The Mechanism of Glucose 6-Phosphate-*D*-*myo*-Inositol 1-Phosphate Cyclase of Rat Testis

THE INVOLVEMENT OF HYDROGEN ATOMS

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Comparison of the initial $^3\text{H}/^{14}\text{C}$ ratios in specifically labelled *D*-glucose 6-phosphates with the final ratios in *myo*-inositol produced by glucose 6-phosphate-*D*-*myo*-inositol 1-phosphate cyclase from rat testis showed that, during the conversion, the hydrogen atoms at C-1 and C-3 were fully retained, one hydrogen atom was lost from C-6, and that at C-5 was apparently retained to the extent of 80–90%. The loss of ^3H could not be stimulated by addition of unlabelled NADH, and when unlabelled substrate was used ^3H from $[^3\text{H}]\text{NADH}$ and $[^3\text{H}]\text{water}$ was not incorporated. Treatment of the enzyme with charcoal abolished the activity, and this was restored to 25–50% of the original activity by NAD^+ . The charcoal-treated enzyme again apparently gave 85% retention of hydrogen with $[5\text{-}^3\text{H}]\text{glucose 6-phosphate}$ as substrate in the presence of NAD^+ alone, but the retention was decreased to 65% with excess of NADH. The results are interpreted as indicating that the cyclization proceeds by an aldol condensation in which C-5 is oxidized by NAD^+ in a tightly-bound ternary complex, and that the apparent loss of ^3H when untreated enzyme is used is due to an isotope effect. It is suggested that after treatment with charcoal some exchange of NADH with an external pool may take place.

myo-Inositol is synthesized in yeast (Chen & Charalampous, 1966), in mould (Pina & Tatum, 1967) and in mammalian testes (Eisenberg, 1967) by glucose 6-phosphate-*D*-*myo*-inositol 1-phosphate cyclase. Direct cyclization without rearrangement of the carbon skeleton, and with retention of configuration at each carbon atom, has been shown by labelling experiments (Eisenberg, Bolden & Loewus, 1964; Chen & Charalampous, 1965). The enzyme uses NAD^+ as cofactor, which suggests an oxidation step in which hydrogen atoms are involved. We have therefore undertaken an

investigation of the retention of hydrogen atoms during the conversion of *D*-glucose 6-phosphate into *D*-*myo*-inositol 1-phosphate in an attempt to clarify the mechanism of the cyclization, which involves the formation of a new carbon-carbon bond. Eisenberg (1967) has proposed two alternative mechanisms involving an aldol condensation (Scheme 1) and an acyloin condensation (Scheme 2) respectively. The aldol condensation (Scheme 1) has been proposed by other workers (Chen & Charalampous, 1967; Loewus & Kelly, 1962). Hauska & Hoffmann-Ostenhof (1967) have described

Scheme 1. Aldol condensation. NAD^+ oxidizes glucose 6-phosphate to 5-oxoglucose 6-phosphate, which undergoes an aldol condensation to give *myo*-inosose-2 1-phosphate; this is reduced by NADH to *myo*-inositol 1-phosphate.

Scheme 2. Acyloin condensation. An NAD^+ -catalysed hydroxylation at C-6 is followed by an acyloin condensation and subsequent dehydroxylation by NAD^+ . This reduction might occur through a 1,2-ene intermediate.

the conversion of [5- ^3H]glucose 6-phosphate into [2- ^3H]myo-inositol by a yeast enzyme. A preliminary account of part of this work has been published (Barnett & Corina, 1967).

