 MS (anti-4, 5, 12a) serum; ■, S. typhimurium TV 163 (chemotype Ra), TV 160 (chemotype Rb), and 395 MR6 (chemotype Rb) and ○, S. typhimurium 395 MR9 (chemotype Rc). Data recorded are minimal concentrations (\(\mu\)g/ml) of LPS causing macroscopic inhibition of the homologous system.

**TABLE 2. Sugar analysis of cell wall lipopolysaccharide of Salmonella typhimurium LT2-M1**

<table>
<thead>
<tr>
<th>Time after the addition of (\beta)-Gal (min)</th>
<th>Mole per cent of detected sugars</th>
<th>No. of repeating units per core stub</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>160</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Grown over night</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

* \(\beta\)-Galactose was added to the culture in the early stationary growth phase.

* Abbreviations: Hep, heptose; Glu, glucose; Gal, galactose; Man, mannose; Rha, rhamnose; and Abe, abequose.

* The ratio (mole per cent of \(L\)-rhamnose + mole per cent of \(D\)-mannose)/(mole per cent of heptose) was used to calculate the amount of repeating units per each core stub (Lindberg, Sarvas, and Mäkelä, unpublished data).

* Samples for methylation analysis hydrolyzed in two steps to minimize destruction of Abe (7).
sugars were used to estimate the amount of O-specific repeating units per core stub (Lindberg, Sarvas, and Mäkelä, unpublished data). The (O-mannose + L-rhamnose)/heptose ratios obtained show that the addition of D-galactose causes a linear increase of O-specific side chain material in the cell wall LPS.

When D-galactose was added to bacteria in the logarithmic phase, samples were taken at 10, 20, 80, and 160 min. The qualitative and quantitative sugar analyses did not reveal any significant differences from the cells in stationary phase.

Methylation analysis of cell wall LPS. LPS isolated from samples taken 10 and 160 min after the addition of D-galactose was subjected to methylation analysis. The LPS preparations were treated with acetic acid at pH 3.5 for 1 hr at 100°C, by which process lipid A was split off. The polysaccharides yielded, in a two-step acid hydrolysis (7), heptose : glucose : galactose : mannose : rhamnose: abequose in the relative molar proportions 2.0:1.0:0.8:0.8:0.8 (10-min sample) and 2.0:2.2:7.4:5.7:5.7:5.7 (160-min sample). The fully methylated polysaccharide, converted to monomeric partially methylated alditol acetates, gave four main and some minor peaks on GLC (Table 3 and Fig. 5). All main peaks were the same as those obtained in the structural investigation of the LPS of S. typhimurium strains 395 MS and LT2 (7, 8).

Mass spectra taken at the ascending part of Table 3. Methyl ethers from the hydrolysates of methylated lipopolysaccharides

<table>
<thead>
<tr>
<th>Sugars</th>
<th>Peak</th>
<th>T°</th>
<th>Molar proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 min</td>
<td>160 min</td>
</tr>
<tr>
<td>D-galactose</td>
<td>A</td>
<td>8.6</td>
<td>6.2</td>
</tr>
<tr>
<td>D-mannose</td>
<td>B</td>
<td>19.5</td>
<td>19.5</td>
</tr>
<tr>
<td>D-glucose</td>
<td>C</td>
<td>21.3</td>
<td>5.5</td>
</tr>
<tr>
<td>D-galactose</td>
<td>D</td>
<td>3.5</td>
<td>1.6</td>
</tr>
<tr>
<td>2:3:4:6-Tetra-O-methyl-D-galactose</td>
<td>E</td>
<td>1.8</td>
<td>1.5</td>
</tr>
<tr>
<td>2:3:4:6-Tri-O-methyl-D-galactose</td>
<td>F</td>
<td>13.4</td>
<td>20.3</td>
</tr>
<tr>
<td>2:3:4-Tri-O-methyl-D-galactose</td>
<td>G</td>
<td>3.6</td>
<td>2.0</td>
</tr>
<tr>
<td>4:6-Di-O-methyl-D-galactose</td>
<td>H</td>
<td>4.1</td>
<td>0.9</td>
</tr>
<tr>
<td>2:3:4:6-Tri-O-methyl-D-mannose</td>
<td>I</td>
<td>5.1</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* Retention times (7) of the corresponding alditol acetates on the ECNSS-M column relative to 1,3-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol.

