ENZYMATIC INCORPORATION OF *N*-ACETYL-D-MANNOSAMINURONIC ACID AND D-GLUCOSE INTO A POLYSACCHARIDE OF *ESCHERICHIA COLI* 014 K7 H$^-$

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1. Introduction

Several hexosaminuronic acids have been discovered in the acidic polysaccharides of a number of microorganisms, for instance, mannosaminuronic acid in K7 specific polysaccharide of *E.coli* 014 K7 H$^-$ [1], the cell wall polysaccharide of *Micrococcus lysodeikticus* ATCC 4698 [2] and the specific polysaccharide of *Staphylococcus aureus* T [3], and glucosaminuronic acid in the specific polysaccharides of *S.aureus* Smith [4] and *Achromobacter georgiopolitanum* n. sp. [5]. In the studies of the biosynthesis of hexosaminuronic acid-containing polysaccharides, we have demonstrated the occurrence of UDP-HexNAcUA in the cells of *S.aureus* Smith and *M.lysodeikticus* [6], and the enzymatic formation of UDP-ManNAcUA in *E. coli* 014 K7 H$^-$ [7]. Recently, the isolation of UDP-HexNAcUA from *A.georgiopolitanum* n. sp. [8] and *M.lysodeikticus* [9], and the biosynthesis of the polysaccharide of *M.lysodeikticus* cell wall [10] have also been reported.

We report here that, when enzymatically synthesized UDP-[^{14}C]ManNAcUA was incubated in the presence of UDP-Glc with the particulate enzyme of *E.coli* 014 K7 H$^-$, [^{14}C]ManNAcUA was incorporated into the polysaccharide with an equimolar amount of D-glucose [11].

Abbreviations: UDP-HexNAcUA, UDP-*N*-acetylhexosaminuronic acid; UDP-ManNAcUA, UDP-*N*-acetyl-D-mannosaminuronic acid; UDP-Glc, UDP-D-glucose; ManNAcUA, *N*-acetyl-D-mannosaminuronic acid; UDP-GclNAc, UDP-*N*-acetyl-D-glucosamine; UDP-GlcNAcUA, UDP-*N*-acetyl-D-glucosaminuronic acid; UDP-Gal, UDP-D-galactose.

2. Materials and methods

UDP-ManNAcUA and acetyl labeled UDP-[^{14}C]ManNAcUA were prepared enzymatically with the soluble enzyme of *E.coli* from UDP-GlcNAc and acetyl labeled UDP-[^{14}C]GlcNAc, respectively [7]. UDP-[^{14}C]Glc was purchased from RCC, England. UDP-[^{14}C]HexNAcUA was isolated from *S.aureus* Smith cultured in a [^{14}C]glucosamine-containing medium, and composed of UDP-GlcNAcUA (93%) and another component presumed as UDP-ManNAcUA (7%) [6]. UDP-GlcNAc, UDP-Gal and UDP-Glc were purchased from Boehringer.

Preparation of the enzyme was carried out as follows. *E.coli* 014 K7 H$^-$, furnished by Drs. I. and F. Ørskov, Statens Serum Institute, Copenhagen, was grown in a medium containing 3.7% Brain Heart Infusion (Difco). *E.coli* cells from 2 liters of culture were harvested at 60% maximal growth, and suspended in 20 ml of 0.03 M Tris–HCl, pH 7.6. The suspension was treated in a 10 Kc sonic oscillator for 3 min. The homogenate was centrifuged at 20 000 *g* for 20 min and the supernatant was further centrifuged at 100 000 *g* for 60 min. The precipitate, washed by centrifugation and suspended in 0.6 ml of 0.03 M Tris-HCl, pH 7.6, was used as the particulate enzyme.

Assay procedure for the polysaccharide synthesis was as follows. Reaction mixtures, in a final volume of 22 μl, contained 0.3 mM UDP-[^{14}C]ManNAcUA (338 cpm/nmole), 0.5 mM UDP-Glc, 2.8 mM $MnCl_2$, 50 mM HEPES buffer, pH 8.5, and the particulate enzyme (91 μg of protein). The mixture was incubated for 30 min at 25°C, and the reaction was terminated by addition of 10 μl of solvent A, isobutyric acid-0.5 M NH_4OH (5:3). The terminated reaction

Table 1
Nucleotide sugar requirement in the polysaccharide synthesis

Labeled nucleotide	Non-labeled nucleotide	Incorporation
		cpm
UDP-[^{14}C] ManNAcUA	None	23
	UDP-Glc	886
	UDP-Gal	151
	UDP-GlcNAc	134
	UDP-Glc, UDP-Gal and UDP-GlcNAc	845
UDP-[^{14}C] HexNAcUA	None	23
	UDP-Glc	29

Conditions for assay were those described in the text, except that the indicated nucleotides were present in the following concentrations: UDP-[^{14}C]ManNAcUA (*E. coli*, 338 cpm/nmole), 0.3 mM; UDP-Glc, 0.5 mM; UDP-Gal, 0.48 mM; UDP-GlcNAc, 0.5mM; UDP-[^{14}C]HexNAcUA (*S. aureus*, 150 cpm/nmole), 0.3 mM.

mixture was chromatographed on Toyo No.50 filter paper in solvent A for 40 hr and radioactivity remaining at the origin on the paper chromatogram was detected by cutting out the paper, inserting the paper in scintillation solution (4 g of PPO and 0.3 g of dimetyl POPOP in 1 liter of toluene) and counting in a liquid scintillation counter.

