Partial Reactions of D-Glucose 6-Phosphate–1L-*myo*Inositol 1-Phosphate Cyclase

By J. E. G. BARNETT, A. RASHEED and D. L. CORINA

Department of Physiology and Biochemistry, University of Southampton, Southampton SO9 5NH, U.K.

(Received 27 April 1972)

After removal of tightly bound NAD^+ by using charcoal, a preparation of D-glucose 6-phosphate–1L-*myo*inositol 1-phosphate cyclase catalysed the reduction of 5-keto-D-glucitol 6-phosphate and 5-keto-D-glucose 6-phosphate by $[4\text{-}^3\text{H}]\text{NADH}$ to give $[5\text{-}^3\text{H}]\text{glucitol 6-phosphate}$ and $[5\text{-}^3\text{H}]\text{glucose 6-phosphate}$ respectively. The position of the tritium atom in the latter was shown by degradation. Both enzyme-catalysed reductions were strongly inhibited by 2-deoxy-D-glucose 6-phosphate, a powerful competitive inhibitor of inositol cyclase. The charcoal-treated enzyme preparation also converted 5-keto-D-glucose 6-phosphate into $[^3\text{H}]\text{myoinositol 1-phosphate}$ in the presence of $[4\text{-}^3\text{H}]\text{NADH}$, but less effectively. These partial reactions of inositol cyclase are interpreted as providing strong evidence for the formation of 5-keto-D-glucose 6-phosphate as an enzyme-bound intermediate in the conversion of D-glucose 6-phosphate into 1L-*myo*inositol 1-phosphate. The enzyme was partially inactivated by NaBH_4 in the presence of NAD^+ . Glucose 6-phosphate did not increase the inactivation, and there was no inactivation in the absence of NAD^+ . There was no evidence for Schiff base formation during the cyclization. D-Glucitol 6-phosphate (L-sorbitol 1-phosphate) was a good inhibitor of the overall reaction. It did not inactivate the enzyme. The apparent molecular weight of inositol cyclase as determined by Sephadex chromatography was 2.15×10^5 .

A possible mechanism for the enzyme D-glucose 6-phosphate–1L-*myo*inositol 1-phosphate cyclase is shown in Scheme 1. This general mechanism was proposed by Loewus & Kelly (1962), and has been supported by other workers (Chen & Charalampous, 1967; Eisenberg, 1967; Barnett & Corina, 1968; Sherman *et al.*, 1969). The oxidized sugar intermediate, and NADH, are tightly bound to the native enzyme in a ternary complex, since Sherman *et al.* (1969) have shown by using an enzyme preparation from bovine testis and perdeuterated substrates that the hydrogen removed during the oxidation is replaced on the same carbon skeleton.

The inability to dissociate the intermediates from

the enzyme has hitherto led to a lack of definitive evidence for the mechanism proposed in Scheme 1, although Barnett & Corina (1968), using a rat testis enzyme preparation, showed that an isotope effect was observed during the conversion of $[5\text{-}^3\text{H}]\text{glucose 6-phosphate}$ into 1L- $[2\text{-}^3\text{H}]\text{myoinositol 1-phosphate}$, but not during the conversion of $[1\text{-}^3\text{H}]$ -, $[2\text{-}^3\text{H}]$ -, $[3\text{-}^3\text{H}]$ - or $[6\text{-}^3\text{H}]\text{glucose 6-phosphate}$ into *myo*inositol phosphate, indicating that hydrogen movement probably occurred at C-5. In the present paper we describe partial reactions of inositol cyclase which give further support to the mechanism proposed in Scheme 1. A preliminary report of part of this work has been published (Rasheed *et al.*, 1972).

Scheme 1. Proposed mechanism for inositol cyclase

NAD^+ oxidizes glucose 6-phosphate to 5-keto-D-glucose 6-phosphate, which undergoes an aldol condensation to give *myo*inosose-2 1-phosphate; this is reduced by NADH to *myo*inositol 1-phosphate. In the partial reaction, 5-keto-D-glucose 6-phosphate is reduced by $[4\text{-}^3\text{H}]\text{NADH}$ to $[5\text{-}^3\text{H}]\text{glucose 6-phosphate}$ and a small percentage of $[^3\text{H}]\text{myoinositol}$.

