EVIDENCE FOR AN INTERMEDIATE STAGE IN THE
Biosynthesis of the Salmonella O-Antigen*

By A. Wright, M. Dankert,† and P. W. Robbins

DEPARTMENT OF BIOLOGY, DIVISION OF BIOCHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY

Communicated by John M. Buchanan, May 24, 1965

The O-antigen of E-group Salmonella strains such as S. anatum and S. newington consists primarily of long polysaccharide chains attached to a complex core or backbone structure.1 The polysaccharide chains have a basic repeating glycosidic sequence that can be represented simply by the formula

\[(-D-mannosyl-L-rhamnosyl-D-galactosyl-)_n.\]

We have recently reported the enzymatic synthesis of these O-antigen chains from the nucleotide precursors UDP-D-galactose, TDP-L-rhamnose, and GDP-D-mannose.2 In the present paper, evidence is presented for an intermediate stage in the biosynthesis of the S. newington O-antigen in which sugars are transferred from the nucleotide sugar precursors to an acceptor prior to incorporation into lipopolysaccharide. Omission of GDP from the in vitro O-antigen synthesizing system leads to the accumulation of a compound which has been characterized as a rhamnosylgalactosylphosphate-derivative. On subsequent addition of GDP to the system containing this disaccharide intermediate, oligosaccharide repeating units are incorporated into O-antigen chains. The isolation and some properties of the disaccharide intermediate are described.

Particulate enzyme systems that catalyze the synthesis of the S. typhimurium O-antigen have been described by Zeleznick et al.3 and by Nikaido and Nikaido.4 The demonstration of an intermediate stage in the S. typhimurium system similar to that described in the present paper appears in the accompanying paper by Weiner et al.5

Materials and Methods.—The bacterial strain used for this study was the lysogenic strain of Salmonella anatum, A1 (e14) (= Salmonella newington).1 A1 (e14) cells grown as previously described

---

3 Robbins, P. W., A. Wright, and J. L. Bellows, these PROCEEDINGS, 52, 1302 (1964).
7 Osborn, M. J., these PROCEEDINGS, 50, 499 (1963).
BIOCHEMISTRY: WRIGHT, DANKERT, AND ROBBINS
PROC. N. A. S.

and treated with EDTA were used throughout this work. The sources of nucleotide sugars UDP-Gal-C14, UDPG, GDPM, and TDPRh were as previously described. Since EDTA-treated cells contain UDPGal-4-epimerase activity, UDPG was used as the source of UDPGal. This substrate is referred to in the text as UDPG-Gal. Studies with labeled UDPG-Gal show that galactose is the only sugar incorporated from this substrate under the conditions described in the present paper. TDPRh-C14 was prepared enzymatically from glucose-C14 and TTP with a sonic extract of JR-11, a mutant strain of E. coli C600 lacking phosphoglucose isomerase. The procedures for isolating and analyzing lipopolysaccharide and for measuring the incorporation of C14 from a nucleotide sugar into cell wall-membrane fractions have been described previously. Large-scale enzymatic reactions were stopped by the addition of 1 vol of 1 N acetic acid. The precipitates were washed with 1 N acetic acid and then with water by centrifugation and resuspension. The following chromatographic solvents and electrophoresis buffers were used. Solvent 1, n-butanol-pyridine-water (6:4:3); solvent 2, 1 M ammonium acetate-ethanol (3.5:7); pH 7.5; solvent 3, n-butanol-pyridine-0.05 M morpholinium tetraborate, pH 8.6 (7:5:2); buffer 1, 0.05 M triethylammonium acetate-0.1 M acetic acid, pH 4.0; buffer 2, 0.05 M sodium borate, pH 9.2. Chromatographic and electrophoretic separations were carried out on Whatman no. 1 or 3 MM paper. Radioactivity was located with a Vanguard 4-pi windowless strip scanner or by radiography.

