# ENZYMIC SYNTHESIS OF INOSINE 5'-DIPHOSPHATE GLUCOSE AND INOSINE 5'-DIPHOSPHATE MANNOSE[1,2]

H. Verachtert, S. T. Bass and R. G. Hansen

Department of Biochemistry
Michigan State University
East Lansing, Michigan

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Enzyme preparations from mammalian sources catalyze the formation of two new hexose-nucleotides, IDP-glucose from ITP and glucose-1-P and IDP-mannose from ITP and mannose-1-P. The reactions are of interest in view of the fact that ITP is the only ribonucleoside triphosphate whose incorporation into sugar linked nucleotides has not thus far been described, although its occurrence in mammalian tissue has been demonstrated (Tancredi and Bianchi, 1958; Telepneva, 1960; Dianzani Mor, 1960; Davey, 1962). It has also been observed that extracts of lactating rat mammary gland in the presence of hexose-1-phosphates and nucleoside triphosphates catalyze the formation of UDP-glucose, GDP-glucose and GDP-mannose (Carlson and Hansen, 1960) and of CDP-glucose, TDP-glucose and ADP-glucose (Verachtert, Bass and Hansen, in preparation). Studies on the biosynthesis and the complete characterization of the different reaction products were undertaken. The present communication describes the formation and the identification of IDP-glucose and IDP-mannose.

The enzyme used was a 200- to 300-fold purified preparation from either lactating rat mammary gland, rat liver or calf liver. The tissue was homogenized in 0.03 N KOH containing 0.005 M EDTA. The extracts were centrifuged, the pH adjusted to 7 and then treated successively with protamine sulfate,

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ammonium sulfate, calcium phosphate gel and DEAE cellulose. This resulted in a 300-fold purification, based upon GDP-glucose pyrophosphorylase.

In a typical experiment, 1 μmole of ITP was incubated with 1 μmole of either glucose-1-P or mannose-1-P, 2 μmoles of magnesium acetate and 50 μg of enzyme protein in a total volume of 0.3 ml of 0.3 N Tris-acetate buffer, pH 7.8. To terminate the reaction, the mixtures were heated at 100° for two minutes, cooled and evaporated to dryness. The residues were taken up in 100 μl of water and spotted on Whatman number 1 or polyethyleneimine treated Whatman number 1 papers, which were prepared by the method described by Randerath (Randerath, 1963). The developing solvents were ethanol-ammonium acetate (7:3) pH 7.5 or 3.8 with the normal papers and 0.3 N LiCl with the treated papers. Under these latter conditions the hexose-nucleotides can be separated from mono-, di- and triphosphates more effectively in a shorter time than with the usual solvents for nucleotides (in preparation). When the incubation mixtures contained enzyme, ITP, $Mg^{++}$ and either glucose-1-P or mannose-1-P, a new spot could be detected on the chromatograms when viewed under ultraviolet light.

For isolation of the reaction products the amounts of substrates and enzymes were increased proportionately and the products separated and purified either by adsorption on charcoal followed by paper chromatography or adsorption on Dowex-Cl and elution with LiCl. Both products resulting from incubation of ITP and glucose-1-P or ITP and mannose-1-P exhibited absorption spectra typical of inosine, with maxima at 248.5 mμ at pH 6 and 253 mμ at pH 11. The results of chemical analysis of the products are shown in Table I.

Neither product had reducing properties when treated with ammoniacal silver nitrate. However, under the conditions of the reducing sugar determination (Park and Johnson, 1949) one-third equivalent of IDP-mannose was analyzed as free reducing power (Table I). Chromatography of the reaction mixture before the addition of the ferric ion solution revealed the presence of IDP-mannose and a smaller quantity of IDP. Thus the free reducing power should probably be attributed to the partial splitting of IDP-mannose under alkaline conditions. No reducing activity was observed with IDP-glucose under the same conditions.

Both products released one equivalent of reducing power upon hydrolysis at 100° for 15 minutes at pH 2. The sugars were identified by paper chromatography with butanol-ethanol water (52-32-16) as the solvent, and two dimensional chromatography with phenol-water as the solvent in the first direction and butanol-propionic acid as the solvent in the second direction (Benson et al., 1950). Glucose from IDP-glucose and mannose from IDP-mannose were found as judged by comparison with authentic standards. During acid hydrolysis, the intermediate formation of IDP and IMP was observed on the paper chromatograms.

