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Biosynthesis of Uridine Diphosphate N-Acetyl-*L*-Fucosamine in a Cell-Free System from *Salmonella arizonae* O:59

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Abstract—The conversion of uridine diphosphate N-acetyl-*D*-glucosamine into uridine diphosphate N-acetyl-*L*-fucosamine was demonstrated with enzymes from cytoplasmic fraction of *Salmonella arizonae* O:59 cells in the presence of NAD^+ ($NADP^+$) and NADPH. The reaction product was identified by ion-pair, reverse-phase HPLC with the use of synthetic nucleoside diphosphate sugar standards under conditions specially developed for separation of uridine diphosphate 2-acetamido-2,6-dideoxyhexoses. *L*-Fucose dehydrogenase from porcine liver was shown to be applicable for determination of N-acetyl-*L*-fucosamine, this enzyme being used to confirm *L*-configuration of the amino sugar residue in the sugar nucleotide formed.

Key words: nucleoside diphosphate sugars, 2-acetamido-2,6-dideoxyhexoses, uridine diphosphate N-acetyl-*L*-fucosamine, biosynthesis, HPLC, *L*-fucose dehydrogenase

2-Acetamido-2,6-dideoxyhexoses are constituents of numerous bacterial cell surface polysaccharides including O-specific polysaccharides of many Gram-negative bacteria and capsular polysaccharides in a number of Gram-positive, pathogenic microorganisms. Such kind of monosaccharide components was not found in glycoconjugates from eukaryotic organisms, whereas six different 2-acetamido-2,6-dideoxyhexoses with *L*- and *D-galacto*-, *L*- and *D-gluco*-, *L-manno*-, and *L-talo*-configuration were identified in bacterial polysaccharides [1]. The first of these monosaccharides, N-acetyl-*L*-fucosamine (*L*-FucNAc), is distributed widely in O-antigenic polysaccharides (for review see [2, 3]), and it is also a component of capsular polysaccharides of streptococci [4] and staphylococci [5].

Some evidence exists that surface carbohydrate structures containing 2-acetamido-2,6-dideoxyhexoses may serve as virulence factors of pathogenic bacteria. It was shown that mutants of *Pseudomonas aeruginosa* devoid of O-antigen were 1000-fold less virulent than the wild type bacteria [6]. Polysaccharide capsules of *Staphylococcus aureus* type 5 render the pathogen to be more resistant to phagocyte uptake and enhance its virulence [7].

Until recently, the information on pathways leading to the biosynthesis of *L*-FucNAc and other bacterial 2-acetamido-2,6-dideoxyhexoses was very limited. In accordance with a biogenetic classification of bacterial polysaccharide components, suggested in review [8], all the monosaccharides mentioned belong to a subgroup of N-acetyl-*D*-glucosamine (GlcNAc). It was proposed that *L*-FucNAc and its *D*-analog (D-FucNAc) are produced as UDP-derivatives from UDP-GlcNAc through enzymic dehydration, epimerization, and reduction of the acetamidosugar residue—reactions similar to the well-documented transformations of nucleoside diphosphate hexoses [9, 10].

We were able to demonstrate [11, 12] UDP-GlcNAc to be an efficient substrate for the nucleotide-activated form of *D*-FucNAc biosynthesis in cells of Gram-positive microorganism *Streptomyces chrysomallus* sp. 2. UDP-2-acetamido-2,6-dideoxyhexos-4-ulose was registered as an intermediate during conversion of UDP-GlcNAc into UDP-*D*-FucNAc. In recent years, several publications have appeared dealing with characterization of genes involved in the biosynthesis of some bacterial polysaccha-

Abbreviations: NDPS) nucleoside diphosphate sugars; *D*-FucNAc) 2-acetamido-2,6-dideoxy-*D*-galactose (N-acetyl-*D*-fucosamine); *L*-FucNAc) 2-acetamido-2,6-dideoxy-*L*-galactose (N-acetyl-*L*-fucosamine); GlcNAc) N-acetyl-*D*-glucosamine; ManNAc) N-acetyl-*D*-mannosamine; *L*-PneNAc) 2-acetamido-2,6-dideoxy-*L*-talose (N-acetyl-*L*-pneumosamine).