Results.—TDPRh-C14 incorporation: Previously reported experiments demonstrated that the incorporation of galactosyl residues from UDPG-Gal-C14 was stimulated strongly by the addition of TDPRh and GDPM. Comparable experiments with TDPRh-C14 are shown in Figure 1. Little or no incorporation takes place when cells are incubated with TDPRh-C14 alone or with TDPRh-C14 and GDPM. A rapid but limited incorporation occurs when cells are incubated with TDPRh-C14 and UDPG-Gal, and extensive synthesis takes place when all three nucleotide sugars are present. The dependence of the last two incorporation reactions on the concentration of TDPRh-C14 in the incubation system is shown in Figure 2. As can be seen, the enzymatic system that catalyzes the limited reaction of TDPRh with UDPG-Gal is saturated by a very low concentration of TDPRh-C14. Evi-

![Fig. 1.—Time course of TDPRh-C14 incorporation by EDTA-treated cells. Incubation mixtures contained 50 μmoles of Tris hydrochloride, pH 7.8; 20 μmoles of MgCl2; 5 μmoles of EDTA, added with the enzyme; 52 μmoles of TDPRh-C14 (13,000 counts); 100 μmoles of UDPG-Gal and 500 μmoles of GDPM, where indicated; and 0.5 ml of EDTA-treated cells in a total volume of 1 ml. Incubation was at 37°, and aliquots (0.1 ml) were taken at the indicated times and analyzed by the procedure described in the text. Zero time samples were obtained by preparing incubation mixtures at 0°.](image1)

![Fig. 2.—Incorporation of rhamnose as a function of TDPRh concentration. Incubation mixtures were as described in Fig. 1, except that 100 μmoles/0.1 ml each of UDPG-Gal and GDPM were added, where indicated. The TDPRh-C14 specific activity was 2880 cpm/μmole. Incubations were at 37° for 30 min.](image2)
FIG. 3.—Time course for rhamnose-C\textsuperscript{14} incorporation in the presence and absence of added GDPM. A single incubation mixture was prepared containing 250 \( \mu \)moles of Tris hydrochloride, pH 7.8; 100 \( \mu \)moles of MgCl\(_2\); 5.5 \( \mu \)moles of TDPRh-C\textsuperscript{14} (366,000 cpm); 2.5 \( \mu \)moles of UDPGal; and 2.5 ml of EDTA-treated cells in a total volume of 5.0 ml. Incubation was at 37\(^\circ\). Zero time samples were prepared separately at 0\(^\circ\). After mixing, the incubation mixture was divided into two equal portions. GDPM (0.2 ml; 10 \( \mu \)mole/ml) was added to one sample at 12 min (+GDPM); 0.2 ml of water was added to the second sample at the same time (−GDPM). Total incorporated counts were measured by the Millipore method with 0.1-ml aliquots. Total extractable counts were measured with 0.2-ml aliquots by the following method. The sample was diluted to 8 ml with 1 M acetic acid in a Spinco ultracentrifuge tube. The contents of the tube were centrifuged at 105,000 \( \times g \) for 10 min and the supernatant fluid was discarded. The pellet was resuspended in 3 ml of water by brief sonication (30–60 sec) with an MSE probe-type sonicator. The suspension was extracted three times with n-butanol, and the combined extracts (5–6 ml) were washed with 1 ml of water. The radioactivity in aliquot (1 ml) of the butanol layer was measured in a liquid scintillation counter.

dence that this limited reaction probably represents the synthesis of an antigen precursor is shown in Figure 3. Preliminary experiments had shown that the incorporation product formed in the absence of GDPM was, to a large extent, extractable with butanol and that antigen was not extractable. The incubations were carried out at a concentration of TDPRh-C\textsuperscript{14} at which the rate and extent of incorporation would be approximately the same in the presence or absence of GDPM. As can be seen, butanol-extractable material accumulates early in the reaction but disappears rapidly after the addition of GDPM. These results suggest that an extractable rhamnosyl-galactosyl-disaccharide derivative is synthesized in the absence of GDPM and that this disaccharide derivative is incorporated into non-extractable 0-antigen after the addition of GDPM.

Further evidence in support of the formation of a disaccharide derivative came from a detailed study of the materials synthesized in the presence and absence of GDPM and isolated by the classical phenol procedure.\textsuperscript{1} A striking difference between the two products was that the former was 90 per cent nondialyzable, whereas the latter was 80–90 per cent dialyzable. Identification of the major component of the dialyzable fraction as the disaccharide rhamnosyl-galactose is described below. A detailed study of the nondialyzable fractions has been carried out and
will be described elsewhere. The results show that in the presence of GDPM, labeled rhamnose is incorporated into long 0-antigen chains, whereas in the absence of GDPM it is found solely as nonreducing terminal units.

