Preliminary Notes

PN 1279

Enzymic transfer of rhamnosyl units from thymidine diphosphate rhamnose to bacterial cell-wall fragments

In microorganisms, rhamnose occurs as a constituent of the cell-wall heteropolysaccharides[1,2], as the carbohydrate moiety of glycolipids[3,4], and as the carbohydrate component of certain endotoxins[5]. The thymidine diphosphate hexose pathway is now well established as the route of biosynthesis of rhamnose[2,3,6]. In an earlier report from this laboratory[7], it was suggested that thymidine diphosphate rhamnose serves as a donor of rhamnosyl units for the synthesis of cell-wall polysaccharides. Experimental verification of this suggestion has now been achieved. ^{14}C-labeled rhamnose from thymidine diphosphate rhamnose was transferred by an enzyme extract from *Streptococcus faecalis* to cell-wall fragments. The acceptor compound in the cell wall was a rhamnose-containing polysaccharide. Examination of an acid hydrolysate of a preparation of the polysaccharide showed that glucose, galactose, galactosamine and some yet unidentified components, in addition to rhamnose, were present in the hydrolysate. A detailed structural characterization of the cell-wall polysaccharide is in progress. Herein are presented the results of experiments on the transfer of rhamnosyl units from thymidine diphosphate rhamnose to the cell-wall fragments.

The thymidine diphosphate rhamnose labeled with ^{14}C in the rhamnose moiety was prepared from thymidine triphosphate and D-α-glucose-1-*P* uniformly labeled with ^{14}C by use of the thymidylyltransferase and related enzymes from *S. faecalis*[2]. Since it is difficult to separate the thymidine diphosphate rhamnose from thymidine diphosphate glucose, the preparation of labeled thymidine diphosphate rhamnose employed in these experiments also contained about 20% of labeled thymidine diphosphate glucose. Enzyme extracts were prepared by breaking washed *S. faecalis* cells with a Branson sonifier in a buffer solution of 0.05 M Tris or 0.1 M phosphate containing 0.01 M magnesium chloride and 0.01 M mercaptoethanol. The material from the sonifier was employed as the enzyme preparation and as a source of cell-wall fragments with endogenous acceptor cites for the rhamnosyl units. In an attempt to increase the number of available acceptor sites, acid-modified cell-wall fragments of *S. faecalis* were also added in Expt. 3. The cell-wall fragments from 2 l of *S. faecalis* culture were obtained by differential centrifugation and were hydrolyzed with 0.1 N sulfuric acid for 1 h at 100°. The resulting acid-modified cell-wall fragments were exhaustively washed with water and adjusted to pH 7 with phosphate buffer and included in the incubation mixture.

A typical reaction mixture was prepared as follows: A sample of ^{14}C-labeled thymidine diphosphate rhamnose preparation (total radioactivity 30000 counts/min) was dissolved in 0.5 ml of phosphate buffer of pH 7 and 1.5 ml of enzyme extract and cell-wall fragments were added. After incubation at room temperature for 6 h,

the cell-wall fragments were reisolated by centrifugation and washed three times with phosphate buffer and twice more with water to insure removal of any labeled thymidine diphosphate hexoses adsorbed or occluded in the cell-wall fragments. Control reaction mixtures were also prepared with enzyme extract inactivated by heat or by phosphotungstate. In all experiments the cell-wall fragments from the final washing were transferred to planchets, taken to dryness in a vacuum desiccator and the radioactivities were measured in a windowless gas-flow counter. Data from three experiments are recorded in Table I.

TABLE I

RADIOACTIVITIES (counts/min) OF CELL-WALL FRAGMENTS AFTER INCUBATION WITH [^{14}C]deTDP-RHAMNOSE AND *S. faecalis* ENZYME EXTRACT

	Expt. 1	*Expt. 2*	*Expt. 3*
Complete system	674	368	411
Inactive enzyme extract	49	27	17

The labeled cell-wall fragments from Expt. 3 were suspended in 2 ml of 1 N hydrochloric acid and heated at 100° for 2 h. Qualitative paper chromatograms developed by two ascents in a solvent system of *n*-butyl alcohol–pyridine–water (45:25:35, v/v) showed that rhamnose (apparent R_F value 0.79) and glucose (apparent R_F value 0.56) as well as several other reducing components were present in the hydrolysate. The compounds in the remaining hydrolysate were separated on preparative chromatograms and the areas of the chromatogram at apparent R_F value of 0.79, 0.56, and 0.20 corresponding to rhamnose, glucose and a blank were extracted. The rhamnose, glucose, and blank solutions were concentrated in a vacuum desiccator, transferred to planchets, and taken to dryness. The radioactivity values in the samples determined in a windowless gas-flow counter were as follows: 210 counts/min in the rhamnose, 33 counts/min in the glucose and 18 counts/min in the blank. The rhamnose and glucose were transferred from the planchets to test tubes and reacted with ethyl mercaptan in hydrochloric acid as described previously[2]. The reaction mixtures were chromatographed on paper in a solvent system of *n*-butyl alcohol–acetic acid–water (5:1:4, v/v). Diethyl dithioacetal of rhamnose was located at R_F value of 0.85 and diethyl dithioacetal of glucose at R_F value of 0.77. Rhamnose and glucose migrated with R_F values of 0.38 and 0.20, respectively, in this solvent system. The diethyl dithioacetals were extracted from the paper directly into planchets. On evaporation of the water, crystalline diethyl dithioacetal of rhamnose was obtained with m.p. 132° and an X-ray-diffraction pattern of authentic diethyl dithioacetal derivative of rhamnose. This derivative was radioactive, 245 counts/min for total sample recovered. The diethyl dithioacetal of glucose was not obtained in crystalline form. Some radioactivity (60 counts/min per total sample) was found in this amorphous material indicating that incorporation of radioactive glucose from labeled thymidine diphosphate glucose to cell-wall fragments had also occurred.

