ENZYMATIC INCORPORATION OF N-ACETYLGLUCOSAMINE INTO CELL WALL LIPOPOLYSACCHARIDE IN A MUTANT STRAIN OF SALMONELLA TYPHIMURIUM*

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Previous studies (Osborn et al., 1962; Nikaido, 1962; Rosen et al., 1964; Rothfield et al., 1964) on the biosynthesis of the cell wall lipopolysaccharide of Salmonella typhimurium have dealt with enzymatic incorporation of glucose and galactose into the lipopolysaccharides of two mutant strains, which are unable to synthesize UDP-glucose and UDP-galactose, respectively. In such mutants, biosynthesis of the lipopolysaccharide is interrupted at an early stage, and the resulting incomplete polysaccharide comprises only the innermos "backbone" region of the normal polymer. This part of the molecule contains L-glycero-D-mannoheptose (Osborn and Horecker, unpublished observations) phosphate, 3-deoxyoctulosonate, (Osborn, 1963) and O-phosphorylethanolamine (Grollman and Osborn, 1964). All of these have been identified as components of the polysaccharide formed by a mutant unable to synthesize UDP-glucose (Fraenkel et al., 1963; see also Fukasawa et al., 1962 and Sandararajan et al. 1962); the polysaccharide isolated from a UDP-galactose-deficient strain which

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lacks the enzyme UDP-galactose-4-epimerase, contains glucose in addition to the above components (Osborn et al., 1962). Galactose, mannose, rhamnose and abequose, which are major constituents of the wild type polysaccharide, are absent in both mutant strains.

Three nucleotide sugar-lipopolysaccharide transferase systems have been identified which catalyze incorporation of glucose and galactose into the incomplete lipopolysaccharides of these mutants. The reactions may be summarized as follows, where "LPS" refers to the incomplete lipopolysaccharide of the UDP-glucose deficient mutant, containing only heptose, phosphate, ethanolamine and 3-deoxy-octulosonate as polysaccharide components:

(1) UDP-glucose + LPS $\xrightarrow{\text{UDP-glucose transferase I}}$ Glu-LPS [1/]

(2) UDP-galactose + Glu-LPS $\xrightarrow{\text{UDP-galactose transferase}}$ Gal-α-1, 3-Glu-LPS

(3) UDP-glucose + Gal-α-1, 3-Glu-LPS $\xrightarrow{\text{UDP-glucose transferase II}}$

Glu-Gal-α-1, 3-Glu-LPS

These enzyme proteins and the acceptor lipopolysaccharides are present in the cell wall-membrane fraction of cells disrupted by sonic oscillation. Reactions (1) (Rothfield et al., 1964) and (2) (Osborn et al., 1962; Rosen et al., 1964) were demonstrated in particulate fractions isolated from the UDP-glucose-deficient and UDP-galactose-deficient mutants, respectively. The second UDP-glucose transferase system has recently been detected in the latter organism; this enzyme catalyzes the transfer of glucose to previously incorporated galactose residues, according to equation (3). Details of this reaction will be published elsewhere (Hilberman et al., 1964).

1/ Abbreviations used: Glu, glucose; Gal, galactose; GNAC, N-acetyl-glucosamine; Tris, tris (hydroxymethyl) aminomethane; TCA, trichloracetic acid.

TABLE I

Incorporation of N-Acetylglucosamine-C^{14} into Cell Wall Fraction of UDP-Galactose-4-Epimeraseless Mutant

Substrate	Additions	Incorporation
		mμmoles
UDP-N-Acetyl Glucosamine-C^{14}	None	0.05
"	UDP-Galactose + UDP-Glucose	1.13
"	UDP-Galactose	0.12
"	UDP-Glucose	0.04
UDP-Glucose-C^{14}	None	0.87
"	UDP-Galactose	2.80
UDP-Galactose-C^{14}	None	12.0

Incubation mixtures contained 80 mM Tris buffer, pH 8.5, 12 mM $MgCl_2$, 4 mM EDTA, C^{14}-labeled nucleotide sugar (0.19 mM UDP-glucose-C^{14} (416 cpm/mμmole) 0.20 mM UDP-galactose-C^{14} (255 cpm/mμmole) or 0.042 mM UDP-N-acetylglucosamine-C^{14} (1180 cpm/mμmole)) and the particulate fraction sedimenting at 12,000 x g (1.0 mg of protein). Nonradioactive UDP-glucose (0.16 mM) and UDP-galactose (0.17 mM) were present where indicated. The final volume was 0.25 ml. The reaction mixtures were incubated for 15 minutes at 37° in the absence of UDP-N-acetylglucosamine-C^{14}, then this component was added where indicated, and the incubation continued for an additional 60 minutes. The reaction was terminated by addition of 2.0 ml 5% TCA, and acid-insoluble radioactivity determined as described previously (Osborn et al., 1962).