Experimental

Materials

Chemicals. D-Glucitol 6-phosphate and 2-deoxy-D-glucose 6-phosphate were purchased from Sigma Ltd. (Kingston, Surrey, U.K.), D-galactose 6-phosphate was from Wessex Biochemicals Ltd. (Bournemouth, Hants., U.K.), *p*-bromophenacyl bromide was from Koch-Light Ltd. (Colnbrook, Bucks., U.K.) and all other chemicals were from BDH Chemicals Ltd. (Poole, Dorset, U.K.) except where stated.

Methods

Scintillation counting. Radioactivity was measured with a Beckman scintillation counter by using Nuclear Enterprises (Edinburgh, U.K.) NE 250 dioxan-based scintillation fluid. Efficiency for tritium was 50%.

[4-³H]NADH. NAD⁺ was reduced by sodium dithionite in [³H]H₂O (The Radiochemical Centre, Amersham, Bucks., U.K.) by the method of Wilton *et al.* (1968). After removal of excess of [³H]H₂O, the NADH content was determined from the *E*₃₄₀. Conversion was 75% and the specific radioactivity was 4.6×10^6 c.p.m./μmol.

Preparation of D-xyllo-5-hexosulose 6-phosphate (5-ketoglucose 6-phosphate) (Scheme 2). Methyl 2,3,4-tri-*O*-benzoyl-6-bromo-6-deoxy-α-D-glucopyranoside (10g) (I), m.p. 118°C, [α]_D²⁰ +56°

(c 5.0 in chloroform), prepared from methyl 2,3-di-*O*-benzoyl-4,6-benzylidene-α-D-glucopyranoside by the method of Hanessian (1966), was shaken at room temperature with AgF (18g) in dry pyridine (75ml) in the dark for 80h. The slurry was then poured into ether (400ml) and the ether layer decanted off. The residue was washed with a further portion of ether (200ml) and the pooled extracts were filtered through Celite and washed with HNO₃, water and Na₂S₂O₃ until all the silver was removed. Pyridine was then removed by washing with 6M-HCl, saturated NaHCO₃ and water and the ether layer was dried over Na₂SO₄. After removal of the ether the methyl 2,3,4-tri-*O*-benzoyl-6-deoxy-α-D-xyllo-hex-5-enopyranoside crystallized from ether-light petroleum (40–60°C) and finally from chloroform-methanol, m.p. 99–101°C, [α]_D²⁰ +49° (c 5.0 in CHCl₃); yield 4.7g (55%) (Found: C, 68.7; H, 4.9. C₂₈H₂₄O₈ requires C, 68.8; H, 4.95%).

Methyl 2,3,4-tri-*O*-benzoyl-6-deoxy-α-D-xyllo-hex-5-enopyranoside (16g) (II) and acetic acid-free lead tetra-acetate were dissolved in dry benzene (150ml) and shaken for 3 weeks at room temperature in the presence of a strong light (100W tungsten bulb at 5cm distance). The solution was filtered and washed with 2M-acetic acid, NaHCO₃ and water, and dried over Na₂SO₄. Methyl 5,6-di-*O*-acetyl-2,3,4-tri-*O*-benzoyl-α-D-glucopyranosid-5-ulose, which crystallized from chloroform, had m.p. 104–107°C, [α]_D²⁰ +25° (c 5 in chloroform) (Found: C,

Scheme 2. Preparation of 5-keto-D-glucose 6-phosphate
Compounds (I)–(V) are described in the Experimental section.

63.1; H, 5.5. $C_{32}H_{30}O_{12}$ requires C, 63.3; H, 5.1%). (Note that this compound may be the isomeric *L-ido* compound.)

