BIOSYNTHESIS OF THE DETERMINANT 34 OF THE SALMONELLA O-ANTIGEN*

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The O-antigenic polysaccharides of group E Salmonella contain D-galactosyl-D-mannosyl-L-rhamnose repeating units, the anomeric change and the modification of which are brought about in the organisms by the presence of specific bacteriophages (Robbins and Uchida, 1962; Uchida et al., 1963). In cells carrying the O-antigen 3, 10 the galactosyl-mannose is linked by α-1,6-linkage, and the galactose unit has an O-acetyl residue. In cells lysogenic for the phage ε^{15}, which carry the O-antigen 3, 15, the galactosyl-mannose is linked by β-1,6-linkage, and O-acetyl residues are absent. The doubly lysogenic cells for the phages ε^{15} and ε^{34} carry the O-antigen 3, 15, 34, and the polysaccharide contains β-D-galactosyl groups which are modified by the

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attachment of an α-D-glucosyl residue to the position 4 of the galactose. In cells lysogenic for ε^{34} alone, on the other hand, the determinant 34 is not formed and the O-antigen remains unchanged (Uetake and Hagiwara, 1960; 1961), possibly because of lack of the β-galactosyl residue in the polysaccharide. These findings suggest that the genetic material of the phage ε^{34} may play a role in the expression of enzymatic activity of the specific transglucosylation which requires the β-glycosidic configuration of the galactosyl residue as acceptor. This communication presents evidence that the phage ε^{34} is involved in the expression of enzymatic activity catalyzing the transfer of glucose from UDPG* to synthesize the determinant 34.

Bacterial strains used were the lysogenic derivatives of S. anatum strain 1 (A_1). The cells were grown in tryptone-yeast extract medium. The exponentially growing cells were harvested, and cell-envelope fractions** were prepared according to the method of Rothfield et al. (1964). UDPG-C^{14} (4×10^6 cpm/μmole) was synthesized enzymatically from radioactive glucose with the use of UDPG pyrophosphorylase.

As shown in Table 1, when the cell-envelope fraction prepared from $A_1(\varepsilon^{15}, \varepsilon^{34})$ was incubated with UDPG-C^{14}, radioactivity was incorporated into an acid-insoluble form. The incorporation of radioactivity with the cell-envelope fraction obtained from $A_1(\varepsilon^{15})$ was only one eighth of that with the one from $A_1(\varepsilon^{15}, \varepsilon^{34})$. This finding indicates that the specific incorporation of radioactivity from UDPG-C^{14} may be catalyzed by an enzyme of which formation is directed by the ε^{34} genome. The incorporation of radioactivity increased with time during the first 50 minutes of incubation, and thereafter completely ceased (Fig. 1).

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* uridine diphosphate D-glucose
** cell wall-membrane fractions

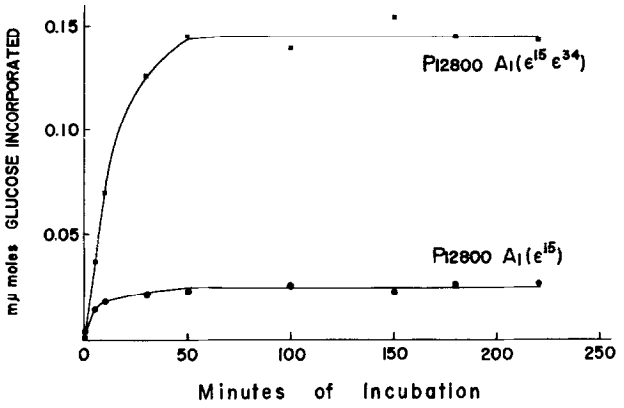

Fig. 1. The time course of glucose-C^{14} incorporation with the particulate enzyme systems. The reaction conditions and procedures were as described in the legend to Table 1. Cell-envelope fractions, (0.1 mg protein) was used. The reaction was stopped at intervals by the addition of trichloroacetic acid.

Table 1. Incorporation of radioactivity from UDPG-C^{14} into cell-envelope fractions.

Cell-envelope	Protein (mg)	C^{14} incorporated into cell-envelope (cpm)		Δ cpm
		0 min	30 min	
P12800$A_1(\varepsilon^{15})$	0.05	11	70	59
	0.1	9	124	115
P12800$A_1(\varepsilon^{15},\varepsilon^{34})$	0.05	8	470	462
	0.1	12	975	963

Cell-envelope fractions were prepared from sonicates of $A_1(\varepsilon^{15})$ and of $A_1(\varepsilon^{15},\varepsilon^{34})$, as a fraction sedimentable between 1,200 and 12,800 x g. Reaction mixtures contained 80 mM Tris-HCl buffer pH 8.0, 20 mM $MgCl_2$ and 0.062 mM UDPG-C^{14}(4 x 10^6 cpm/μmole) in a total volume of 125 μl including the cell-envelope fractions indicated. The mixture was incubated for 30 min. at 37°C. At the end of reaction an equal volume of 10% trichloroacetic acid was added to the mixture at 0°C. The mixture was filtered through Millipore filter (HA), followed by washing with 5% trichloroacetic acid. The membranes were attached to planchets and counted in a Nuclear Chicago gas flow counter. The cell- envelope fraction loses its enzymatic activity appreciably upon freezing and thawing.