MATERIALS AND METHODS

D-[3- ^3H]Glucose. 1,2:5,6-Di-*O*-isopropylidene- α -D-ribohexofuranos-3-ulose (100mg.) (Brimacombe, Bryan, Husain, Stacey & Tolley, 1967) was dissolved in LiAlH_4 -dried tetrahydrofuran. Then LiCl (50mg.) and NaB^3H_4 (3mg.; 53mc/m-mole) were added, the mixture was refluxed for 3hr. and cooled, and water (5ml.) was added. The tetrahydrofuran layer was separated and the aqueous layer was re-extracted with tetrahydrofuran. Thin-layer chromatography on Kieselgel G with ethyl acetate–light petroleum (b.p. 40–60°) (3:2, v/v) as solvent showed three spots corresponding to the starting material and 1,2:5,6-di-*O*-isopropylidene-D-*allo*-furanose and D-*gluco*-furanose (Collins, 1965). The combined organic layers were evaporated to dryness *in vacuo* and 0.5N- H_2SO_4 (2ml.) was added. The mixture was refluxed for 30min., cooled, deionized with Amberlite IRA 400 (CO_3^{2-} form), evaporated to dryness and chromatographed for 72hr. on Whatman 3MM paper with ethyl acetate–pyridine–water (8:2:1, by vol.) as solvent; this gave a complete separation of the D-[3- ^3H]glucose (20 μC) from D-[3- ^3H]allose, which was the major product (> 95%).

Other labelled compounds. D-[5- ^3H]Glucose (Barnett & Corina, 1966) and D-[1- ^3H]glucose (Lowenstein, 1961) were synthesized by the established methods. D-[6- ^3H]Glucose, D-[U- ^{14}C]glucose, D-[1- ^{14}C]glucose, D-[6- ^{14}C]glucose, NaB^3H_4 and [^3H]water were obtained from The Radiochemical Centre, Amersham, Bucks. [4- ^3H]NADH was the gift of D. C. Wilton of this Laboratory. It was prepared by the method of Popják, Goodman, Cornforth, Cornforth & Ryhage (1961) for [4- ^3H]NADPH, and had the ^3H label distributed 62.5% on the A-face and 37.5% on the B-face of the nucleotide.

Glucose 6-phosphates. Specifically labelled glucose 6-phosphates were made by the action of ATP and hexokinase on the corresponding labelled glucoses (Barnett & Corina, 1966).

Preparation of an extract containing glucose 6-phosphate-D-myoinositol cyclase activity. The testes (8–10g.) of two hooded rats were homogenized in 2 vol. of ice-cold 0.154M-KCl, pH 7.4, and centrifuged for 80min. at 160000g at 2°, and the supernatant was heated at 60° for exactly 2min. to destroy phosphatases and then fractionated with $(\text{NH}_4)_2\text{SO}_4$. The 30–40%-saturated fraction, which was precipitated at 3° by $(\text{NH}_4)_2\text{SO}_4$ at concentrations between 165.5g./l. and

222.2g./l., was redissolved in 5ml. of 50mm-tris-acetate buffer, pH 7.4, and dialysed against the same buffer containing GSH (1mm) and EDTA (1mm) for 6hr., and then for 12hr. against buffer containing GSH only. The enzyme preparation was either used directly, or stirred with activated charcoal (British Drug Houses Ltd., Poole, Dorset) (100mg./ml.) for 5min. at 4° and then centrifuged at 1500g for 5min. to remove NAD^+ . The supernatant was then used as the enzyme preparation. Attempts to store the enzyme extract as a freeze-dried powder, an acetone-dried powder or a deep-frozen solution were only partially successful. In each case only about 10% of the activity remained after 1 month.

Assay of cyclase activity. [6- ^3H]Glucose 6-phosphate in 50mm-tris-acetate buffer, pH 7.4 (30mm, about 10^7 counts/min.) (0.5ml.), 50mm-tris-acetate buffer, pH 7.4, containing magnesium acetate (5mm) and ammonium acetate (20mm) (1.5ml.), 30mm- NAD^+ in 50mm-tris-acetate buffer, pH 7.4 (0.5ml.), and 0.5ml. of enzyme extract were incubated at 32° for 3hr. myo-Inositol (10mg.) was added, and the reaction was terminated by boiling. The mixture was treated with 0.3M- $\text{Ba}(\text{OH})_2$ (5ml.), neutralized with 1N- H_2SO_4 and centrifuged to remove protein and BaSO_4 , and the supernatant was evaporated at 50° and 15mm. Hg to 3ml. Then 10N-HCl (6ml.) was added, and the mixture was heated to 105° for 18hr. in a sealed tube to hydrolyse D-myoinositol 1-phosphate, deionized and evaporated to dryness *in vacuo*. After chromatography for 18hr. on Whatman 3MM paper with acetone–water (17:3, v/v) as solvent, the inositol band was eluted and evaporated to dryness *in vacuo*. Acetic anhydride (0.5ml.) and HClO_4 (1 drop) were added, and the mixture was left at 20° for 90min. and poured into ice-cold water. After one recrystallization from toluene, the hexa-acetate (m.p. 217°, yield from carrier 30–50%) was counted and the counts corrected to 100% recovery of carrier.