UDP-glucose-C^{14}, UDP-galactose-C^{14}, and UDP-galactose were prepared by the methods described earlier (Rothfield et al., 1964; Osborn et al., 1962). UDP-N-acetylglucosamine-C^{14} was a generous gift from Dr. S. Kornfeld of the National Institutes of Health.

This communication presents evidence that the next step in synthesis of the polysaccharide is the transfer of N-acetylglucosamine from UDP-N-acetylglucosamine to the product of reaction (3). We have found the cell wall-membra fraction of the UDP-galactose-4-epimeraseless mutant to contain a UDP-N-

acetylglucosamine-lipopolysaccharide transferase system, whose activity is dependent on the prior addition of both glucose and galactose to the incomplete, mutant polysaccharide (Table I). Table I also shows parallel experiments in which the incorporation of C^{14}-galactose (reaction (2)) and C^{14}-glucose (reaction (3)) were determined. These results suggested that incorporation of N-acetylglucosamine required prior addition of both glucose and galactose to the incomplete polysaccharide. In order to test this hypothesis, the particulate, enzyme-acceptor complex was first incubated with a mixture of unlabeled UDP-galactose and UDP-glucose, reisolated by centrifugation, washed to remove residual substrates, and finally incubated a second time with UDP-N-acetylglucosamine-C^{14} in the absence of other nucleotide sugars. Controls were included in which one or both nucleotide sugars were omitted from the first incubation. The results (Table II) are in accord with the hypothesis that the product resulting from the combined action of the UDP-galactose trans-

TABLE II

Dependence of N-Acetylglucosamine Incorporation on Prior Addition of Galactose and Glucose

Nucleotide Sugars Present		Incorporation of N-Acetylglucosamine
First Incubation	Second Incubation	
		mμmoles
UDP-Glucose + UDP-Galactose	UDP-N-Acetylglucosamine-C^{14}	0.37
UDP-Galactose	"	0.04
UDP-Glucose	"	0.01
None	"	0.01

Incubation mixtures were as described in Table I. The particulate enzyme fraction (1.0 mg protein) was first incubated with the indicated unlabeled nucleotide sugars for 60 minutes. At this time, 1.0 ml of 50 mM Tris buffer, pH 9.0 containing 1 mM EDTA, was added, the particulate material was recovered by centrifugation (20 minutes at 12,000 x g, 4° C), and washed with 2.0 ml Tris-EDTA, pH 9.0. The enzyme was then incubated for a second 60-minute period with UDP-N-acetylglucosamine-C^{14} alone.

ferase (reaction (2)) and UDP-glucose transferase II (reaction (3)) acts as specific acceptor for subsequent addition of N-acetylglucosamine. Although these data do not allow unequivocal conclusions as to the site of attachment of the enzymatically incorporated N-acetylglucosamine, the reaction is tentatively summarized as shown in equation (4):

(4) UDP-N-acetylglucosamine + Glu-Gal-α-1, 3-Glu-LPS

$$\xrightarrow{\text{UDP-N-acetylglucosamine transferase}} \text{GNAC-Glu-Gal-}\alpha\text{-1, 3-Glu-LPS}$$

Evidence that N-acetylglucosamine is specifically incorporated into

TABLE III

Isolation of the N-Acetylglucosamine Transferase Product

Fraction	Radioactivity	Percent Recovery
	cpm	
Acid precipitated cell wall material	15,000	(100)
Crude lipopolysaccharide (aqueous phase of phenol extract)	16,000	107
Purified lipopolysaccharide (Mg^{++} precipitate)	14,800	99
Lipid-free polysaccharide	12,750	85