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rides containing *L*-FucNAc residues [13-17]. Formation of *L*-FucNAc from UDP-GlcNAc was proposed based on the results of gene sequencing and comparison of amino acid sequences of the gene products with those known for some nucleotide sugar epimerases and oxidoreductases. Nevertheless, functions ascribed to these gene products were not confirmed biochemically. Meanwhile, many enzymes of this group, catalyzing different reactions, reveal high structural homology and their unequivocal identification is frequently difficult.

In this paper we describe the results of study of transformation of UDP-GlcNAc into UDP-*L*-FucNAc, catalyzing with cytoplasmic enzymes from *Salmonella arizonae*[1] serogroup O:59 cells. The microorganism was chosen for investigation to simplify interpretation of the results of biochemical experiments. In many *L*-FucNAc-containing polysaccharides, the polymeric chain includes also a residue of *D*-FucNAc as well as residues of other 2-acetamido-2,6-dideoxyhexoses. In the case of *S. arizonae*, as established earlier in our laboratory [18], the O-specific polysaccharide is composed of trisaccharide repeating units which contain, in addition to *L*-FucNAc, only *D*-GlcNAc and *D*-galactose residues. Identification of enzymic reaction products was simplified also by recent chemical synthesis of UDP-β-*L*-FucNAc [19] and UDP-α-*D*-FucNAc [20] performed in our laboratory.

MATERIALS AND METHODS

Materials. Radiolabeled substrates UDP-[^{14}C]GlcNAc and [^{14}C]fucose were from Amersham (England); nucleotides UDP-GlcNAc, NAD^+, $NADP^+$, and NADPH were from Serva (Germany). Synthetic samples of UDP-β-*L*-FucNAc [19] and UDP-α-*D*-FucNAc [20] were synthesized in our laboratory. The monosaccharide *L*-fucose and the enzyme *L*-fucose dehydrogenase from *Pseudomonas* sp. were from Sigma (USA), and samples of *D*-FucNAc and *L*-FucNAc were generous gifts of Dr. E. V. Vinogradov (Institute of Biological Sciences, NRCC, Ottawa, Canada).

Analytical methods. The following solvent systems were used for paper chromatography: ethanol–1 M ammonium acetate, pH 7.5 (5 : 2 v/v) (A); ethyl acetate–pyridine–acetic acid–water (5 : 5 : 1 : 3 v/v) (B). Paper electrophoresis was carried out in 0.05 M triethylammonium bicarbonate (TEAB) buffer, pH 8.0 (C) with picric acid as a marker. Nucleotides were visualized on paper under a UV lamp, and 0.2% solution of ninhydrin in acetone followed by heating at 110°C for 5 min was used for detection of amino sugars. Radiolabeled substances were determined on a Delta-300 liquid scintillation counter (Tracor, Holland) with toluene scintillator ZhS-8 or with dioxane scintillator ZhS-50 (USSR).

For HPLC of nucleotide sugars, a module system consisting of a pump (Altex, USA), sample injection valve (Rheodyne 7025, USA), UV detector (254 nm) and recorder (Knauer, Germany) were employed. Separation was performed on a reversed-phase Luna C-18(2) column (0.46 × 25 cm, 5 μ) (Phenomenex, USA). Isocratic elution with 20 mM aqueous triethylammonium acetate buffer, pH 6.0, was used at flow rate of 0.3 ml/min. After 3-4 cycles of separation, the column was regenerated by washing with 4% acetonitrile in the elution buffer for 15 min. In experiments with radiolabeled nucleotide sugars, fractions of 0.6 ml were collected and their radioactivity was estimated.

Hydrolysis of nucleotide sugars to N-acetylhexosamines was performed in 0.01 M HCl at 100°C for 15 min. Strong acid hydrolysis to amino sugars was performed in 4 M HCl at 100°C for 5 h, and the acid was removed by evaporation with water prior the paper chromatography.