Measurement of $^3\text{H}/^{14}\text{C}$ ratios. The standard enzyme assay procedure was followed with mixtures of ^{14}C -labelled and ^3H -labelled glucose 6-phosphates as substrates (see Table 2), but portions of the incubation medium were removed at suitable times and chromatographed on Whatman 3MM paper with acetone–acetic acid–water (16:1:3, by vol.) as solvent for 18hr. to repurify the glucose 6-phosphate, which was eluted, evaporated to dryness *in vacuo*, redissolved in 0.05ml. of water and counted. Glucose 6-phosphate was counted in the presence of inositol hexa-acetate and vice versa to eliminate quenching effects. In some cases 30mm- NADH (0.5ml.) was added to the incubation medium to stimulate exchange with enzyme-bound NADH . In these cases only 3mm- NAD^+ (0.5ml.) was added to the incubation medium.

Counting procedure. Radioactivity was measured by

adding the sample to 10ml. of NE 220 scintillation fluid [Nuclear Enterprises (G.B.) Ltd., Edinburgh] in a glass vial. ^3H and ^{14}C were counted simultaneously on a Beckman liquid-scintillation spectrometer. Usually the sample was added in 0.05ml. of water or as the solid. In double-label experiments each tube contained 0.05ml. of water, glucose 6-phosphate and inositol hexa-acetate to minimize quenching effects. The counting efficiency for ^{14}C was 70% (background 11 counts/min.) and for ^3H 13% (background 16 counts/min.); 3.2% of ^{14}C appeared in the ^3H channel and 1.1% of ^3H in the ^{14}C channel, and counts were corrected for this before the ratios were calculated. In all ratio experiments the final count was more than 100 times background.

Attempted incorporation of ^3H from $[4\text{-}^3\text{H}]\text{NADH}$. The standard assay conditions were used with unlabelled glucose 6-phosphate as substrate and normal or charcoal-treated enzyme preparation. $[4\text{-}^3\text{H}]\text{NADH}$ (1.3×10^6 counts/min.) was added to each incubation. In no case was there an observable increase in radioactivity in the isolated *myo*-inositol compared with that isolated from a control incubation with boiled enzyme ($5 \pm 2 \times 10^2$ counts/min.).

*Oxidation of *myo*-inositol to *myo*-inosose-2.* *myo*-Inositol formed from $[\text{U}\text{-}^{14}\text{C}]$ - and $[\text{5-}^3\text{H}]$ - or $[\text{6-}^3\text{H}]$ -glucose 6-

phosphate was oxidized over platinum-charcoal (Koch-Light Laboratories Ltd., Colnbrook, Bucks.) by the method of Heyns & Paulsen (1953). The oxidation was carried to approximately half-completion, and the *myo*-inosose-2 and *myo*-inositol were separated on Whatman 3MM paper with acetone-water (17:3, v/v) as solvent. The $^3\text{H}/^{14}\text{C}$ ratios were then determined.

RESULTS

NAD⁺ requirement of the enzyme. The stimulation of the 18hr.-dialysed enzyme by NAD^+ is given in Table 1. The activity in the absence of added NAD^+ was about 50% of that with 5mm- NAD^+ . Treatment with charcoal decreased the activity to 1.8% in the absence of NAD^+ , but in the normal conditions of 5mm- NAD^+ the activity was restored to only 26% of the original, indicating partial denaturation. NADH could not replace NAD^+ : the activity in 5mm- NADH in the absence of NAD^+ was 1.4% of that with 5mm- NAD^+ .