The particulate enzyme fraction (52.5 mg protein) was incubated with 80 mM Tris buffer, pH 8.5, 12 mM $MgCl_2$, 4 mM EDTA, 0.19 mM UDP-glucose, and 0.20 mM UDP-galactose (final volume 17.7 ml) for 20 minutes at 37°. At this time, UDP-N-acetylglucosamine-C^{14} (1.4×10^5 cpm/μmole) was added to a final concentration of 0.09 mM, and the incubation continued for an additional 90 minutes. The reaction was terminated by addition of 1.0 ml 100% TCA. Lipopolysaccharide was isolated by extraction of the acid-insoluble cell wall fraction with 45% phenol at 68° (Westphal et al., 1952), and purified by precipitation with $MgCl_2$ (Osborn et al., 1962). Lipid-free polysaccharide was obtained by hydrolysis of the dialyzed lipopolysaccharide at pH 3.4 (Osborn, 1963).

lipopolysaccharide was obtained by isolation of the endogenous lipopolysaccharide following incubation of the particulate fraction with UDP-N-acetylglucosamine C^{14} in the presence of unlabeled UDP-glucose and UDP-galactose. The results are shown in Table III. All of the radioactivity incorporated into cell wall material was recovered in the purified lipopolysaccharide, and after mild acid hydrolysis to split polysaccharide from lipid, only the soluble polysaccharide fraction contained C^{14}. Paper electrophoretic analysis of this fraction and of nonradioactive, galactose-deficient mutant polysaccharide is shown in Fig. 1. All of the radioactivity migrated toward the anode with mobility identical to the more slowly migrating fractions of the authentic mutant polysaccharide. The mobility of the components containing N-acetylglucosamine-C^{14} was similar to that observed earlier (Rosen et al., 1964;

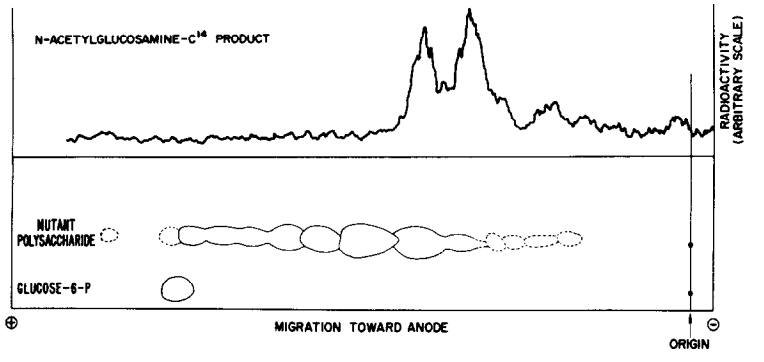

Fig. 1. Paper electrophoretic analysis of lipid-free polysaccharide containing N-acetylglucosamine-C^{14}. Labeled polysaccharide (4,000 cpm) isolated as described in Table III and a parallel sample (150 μg) of nonradioactive mutant polysaccharide (Osborn, 1963) were applied to Whatman No. 1 paper and electrophoresed in pyridine: acetic acid: H_2O (1:10:69), pH 3.5, (140 v/cm, 1 hour) in a Savant high voltage electrophoresis apparatus. Radioactive areas were located by scanning with a Baird-Atomic 4π Scanogram. The nonradioactive polysaccharide was developed with periodate-$AgNO_3$ as previously described (Osborn, 1963).

Rothfield et al., 1964) for the products of galactose and glucose incorporation. After complete acid hydrolysis (1 N HCl 5 hours 100°), all of the radioactivity migrated chromatographically with glucosamine.

Conclusions—The UDP-N-acetylglucosamine-lipopolysaccharide transferase described here extends previous studies (see Osborn et al., 1964 for review) on biosynthesis of the inner, "core" region of the S. typhimurium polysaccharide, and provides additional evidence that synthesis occurs by sequential transfer of monosaccharides from the appropriate nucleotide sugar to the growing chain. No hexosamine has been detected in the incomplete polysaccharides of the UDP-galactose and UDP-glucose-deficient mutants, but the "core" polysaccharides formed by rough and GDP-mannose-deficient strains have recently been found to contain N-acetylglucosamine in addition to glucose and galactose (Osborn et al., 1964). From the biosynthetic studies a tentative sequence for this portion of the polysaccharide can be written as shown in equation (4). The present results are consistent with the postulated attachment of N-acetylglucosamine to glucose, but do not exclude linkage to another residue. Further studies are in progress to determine the site of attachment of N-acetylglucosamine.

Edstrom and Heath (1964) have also demonstrated reactions (2), (3) and (4) in an epimeraseless mutant of E. coli 0111. The similarity in structure of the "core" polysaccharide of this organism to that of S. typhimurium is of particular interest, since the composition and structure of the outer, O-antigenic side chains of the two polysaccharide are entirely different.

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