b Since a considerable proportion of 2,4-di-O-methyl-abequose was lost during the methylation analysis, the per cent of the methylated sugars is given relative to that of 2,3-di-O-methyl-L-rhamnose, assumed to be the same as the molar per cent of L-rhamnose in the original lipopolysaccharide. The figures for the methyl ether of L-rhamnose in the 10- and 160-min samples are adjusted to the value (19.3) found for this ether in the wild-type strain S. typhimurium LT2. The percentages of the other methylated sugars are given relative to the 2,3-di-O-methyl-L-rhamnose derivative.

c Not detected.

FIG. 4. Changes in sugar composition of cell wall lipopolysaccharide from Salmonella typhimurium LT2-M1 at different times after the addition of D-galactose to a culture in the early stationary growth phase. The figures represent mole per cent of detected sugars. Heptose (○), D-glucose (∆), D-galactose (●), and D-mannose (■).

FIG. 5. Gas-liquid chromatographic separation of methylated sugars, as their alditol acetates, obtained from the hydrolysate of the fully methylated lipopolysaccharide from Salmonella typhimurium LT2-M1 10 min after the addition of D-galactose. The identities of the designated peaks are given in Table 3.
peak D gave the expected fragments of a 1,2,5-
tri-O-acetyl-3,4,6-tri-O-methylhexitol which, from its retention time (T = 1.98), was identified
as derived from 3,4,6-tri-O-methyl-D-glucose.
The signals obtained in the mass spectra taken at
the latter half of peak D showed the presence of a 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylhexitol which,
from its retention time (T = 2.08), was identified as derived from 2,4,6-tri-O-methyl-D-mannose. Peak F was identified as the 2,3,4-
tri-O-methyl-D-glucose derivative. This sugar is
assumed to derive from the core polysaccharide,
i.e., as Glu-I with Gal-II linked to C-6 (Fig. 1).
Peak H gave the mass spectrum of a 1,3,4,5-
tetra-O-acetyl-2,6-di-O-methyl-hexitol which,
from its retention time (T = 3.65), must be
derived from 2,6-di-O-methyl-D-galactose. The
3,6-di-O-methyl-D-galactose (peak I) and the
2,4-di-O-methyl-D-glucose (peak K) are probably
derived from the core.

The average number of repeating units in the
O-specific side chains was calculated from the
amount of 2,4,6-tri-O-methyl-D-mannose (rep-
resenting the terminal mannose of the O side
chain) compared to the amount of 4,6-di-O-
methyl-D-mannose and 2,4,6-tri-O-methyl-D-
mannose (representing the total amount of
mannose in the O side chain). It was found that
the O-specific side chains from the 10-min sample
contained 11 repeating units and those from the
160-min sample contained 15.

The presence of unsubstituted Glu-I residues
in the core is indicated by a calculation of the
amount of 2,3,4,6-tetra-O-methyl-D-glucose.
Assuming a core structure as shown in Fig. 1,
no nonreducing terminal glucose units, except
for unsubstituted Glu-I and Glu-II residues, are
expected. In the O-specific side chains, the amount
of D-glucose linked to the 4-position of D-galac-
tose [the determinant structure of O-antigen
122 (22)] is estimated from the amount of 2,6-
di-O-methyl-D-galactose. The 10-min sample
thus contained 21.3 minus 3.6% = 17.7% of
terminal D-glucose residues in the core. The 160-
min sample contained 3.5%, whereas the wild-
type strain LT2 displayed no terminal D-glucose
units in the core. It is probable that most of the
terminal glucose is derived from the Glu-I residue
since no serological activity for an intermediate
core structure could be detected (Fig. 3).

DISCUSSION

The addition of D-galactose to bacteria in the
logarithmic and the early stationary phase
resulted in a rapid synthesis of the complete core
polysaccharide and O-specific side chains. This
synthesis was demonstrated with the three sets
of methods used: phage attachment velocities,
passive hemagglutination inhibition, and quan-
titative sugar analysis.

