Enzymatic Synthesis of the Maltose Analogues, Glucosyl Glucosamine, Glucosyl N-Acetylglucosamine and Glucosyl 2-Deoxyglucose by an Extract of Neisseria perflava*

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(Received for publication, February 1, 1961)

It has been shown by Fitting and Doudoroff (1) that maltose phosphorylase causes the reversible cleavage of maltose in the reaction,

\[ 4\text{-O-\(\alpha\)-D-glucosyl D-glucose} + \text{Pi} \rightarrow \beta\text{-glucose-1-P} + \text{glucose} \]

Since xylose could be substituted for glucose to yield 4-O-\(\alpha\)-D-glucosyl \(\alpha\)-xylose (2), it seemed possible that the enzyme could be used for the synthesis of yet other disaccharides possessing the \(\alpha\)-1,4 linkage. The present communication demonstrates that glucosamine, \text{N-acetylglucosamine}, and 2-deoxyglucose can indeed serve as substrates to yield the corresponding maltose analogues. The preparation of these new disaccharides and some of their properties are also reported.

**EXPERIMENTAL PROCEDURE**

**Methods and Materials**

*Cultivation of Bacteria and Preparation of Crude Maltose Phosphorylase—Enzyme was previously prepared from Neisseria meningitidis (1). In the present work a nonpathogenic organism, Neisseria perflava, was used since we were able to show that it contains relatively large amounts of maltose phosphorylase. N. perflava, kindly donated by Dr. J. Mager, was cultivated in a medium containing the following ingredients (\%): Bactopeptone, 1; yeast extract, 0.5; NaCl, 0.5; Na\textsubscript{2}HPO\textsubscript{4}·12H\textsubscript{2}O, 0.5, and glucose, 1. Cultures were grown at 37\(^\circ\) for 20 hours in a New Brunswick shaker incubator operating at 120 r.p.m. One volume of a fresh culture served as starter for 10 volumes of medium. Harvest of bacteria and preparation of extracts were carried out in the cold. Cells were collected in the Sharples centrifuge and the sediment was washed twice with 10 volumes of 0.02 M Tris buffer, pH 7.4, containing 0.5% KCl. Cells were finally suspended in the above buffer at a concentration of 1.5 g (wet weight)/10 ml and disrupted in the Nossal shaker (3) or in a Raytheon 10-ke oscillator. Cell debris was removed by centrifugation at 10,000 \(\times\) g for 20 minutes. About 3 g of extract protein were obtained per 10 liters of culture. This extract served as crude maltose phosphorylase in all experiments.*

* This investigation was supported in part by a research grant (E-1494) from the National Institutes of Health, United States Public Health Service.

The preparation, when stored for 3 months at 20\(^\circ\), did not show any appreciable loss of activity.

**Maltose Phosphorylase Activity**—The rate of arsenolysis of maltose served as the measure of activity (cf. (1)). The reaction mixture in a final volume of 2 ml contained: maltose, 100 \(\mu\)moles; sodium arsenate, pH 6.5, 40 \(\mu\)moles; histidine buffer, pH 6.5, 40 \(\mu\)moles; and crude enzyme, 5 mg of protein. After incubation for 60 minutes at 37\(^\circ\), the reaction was terminated by deproteinization with the Ba(OH)\textsubscript{2}, ZnSO\textsubscript{4} reagents (4) and the glucose formed was measured with glucose oxidase (5). Assays were corrected for weak maltase activity which was present in some extracts. Maltose phosphorylase activity corresponded to 4 to 6 \(\mu\)moles of glucose formed per mg protein per hour.

The crude extract also contained an enzyme which converts \(\beta\)-glucose-1-P to glucose-6-P in the presence of a divalent cation (6). This interfering reaction was eliminated by addition of EDTA.1

**Serum Maltase**—A dialyzed ammonium sulfate fraction prepared from ox serum according to Lieberman and Eto (7) was used.

