MODE OF CONVERSION OF GLUCOSE-6-P TO INOSITOL, AND THE ROLE OF DPN AND NH_4^+ IONS

I. W. Chen and F. C. Charalampous

Department of Biochemistry , School of Medicine
University of Pennsylvania, Philadelphia 4, Pennsylvania

Received September 8, 1964

In a previous publication we described a soluble enzyme system from yeast which catalyzes the biosynthesis of inositol from glucose-6-P in the presence of DPN and Mg^{++} (Chen and Charalampous, 1964). In order to study the mechanism of this reaction we purified this system by a combination of protamine sulfate precipitation, ammonium sulfate fractionation, adsorption on calcium phosphate gel and alumina C_γ followed by selective elution, and extensive dialysis. A 100 fold purification was achieved with a yield of 48 to 50%. The purified enzyme catalyzes the formation of 0.25 μmoles of inositol per mg. of protein per hour under the assay conditions described in Fig. 1 and in the presence of 14 mM NH_4^+. The ratio of optical density at 280 over 260 mμ is 1.83, and the enzyme is practically free of DPN, TPN and thiamine pyrophosphate. (Racker et al., 1953). DPN was determined from the amount of radioactive inositol fromed by the purified yeast enzyme acting on uniformly labeled ^{14}C-glucose-6-P; TPN was determined from the amount of $^{14}CO_2$ produced by the combined action of glucose-6-P dehydrogenase and 6-phosphogluconic acid dehydrogenase acting on 1-^{14}C-glucose-6-P. Thiamine pyrophosphate was determined spectrophotometrically (de la Haba and Racker, 1955).[1] In all these assays the presence of as little as 1 mμmole of coenzyme, contained

1. We are grateful to Dr. E. Racker for a generous gift of a mixture of ribose-5-P, ribulose-5-P, and xylulose-5-P used as substrate in the transketolase assay.

in 1 mg of enzyme protein, could have been detected.

As a result of this purification it was possible to test the biosynthesis of inositol from glucose-6-P without interference by the possible presence of the enzymes of the hexose monophosphate shunt and of the glycolytic pathway with the exception of phosphoglucomutase and phosphohexose isomerase. Furthermore, we could not detect any fruchose-6-phosphate phosphoketolase activity in the purified enzyme system (Racker, 1962).

Requirement for NH_4^+. Using the purified system it was possible to re-examine the ion requirements of the reactions. It was found that NH_4^+ ions exert a strong activating effect. These results are summarized in Fig. 1

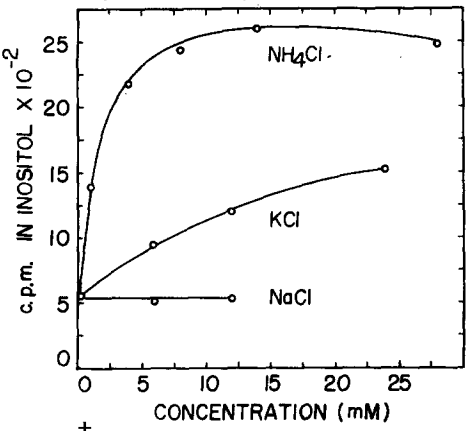

Fig. 1. Effect of NH_4^+ concentration on the biosynthesis of inositol from glucose-6-P. The incubation mixture (0.25 ml) contained 0.8 mM DPN, 2.7 mM $MgCl_2$, 4.0 mM uniformly labeled ^{14}C-glucose-6-P (S.A. 89,600 c.p.m. per μmole), 50 mM Tris buffer pH 7.7, and 0.22 mg of enzyme protein. Incubation was carried out for 30 minutes at 29°C. The radioactive inositol was isolated and counted as described elsewhere (Chen and Charalampous, 1964).

It is seen that maximum activation is obtained with 12 mM NH_4Cl. $(NH_4)_2SO_4$ can substitute for NH_4Cl. K^+ ions are less effective than NH_4^+ ions while Na^+ ions are without effect. The purified enzyme is completely inactivated by heavy metals such as Ag^+ (0.05 mM), Hg^{++} (0.5 mM), Cu^{++} (0.5 mM), Zn^{++} (1.0 mM) and Co^{++} (3.0 mM).