The homogeneity of the culture with respect to
the synthesis of O side chains was indicated by
the results of the ARC determination of phage
C21. If a fraction of the culture had been unable
to respond to the exogenous D-galactose, the
cells of this fraction would still adsorb phage
C21. Since C21 attachment could not be demon-
strated for more than 10 min after the D-galactose
addition, no inactive cells seemed to be present
within the experimental limits of detection.

Attachment of the smooth specific P22 phage,
which is known to require more than one re-
peating unit in a side chain (Lindberg, Sarvas,
and Mäkelä, unpublished data), was demonstra-
table 10 min after the addition of D-galactose.
Forty minutes after the addition, the amount of O
side chain material present was sufficient to
result in an ARC similar to that for the wild-
type strain S. typhimurium LT2. The structural
studies using methylation analysis of the LPS
showed that the O-specific side chains contained
an average of 11 to 15 repeating units.

The rough specific C21 phage (22) attacks only
mutants of chemotypes Rc and Rd. When Gal-I
and Gal-II were linked to the Glu-I of the core
polysaccharide, the C21 phage no longer at-
tached to the bacteria (12). The addition of
D-galactose to the LT2-M1 cells resulted in a
rapid reduction in the accessibility of the C21
receptors. After 10 min, the ARC was only
0.7 x 10^{-11} ml/min compared with 17.0 x 10^{-11}
ml/min obtained before addition of D-galactose.
At later times, no attachment of C21 could be
demonstrated. Both the passive hemagglutination
inhibition and the methylation analysis showed
that terminal structures (Glu-I) of chemotype Rc
were present in the LPS for more than 10 min
after the addition of D-galactose. The immuno-
logical tests still revealed an inhibition of the
homologous S. typhimurium 395 MR9 LPS/
antiserum system after 160 min. A comparison
of the relative amounts of 3,6-di-O-methyl-D-
glucose (assumed to represent a completed core)
and 2,3,4,6-tetra-O-methyl-D-glucose (cor-
rected for the 1,4-linked glucose of the O side
chain and thus assumed to represent unsub-
stituted Glu-I residues) showed that, after 10
min, every 25th Glu-I residue and, after 160
min, every 3rd carried a completed O side chain.
The introduction of O-specific side chains reduced
the attachment of the C21 phage, probably
through steric interference.

The FO phage attacks rough mutants of
chemotype Ra, containing a terminal N-acetyl-
D-glucosamine residue (10). Since the amino
sugar is terminal also in smooth strains (16),
they are also susceptible to the FO phage. However, the ARC of the phage is much lower to smooth strains than to complete rfb mutants (nonleaky mutants of chemotype Ra; Lindberg, Sarvas, and Mäkelä, unpublished data). Maximal ARC were recorded after 40 to 80 min; however, the values were low compared with Ra mutants (13). The passive hemagglutination inhibition did not reveal at any time after the addition of D-galactose the presence of sites with the serological specificity of chemotype Ra (Fig. 3). Thus, most of the completed core stubs were immediately substituted by O-specific side chains. Even if every completed core stub carried N-acetyl-d-glucosamine, the receptor sites must be sufficiently close to each other for FO attachment to occur. This requirement was not fulfilled until 20 min after the addition of D-galactose, when it could be estimated that every 10th Glu-I was substituted, suggesting that FO attachment required multiple sites on many side chains.

The rough specific phages 6SR and Br2 have their receptors in the LPS of rough mutants. Both phages seemed more sensitive to interference by O side chains than did the FO phage (Lindberg, Sarvas, and Mäkelä, unpublished data). No attachment of the phages could be demonstrated at any time after addition of D-galactose, indicating that the synthesis of the core LPS and attachment of O side chains did not stop at an intermediate step. This observation was in agreement with the findings for the FO phage. Furthermore, the passive hemagglutination inhibition did not reveal any antigenic specificity which could be attributed to the core structures (Fig. 3).