Labelling experiments. No ^3H was incorporated from $[^3\text{H}]\text{NADH}$ ($5\mu\text{C}$ added to the incubation, 2ml.) or $[^3\text{H}]\text{water}$ (5mc added to the incubation, 2ml.) into *myo*-inositol with unlabelled glucose 6-phosphate under the standard assay conditions.

$^3\text{H}/^{14}\text{C}$ ratios. Table 2 shows the ratios obtained with variously labelled glucose 6-phosphates. Each result is the mean of two or three determinations with the same mixture of substrates and enzyme preparation, and the limits were obtained by dividing the lowest glucose 6-phosphate ratio into the highest *myo*-inositol ratio and vice versa. With $[\text{1-}^3\text{H}]\text{glucose 6-phosphate}$ a slow removal of ^3H was noticed, presumably due to residual glucose 6-phosphate dehydrogenase, and so for this label 2hr. and 3hr. ratios were determined.

The ratios indicate that both the hydrogen atom

Table 1. *Effect of NAD^+ concentration on the yield of inositol*

The substrate was $[\text{6-}^3\text{H}]\text{glucose 6-phosphate}$. The incubations were as described in the text.

Concn. of added NAD^+ (mm)	Radioactivity of inositol, corrected to 100% recovery (counts/min.)	% of activity of complete system
5	15730	100
1	15330	97
0.5	12320	78
0.1	9450	60
0	7770	49

Table 2. *$^3\text{H}/^{14}\text{C}$ ratios of incorporation of variously labelled glucose 6-phosphate into inositol*

Glucose 6-phosphate was incubated and *myo*-inositol hexa-acetate isolated under the conditions described in the text. The results are expressed as the mean of two or three determinations, and the limits on the apparent ^3H retention percentages were obtained by dividing the lowest value for the glucose 6-phosphate ratio into the highest value of the inositol hexa-acetate ratio and vice versa.

Position of label		$^3\text{H}/^{14}\text{C}$ ratio		Apparent retention of ^3H (%)	Radiochemical yield (%)
^{14}C	^3H	Reisolated glucose 6-phosphate	Inositol hexa-acetate		
1	1 (2hr.)	0.39 ± 0.03	0.35 ± 0.02	96 ± 8	1.2 ± 0.2
1	1 (3hr.)	0.335 ± 0.03	0.34 ± 0.005	101 ± 8	0.60 ± 0.04
U	3	0.60 ± 0.03	0.61 ± 0.02	101 ± 6	0.28 ± 0.02
U	5	2.07 ± 0.014	1.70 ± 0.02	85 ± 5	0.149 ± 0.105
U	5	2.91 ± 0.12	2.455 ± 0.06	84 ± 5	0.265 ± 0.005
U	5	4.20 ± 0.10	3.64 ± 0.08	86 ± 4	0.776 ± 0.001
U	5	5.295 ± 0.015	4.825 ± 0.035	90 ± 2	0.24 ± 0.03
6	6	0.58 ± 0.0	0.285 ± 0.005	50 ± 1	0.24 ± 0.04
U	6	1.61 ± 0.01	0.77 ± 0.02	48 ± 2	0.265 ± 0.05

Table 3. *Effect of treatment with charcoal on $^3\text{H}/^{14}\text{C}$ ratios of $[5\text{-}^3\text{H}]$ - and $[6\text{-}^3\text{H}]$ - and $[\text{U-}^{14}\text{C}]$ -glucose 6-phosphates*

Either 'normal' or 'charcoal-treated' enzyme was used in media containing either 30 mM-NAD⁺ ('NAD⁺') or 3 mM-NAD⁺ and 30 mM-NADH ('NADH').