In order to identify the radioactive product, a large scale run with 2.3 μmoles of UDPG-C^{14} (9.2 x 10^6 cpm) and 56.5 mg protein of the $A_1(\varepsilon^{15}, \varepsilon^{34})$ cell-envelope fraction was carried out. The lipopolysaccharide of the cell-envelope was isolated and purified by 45% hot phenol extraction, dialysis, and ultracentrifugation. About 7% of the radioactivity added to the reaction mixture was incorporated into 75% ethanol-precipitable and water-soluble but phenol-insoluble material. Of the total radioactivity incorporated, 86% was recovered as non-dialyzable material. After centrifugation at 59,310 x g for 30 minutes, more than 80% of radioactivity was recovered in pellets. The purified product thus obtained contained carbohydrates, but no protein (tested by the Lowry method) and no UV-absorbing substance were detected. When the radioactive product was mixed with anti-34 serum, 77% of the radioactivity was found in the precipitates, while 7% remained in the supernatant; the remainder was lost while washing the precipitates. It was proved, therefore, that the radioactivity transferred from UDPG-C^{14} was incorporated into the antigenic lipopolysaccharide endogenously present in the cell-envelope fraction.

Complete acid hydrolysis of the radioactive lipopolysaccharide revealed that only glucose contained radioactivity (Fig. 2), while partial acid hydrolysis yielded several radioactive oligosaccharides (Fig. 3). The area of peak 2 in Fig. 3 which corresponds in mobility to galactosyl-mannose and glucosyl-galactose (Robbins and Uchida, 1962) was eluted and subjected to paper electrophoresis. As shown in Fig. 4, two spots of carbohydrate corresponding in mobility to galactosyl-mannose and glucosyl-galactose were visualized by spraying the paper strip with color reagent. The radioactivity was present entirely at the spot of glucosyl-galactose. It has already been proved that α-1,4-D-glucosyl-D-galactose obtained from the $A_1(\varepsilon^{15},\varepsilon^{34})$ lipopolysaccharide is the essential component of the determinant 34 (Uchida et al., 1963).

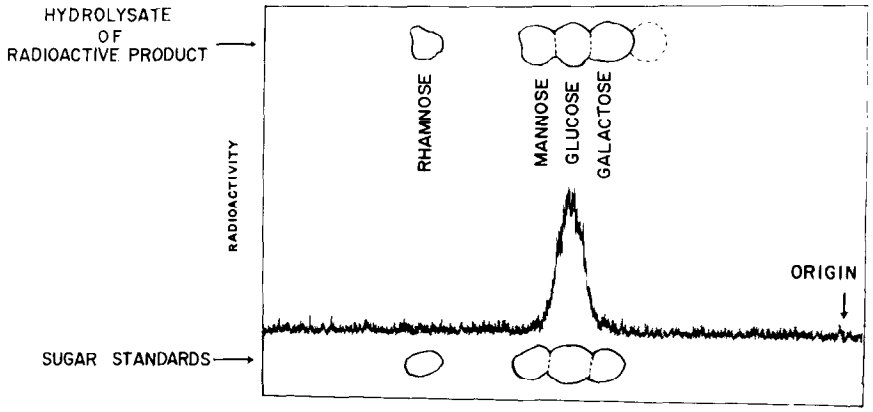

Fig. 2. Chromatogram of hydrolysate of radioactive lipopolysaccharide. Hydrolysis was carried out with 1N HCl at 100°C for 2 hours. Of the radioactivity, 93% was recovered in a soluble form. The hydrolysate was passed through a Dowex-1 (formate form) column, and 91% of the radioactivity was recovered in the effluent. The condensed effluent was applied to Whatman No. 1 paper and subjected to descending paper chromatography with butanol-pyridine-H_2O (6:4:3). Radioactivity was located with an Aloca 4 pi windowless Scanogram. Carbohydrate was located by means of either $AgNO_3$-NaOH or aniline hydrogen phthalate.