Bacterial strain. Cells of *S. arizonae* serogroup O:59, strain 40024, were grown at the I. I. Mechnikov Institute of Vaccines and Sera, Ministry of Health of the Russian Federation. Hottinger tryptic broth supplemented with 0.5% of glucose was used; the cells were grown at 37°C for 6 h, harvested by centrifugation, and washed twice with physiological solution and once with 50 mM Tris-HCl, pH 7.9, supplemented with 1 mM DTT and 2 mM EDTA.

Procedure for the enzyme preparation. Washed cells were suspended in the same buffer and were disrupted on ice by ultrasonic disintegrator UZDN-1 (USSR) at 22 kHz for 6 × 15 sec bursts. The cell debris was removed by centrifugation at 10,000*g* for 30 min, and membranes were removed by centrifugation of the supernatant at 105,000*g* for 60 min on L5-65 model centrifuge (Beckman, USA). The cytoplasmic fraction was used as an enzyme preparation.

Conversion of UDP-GlcNAc into UDP-2-acetamido-2,6-dideoxyhexose. The enzymic reaction was carried out in a total volume of 200 μl. The mixture contained 0.25 mM UDP-GlcNAc (supplemented if necessary with UDP-[^{14}C]GlcNAc to specific radioactivity of 1 or 20 mCi/mmol), 2.5 mM UMP, 0.25 mM NAD^+, 0.1 mM NADPH, 10 mM $MgCl_2$, 25 mM Tris-HCl, pH 7.9, and 100 μl of the enzyme preparation. After incubation for 2 h at 37°C, an equal volume of ethanol was added with ice cooling, sedimented protein was removed by centrifugation at 6000 rpm for 15 min, and nucleotide sugars were isolated from the supernatant by paper chromatography in system A.

Isolation of *L*-fucose dehydrogenase from porcine liver and assay of its activity. The enzyme was prepared

[1] At present there is no generally accepted recommendations on nomenclature of *Salmonella* genus bacteria. In this paper, we use the old name of the microorganism as a species that is considered as possible (see http:/www.bacterio.cict.fr/salmonellanom.html).

from fresh porcine liver by fractionation of the liver homogenate with protamine sulfate and ammonium sulfate as described [21]. Enzyme activity was assayed at 25°C in 0.5 M Tris-HCl, pH 8.5, with 1 mM NAD^+ and 1 mM *L*-fucose. The enzyme reaction was followed by NADH content increase detected at 340 nm. The products of the oxidation were analyzed by paper electrophoresis (system C).

RESULTS

The enzymic conversion of nucleoside diphosphate hexoses into derivatives of 6-deoxyhexoses is known to occur with participation of oxidized and reduced pyridine nucleotides [9, 10]. Taking this into account, the incubation of UDP-GlcNAc with the cytoplasmic fraction from *Salmonella arizonae* O:59 cells was performed in the presence of NAD^+ and NADPH, as well as Mg^{2+} ions, usually required for such reactions, and UMP as an inhibitor of nonspecific pyrophosphatases. Several methods were used to characterize the enzymic reaction products formed.

As shown in our previous study of UDP-*D*-FucNAc biosynthesis [11], the most convenient method for isolation of nucleoside diphosphate sugar (NDPS) fraction from the incubation mixture is paper chromatography in system A. The procedure allows to separate NDPS fraction from the products of their degradation (UMP, hexose phosphates, monosaccharides) as well as from pyridine cofactors, which show lower mobility in this system.