Position of ^3H label on glucose	System	$^3\text{H}/^{14}\text{C}$ ratio		Apparent retention of ^3H (%)	Radiochemical yield (%)
		Reisolated glucose 6-phosphate	Inositol hexa-acetate		
C-5	Normal (NAD ⁺)	2.07 $\pm$ 0.14	1.70 $\pm$ 0.02	85 $\pm$ 5	0.149 $\pm$ 0.015
	Normal (NADH)	2.07 $\pm$ 0.14	1.68 $\pm$ 0.02	84 $\pm$ 5	0.150 $\pm$ 0.02
	Charcoal (NADH)	2.07 $\pm$ 0.14	1.29 $\pm$ 0.04	62.5 $\pm$ 4.5	0.092 $\pm$ 0.03
C-5	Normal (NAD ⁺)	3.34 $\pm$ 0.016	2.69 $\pm$ 0.05	81.5 $\pm$ 3	0.32 $\pm$ 0.02
	Charcoal (NAD ⁺)	3.34 $\pm$ 0.016	2.73 $\pm$ 0.003	83 $\pm$ 2	0.16 $\pm$ 0.015
	Charcoal (NADH)	3.34 $\pm$ 0.016	2.185 $\pm$ 0.015	65 $\pm$ 2	0.0425 $\pm$ 0.002
C-5	Normal (NAD ⁺)	2.91 $\pm$ 0.12	2.45 $\pm$ 0.06	84 $\pm$ 5	0.265 $\pm$ 0.005
	Charcoal (NADH)	2.91 $\pm$ 0.12	1.79 $\pm$ 0.02	61 $\pm$ 2.5	0.042 $\pm$ 0.002
C-6	Normal (NAD ⁺)	1.607 $\pm$ 0.013	0.77 $\pm$ 0.02	48 $\pm$ 2	0.265 $\pm$ 0.005
	Charcoal (NADH)	1.607 $\pm$ 0.013	0.82 $\pm$ 0.005	51 $\pm$ 1	0.021 $\pm$ 0.001

at C-1 and that at C-3 are retained during the cyclization. One of the two hydrogen atoms was retained at C-6 (50% retention), and the $^3\text{H}/^{14}\text{C}$ ratio fell to about 85% when $[5\text{-}^3\text{H}]$ glucose 6-phosphate was used.

The ratios when $[5\text{-}^3\text{H}]$ glucose 6-phosphate was used varied slightly with different enzyme preparations. The ratio after treatment with charcoal and in the presence of NADH was particularly low (Table 3).

The ^3H label from C-5 of glucose 6-phosphate was located at C-2 of the *myo*-inositol isolated from the incubation of $[5\text{-}^3\text{H}]$ glucose 6-phosphate; this is known because the *myo*-inositol lost more than 90% of its ^3H on catalytic oxidation with 10% platinum-charcoal (Heyns & Paulsen, 1953), which converts *myo*-inositol into *myo*-inosose-2.

DISCUSSION

The incorporation of exactly half of the ^3H label at C-6 of glucose 6-phosphate into *myo*-inositol confirms the finding of Chen & Charalampous (1967), using yeast enzyme, that the rates of incorporation of radioactivity from ^{14}C glucose 6-phosphate and $[6\text{-}^3\text{H}]$ glucose 6-phosphate into *D-myo*-inositol 1-phosphate were equal assuming that only one of the two hydrogen atoms could be incorporated. This assumption follows from the further assumption that the enzyme obeys usual specificity rules and distinguishes between the two hydrogen atoms at C-6. Chen & Charalampous (1967) also showed that ^3H was eliminated into the medium at exactly half the rate of incorporation into inositol 1-phosphate, showing a considerable isotope effect. The precise ratio of 50 ± 1 (Table 2) would still be maintained

if these observations applied to the mammalian enzyme, since the slower-reacting molecules in which the ^3H is eliminated do not contribute to the final $^3\text{H}/^{14}\text{C}$ ratio in *myo*-inositol.