The labeled cells from Expt. 2 were suspended in 1 ml of 0.02 N hydrochloric acid. An aliquot of 0.1 ml of the suspension was added to 1.5 mg of uridine diphosphate glucose and both suspensions were heated at 100° for 15 min. In the hydrolysate of the aliquot sample, uridine monophosphate and uridine diphosphate but no uridine

diphosphate glucose were detected by paper chromatography in ethanol (7 parts) and 1 M ammonium acetate of pH 7.5 (3 parts). Evidently the conditions of hydrolysis were sufficient to cleave the uridine diphosphate glucose to uridine monophosphate and uridine diphosphate and to glucose-1-*P* and glucose. It is to be expected that any labeled thymidine diphosphate rhamnose adsorbed to the cell-wall fragments would also be hydrolyzed by this method since the latter compound is hydrolyzed at essentially the same rate as uridine diphosphate glucose. The hydrolysate of the total mixture was chromatographed in the above solvent and the areas of the chromatogram which would contain rhamnose and glucose were extracted and concentrated to dryness. Radioactivity was not detectable in these fractions indicating that labeled thymidine diphosphate hexoses were not adsorbed to the cell-wall fragments. The mild acid treatment did yield a water-soluble product which was radioactive and which moved in the ethanol–ammonium acetate solvent system as a relatively broad band with R_F value of 0.19 to 0.44. This band was extracted from the chromatogram and hydrolyzed in 1 N hydrochloric acid for 2 h. The conditions of hydrolysis were sufficient for breaking glycosidic bonds and rhamnose, glucose, galactose, and galactosamine were detected by chromatographic techniques and appropriate spray reagents in the hydrolysate. The rhamnose area from a preparative chromatogram of the hydrolysate was extracted and taken to dryness in a planchet. Radioactivity measurements showed that the rhamnose was radioactive; however, not all the radioactivity in the polysaccharide fraction was recovered in the rhamnose.

The results in Table I show that a transfer of ^{14}C-labeled glycosyl units from thymidine diphosphate hexoses to cell-wall fragments did indeed occur. Attempts to increase the amount of incorporation of radioactivity into the fragments have not yet been successful. In 8 experiments thus far completed, a small but significant radioactivity has always been found in the cell-wall fragments. That labeled rhamnose was transferred as intact units is amply demonstrated by the experiments on the recovery of radioactive rhamnose from the cell-wall fragments. Thus, thymidine diphosphate rhamnose functions as a donor of rhamnosyl units for the synthesis of polysaccharides in the bacterial cell wall as well as for the synthesis of rhamnose-containing glycolipids[8] and glycosides[9].

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Department of Biochemistry and Nutrition,
University of Nebraska, Lincoln, Nebr. (U.S.A.)

JOHN H. PAZUR
JOHN S. ANDERSON

[1] M. R. SALTON AND J. Y. PAVLIK, *Biochim. Biophys. Acta*, 39 (1960) 398.
[2] J. H. PAZUR AND E. W. SHUEY, *J. Biol. Chem.*, 236 (1961) 1781.
[3] L. GLASER AND S. KORNFELD, *J. Biol. Chem.*, 236 (1961) 1795.
[4] G. HAUSER AND M. L. KARNOVSKY, *J. Biol. Chem.*, 224 (1957) 91.
[5] O. WESTPHAL, *Ann. Inst. Pasteur*, 98 (1960) 789.
[6] T. OKAZAKI, L. OKAZAKI, J. L. STROMINGER AND A. M. MICHELSON, *J. Biol. Chem.*, 237 (1962) 3014.
[7] J. H. PAZUR AND E. W. SHUEY, *J. Am. Chem. Soc.*, 82 (1960) 5009.
[8] M. BURGER, L. GLASER AND R. M. BURTON, *Biochim. Biophys. Acta*, 56 (1962) 172.
[9] G. A. BARBER AND E. F. NEUFELD, *Biochem. Biophys. Res. Commun.*, 6 (1961) 44.

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