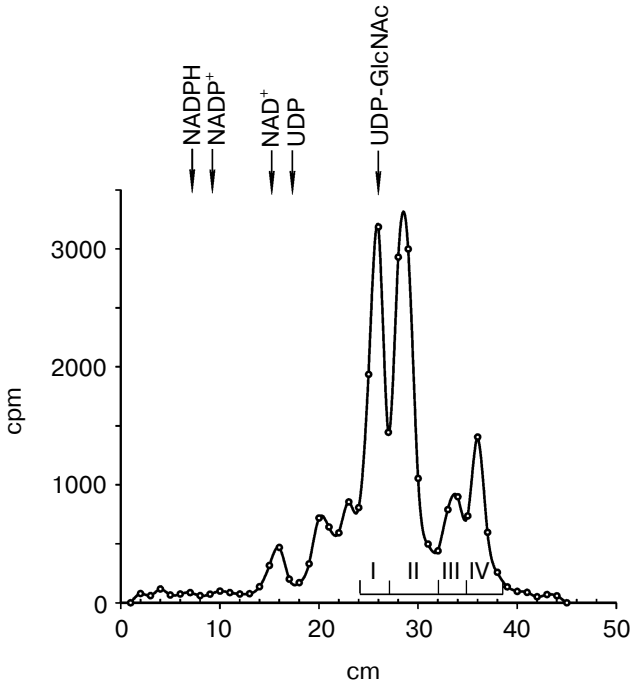

Fig. 1. Distribution of radioactivity in paper chromatography analysis (system A) of incubation mixture with UDP-[^{14}C]GlcNAc. Arrows indicate mobility of non-radioactive standards. Zone I, 24-27 cm; zone II, 27-32 cm; zone III, 32-35 cm; zone IV, 35-39 cm.

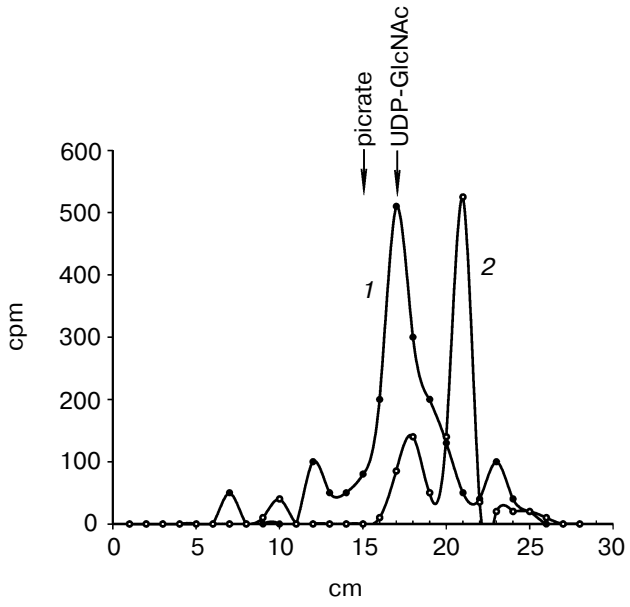

Fig. 2. Distribution of radioactivity in paper electrophoresis (system C) of products present in zones II (*1*) and III (*2*) of the paper chromatogram shown in Fig. 1.

Analysis of the incubation mixture with UDP-[^{14}C]GlcNAc as a substrate revealed the presence of the bulk of radioactivity in two not completely resolved components (Fig. 1). Mobility of the first of these components (zone I) coincided with that of standard UDP-GlcNAc, the second one (zone II) showed slightly higher chromatographic mobility ($R_{\text{UDP-GlcNAc}}$ 1.1). Appearance of a similar product was noticed by us earlier in study of UDP-2-acetamido-2,6-dideoxyhexose biosynthesis with cell-free preparation from *Streptomyces chrysomallus* [11]. Thus, it is reasonable to assume the substance of zone II to be the nucleotide sugar expected.

In addition to these two main components, two minor radioactive components with higher chromatographic mobility were revealed also in the incubation mixture (zones III and IV). The component from zone II was found (Fig. 2) to move during paper electrophoresis as a nucleoside diphosphate sugar, whereas the electrophoretic mobility of the substance from the zone III was characteristic for that of glycosyl phosphate, being obviously a product of NDPS degradation.

In additional experiments (data not shown), it was found that formation of the product present in zone II from UDP-GlcNAc was quite efficient when $NADP^+$ was used instead of NAD^+. The absence of both NAD^+ and $NADP^+$ resulted in drastic decrease in enzymic UDP-GlcNAc transformation. In the case of the absence of