Reduction of DPN during the biosynthesis of inositol from glucose-6-P. Although DPN is necessary for the biosynthesis of inositol from glucose-

6-P it was not possible to demonstrate its reduction when the crude enzyme system was used. However, using the purified enzyme we were able to demonstrate the formation of DPNH during this reaction. Fig. 2 summarizes the results.

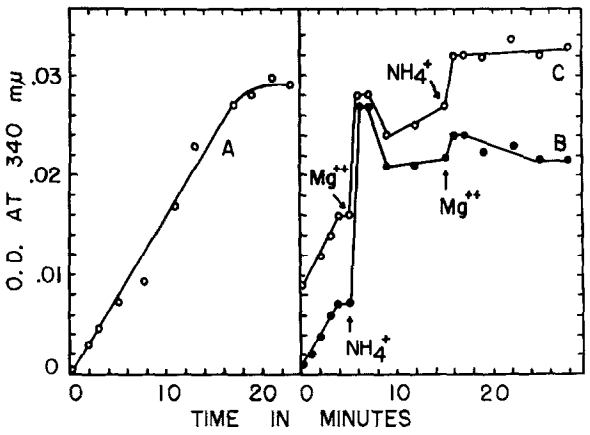

Fig. 2. Reduction of DPN during the biosynthesis of inositol from glucose-6-P. The incubation mixtures contained the components described in Fig. 1 except that 0.7 mg of enzyme protein was used in a final volume of 1.2 ml. In Curve A the reaction mixture contained all the components from the very beginning, while in curves B and C the $MgCl_2$ and NH_4Cl were added at different times as shown in the graph. In all cases the optical density was measured against blanks of comparable composition but without glucose-6-P.

Contribution of the carbon atoms of glucose-6-P to the biosynthesis of inositol. The successful purification of this enzyme from yeast offers a unique opportunity to determine whether the conversion of glucose-6-P to inositol involves the utilization of the carbon chain as an intact six carbon unit or whether fragmentation and reassembly occurs prior to cyclization. In this study 1-^{14}C-glucose-6-P, purchased from the Nuclear Chicago Corporation, and 6-^{14}C-glucose-6-P prepared enzymatically (Chen and Charalampous, 1964) were used separately for the biosynthesis of inositol. The 1-^{14}C-glucose-6-P contained 93% of the ^{14}C in C-1 as determined by the $^{14}CO_2$ produced enzymatically by the combined action of glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase. The 6-^{14}C-glucose-6-P contained 99.0% of the ^{14}C in C-6. This was established by dephosphorylation with

alkaline phosphatase followed by HIO_4 oxidation (Reeves, 1941) and counting the formaldehyde-dimedon derivative. The incubation mixtures for the biosynthesis of inositol contained the components described in Fig. 1 except that the final volume was 10 ml and the concentration of NH_4Cl was 14 mM. At the end of 4 hours incubation 20 mg of carrier inositol were added and the radioactive inositols were isolated and crystallized to constant specific activity. Their m.p. was 228°. The radioactive inositols were converted to the corresponding D-glucuronic acids by the kidney enzyme. After adding 150 μmoles of carrier sodium D-glucuronate the radioactive glucuronates were isolated and crystallized to constant specific activity (Charalampous, 1960). Their purity was confirmed by quantitative paper chromatography using n-butanol: acetic acid: water (100:21:50) as solvent system. A portion of the two samples of the radioactive glucuronates was oxidized with sodium periodate and the CO_2 was collected and counted (Imai, 1963). The remaining portions of the radioactive glucuronates were reduced to L-gulonolactones and, after adding 456 μmoles of carrier L-gulonolactone, they were crystallized to constant specific activity (Charalampous, 1960). Their m.p. was 188°. Portions of the gulonolactones were oxidized with sodium periodate (Imai, 1963) and the resulting CO_2 and formaldehyde were isolated and counted (Dyer, 1956). The results are summarized in Tables I and II.

These results demonstrate unequivocally that in the biosynthesis of inositol, catalyzed by the purified yeast enzyme, the carbon chain of glucose-6-P is used as an intact 6 carbon unit. Thus the C-1 of glucose-6-P becomes C-6 of inositol to the extent of 99% while the C-6 of glucose-6-P becomes C-1 of inositol to the extent of 100%. The previous results obtained in studies with whole yeast (Charalampous 1957) must reflect randomization of the ^{14}C of glucose-6-P by re-

actions not directly involved in the biosynthesis of inositol.