**Analytical Methods**—Reducing aldose was measured iodometrically (8), glucose with glucose oxidase (5), 2-deoxyglucose with thiobarbiturate (9), glucosamine with the Elson and Morgan reagent (10), \text{N-acetylglucosamine} according to Aminoff et al. (11) and also according to Reissig et al. (12). Protein was determined colorimetrically according to Lowry et al. (13) and turbidimetrically according to Stadtman et al. (14). Pi was measured by the method of Fiske and SubbaRow (15).

The disaccharides to be described in the present communication were determined by assay of the glucose released after hydrolysis in 2 \(\times\) HCl for 1 hour at 98\(^\circ\).

**Chemicals**—Phosphate buffer contained both Na\textsuperscript{+} and K\textsuperscript{+}. \(\beta\)-Glucose-1-P was prepared by phosphorylation of maltose (16). Mannosamine and \text{N-acetylmannosamine} were kindly donated by Dr. S. Roseman. Other materials were commercial preparations.

**RESULTS**

A variety of sugars were tested in the maltose phosphorylase system to establish whether they react with \(\beta\)-glucose-1-P to

1 The abbreviation used is: EDTA, ethylenediaminetetraacetate.
Glucosyl transfer from $\beta$-glucose-1-P to aldohexoses

The reaction mixture in a final volume of 1 ml contained $\beta$-glucose-1-P, 10 pmoles; aldohexose, 20 pmoles; EDTA, 5 pmoles; histidine buffer, pH 6.5, 50 pmoles; and crude maltose phosphorylase, 5 mg of protein. The system was incubated for 180 minutes at 37°, and the reaction was stopped with 5% trichloroacetic acid.

<table>
<thead>
<tr>
<th>Hexose added*</th>
<th>$P_i$ formed from $\beta$-glucose-1-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>&lt;0.2 pmol</td>
</tr>
<tr>
<td>$d$-Glucose</td>
<td>8.5 pmol</td>
</tr>
<tr>
<td>$d$-Glucosamine</td>
<td>8.4 pmol</td>
</tr>
<tr>
<td>N-Acetyl-$d$-glucosamine</td>
<td>8.4 pmol</td>
</tr>
<tr>
<td>$\alpha$ 2-Deoxyglucose</td>
<td>8.3 pmol</td>
</tr>
<tr>
<td>$d$-Mannosamine</td>
<td>&lt;0.2 pmol</td>
</tr>
<tr>
<td>N-Acetyl-$d$-mannosamine</td>
<td>&lt;0.2 pmol</td>
</tr>
</tbody>
</table>

* $d$-Mannose, $d$-galactose, $d$-ribose, and several other sugars had been shown previously not to react in such a system (1).

† 2-Deoxyglucose, 8.1 pmoles, was shown to be consumed.

yield disaccharides. As shown in Table I, addition of glucosamine, N-acetylglucosamine and 2-deoxyglucose caused the specific release of $P_i$ from $\beta$-glucose-1-P. Paper chromatography indicated that the corresponding disaccharides were formed. On the basis of these observations, maltose phosphorylase was employed for the preparation of the hitherto unknown disaccharides.

Preparation of Glucosyl Glucosamine—This disaccharide could be synthesized by a condensation of $\beta$-glucose-1-P with glucosamine. However, this reaction would require the preparation of large amounts of $\beta$-glucose-1-P. The disaccharide was therefore synthesized by an exchange reaction between maltose and glucosamine in the presence of catalytic amounts of $P_i$ (see Equation 1).

