INOSITOL 1-PHOSPHATE AS INTERMEDIATE IN THE CONVERSION OF GLUCOSE 6-PHOSPHATE TO INOSITOL*

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The conversion of glucose-6-P to inositol by a partially purified enzyme system from yeast and the cofactor requirements of this system have been described in previous publications (Chen and Charalampous, 1964). We have now been able to demonstrate the accumulation, in the reaction mixture, of a phosphorylated intermediate which was isolated by paper chromatography and identified as inositol-1-P. The accumulation of this compound during the biosynthesis of inositol from uniformly labeled ^{14}C-glucose-6-P is shown in Fig. 1.

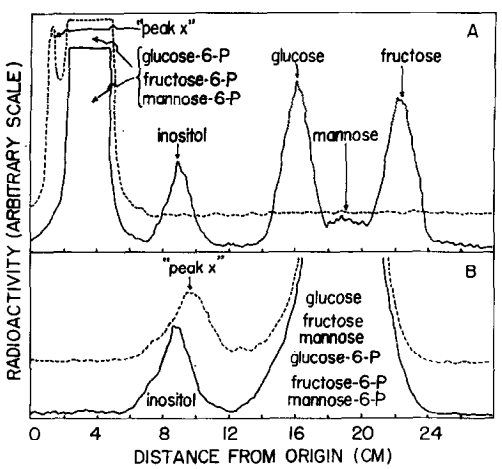

Fig. 1. ^{14}C-tracings of paper chromatograms of reaction mixtures at the end of the incubation. The complete system (0.25 ml) contained 0.8 mM DPN, 1.2 mM $MgCl_2$, 14 mM ammonium acetate, 4.0 mM uniformly labeled ^{14}C-glucose-6-P (S.A. 4.17 x 10^6 c.p.m. per μmole), 50 mM Tris-acetate buffer pH 7.7, and 0.26 mg of enzyme protein. After 30

*Throughout this paper the term inositol and the various inositol phosphates refer to myo-inositol and its phosphates.

minutes incubation at 29° C the reaction tubes were placed in a boiling water bath for one minute and they were immediately chilled in an ice bath. Amberlite IR-120 resin was added in the tubes and, after centrifugation, aliquots from the supernatant fluids were chromatographed on Whatman No. 1 paper in two solvent systems: A. phenol: water (4:1 w/w), descending, 27 hours; B. methanol: formic acid: water (80:15:5), ascending, 21 hours. After drying, the chromatograms were scanned for radioactivity in the Baird-Atomic 4 π Scanogram. Known standards were used as reference compounds and they were detected by staining with silver (Anet and Reynolds, 1954). The "continuous" tracings are from the complete system; the "broken-line" tracings are from the complete minus $MgCl_2$.

It is seen that the omission of $MgCl_2$ from the incubation mixture results in the appearance of "peak X". If the chromatogram in Solvent A is allowed to develop for 6 days one obtains complete separation of "peak X" from the sugar phosphates. If "peak X" is eluted from chromatogram A and rechromatographed in Solvent B one obtains "peak X" of chromatogram B. Similarly, rechromatography of "peak X" of chromatogram B in solvent A produces "peak X" of chromatogram A.

Identification of "peak X" as inositol-1-P. This was achieved as follows: a) Sequential quantitative paper chromatography. The reaction mixture was chromatographed in solvent B (Fig. 1) together with standard L-myo-inositol-1-P and inositol-2-P as reference compounds (purchased from CALBIOCHEM). The radioactive area of "peak X" corresponding to the two standard inositol phosphates was eluted and rechromatographed in solvent A (Fig. 1) for 6 days. The radioactive compound migrated exactly like L-myo-inositol-1-P. It was eluted and rechromatographed in the solvent system of Pizer and Ballou (Pizer and Ballou, 1958) at 38°C. This system separates (±)-inositol-1-P, inositol-2-P, inositol-5-P, pinitol-4-P, and inositol 1, 2-phosphate. The radioactive compound migrated exactly like L-myo-inositol-1-P. These results are shown in Table 1.

Table I

Chromatographic identification of the radioactive "peak X" as inositol-1-P

Compound	Distance in mm. from origin		
	A*	B*	C*
inositol	440	86	216
L-myo-inositol-1-P	52	97	121
inositol-2-P	98	99	155
Radioactive "peak X"	52	97	121

*A. phenol:water (4:1 w/w); B. methanol: formic acid:water (80:15:5 v/v); C. isopropyl alcohol: ammonia:water(70:10:20 v/v).

b) Dephosphorylation with alkaline phosphatase and identification of inositol. The radioactive compound of "peak X" is completely retained on Dowex-1 resin. After treatment with phosphatase it is quantitatively converted to a neutral compound which was identified as inositol by paper chromatography in 3 different solvent systems (Chen and Charalampous, 1964), and by crystallization, after addition of carrier inositol, to constant specific activity (m.p. 227°C).

c) Kinetic studies of the conversion of ^{14}C-inositol phosphate (peak X) to inositol by the purified yeast enzyme. In preliminary experiments it was observed that L-myo-inositol-1-P and inositol-2-P were converted to inositol by the purified yeast enzyme at quite different rates. It was, therefore, useful to compare these rates with that obtained when ^{14}C-inositol-1-P was the substrate. The results are summarized in Table II.