P22 attachment and the passive hemagglutination inhibition proved to be sensitive criteria for the detection of small amounts of O-antigenic side chains. However, it has been previously demonstrated (Lindberg, Sarvas, and Mäkelä, unpublished data) that serological tests were more sensitive than phage P22 attachment for the detection of O-specific material. The serological and chemical analyses detected residual Glu-I structures in the core where no phage C21 attachment was observed. Attachment of the FO phage requires the presence of terminal N-acetyl-d-glucosamine units on the core (10). The serological specificity of this terminal amino sugar in rough mutants has been termed RII (1). LPS preparations from bacteria which adsorbed the FO phage displayed no inhibition of the homologous S. typhimurium TV 163 (chemotype Ra) LPS/antiserum system in concentrations up to 256 μg of LPS/ml. Semirough (SR) strains, in which most core stubs probably are capped by a repeating unit, also yield an LPS for which no serological RII specificity has been detected either by complement fixation (14) or passive hemagglutination inhibition. Thus, FO attachment seemed to be more sensitive for the detection of the N-acetyl-D-glucosamine of the core than the serological tests used.

The data obtained in the quantitative sugar and methylation analyses were used for a calculation of the amount of O-antigenic side chains synthesized. The sugar analysis presented in Table 2 shows that the amount of abequose is not stoichiometric with the amounts of D-mannose and L-rhamnose. This lack of stoichiometry has been shown to be due to the destruction of part of the abequose during acid hydrolysis of the LPS (7). The amount of heptose was used for an estimation of the LT2-M1 (Rc) structure, whereas the amounts of L-rhamnose and D-mannose represented the O side chains. The addition of D-galactose initially causes a linear increase in the amount of O side-chain sugars.

Measurements of the phage attachment velocities were performed on bacteria in the logarithmic growth phase, whereas the hemagglutination-inhibition tests and sugar analyses were made on LPS preparations extracted from bacteria in the early stationary phase. The samples from cultures to which D-galactose was added during the stationary phase had a bacterial density five times greater than those from log-phase cultures. Therefore, only the former samples yielded sufficient LPS from isolated cell walls for both the chemical and immunological experiments. LPS prepared from log-phase cells was also analyzed by GLC. No significant differences in carbohydrate composition between log- and stationary-phase cells were observed. It is interesting to note that synthesis of the complete core and O side chain also occurred rapidly in nongrowing cells.

By using the ratio (2,4,6-tri-O-methyl-D-mannose + 4,6-di-O-methyl-D-mannose)/(2,4,6-tri-O-methyl-D-mannose), it could be estimated that the average O side chain in the 10-min sample contained 11 repeating units, whereas the 160-min sample contained 15 repeating units. In a previous paper, the average number of repeating units in the wild-type strain S. typhimurium LT2 was estimated to be eight per O side chain (8). The immunological results and the determinations of the phage ARC suggested that as soon as a complete core stub had been synthesized it was substituted by an O side chain. This would mean that the synthesis of the O side chains is either proportional to the synthesis of the core or that an excess of O-antigenic hapten is synthesized. However, no quantitative measurements of the rate of the "hapten" synthesis could be done. The increased number of
repeating units in the O side chains of the D-galactose-supplemented LT2-M1 bacteria, as compared to the parent strain, could indicate that the synthesis and polymerization of the repeating units occur at a higher rate than the synthesis of the complete core polysaccharide.

A calculation of the number of Glu-I residues substituted by an O side chain can be done by using the data on the sugar composition and assuming that every O side chain is built by an average of 15 repeating units. Ten minutes after the addition of D-galactose, every 25th Glu-I residue carries a side chain; after 40 min, every 10th; and, after 160 min, every 3rd. D-Glucose linked (1 → 4) to D-galactose in the repeating unit is the immunodominant sugar of O factor 12 (22). In the wild-type strain LT2, about 50% of the D-galactose residues carried D-glucose (Table 3). The corresponding figures for the 10- and 160-min samples were 18 and 10%, respectively. Present evidence indicates that the O-antigenic side chain is formed by polymerization of abequose → mannose-rhamnose-galactose tetrasaccharide repeating units from a lipid-bound intermediate to an O-specific hapten (17, 18). The O-specific hapten is then transferred from the antigen carrier lipid to the completed LPS core. The D-glucose residue of O factor 122 is probably transferred to the O side chain (19; H. Nikaido, personal communication). This suggestion is strengthened by the fact that a purified haptenic fraction does not react with O 122 antibodies in passive hemagglutination inhibition (unpublished data). The discrepancy in the amounts of (1 → 4) linked D-glucose between the three different LPS preparations was made apparent only in the methylation analysis; in the serological tests all showed antigen 122 activity.

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