The constancy of the ratio with the label at C-1 and C-3 indicates that the hydrogen atoms at these positions are not involved in the cyclization. When the normal untreated enzyme preparation was used, the ratio with $[5\text{-}^3\text{H}]$ glucose 6-phosphate was consistently near 85% (Tables 2 and 3). This loss might be accounted for by exchange of $[4\text{-}^3\text{H}]$ NADH formed during the reaction by oxidation at C-5 according to Scheme 1 with NADH in the medium, but all attempts to stimulate the exchange by incubation with small amounts of NAD⁺ and large amounts of NADH failed. Further, incubation with excess of $[4\text{-}^3\text{H}]$ NADH in the medium failed to give any *myo*-inositol containing ^3H . There was therefore no indication that NADH could be exchanged. The 18 hr.-dialysed enzyme alone had about 50% of the activity of that with 5 mM-NAD⁺, indicating tight binding to the enzyme, but the stimulation by added NAD⁺ (Table 1) indicates either that NAD⁺ was not quantitatively bound, or that the enzyme was stimulated by NAD⁺ in an additional way.

During the investigation of NAD⁺ requirements a charcoal-treated enzyme preparation was made. This gave the same $^3\text{H}/^{14}\text{C}$ ratios as the untreated enzyme in the presence of NAD⁺ (Table 3), but in the presence of a tenfold excess of NADH, the retention with $[5\text{-}^3\text{H}]$ glucose 6-phosphate decreased to 65%. The retention with $[6\text{-}^3\text{H}]$ glucose 6-phosphate was unaltered. The stimulation of loss by NADH suggests that some exchange of enzymic NADH was taking place with the external pool,

but no incorporation was observed in an experiment with unlabelled substrate and $[4\text{-}^3\text{H}]\text{NADH}$.

Tightly bound NADH has been observed several times in enzymes that catalyse simultaneous oxidation and reduction reactions. Thus no exchange of ^3H from added $[4\text{-}^3\text{H}]\text{NADH}$ was observed with UDP-glucose 4-epimerase (Maxwell & Szulmajster, 1960), and this enzyme has subsequently been shown to contain tightly bound NADH (Wilson & Hogness, 1964). If the NADH was very tightly bound in a ternary complex with enzyme and substrate some alternative explanation must be sought for the low $^3\text{H}/^{14}\text{C}$ ratios when $[5\text{-}^3\text{H}]\text{glucose 6-phosphate}$ was used. This might be due to an isotope effect between ^3H and ^1H either during the transfer of ^3H from the sugar to the NAD^+ or during the reduction of the cyclized intermediate ketone (Scheme 1) by NADH. Since the ^3H and ^{14}C atoms are on different molecules, slower reaction of the former would lead to the observed low ratio in the $[5\text{-}^3\text{H}]\text{glucose 6-phosphate}$ experiments. The rather small effect noticed with the normal enzyme suggests that these reactions are only partially rate-limiting, as would be expected from the large isotope effect noticed by Chen & Charalampous (1967) at C-6 with the yeast enzyme. The even lower ratio for the ^3H at C-5 in the charcoal-treated enzyme experiments might be explained by partial denaturation of the enzyme leading to different interatomic distances, since after such treatment full activity could not be restored by NAD^+ . Hauska & Hoffmann-Ostenhof (1967) in parallel experiments of $[^3\text{H}]\text{glucose 6-phosphate}$ and $[^{14}\text{C}]\text{glucose 6-phosphate}$ with yeast cyclase found that the ^3H was incorporated at 95% of the rate of the ^{14}C . This may be a genuine species difference, or may be due to the use of parallel experiments rather than a mixed label.

The obligatory requirement for NAD^+ , the failure to incorporate $[^3\text{H}]\text{water}$ or $[4\text{-}^3\text{H}]\text{NADH}$, the complete retention of ^3H at C-1 and C-3, the loss of exactly 50% of the ^3H at C-6 and the

decrease in the $^3\text{H}/^{14}\text{C}$ ratio with $[5\text{-}^3\text{H}]\text{glucose 6-phosphate}$ are entirely consistent with the aldol condensation involving oxidation by NAD^+ at C-5 in a tightly bound ternary complex (Scheme 1). It is not consistent with the acyloin condensation (Scheme 2), which requires only movement of hydrogen at C-6 and which cannot explain the low ratios with $[5\text{-}^3\text{H}]\text{glucose 6-phosphate}$.

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