The reaction mixture in a final volume of 60 ml contained: maltose, 3 mmoles; glucosamine, 1.2 mmoles; $P_i$, 0.3 mmole; Tris buffer, pH 7.0, 3 mmoles; EDTA, 0.12 mmole, and crude enzyme, 390 mg of protein. The Tris buffer was added to decrease the pH to 5.0. Flocculating protein was removed by centrifugation. Part of the supernatant solution, containing 0.4 mmole of disaccharide, was put on a Dowex 50-H+ column (200 to 400 mesh) (30 x 250 mm). Distilled water, 500 ml, was passed slowly through the column, removing glucose and maltose. The column was eluted with 0.3 N HCl, as suggested by Gardell (18) for separation of amino sugars. The flow rate was adjusted to 0.5 ml per minute. Elution of sugars was followed by reductometry (19). After 150 ml of acid had emerged from the column, glucosyl glucosamine appeared in the effluent and was collected in a volume of 90 ml. Elution of the monosaccharide, glucosamine, started only after a further amount of 300 ml of acid passed through the column. Since the disaccharide was eluted much earlier than the monosaccharide, elution of the former can probably be accelerated, without endangering separation.

The pooled fractions containing the disaccharide were deacified with Amberlite IRA 400 CO$_3^-$ anion exchanger. The solution was evaporated to dryness under reduced pressure at room temperature, keeping the pH at about 5 by intermittent addition of HCl. To remove the last traces of water, methanol was twice added and evaporated. Dried crystalline material, 160 mg, containing 350 pmoles of glucosyl glucosamine hydrochloride, was obtained. The properties of this disaccharide are summarized in Table II.

Preparation of Glucosyl N-Acetylglucosamine—Although this disaccharide could be prepared enzymatically, as shown in Table I, the chemical N-acetylation of pure glucosyl glucosamine appeared to be the method of choice.

Glucosyl glucosamine, 30 pmoles, was N-acetylated and purified according to the method of Crompton (20). N-Acetyl amino derivative, 27.5 pmoles, was obtained. The product had the same chromatographic migration rate as the disaccharide prepared by enzymatic condensation of $\beta$-glucose-1-P with N-acetylglucosamine.

Information on the glucosidic linkage in the acetylated disaccharide was obtained by testing the compound in the assay for N-acetyl amino sugars (11). It has been shown that in this assay, N-acetylglucosamine substituted at carbon 4 by a methyl or a glycosyl group reacted only to a small extent or not at all, whereas substitution at other carbon positions did not interfere with and sometimes even augmented the reaction (21, 22). When glucosyl N-acetylglucosamine was tested, the color yield was only 12% of that of an equimolar amount of free N-acetylglucosamine. This observation indicates that in glucosyl N-acetylglucosamine and in glucosyl glucosamine, from which the former was prepared, the glucosidic linkage is on carbon 4. Other properties of glucosyl N-acetylglucosamine are given in Table II.

TABLE II
Analyses of disaccharides synthesized

All analyses were carried out on the purified disaccharides. Descending chromatography was performed on Whatman No. 1 paper.

Hydrolysis by serum maltase was tested in 1 ml of a mixture containing: disaccharide, 10 pmoles; phosphate buffer, pH 4.0, 40 pmoles; and serum maltase, 10 mg of protein. The mixture was incubated at 37° for 3 hours. The glucose and the corresponding aldohexose formed were determined by independent methods. The percentage of hydrolysis was calculated from the amount of glucose formed.

<table>
<thead>
<tr>
<th>Disaccharide</th>
<th>$t_{25}^{\text{R}}$</th>
<th>Chromatographic migration</th>
<th>Hydrolysis by serum maltase</th>
<th>Ratio of products formed (glucose to corresponding aldohexose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucosyl glucosamine</td>
<td>+147</td>
<td>0.31</td>
<td>0.58</td>
<td>100</td>
</tr>
<tr>
<td>Glucosyl N-acetylglucosamine</td>
<td>+110</td>
<td>0.62</td>
<td>0.96</td>
<td>83</td>
</tr>
<tr>
<td>Glucosyl 2-deoxyglucose</td>
<td>+123</td>
<td>1.00</td>
<td>1.20</td>
<td>100</td>
</tr>
</tbody>
</table>