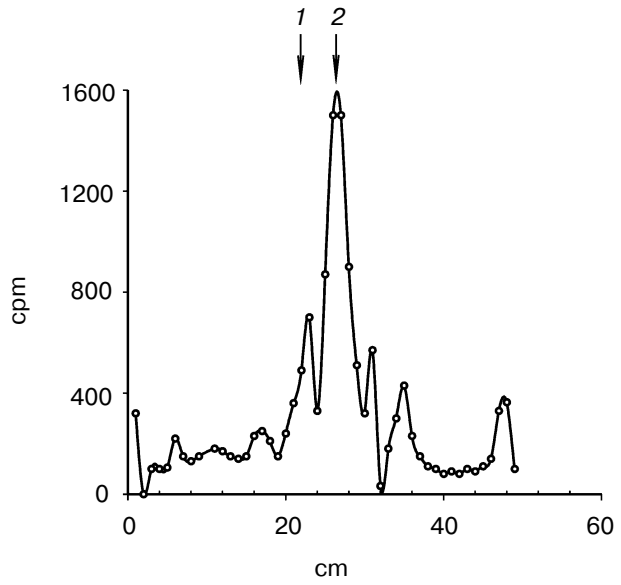

Fig. 3. Distribution of radioactivity in paper chromatography (system B) of strong acid hydrolyzate of NDPS fraction. Standards: *1*) *D*-glucosamine; *2*) *L*-fucosamine.

NADPH in the incubation mixture, the product of zone II was not detected at all.

For a preliminary identification of amino sugar residue in the reaction product, a total NDPS fraction (zones I + II from paper chromatogram in system A) was subjected to strong acid hydrolysis, followed by paper chromatography in system B, which is well suited for separation of different amino sugars. The main ninhydrin-positive product coincided in its mobility with fucosamine (Fig. 3), as could be expected in the case of UDP-GlcNAc conversion into UDP-FucNAc.

For final identification of the reaction product, the NDPS fraction was analyzed by HPLC. First of all, the application of this method was investigated for separation of UDP-GlcNAc and synthetic samples of UDP-α-*D*-FucNAc and UDP-β-*L*-FucNAc available to us. From different HPLC procedures suggested for nucleotide sugars separation [22-26], recently described [26] ion-pair, reverse phase chromatography was chosen as a basic approach. This method enabled good separation of nucleoside diphosphate hexoses and nucleoside diphosphate-6-deoxyhexoses with different nucleotide moiety but was not used before for UDP-2-acetamido-2,6-dideoxyhexose identification. After experimentation, the conditions were found (see "Materials and Methods") for efficient separation of these compounds using isocratic elution with 20 mM aqueous triethylammonium acetate, pH 6.0. Under these conditions, the elution times for UMP, UDP, UDP-Glc, UDP-GlcNAc, UDP-*L*-FucNAc, and UDP-*D*-FucNAc were found to differ significantly (Fig. 4a, standards), being 28, 30, 34, 38, 63, and 67 min, respectively. As can be seen, the presence of a methyl group at C-5 of hexose residue results in considerable increase of the elution time.

For the NDPS fraction obtained from UDP-GlcNAc with enzymes from *S. arizonae*, the analysis under conditions described (Fig. 4a) demonstrated unequivocally the nucleotide product to be UDP-derivative of N-acetyl-*L*-fucosamine. Additional components of the mixture with lowered elution time are probably the products of NDPS decomposition during their isolation by paper chromatography.

In an analogous experiment with UDP-[^{14}C]GlcNAc as a substrate (Fig. 4b), in addition to start-

