FORMATION OF GUANOSINE DIPHOSPHATE RHAMNOSE AND GUANOSINE DIPHOSPHATE TALOMETHYLOSE FROM GUANOSINE DIPHOSPHATE MANNOSE

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A polysaccharide containing D-rhamnose and D-talomethylose has been obtained from a gram-negative bacterium (strain GS) isolated from soil (Markovitz, 1961). Crude extracts of strain GS have now been found to convert GDP-D-mannose (Sigma Chemical Co., St. Louis, Mo.) to GDP-rhamnose and GDP-talomethylose in the presence of TPNH.

Extracts of bacteria were prepared by sonic disruption for 30 min in a Raytheon 9 kc sonic disintegrator, centrifuged at 34,800 x g for 10 min and the supernatant liquid was used as the enzyme preparation (20 to 30 mg protein per ml). Figure 1 illustrates the GDP-D-mannose induced oxidation of TPNH. The correction for TPNH oxidation by the enzyme preparation was 0.01 O.D. unit per min. Preincubation of the extract with GDP-D-mannose for 15 min followed by addition of TPNH caused a greater and more rapid oxidation of TPNH than when GDP-D-mannose and TPNH were added to the enzyme preparation simultaneously (Figure 1). Such results suggest that the compound catalyzing the oxidation of TPNH is derived from GDP-D-mannose. In experiments in which GDP-D-mannose was limiting (GDP-D-mannose, 0.04 μmole/ml; TPNH, 0.15 μmole/ml) spectrophotometric data indicated that each μmole of GDP-D-mannose oxidized 0.75 μmole of TPNH. The stoichiometry observed makes it unlikely that GDP-aldeheptose, found in commercial preparations of GDP-D-mannose at a level of about 8% (Ginsburg, 1960A), is the compound catalyzing this oxidation. Hydrolysis of GDP-D-mannose in 0.01 N HCl for 30 min destroyed the ability of the nucleotide to catalyze TPNH oxidation.

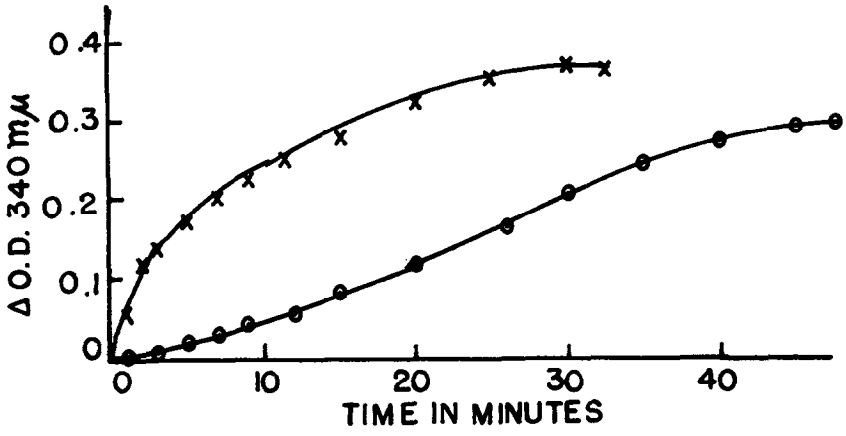

Fig. 1. TPNH oxidation catalyzed by GDP-D-mannose and the effect of preincubation of GDP-D-mannose with enzyme on TPNH oxidation. The reaction mixture contained 155 μmole of Na_2HPO_4-KH_2PO_4, pH 8.0, 6 μmole $MgCl_2$, 0.6 μmole TPNH, 0.9 μmole GDP-D-mannose, and 0.1 ml of enzyme in a final volume of 3.0 ml, room temperature. Values have been corrected for the oxidation rate of TPNH in an identical reaction mixture without GDP-D-mannose. The TPNH oxidation rate in the control mixture was 0.01 O.D. unit per minute. O, TPNH added at zero time; X, TPNH added after 15 min incubation at room temperature.

