BIOSYNTHESIS OF CELL WALL POLYSACCHARIDE IN MUTANT STRAINS OF SALMONELLA. IV. SYNTHESIS OF S-SPECIFIC SIDE-CHAIN*

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Strain TV208 of Salmonella typhimurium was first isolated as a typical rough mutant (Subbaiah and Stocker, 1964). It cannot synthesize TDP-L-rhamnose ($TDPRh^+$) (Nikaido et al., 1964), and therefore fails to synthesize the "S-specific side-chain" portion of the complete LPS, which contains L-rhamnose, D-mannose, D-galactose, abequose and probably D-glucose (Kauffmann et al., 1960; Staub, 1964). The R_{II}-type LPS (Beckmann et al., 1964) produced by this mutant then represents the central portion of the complete LPS, i.e. the portion devoid of L-rhamnose. A preceding paper of this series (Nikaido, 1965) presented evidence that a crude particulate fraction from this mutant could transfer D-galactose, and then L-rhamnose, onto this incomplete LPS. We now report on the incorporation of other constituents of the S-specific side-chain, and on the interdependence of various incorporation reactions.

Most of the procedures used have been described previously (Nikaido, 1965). "Enzyme" preparations were made by centrifuging the sonic extracts of TV208 at

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+Abbreviations used: LPS, lipopolysaccharide; Tris, tris-(hydroxymethyl) aminomethane; UDPGal, uridine diphosphate D-galactose; TDPRh, thymidine diphosphate L-rhamnose; GDPM, guanosine diphosphate D-mannose; CDPA, cytidine diphosphate abequose; TCA, trichloroacetic acid.

18,000 x g after the removal of intact cells by low speed centrifugation, and washing the 18,000 x g pellet in large volumes of Tris-HCl buffer, pH 7.5. Incorporation of sugars was determined after precipitation, with TCA, of the fragments of the cell wall-cell membrane complex which contains LPS. Nucleotide-sugars were obtained as follows: UDPGal and TDPRh, both labeled and unlabeled, as described previously (Nikaido, 1965); GDPM-H^3, chemically synthesized (Roseman et al., 1961) from α-D-mannose-1-phosphate which had been tritiated by the Wilzbach procedure and then purified extensively; GDPM, from Sigma; CDPA-C^{14}, enzymatically synthesized (Nikaido and Nikaido, 1965) from CDP-D-glucose-C^{14} which was made chemically (Roseman et al., 1961); CDPA, as described already (Nikaido and Jokura, 1961). All nucleotide-sugars were labeled exclusively in their sugar moieties.

Table I shows the incorporation of various sugars in the presence of "enzyme", which also contains the incomplete LPS serving as acceptors in the sugar transfer reactions. Two observations can be made from these results. (1) Minimal requirement for incorporation. UDPGal was necessary for the incorporation of rhamnose, both UDPGal and TDPRh for mannose incorporation, and all three nucleotide-sugars (UDPGal, TDPRh, and GDPM) for abequose incorporation. Taken together with our previous findings on the sequential transfer of galactose and rhamnose (Nikaido, 1965), these results suggest that the sugars are added in a stepwise manner, with galactose first, followed by rhamnose, mannose, and abequose in this order (cf. Fig. 2).

It is interesting to compare this sequence of enzymatic addition (abequosyl-mannosyl-rhamnosyl-galactose) with the sequence inferred from chemical and immunological studies on the structure of the complete LPS from group B Salmonella such as S. typhimurium. Since galactosyl-mannosyl-rhamnose appears

Table I. Incorporation of various sugars into TCA-insoluble fraction catalyzed by "enzyme" from TV208.