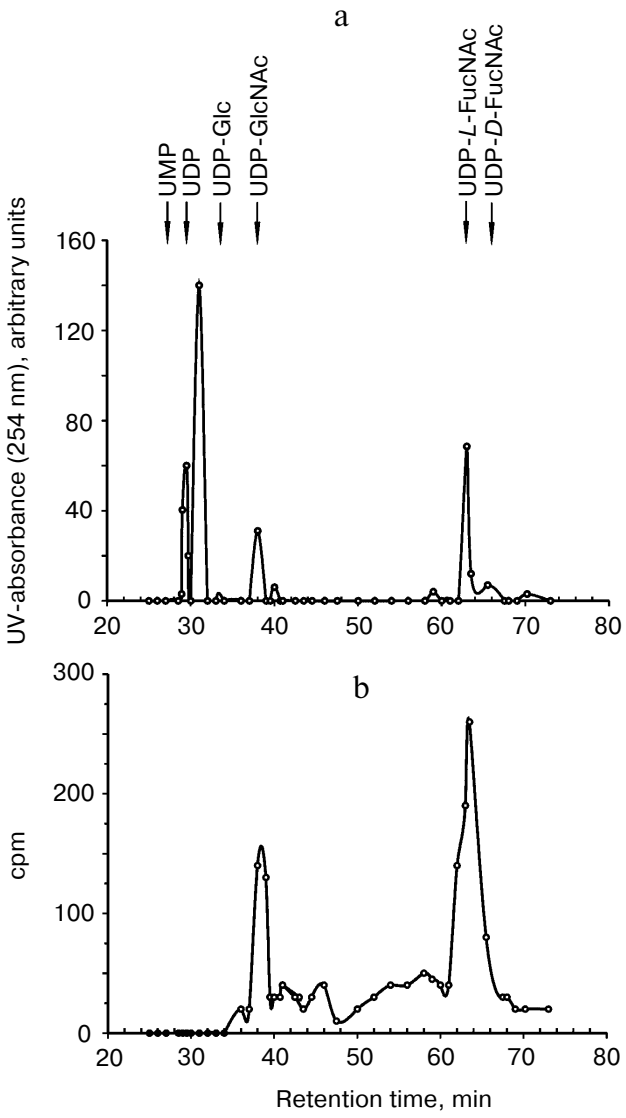

Fig. 4. Ion-pair reversed-phase HPLC of NDPS fraction (conditions are described in "Materials and Methods"). a) Incubation mixture with non-radioactive UDP-GlcNAc (detection by UV-absorbance; arrows show elution time of standards). b) Incubation mixture with UDP-[^{14}C]GlcNAc (fractions being assayed for radioactivity).

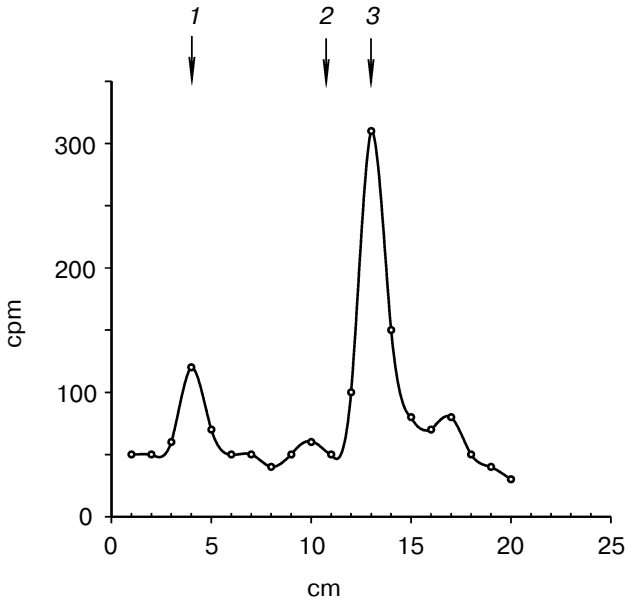

Fig. 5. Distribution of radioactivity in paper electrophoresis of incubation mixture with porcine liver *L*-fucose dehydrogenase and [^{14}C]FucNAc isolated from UDP-[^{14}C]FucNAc prepared by biosynthesis. Standards: *1*) [^{14}C]FucNAc; *2*) picric acid; *3*) *L*-[^{14}C]fuconate.

ing material, UDP-[^{14}C]-*L*-FucNAc was registered as a single product of the transformation. The degree of conversion of UDP-[^{14}C]GlcNAc was calculated as 68 and 64.5% from peak areas and from radioactivity distribution in eluates.

Further confirmation of *L*-configuration of the 2-acetamido-2,6-dideoxysugar formed was obtained with the use of porcine liver *L*-fucose dehydrogenase (EC 1.1.1.122) [21]. In the presence of NAD^+, the enzyme catalyses *L*-fucose oxidation to *L*-fuconolactone, which is converted rapidly to *L*-fuconic acid under the reaction conditions. This enzyme is used frequently for *L*-fucose microassay [27-29] and was shown to be able to oxidize a number of monosaccharides, *L*-fucose structural analogs.