In order to determine whether or not nucleotide-linked methylpentoses are derived from GDP-D-mannose and TPNH the following reaction mixture was incubated for 2 hrs at room temperature; 50 μmoles GDP-D-mannose, 20 μmoles TPNH, 5 μmoles DPN, 50 μmoles glucose-6-phosphate, 7 μmoles $MgCl_2$, 175 μmoles Na_2HPO_4-KH_2PO_4, pH 7.5, 20 mg glucose-6-phosphate dehydrogenase (yeast, Sigma Chemical Co., St. Louis, Mo.) and an enzyme preparation from strain GS containing approximately 60 mg protein. Nucleotides were absorbed on one gm acid-washed Norit A (Crane, 1958; Ito and Strominger, 1960) and after thorough washing with distilled water the Norit A was heated with 0.01 N HCl at 100°C for 30 min to liberate the monosaccharides linked to nucleotides. The Norit A was washed with water and with 10% ethanol. Preliminary paper chromatography of both the 0.01 N HCl fraction and the 10% ethanol fraction revealed the same components. After paper chromatography of both fractions in acetic acid:n-butanol:water (1:4:1) the components visualized with an aniline oxalate spray were eluted and the

following substances were identified in the quantities indicated: rhamnose, 0.87 μmole; talomethylose, 0.43 μmole; ribose, 1.0 μmole; mannose, 5.2 μmole. Low yields of methylpentose may have been due to destruction of GDP-D-mannose by the crude enxyme preparation.

The isolated rhamnose and talomethylose were identified by paper chromatography in 4 solvent systems with authentic rhamnose, talomethylose and fucose as standards: A, acetic acid:n-butanol:water (1:4:1); B, 2-butanone saturated with water (Krauss, et al, 1960); C, tert.-amyl alcohol:isopropanol: water (8:2:3) (Cifonelli and Smith, 1954); D, pyridine:n-butanol:water (4:6:3). In addition, both isolated methylpentoses reacted in the specific test for methylpentoses (Dische and Shettles, 1948) to produce a chromophore with an absorption spectrum identical to that given by authentic L-rhamnose. The specific test for methylpentoses (Dische and Shettles, 1948) was also used to estimate the isolated rhamnose and talomethylose using appropriate standards. The isolated ribose was identified by paper chromatography in solvents A, B, C, D and water-saturated phenol-1% ammonia containing 0.04% 8-hydroxyquinoline (Block et al., 1958) and was found to react in the orcinol test (Schneider, 1957) to give a chromophore with an absorption spectrum identical to that given by authentic ribose. Mannose was tentatively identified by chromatography in solvent A, and distinguished from glucose enzymatically in an assay with ATP, hexokinase, glucose-6-phosphate dehydrogenase and TPN. As a control, the crude enzyme preparation itself, after incubation for 2 hrs at room temperature, was examined to determine the content of nucleotide-linked monosaccharides. Only two spots were visualized with the aniline oxalate spray after paper chromatography in solvent A . These spots had mobilities coinciding with those of glucose and ribose, the latter giving a pink color with aniline oxalate spray characteristic of pentoses The spot corresponding to ribose was estimated visually to represent about 25% of the ribose present in the nucleotide fraction isolated after incubation of GDP-D-mannose and the TPNH generating system with the enzyme preparation.

In another experiment the TPNH generating system was inactivated prior to addition of GDP-D-mannose (10 μmoles) and the enzyme preparation (approximately

30 mg protein). The following compounds were isolated from Norit A hydrolysates: mannose, 0.16 μmole; ribose, 0.31 μmole; rhamnose, 0.21 μmole; talomethylose, 0.22 μmole. As a control the crude enzyme preparation, after incubation for 2 hrs at room temperature, was heat-inactivated and combined with the complete incubation mixture. This control was carried through the entire procedure and 0.86 μmole of mannose was isolated from Norit A hydrolysates in addition to 0.21 μmole glucose (determined enzymatically with ATP, hexokinase, glucose-6-phosphate dehydrogenase and TPN) and 0.05 μmole of the component migrating like ribose. Neither rhamnose nor talomethylose was obtained from the Norit A hydrolysates of the heat-inactivated control mixture. Other incubated controls, containing the enzyme preparation and TPNH generated enzymatically (but without GDP-D-mannose), did not contain nucleotide-linked methylpentoses. From this experiment it was concluded that an intact TPNH-generating system was not required and that the nucleotide-linked methylpentoses did not arise from endogenous precursors.