* Not measured. After acid hydrolysis the molar ratio of products was 0.94.
Preparation of Glucosyl 2-Deoxyglucose—This disaccharide was prepared by the enzymatic condensation of 2-deoxyglucose with β-glucose 1-P. The reaction mixture, in a final volume of 40 ml contained: β-glucose-1-P, 540 μmoles; 2-deoxyglucose, 320 μmoles; EDTA, 270 μmoles; and crude enzyme, 270 mg of protein, which had been dialyzed for 6 hours at 5°C against distilled water. After incubation for 210 minutes at 37°C, the reaction was terminated with the Ba(OH)₂, ZnSO₄ reagents (4). The mixture was centrifuged, the supernatant solution was saved and the precipitate was extracted with 140 ml of 75% ethanol. The two supernatant solutions were combined and concentrated to a volume of 3 ml under reduced pressure at 30°C. The material was separated by descending chromatography on 4 sheets of Whatman No. 3 MM paper with butanol-ethanol-water (4:1:1) as the developing system. The areas containing only glucosyl 2-deoxyglucose were eluted with distilled water. A total of 360 μmoles of the disaccharide were obtained in a volume of 7.5 ml.

When the purified disaccharide was reduced by borohydride (23), subsequent hydrolysis yielded 1 mole of glucose per mole of disaccharide. It was thus shown that in the disaccharide obtained glucose constitutes the nonreducing moiety. Information on the linkage in the disaccharide was obtained by testing the compound in the assay for 2-deoxy sugars (9). In this assay, the disaccharide reacted only to an extent of 6% of that of free 2-deoxyglucose. Since the method requires cleavage by periodate between carbon 3 and 4 (9), it follows that one of these carbons in the 2-deoxyglucose moiety of the disaccharide is substituted by the glucosidic linkage. Other analyses of glucosyl 2-deoxyglucose are given in Table II.

**DISCUSSION**

It was shown previously that maltose phosphorylase catalyzes the synthesis of 4-O-α-glucosyl d-glucose and 4-O-α-d-glucosyl d-xylene (2). All tests performed indicate that the new sugars described in the present communication also are disaccharides possessing the α-1,4 glucosidic linkage.

Although a crude enzyme preparation served in these studies, it appears justified to ascribe the observations on substrate specificity solely to the action of maltose phosphorylase. Some of these observations deserve further comment. Whereas glucose readily reacted with β-glucose-1-P, mannose was shown to be inert (1). It was therefore evident that the enzyme had stereo-specific requirements towards carbon two of the acceptor aldose. Since 2-deoxyglucose served as substrate, it could be deduced that a hydroxyl group at carbon 2 is not required, and furthermore, that its presence in the mannose configuration renders the molecule unavailable for enzyme action. This concept is confirmed and amplified by the finding that the hydroxyl group at carbon 2 in glucose may be substituted by an amino group or even an acetylamino group while the molecule retains its activity as substrate. Such substitutions are, however, not permissible in the mannose configuration as demonstrated by the inactivity of mannosamine and N-acetylmannosamine.

Although the disaccharides described have not yet been tested in biological systems, it is possible that they might find application as maltose analogues in studies of permeability, enzyme induction, and substrate specificity of α-glucosidases.

**SUMMARY**

An extract of Neisseria perfrava catalyzed the synthesis of three new maltose analogues, glucosyl glucosamine, glucosyl N-acetylglicosamine, and glucosyl 2-deoxyglucose. Methods for the preparation of these disaccharides in pure form were developed. Some of the physical and chemical characteristics of the new disaccharides were reported. The requirements of maltose phosphorylase towards the configuration at carbon 2 of the acceptor aldose were analyzed.

**REFERENCES**

Enzymatic Synthesis of the Maltose Analogues, Glucosyl Glucosamine, Glucosyl N-Acetylglucosamine and Glucosyl 2-Deoxyglucose by an Extract of Neisseria perflava
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