We studied the ability of *L*-FucNAc and *D*-FucNAc to interact with the enzyme. It was found that *L*-FucNAc could be oxidized by the enzyme from porcine liver but not by *L*-fucose dehydrogenase from *Pseudomonas* sp. The initial velocity of the reaction, determined by measurement of NADH formed, was found to be approximately 10% of that for *L*-fucose and, consequently, the reaction may be used for determination of *L*-FucNAc. In contrast, *D*-FucNAc was not able to participate in the enzymic reaction, as NADH appearance could not be detected even after incubation for 15 h.

A monosaccharide, liberated from UDP-[^{14}C]FucNAc after mild acid hydrolysis, was treated with *L*-fucose dehydrogenase and NAD^+. After incubation for 15 h, the reaction mixture was analyzed by paper electrophoresis (Fig. 5). The main radioactive product present in the incubation mixture was found to correspond to *L*-fuconic acid in electrophoretic mobility. This result confirms the *L*-configuration of FucNAc residue in the isolated NDPS.

In conclusion, we were able to demonstrate for the first time the conversion of UDP-GlcNAc into UDP-*L*-FucNAc catalyzed by cytoplasmic enzymes from *Salmonella arizonae* O:59 cells, and the structure of the product formed was confirmed by two independent methods.

DISCUSSION

The presently known pathways for biosynthesis of nucleoside diphosphate-6-deoxyhexoses from nucleoside diphosphate hexoses are multistep processes [9, 10]. The first step consists of conversion of the hexose derivatives (dTDP-*D*-glucose, GDP-*D*-mannose) to the corresponding derivatives of 6-deoxyhexos-4-uloses. Transition from the latter to different 6-deoxyhexose derivatives includes stereospecific reduction of the C-4 keto group to yield a center of *D*- or *L*-configuration. This reaction may be preceded by epimerization at C-3 and C-5 (or epimerization at one of the chiral centers mentioned), resulting in formation of all the diversity of 6-deoxyhexoses in Nature.

Biosynthesis of 2-acetamido-2,6-dideoxyhexoses proceeds through intermediate derivatives of hexos-4-uloses as well (Fig. 6). Thus, UDP-*D*-FucNAc biosynthesis from UDP-GlcNAc includes intermediate (A), that gives after reduction the product differing from the starting NDPS by the configuration at C-4 of the amino sugar.

Transformation of UDP-GlcNAc into UDP-*L*-FucNAc is a more complicated process as it includes inversion of configuration at C-2, C-3, and C-5 of the amino sugar.

Initially, we supposed [8] this process to start from C-2 epimerization of UDP-GlcNAc leading to UDP-N-acetyl-*D*-mannosamine (UDP-ManNAc). This enzymic reaction was demonstrated in a number of microorganisms [30-35]. Conversion of UDP-ManNAc to UDP-*L*-FucNAc could proceed by a route analogous to the biosynthesis of GDP-*L*-fucose from GDP-*D*-mannose, i.e., including a precursor 4-keto-sugar (B), its epimerization at C-3 and C-5 to get a derivative of *L-xylo*-hexos-4-ulose, and finally reduction of the latter to UDP-*L*-FucNAc. An analogous pathway has been proposed by other authors [16].

When most of the experiments described in this paper were completed, a publication of researchers from Canada appeared [36]. This group studied enzymic reactions catalyzed by recombinant proteins (expressed in *Escherichia coli*) which have been proposed earlier, based on the molecular genetic data, to be involved in UDP-*L*-FucNAc biosynthesis. Genes for recombinant enzyme