3. Results and discussion

It has been reported that K7 specific substance contained mannosaminuronic acid of 15–20% at least and glucose, as main components, together with glucosamine and galactose, as minor components.

Table 1 shows the substrate requirement in the polysaccharide synthesis. The incorporation of [^{14}C] ManNacUA from UDP-[^{14}C] ManNAcUA required the presence of UDP-Glc. Since there was no extra stimulation of the incorporation of [^{14}C] ManNAcUA in the presence of two other non-labeled nucleotides, UDP-GlcNAc and UDP-Gal, these two nucleotides would not be required as substrate in the polysaccharide synthesis, and the small amount of

Table 2
Stoichiometry of the incorporation of glucose and ManNAcUA into polysaccharide

Incubation time	UDP-[^{14}C]Glc			UDP-[^{14}C]ManNAcUA			Ratio glucose ManNAcUA (A/B)
	+ UDP-ManNAcUA	– UDP-ManNAcUA	Difference (A)	+ UDP-Glc	– UDP-Glc	Difference (B)	
min	nmoles	nmoles	nmoles	nmoles	nmoles	nmoles	
15	0.62	0.12	0.50	0.48	0.03	0.45	1.12
30	1.04	0.17	0.87	0.79	0.05	0.74	1.18
60	1.49	0.25	1.24	1.20	0.04	1.16	1.07
120	2.29	0.55	1.74	1.79	0.11	1.68	1.04

The complete reaction mixtures, in a final volume of 86 μl, contained 3.2 mM $MnCl_2$, 50 mM HEPES buffer, pH 8.5, the particulate enzyme (360 μg of protein) and the indicated nucleotides present in the following concentrations: UDP-[^{14}C]Glc (300 cpm/nmole), 0.5 mM; UDP-ManNAcUA, 0.3 mM; UDP-[^{14}C]ManNAcUA (322 cpm/nmole), 0.3 mM; UDP-Glc, 0.5 mM. The mixtures were incubated at 25°C. Aliquots (16 μl) of each reaction mixture were removed at the indicated intervals.

Table 3
Distribution in several bacteria of the enzyme which synthesizes polysaccharide from UDP-ManNAcUA and UDP-Glc

Bacteria	Incorporation
	cpm
E. coli 014 K7 H^-	886
E. coli K12	23
E. coli B	23
S. aureus Smith	23
S. aureus H	23
M. lysodeikticus ATCC 4698	23

Cells were harvested at 60% maximal growth and disrupted by the treatment in a sonic oscillator (*E. coli* and *S. aureus*) or by grinding with alumina (*M. lysodeikticus*). Then the particulate enzyme was separated as described in the text. The assay of polysaccharide synthesis was carried out as described in the text with the particulate enzyme (91 μg of protein) from each strain.

incorporation of [^{14}C] ManNAcUA in the presence of either UDP-GlcNAc or UDP-Gal is probably ascribable to the contamination of UDP-Glc in UDP-GlcNAc or epimerization of UDP-Gal to UDP-Glc. On the other hand, [^{14}C] HexNAcUA from UDP-[^{14}C] HexNAcUA, isolated from *S.aureus* and composed chiefly of UDP-[^{14}C] GlcNAcUA, was scarcely incorporated into the polysaccharide in either the presence of absence of UDP-Glc. As shown in Table 2, [^{14}C] glucose was incorporated from UDP-[^{14}C] Glc into polysaccharide in the presence of enzymatically synthesized UDP-ManNAcUA. In the presence of the latter nucleotide a little incorporation of radioactivity was observed, probably suggesting the formation of a glucan, such as glycogen, as described earlier [12]. All these results indicate that both UDP-ManNAcUA and UDP-Glc were necessary for the polysaccharide synthesis as substrate.

Table 2 also shows the stoichiometry of the incorporation of glucose and ManNAcUA into the polysaccharide, namely, 1.04–1.18 moles of glucose per mole of ManNAcUA.

Most of the reaction product, after solubilization by treatment in 0.01 N HCl for 10 min at 100°C, was excluded from Sephadex G-75, providing evidence for the synthesis of a polysaccharide of molecular weight of over 50 000.

The possible occurrence of this enzyme was investigated in several strains including those known to have HexNAcUA in their cell envelopes. As shown in Table 3, the activity of the formation of the polysaccharide was detected only in *E.coli* 014 K7 H^-.

The results described above indicate that the enzymatically synthesized UDP-ManNAcUA serves as a precursor of the polysaccharide in *E.coli* 014 K7 H^-, and that the polysaccharide, which seems to be backbone of K7 specific polysaccharide antigen of this organism although immunochemical identification remains to be done, is probably composed of equimolar amounts of ManNAcUA and glucose.

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