**Isolation and properties of disaccharide and disaccharide intermediate:** Recent chemical studies in our laboratory have led to the isolation and characterization of the 1,3-L-rhamnosyl-\( \beta \)-galactose disaccharide derived from the *S. newington* 0-antigen.\(^5\) This disaccharide was compared with dialyzable labeled material prepared by direct phenol extraction of cells incubated with TDPRh-C\(^{14}\) and unlabeled UDPG-Gal or unlabeled TDPRh and UDPG-Gal-C\(^{14}\). The results of these comparisons are as follows:

(a) Both labeled compounds have the same mobility on chromatography in solvent 1 and on electrophoresis in buffer 2 as an authentic sample of rhamnosylgalactose prepared by acetolysis of purified *S. newington* lipopolysaccharide.

(b) Complete acid hydrolysis (1 N HCl for 1 hr at 100\(^\circ\)) gives only rhamnose-C\(^{14}\) from the rhamnose-labeled compound and only galactose-C\(^{14}\) from the galactose-labeled compound. Borohydride reduction followed by acid hydrolysis of the two compounds gives rhamnose-C\(^{14}\) in one case and galactitol-C\(^{14}\) in the other case, as shown by chromatography in solvent 3. The complete conversion of galactose to galactitol proves that galactose is present at the reducing end of the molecule.

(c) Both labeled compounds are acetylated by the enzyme 0–10 transacetylase which is highly specific for the rhamnosylgalactose structure in the *S. anatum* 0-antigen.\(^5\)

It seems likely that the usual phenol procedure\(^1\) produces the free disaccharide by hydrolysis of the intermediate. The unhydrolyzed disaccharide derivative can be extracted from cells with n-butanol, chloroform-methanol (2:1), or solvent 2. The extracted material is almost insoluble in water but behaves as a single charged component on chromatography and electrophoresis. Treatment with dilute acid (0.01 N H\(_2\)SO\(_4\) at 100\(^\circ\)) gives 50 per cent hydrolysis in 8 min. The radioactive hydrolysis product is rhamnosylgalactose. As expected, treatment of the butanol extract with aqueous phenol under the conditions used for extracting lipopolysaccharide also produces the disaccharide. Incubation of the extract with 0.1 M Tris hydrochloride at pH 9 and 37\(^\circ\) gives a charged radioactive material which on treatment with purified alkaline phosphatase gives rhamnosylgalactose. Alkaline phosphatase has no action without prior incubation of the intermediate in Tris. Since the free disaccharide is liberated from the disaccharide intermediate by mild acid hydrolysis, the substance produced by treatment with Tris is probably rhamnosylgalactosyl-1-phosphate.

**Two-step incubation experiment:** Since most of the incorporated radioactivity can be accounted for as a disaccharide derivative when cells are incubated with UDPG-Gal and TDPRh, an experiment was carried out to demonstrate directly that this disaccharide derivative could be incorporated into antigen. Cells were labeled by incubation with UDPG-Gal and TDPRh-C\(^{14}\) and then were washed thoroughly to remove nucleotides. There was no loss of incorporated radioactivity after second incubations carried out in the presence or absence of GDPM. Addition of GDPM had the following effects: (a) it reduced the butanol soluble fraction from 80 per cent to less than 10 per cent of the incorporated material, and (b) it decreased the dialyzable fraction of the phenol extracted labeled product from 90
Discussion.—The results presented in this paper support our previous observations that the *Salmonella newington* 0-antigen is synthesized *in vitro* from the three nucleotide sugar precursors UDPGal, TDPRh, and GDPM. Rhamnose incorporation is dependent on the presence of UDPGal, and mannose is incorporated in the presence of both UDPGal and TDPRh. It is of interest that this order of addition is identical to that in *S. typhimurium* which also has a mannosyl-rhamnosyl-galactosyl repeating sequence. In the presence of only two of the precursors, UDPGal and TDPRh, a product is formed which is a precursor of 0-antigen. On the basis of our results we postulate that this precursor is a rhamnosylgalactosylphosphate-acceptor.
complex. The synthesis of this derivative points to a mechanism for polysaccharide biosynthesis more complex than those generally accepted. This mechanism could have at least four intermediate steps as follows: (1) transfer of galactose-1-phosphate from UDPGal to the acceptor; (2) transfer of rhamnose from TDPRh to the galactosylphosphate-acceptor; (3) transfer of mannose from GDPM to the rhamnosylgalactosylphosphate-acceptor; (4) 0-antigen synthesis from the mannosylrhamnosylgalactosyl precursor units.

The nature of the acceptor described in this work is not known, but its solubility in various organic solvents suggests that it might be a lipid or a compound with the physical properties of a lipid. At present it is not known whether 0-antigen chains are formed on the acceptor and then transferred to lipopolysaccharide or whether the trisaccharide is transferred to some other point where chain growth occurs. The soluble antigen\textsuperscript{10} which is found in cells may be the result of transfer of the trisaccharide units to a substance other than lipopolysaccharide core or it may represent yet another step in the synthesis of the lipopolysaccharide.