TABLE I

Biosynthesis of Inositol from Specifically Labeled ^{14}C-Glucose-6-P and Its Conversion to D-Glucuronate and L-Gulonolactone

Isolated Compounds*	Substrates Used*			
	1-^{14}C-glucose-6-P		6-^{14}C-glucose-6-P	
	S.A.	Recovery	S.A.	Recovery
	(c.p.m./μ mole)	%	(c.p.m./μ mole)	%
Inositol	1.34×10^5	22.0	1.28×10^5	23.0
D-Glucuronate	1.27×10^5	3.9	1.17×10^5	3.6
L-Gulonolactone	1.19×10^5	1.6	1.09×10^5	1.9

*Recovery is expressed as % of the ^{14}C used in the respective substrates. The specific activity of both samples of glucose-6-P was 1.25×10^6 c.p.m. per μmole. The specific activities of the isolated compounds have been corrected for the amount of each carrier added.

TABLE II

Periodate Oxidation of ^{14}C-labeled D-Glucuronate and L-Gulonolactone

Label in glucose-6-P	Compound Oxidized	Products of Oxidation			
		CO_2 (C-1 of inositol)		Formaldehyde (C-6 of inositol)	
		amount oxidized	amount recovered	amount oxidized	amount recovered
		c.p.m.	c.p.m.	c.p.m.	c.p.m.
1-^{14}C	D-glucuronate	19,470	0	—	—
6-^{14}C	D-glucuronate	15,000	13,800	—	—
1-^{14}C	L-gulonolactone	37,400	0	311,000	320,000
6-^{14}C	L-gulonolactone	13,310	13,240	110,000	0

Similar conclusions were reached by other investigators in studies using the whole rat (Imai, 1963), higher plants (Loewus and Kelly, 1962; Kindl and H-Ostenhof, 1964), and crude testis homogenates (Eisenberg et al., 1964). Although these conclusions are strengthened by our data presented in this communication, conclusive proof must await the purification of the enzyme system from these various

tissues in order to eliminate interpretational difficulties arising from the complexity of those systems.

The demonstration that DPN is reduced during the biosynthesis of inositol from glucose-6-P is evidence for the occurence of an oxidation step in this conversion. It can be calculated from the data of Fig. 2 that the steady state concentration of DPNH is 6 mμmoles per ml when the rate of inositol biosynthesis is 120 mμmoles per hour. Since the overall conversion of glucose-6-P to inositol does not involve a net oxidation or reduction it must be concluded that the DPNH is oxidized in a subsequent step involving reduction of the intermediate formed during the first oxidative step.

ACKNOWLEDGMENT

This research was supported by U.S. Public Health Grant CA-02228-11 from the United States Public Health Service.

REFERENCES

Charalampous, F.C., J. Biol. Chem., 225, 595 (1957)
Charalampous, F.C., J. Biol. Chem., 235, 1286 (1960)
Chen, I.W., and Charalampous, F.C., J. Biol. Chem., 239, 1905 (1964)
de la Haba, G., and Racker, E., in S.P. Colowick and N.O. Kaplan (Editors), Methods in Enzymology, Vol. I, Academic Press, Inc., New York, 1955, p. 375
Dyer, J.R., in D. Glick (Editor), Methods of Biochemical Analysis, Vol. III, Interscience Publishers, New York, 1956, p. 134
Eisenberg, F. Jr., Bolden, A.H., and Loewus, F.A., Biochem. Biophys. Res. Commun., 14, 419 (1964)
Imai, Y., J. Biochem. (Tokyo), 53, 50 (1963)
Kindl, H., and Hoffmann-Ostenhof, O., Monatshefte Fur Chemie, 95, 548 (1964)
Kindl, H., and Hoffmann-Ostenhof, O., Biochem. Z., 339, 374 (1964)
Loewus, F.A., and Kelley, S., Biochem. Biophys. Res. Commun., 7, 204 (1962)
Racker, E., de la Haba, G., and Leder, I.G., J.Am. Chem. Soc., 75, 1010 (1953)
Racker, E., in S.P. Colowick and N.O. Kaplan (Editors), Methods in Enzymology, Vol. V, Academic Press, Inc., New York, 1962, p. 276