An attempt was made to isolate and identify the intact nucleotides derived from GDP-D-mannose and to determine whether or not TPNH was required for their formation. Fourteen μmoles of GDP-D-mannose and 0.7 ml of enzyme preparation (approximately 15 mg protein) were added to each of two tubes (Tube 1 and Tube 2). After 30 min incubation at room temperature, 5.1 μmoles TPNH in 0.5 ml were added to tube 1 while 0.5 ml water was added to tube 2. The incubation was continued for 1.5 hrs. The reaction was terminated by addition of about 5 volumes ethanol and sufficient ammonium acetate to obtain complete precipitation of polysaccharides. Precipitated material was removed by centrifugation and the supernatant fluids were concentrated at 40°C in a Flash-evaporator. The concentrated supernatant fluids were streaked on Whatman #1 paper and chromatographed in neutral 1 M ammonium acetate:ethanol (3:7) (Paladini and Leloir, 1952). Areas of the paper absorbing ultraviolet light were cut out and eluted. Tube 1 (GDP-D-mannose + TPNH) contained a fraction that migrated like GDP-D-mannose and gave a typical ultraviolet absorption spectrum for compounds containing guanosine. Analysis of this fraction for guanosine (assuming

a molar absorbancy of 13.6×10^3 for guanosine at 250 mμ and pH 7.0), 0.03 N HCl-labile carbohydrate (Park and Johnson, 1949) and methylpentose (Dische and Shettles, 1948; L-rhamnose as standard) gave 2.4 μmoles, 2.1 μmoles and 0.86 μmoles respectively, indicating a mixture of guanosine-linked compounds. This fraction was hydrolyzed at 100°C with 0.03 N HCl for 30 min and subsequently chromatographed in solvent A. Visualization of the spots with aniline oxalate revealed the presence of rhamnose, talomethylose, mannose, and a component giving an orange color with an $R_{rhamnose} = 0.15$. A trace of another component that migrated like ribose (pink color; $R_{rhamnose} = 0.81$) was also seen but was present in much smaller quantity than was noted in previous experiments. Tube 2 (GDP-D-mannose) had little if any (<10%) guanosine-containing compounds that contained either methylpentose or 0.03 N HCl-labile carbohydrate.

Spectrophotometric, colorimetric, and chromatographic analyses indicate that strain GS contains enzymes that convert GDP-D-mannose to GDP-rhamnose and GDP-talomethylose only in the presence of TPNH. The stoichiometry of the overall reaction can be written as follows:

$$2 \text{ GDP-D-mannose} + 2 \text{ TPNH} + 2 \text{ H}^+ \longrightarrow$$
$$\text{GDP-rhamnose} + \text{GDP-talomethylose} + 2 \text{ TPN} + 2 \text{ H}_2\text{O}$$

The above equation does not require the formation of equal quantities of GDP-rhamnose and GDP-talomethylose in view of the possibility that separate enzymes may be involved. Since the polysaccharide synthesized by strain GS contains D-rhamnose and D-talomethylose (Markovitz, 1961) it seems likely that the GDP-linked methylpentoses are the D isomers, but this remains to be established. The origin of the ribose is uncertain. An enzymatic system was used for generating TPNH in experiments in which substantial quantities of ribose were identified chromatographically but GDP-ribose was not isolated.

The formation of GDP-rhamnose and GDP-talomethylose from GDP-D-mannose appears to proceed in a fashion similar to the formation of GDP-L-fucose from GDP-D-mannose (Ginsburg, 1960B) and to the formation of TDP-L-rhamnose from TDP-glucose (Pazur and Shuey, 1961; Glaser and Kornfeld, 1961). This is the

first report of two methylpentose-containing nucleotides in which the methylpentoses are 4-epimers.

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