Anderson et al. have recently presented evidence for an intermediate stage in the biosynthesis of mucopeptide involving a lipid acceptor.\textsuperscript{11} As in the system reported here, the repeating sequence is carried by the lipid acceptor prior to its incorporation into polysaccharide. Rothfield et al. have shown that a lipid fraction is also required for the transfer of sugars to the R-specific component of lipopolysaccharide.\textsuperscript{12, 13} Although this lipid does not seem to act as an intermediate acceptor of sugars, it might be of importance in the formation of lipopolysaccharide-bound 0-antigen.

Infection of \textit{S. anatum} by the temperate bacteriophage \textit{e}\textsuperscript{15} brings about the synthesis of an 0-antigen with a $\beta$-d-galactosyl linkage in place of the $\alpha$-d-galactosyl linkage present in the usual \textit{S. anatum} 0-antigen. This change can readily be explained in terms of the proposed mechanism if it is assumed that the phage is able to control the final transfer of trisaccharide precursor units, since galactose is present at the activated reducing end of the intermediate. Experiments are currently being carried out to investigate the phage conversion problem and define the chemical structure of the intermediate.

\textit{Summary.}—The \textit{Salmonella newington} 0-antigen is made up of mannosyl-rhamnosyl-galactosyl repeating units. The experiments presented in this paper suggest that 0-antigen chains are not the primary acceptors of sugars from the nucleotide sugar precursors but that these are first transferred to an acceptor where the 0-antigen repeating sequence is formed. Subsequent transfer from this acceptor results in formation of 0-antigen.

The following abbreviations are used: UDPG, uridine diphosphate-D-glucose; UDPGal, uridine diphosphate-D-galactose; GDPM, guanosine diphosphate-D-mannose; TDPRh, thymidine diphosphate-L-rhamnose; TTP, thymidine triphosphate; EDTA, (ethylene dinitrilo) tetraacetic acid disodium salt.

* This investigation was supported by USPHS research grant AM 06803-03 from the National Institute of Arthritis and Metabolic Diseases.
† Fellow of the Consejo Nacional de Investigaciones Científicas y Tecnicas, Republica Argentina.
\textsuperscript{2} Robbins, P. W., A. Wright, and J. L. Bellows, these \textit{Proceedings}, \textit{52}, 1302 (1964).
AN ENZYME AGGREGATE IN THE TRYPTOPHAN PATHWAY OF
NEUROSPORA CRASSA*

BY J. A. DEMOSS AND JUDITH WEGMAN

BIOLOGY DEPARTMENT, REVELLE COLLEGE, UNIVERSITY OF CALIFORNIA (SAN DIEGO)

Communicated by Bruno Zimm, May 21, 1965

Tryptophan biosynthesis in Neurospora crassa is controlled by four unlinked genetic loci. Biochemical studies have implicated the reaction sequence shown in Figure 1. Studies with the various classes of tryptophan auxotrophs have shown that tryp-3 mutants lack tryptophan synthetase and that tryp-4 mutants lack PR-transferase. The tryp-1 locus apparently controls both PRA isomerase and InGP synthetase since the majority of the mutations at this locus lead to the loss of both of these activities. The initial step in this sequence, the conversion of chorismic acid to anthranilic acid, has only recently been demonstrated in extracts from N. crassa. This activity has been purified 85-fold with good recovery and is, therefore, assumed to be catalyzed by a single enzyme, anthranilate synthetase. From their nutritional characteristics, it is clear that tryp-2 mutants can convert anthranilate to tryptophan but are blocked in the synthesis of anthranilate, presumably due to the absence of anthranilate synthetase.

While genetic studies have demonstrated that all tryptophan auxotrophs of N. crassa map at one of these four genetic loci, a very unusual class of mutants, designated the tryp-1C class, has been observed. Mutants in this class map within the tryp-1 locus but have the nutritional characteristics of tryp-2 mutants, i.e., they can grow on anthranilate as well as indole and tryptophan. Thus, it appeared that mutations at the tryp-1 locus may lead to the loss of PRA isomerase and InGP synthetase or, alternatively, in the case of tryp-1C mutants, of anthranilate synthetase.

To explore this unusual gene-enzyme relationship the effects of mutations at the various tryptophan loci on the enzymes involved in tryptophan biosynthesis have