Labeled Nucleotide	Non-labeled Nucleotide	Incorporation (mμmoles) Exp. 1	Exp. 2
	None	2.3	0.9
	TDPRh	3.3	2.9
UDPGal	TDPRh, GDPM	4.2	3.3
(2.3×10^5)*	TDPRh, GDPM, CDPA	7.9	5.3
	GDPM, CDPA	2.2	1.5
	None	0.4	0.7
	UDPGal	3.1	3.0
TDPRh	UDPGal, GDPM	3.3	3.3
(6.4×10^5)	UDPGal, GDPM, CDPA	5.0	5.6
	GDPM, CDPA	0.5	0.7
	None	<0.1	0.1
	UDPGal	<0.1	0.1
GDPM	UDPGal, TDPRh	1.3	2.1
(1.7×10^5)	UDPGal, TDPRh, CDPA	3.4	3.4
	TDPRh, CDPA	0.1	0.5
	None	<0.01	<0.01
	UDPGal	<0.01	<0.01
CDPA	UDPGal, TDPRh	<0.01	<0.01
(6.5×10^5)	UDPGal, TDPRh, GDPM	0.17	0.25
	TDPRh, GDPM	0.06	0.05

*Specific radioactivity of nucleotide-sugars in c.p.m./μmole.

Reaction mixture contained, in a total volume of 0.3 ml., Tris-HCl buffer, 15 μmoles; $MnCl_2$, 0.5 μmole; "enzyme". 4.5 mg protein; and various nucleotide-sugars as indicated. The amount of labeled or unlabeled nucleotide-sugars was as follows: UDPGal, 35 mμmoles; TDPRh, 10.5 mμmoles; GDPM, 25 mμmoles; and CDPA, 20 mμmoles. 10% TCA was added after 15 minutes at 37°C. Precipitates were washed four times with ice-cold 5% TCA, and the TCA-insoluble radioactivity was measured either with a Nuclear-Chicago low background gas flow counter or with a Tri-Carb liquid scintillation counter. In the former case correction was made for self-absorption, and in the latter case an internal standard was used for correction of quenching.

to form a part of the "repeating unit" in the S-specific side-chain of the LPS (Staub, 1964), and since the reducing group of rhamnose is presumably linked to the galactose moiety of the next repeating unit, the sequence mannosyl-rhamnosyl galactose should also be present in S. typhimurium LPS. Furthermore, immunochemical evidence suggests that abequose is linked to mannose (Staub, 1964). Therefore, the sequence of enzymatic addition is exactly the sequence believed to comprise a major portion of the S-specific side-chain, indicating that the most

proximal portion of the chain contains the same sequence of monosaccharides as its remaining portion.