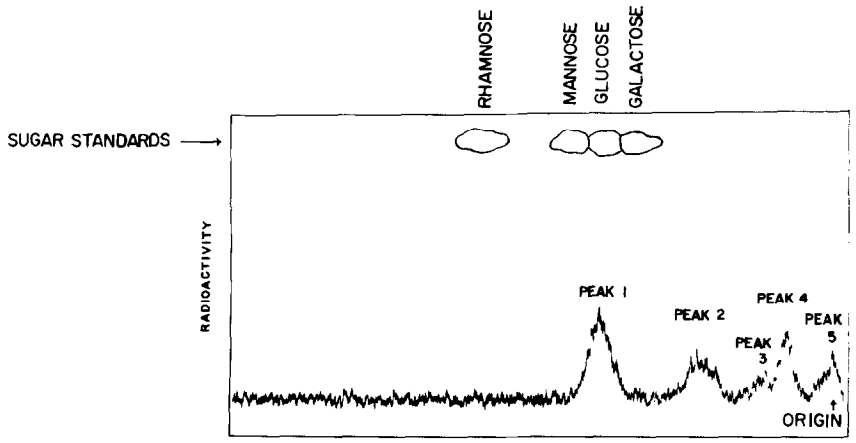

Fig. 3. Chromatogram of partially hydrolyzed radioactive product. The purified radioactive product was hydrolyzed with 0.5N H_2SO_4 at 100°C for 25 min. After neutralization with $Ba(OH)_2$ and centrifugation, the supernatant fluid was dried in vacuo. The residues were dissolved in water and subjected to descending paper chromatography on Whatman No. 1 paper with butanol-pyridine-H_2O (6:4:3). Radioactivity and carbohydrate were located as described in Fig. 2.

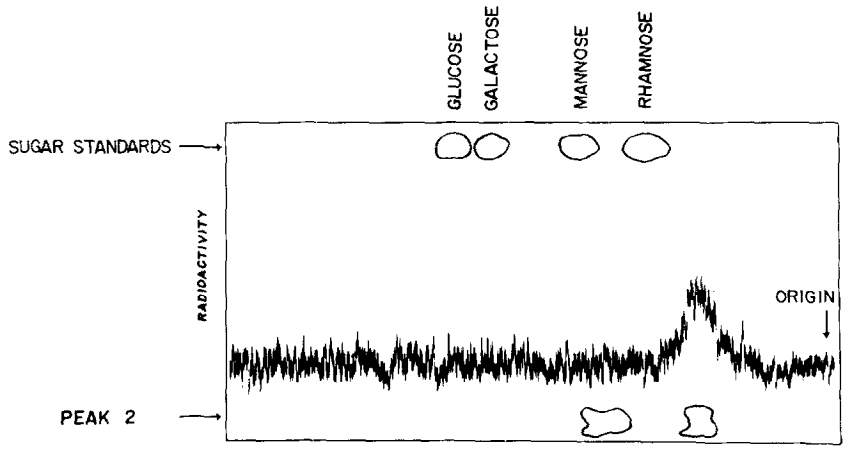

Fig. 4. Paper electrophoresis of radioactive carbohydrates of peak 2 in the figure 3. Chromatographically separated radioactive carbohydrates (peak 2 in Fig. 3) was eluted and condensed. An aliquot was applied to Whatman No. 1 paper, and electrophoresis was performed in 0.05 M sodium borate at 600 volts for 6 hours. Radioactivity was located as described in Fig. 2. Carbohydrate was located by means of 5% ethanolic lactic acid spray prior to the treatment with aniline hydrogen phthalate. The disaccharide glucosyl-galactose migrates slower than rhamnose, and galactosyl-mannose appears between mannose and rhamnose areas (Robbins and Uchida, 1962).

Together with the findings that the incorporation of glucose-C^{14} into cell-envelope was obtained specifically with the cell-envelope of $A_1(\varepsilon^{15},\varepsilon^{34})$ but not with that of $A_1(\varepsilon^{15})$, the presence of radioactive glucosyl-galactose residues in the lipopolysaccharide supports the hypothesis that the phage ε^{34} brings about the formation of a trans-glucosylase which is responsible for the synthesis of the determinant 34.

Weiner et al. (1965) and Wright et al. (1965) have reported recently that lipid-linked intermediates are involved in the synthesis of O-antigen polysaccharides. In our case, addition of glucose to the antigenic lipopolysaccharide occurred in the absence of nucleotide sugars other than UDPG, which are required for the biosynthesis of O-antigen side chain composed of galactose, mannose and rhamnose (Robbins et al., 1964; Zeleznick et al., 1965; Nikaido and Nikaido, 1965). Although the possibility can not be excluded that addition of glucose

requires the lipid-linked intermediate, our results suggest that the transglucosylation may occur without simultaneous de novo synthesis of the O-antigen intermediate.

Work towards the more rigorous identification of the product and solubilization of the enzyme system is now in progress.

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