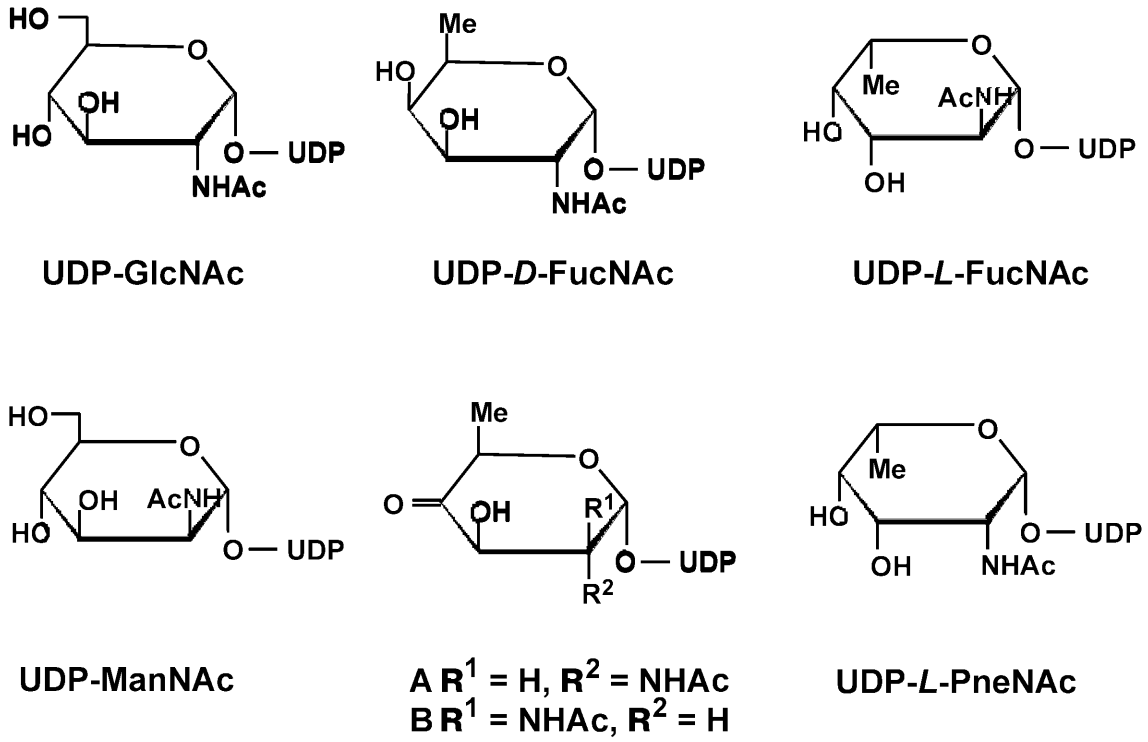

Fig. 6. Structures of some UDP-2-acetamido-2-deoxyhexoses, UDP-2-acetamido-2,6-dideoxyhexoses, and UDP-2-acetamido-2,6-dideoxyhexos-4-uloses. A) UDP-2-acetamido-2,6-dideoxy-*D-xylo*-hexos-4-ulose; B) UDP-2-acetamido-2,6-dideoxy-*D-lyxo*-hexos-4-ulose.

production originated from *Staphylococcus aureus* type 5 and from *P. aeruginosa* O:11. Results obtained allowed the authors to propose another mechanism for transformation of UDP-GlcNAc into UDP-*L*-FucNAc with C-2 epimerization of the amino sugar being the last step of the process. The use of three individual recombinant enzymes allowed the scientists from Canada to demonstrate convincingly that the first enzyme carries out UDP-GlcNAc conversion to compound (A) and subsequent epimerization at C-3 and C-5 of the intermediate leading to equilibrium mixture of 2-acetamido-2,6-dideoxyhexos-4-ulose derivatives. A minor component of the resulting mixture with *L-lyxo*-configuration serves as a substrate of the second enzyme, and its reaction product was identified unequivocally as UDP-derivative of 2-acetamido-2,6-dideoxy-*L*-talose (N-acetyl-*L*-pneumosamine, *L*-PneNAc). The authors assume that epimerization at C-2 of UDP-*L*-PneNAc, leading to UDP-*L*-FucNAc, represents the final step of the biosynthetic pathway.

It has to be mentioned that identification of the final product of the enzymic transformation as UDP-*L*-FucNAc was not so convincing as for the intermediates mentioned. The data presented implicate only appearance of UDP-2-acetamido-2,6-dideoxyhexose, whose electrophoretic mobility was different from that for UDP-*L*-PneNAc. The authors have specially noticed high lability of this compound. In our work, we were quite successful in isolation of UDP-*L*-FucNAc by paper chromatography and HPLC; it was found to be only slightly less stable than UDP-GlcNAc.

Further work is necessary to clarify the mechanism of the conversion of UDP-GlcNAc into UDP-*L*-FucNAc with enzymes from *S. arizonae*.

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