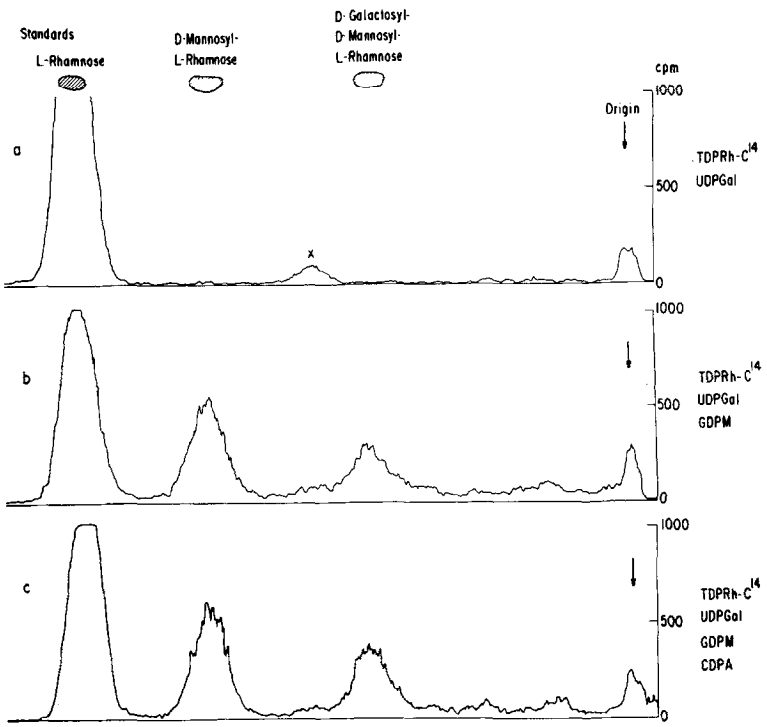

Fig. 1. Paper chromatography of partial hydrolyzates of the products of rhamnose-C^{14} incorporation.
"Enzyme" from TV208 (12 mg protein) was incubated at 37°C for 30 minutes with Tris-HCl buffer, pH 7.5, 30 μmoles; $MgCl_2$, 1 μmole; and nucleotides as shown, in a total volume of 0.52 ml. The amount of nucleotide-sugars added was: TDPRh-C^{14} (specific radioactivity, 1.0×10^7 c.p.m./μmole), 10 mμmoles; UDPGal, 43 mμmoles; GDPM, 100 mμmoles; and CDPA, 22 mμmoles. Crude LPS was isolated as described (Nikaido, 1965), and was hydrolyzed for 20 minutes in N H_2SO_4, and the hydrolyzate was chromatographed on Whatman No. 1 paper with n-butanol-pyridine-water (6:4:3). The radioactivity was recorded with a Vanguard 4 pi scanner. Oligosaccharides used as markers were purified from LPS of S-form S. typhimurium following partial acid hydrolysis.

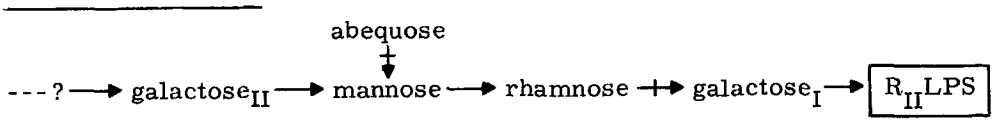

Fig. 2. Hypothetical structure of incorporation products.

⊣⊢, acid-labile linkages.

(2) Requirement for maximal incorporation. It is seen that the addition of all four nucleotide-sugars was necessary for the maximal incorporation of any sugar unit. The easiest explanation would be to assume that the addition of the second galactose (galactose$_{II}$ of Fig. 2) on to the mannose unit requires the previous addition of abequose to the mannose; therefore extensive elongation of the side-chain, involving many repeating units, would be possible only in the presence of all four nucleotide-sugars. However, this hypothesis is not sufficient to explain the results, for although abequose itself was incorporated only in small amounts (0.2 mμmole), the presence of non-labeled CDPA stimulated the incorporation of other sugars to a much greater extent (2 mμmoles). It is possible that the presence of some nucleotide-sugars activates the enzymes transferring other sugars, which might be a useful mechanism for the metabolic control of LPS synthesis.

The product of incorporation has been closely examined in the case of rhamnose incorporation, and was found to be typical LPS (Nikaido, 1965). More recently, products of rhamnose incorporation were partially hydrolyzed with acid and were chromatographed on paper together with two authentic oligosaccharides, which had been isolated from the complete LPS of S-form S. typhimurium. When the TDPRh-C^{14} incubation mixture contained UDPGal and GDPM, with or without CDPA, both mannosyl-rhamnose-C^{14} and galactosyl-mannosyl-rhamnose-C^{14} were found in partial hydrolyzates of the incorporation products (Fig. 1b, 1c). This finding supports the assumption that the sequences shown in Fig. 2 were actually synthesized in vitro; the observed oligosaccharides would be expected since the very acid-labile abequosyl linkages would be split off immediately, followed by the acid-labile rhamnosyl linkages. Fig. 1b shows also that galactose can be added on a mannose unit which is not substituted with abequose. In contrast to these results, only one oligosaccharide (X) was found

when incubation was done with TDPRh-C^{14} and UDPGal (Fig. 1a). X was also isolated from the incubation mixture containing both UDPGal-C^{14} and TDPRh-C^{14}; acid hydrolysis and chromatography before and after $NaBH_4$ reduction indicated X to be rhamnosyl-galactose (Nikaido, 1965). This finding also supports the structure proposed in Fig. 2.

In conclusion, D-galactose, L-rhamnose, D-mannose, and abequose were incorporated in vitro into the incomplete LPS contained in particulate fractions of a mutant of S. typhimurium. We assume that these sugars are transferred, one by one, in a sequence specific for the S-specific side-chain, as shown in Fig. 2. A part of this sequence was established by the isolation of oligosaccharides from partially hydrolyzed incorporation products. Very recently, Zeleznick et al. (1965) and Robbins et al. (1964) independently reported the in vitro incorporation of D-mannose, L-rhamnose and D-galactose in other strains of Salmonella.

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