It is seen that the rate of dephosphorylation of the radioactive inositol phosphate (peak X) is identical to that of L-myo-inositol-1-P and 2.5 times greater than that of inositol-2-P. The dephosphorylation reaction requires only Mg^{++} as cofactor. Neither DPN nor NH_4^+ exert any effect.

Table II

Rate of dephosphorylation of various inositol phosphates by the yeast enzyme system

Substrate	μmoles of ^{14}C-inositol and Pi formed	
	15 min.	30 min.
^{14}C-inositol phosphate	0.100	0.210
L-myo-inositol-1-P	0.101	0.194
inositol-2-P	0.040	0.079

The incubation mixture (0.25 ml) contained 4.0 mM substrate, 50 mM Tris-acetate buffer pH 7.7, 1.2 mM $MgCl_2$ and 0.17 mg of enzyme protein. The ^{14}C-inositol phosphate was diluted with L-myo-inositol-1-P to give a S.A. of 15,000 c.p.m. per μmole. The incubation was carried out at 29°C and the reaction rate was followed by measuring the amount of Pi released from the non-radioactive substrates. When ^{14}C-inositol phosphate was the substrate the deproteinized reaction mixture was passed through a column of Dowex-1 resin in order to remove unreacted ^{14}C-inositol phosphate. Aliquots from the neutral fraction containing ^{14}C-inositol were plated and counted. The μmoles of ^{14}C-inositol thus formed were calculated from the initial S.A. of the ^{14}C-inositol phosphate mentioned above.

In order to confirm that inositol-1-P is the true intermediate and not a secondary product resulting from phosphate migration of another inositol phosphate the following experiment was performed. The isolated ^{14}C-inositol-1-P (peak X) as well as standard L-myo-inositol-1-P and inositol-2-P were added, separately, to the complete mixture described in Fig. 1. The reaction was stopped immediately by heat inactivation, and the reisolation of the above compounds was carried out according to the procedure described before. It was found that all the radioactivity was recovered as inositol-1-P. Furthermore, there was no interconversion of L-myo-inositol-1-P and inositol-2-P. The conditions used were chosen so that as little as 1% phosphate migration could have been detected. Additional evidence that inositol-1-P is the only form of

inositol phosphate present, when $MgCl_2$ is omitted from the incubation mixture, was obtained by treating half of the incubation mixture with alkaline phosphatase until all the acidic radioactive compounds were converted to neutral ones, and comparing the counts obtained as free inositol with those present as inositol-1-P in the untreated portion of the incubation mixture. It was found the ^{14}C-inositol-1-P accounts for more than 99% of the counts released as ^{14}C-inositol by phosphatase.

The cofactors necessary for the conversion of glucose-6-P to inositol-1-P and inositol are shown in Table III.

Table III

Cofactor requirements for the biosyntnesis of inositol-1-P and inositol from glucose-6-P

Additions	Isolated Products	
	inositol-1-P	inositol
	c. p. m.	c. p. m.
Complete*	30,000	185,000
" - Mg^{++}	230,000	2,000
" - NH_4^+	17,000	38,000
" - DPN	500	900
" - Mg^{++} - NH_4^+	47,000	400

*The composition and incubation conditions are those of Fig. 1.

It is seen that DPN is required for the biosynthesis of inositol-1-P and inositol. However the presence of Mg^{++} ions prevents the accumulation of inositol-1-P and allows the appearance of equivalent amounts of inositol. NH_4^+ ions accelerate five fold the rate of inositol-1-P biosynthesis but have no effect on the conversion of inositol-1-P to inositol.

Summary The isolation and identification of inositol-1-P as one of the intermediates in the conversion of glucose-6-P to inositol demonstrate that the enzymic oxido-reduction and cyclization of glucose-6-P occur without loss of the phosphate group to form inositol-1-P which is then dephosphorylated to inositol.

This represents the first demonstration of the biosynthesis de novo of inositol-1-P which is the form present in various natural phosphoinositides (Brockerhoff and Ballou, 1961).

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REFERENCES

Anet, E.F.L., and Reynolds, T.M., Nature, 174, 930 (1954)
Brockerhoff, H., and Ballou, C.E., J. Biol. Chem., 236, 1907 (1961)
Chen, I.W., and Charalampous, F.C., J. Biol. Chem. 239, 1905 (1964)
Chen, I.W., and Charalampous, F.C., Biochem. Biophys. Res. Commun., 17, 521 (1964)
Pizer, F.L., and Ballou, C.E., J. Am. Chem. Soc., 81, 915 (1959)