TABLE I

Analysis of IDP-glucose and IDP-mannose synthesized enzymically

| | IDP-glucose | IDP-mannose |
|---|---|---|
| Inosine (1) | 1.0 | 1.09 |
| Acid labile phosphorus (2) | 0.97 | 0.95 |
| Inorganic free phosphorus (2) | 0.0 | 0.0 |
| Total phosphate (3) | 2.0 | 2.0 |
| Reducing sugar (4) | 0.0 | 0.30 |
| Reducing sugar after hydrolysis (5) | 1.03 | 0.98 |
| Glucose-1-P (6) | 0.94 | 0.00 |

(1) Calculated for inosine at 250 mμ at pH 6 (with ε max. of 12,200).
(2) Method of Fiske and SubbaRow (Fiske and SubbaRow, 1925).
(3) Method of King (King, 1932).
(4) Method of Park-Johnson (Park and Johnson, 1949).
(5) Reducing sugar after hydrolysis at pH 2 for 15 minutes at 100°.
(6) Determined with snake venom pyrophosphatase, TPN, phosphoglucomutase and glucose-6-P dehydrogenase.

Similar hydrolysis curves were found for IDP-glucose and IDP-mannose when heated at 100° in 0.01 N HCl. Hydrolysis of both products was complete in 5 to 10 minutes.

Both products were also incubated for one hour in Tris-acetate buffer, pH 7.8 with snake venom, containing 5'-nucleotidase. By paper chromatography the products formed were either glucose-1-P or mannose-1-P, inorganic phosphate and only one ultraviolet absorbing spot corresponding to inosine. The quantitative analysis of the products formed under these conditions is given in Table II.

TABLE II

Analysis of products resulting from incubation of either IDP-glucose or IDP-mannose with snake venom pyrophosphatase containing 5'-nucleotidase

| | IDP-glucose | IDP-mannose |
|---|---|---|
| Inorganic free phosphate (1) | 1.0 | 1.0 |
| Total phosphate (1) | 1.92 | 2.07 |
| Reducing sugars (2) | 0.0 | 0.0 |
| Reducing sugar after acid hydrolysis (3) | 0.94 | 0.96 |

(1) Method of Fiske and SubbaRow (Fiske and SubbaRow, 1925).
(2) Method of Park-Johnson (Park and Johnson, 1949).
(3) Reducing sugar after hydrolysis at pH 2 for 15 minutes at 100°.

The results clearly indicate that the products contain inosine-5'-phosphate, and either glucose-1-P or mannose-1-P, bound to inosine-5'-phosphate through a pyrophosphate linkage.

For final confirmation IDP-glucose was chemically synthesized from inosine-5'-phosphate and glucose-1-P essentially by the method of Khorana (Roseman, Distler, Moffat, Khorana, 1961). The chemically prepared product and the enzymically prepared product behaved similarly on treatment with snake venom enzymes, hydrolysis and paper chromatography. The synthesis of IDP-mannose was not attempted.

The similarity in structure of IDP-glucose with other known sugar-nucleotides was further established by the products IMP and cyclic glucose 1,2-monophosphate after treatment with ethanol-$NH_4OH$ (7.5:3). Under these conditions UDP-glucose (see also Leloir, 1951) ADP-glucose, CDP-glucose, GDP-glucose and TDP-glucose are split into the corresponding monophosphates and cyclic glucose 1,2-monophosphate.

Furthermore, it was observed that on incubation of these hexose nucleotides with $Mg^{++}$ in Tris at pH 7.8, a similar degradation occurred. Under both circumstances IDP-mannose and GDP-mannose are unaffected.

On incubation of either IDP-glucose or IDP-mannose with the same enzyme preparation in the presence of PP and $Mg^{++}$ the formation of ITP and either glucose-1-P or mannose-1-P was observed.

IDP-glucose pyrophosphorylase can be separated from UDP-glucose, CDP-glucose and TDP-glucose pyrophosphorylases as these latter enzymes require higher concentrations of $(NH_4)_2SO_4$ for precipitation. Moreover, on chromatography on a DEAE-cellulose column the pyrimidine nucleotide pyrophosphorylases fractionate differently than IDP-glucose pyrophosphorylase. After fractionation with either $(NH_4)_2SO_4$ or DEAE-cellulose GDP-glucose and IDP-glucose pyrophosphorylase activity are still unresolved. Preliminary data indicate that these latter reactions are catalyzed by individual enzymes as the ratio of activity GDP-glucose/IDP-glucose of preparations thus far obtained has varied between 5 and 50. While these results are not definitive they do indicate that the GDP-glucose and IDP-glucose pyrophosphorylases may be two different enzymes. Further fractionation of these enzymes is now in progress.

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