Subsequently it was found difficult to reproduce this reaction, and mixtures of the diacetate and starting material were obtained. In a typical experiment 30% was converted, as shown by g.l.c. The remaining starting material in these preparations was hydrolysed to 6-deoxy-5-keto-D-glucose and separated during subsequent chromatography.

Methyl 5,6-di-*O*-acetyl-2,3,4-tri-*O*-benzoyl- α -D-glucopyranoside-5-ulose (2g) (III) was dissolved in dry chloroform (15ml) at -10°C and 2M-sodium methoxide in methanol (3ml) was added, followed after 10min by Amberlite IR 120 (H^{+} form) (5g) suspended in 10ml of water. The aqueous layer was separated and the chloroform layer washed with a further 20ml of water, and the combined aqueous extracts were evaporated *in vacuo* to syrupy methyl α -D-glucopyranoside (250mg, 40%).

Methyl α -D-glucopyranoside (100mg) (IV) was redissolved in 15ml of 0.05M- H_2SO_4 and left for 2h at room temperature. Amberlite IRA 400 (CO_3^{2-} form) was added to neutrality and the solution was freeze-dried to give syrupy 5-keto-D-glucose, which was purified by paper chromatography on Whatman 3MM paper with butanol-ethanol-water (4:1:5, by vol., upper phase) as solvent. The band corresponding to 5-keto-D-glucose (R_{glucose} 2.73) was eluted and freeze-dried to a syrup, which was used for the preparation of the phosphate. For determination of the optical rotation, the keto sugar was rechromatographed, eluted from the paper with 1ml of water, and the rotation was observed and the weight determined after freeze-drying: $[\alpha]_D^{22} +5.5^{\circ}$ (c 0.62 in water); Kiely & Fletcher (1969) found $[\alpha]_D -14.6^{\circ}$ (c 3.12 in water).

Syrupy 5-keto-D-glucose (50mg) (V) was dissolved in 5ml of 0.1M-Tris acetate buffer, pH 7.4, containing 5mM-magnesium acetate, 60mM-ATP and 100 units of baker's-yeast hexokinase (Seravac, Maidenhead, Berks.). The mixture was incubated at 35°C for 16h. 5-Keto-D-glucose 6-phosphate was purified by paper chromatography on Whatman 3MM paper with acetone-water (17:3, v/v) as solvent. The area corresponding to 5-keto-D-glucose 6-phosphate ($R_{\text{glucose 6-phosphate}}$ 2.15) was eluted and concentrated by freeze-drying. The phosphate was stored as a solution (10mg/ml) at -20°C .

G.l.c. of the intermediates in the preparation of 5-keto-D-glucose 6-phosphate. (a) Early stages in the preparation of 5-keto-D-glucose were monitored by g.l.c. Reaction mixtures were redissolved in ethyl acetate before chromatography with a Pye 104 gas-liquid chromatograph, on a 5ft (1.5m) $\times$ $\frac{1}{8}$ in (6mm) glass column packed with 3% E-30 on Diatomite C, 100-200 mesh. Conditions of chromatography

were: N_2 carrier flow rate, 80ml/min; column temperature, 260°C ; injection-port temperature, 340°C . The relative mobilities were: methyl 2,3,4-tri-*O*-benzoyl-6-deoxy- α -D-xylo-hex-5-enopyranoside, 1.00 (retention time 18.5min); methyl 5,6-di-*O*-acetyl-2,3,4-tri-*O*-benzoyl- α -D-glucopyranoside, 0.58; methyl 2,3-di-*O*-benzoyl-4,6-benzylidene- α -D-glucopyranoside, 1.49; methyl 2,3,4-tri-*O*-benzoyl-6-bromo-6-deoxy- α -D-glucopyranoside, 1.89. Yields were also estimated by comparisons of peak areas.

(b) 5-Keto-D-glucose was converted into the trimethylsilyl derivative and analysed by g.l.c. on the 3% E-30 column used above, but with a column temperature of 160°C and a carrier-gas flow rate of 50ml/min. One major peak was eluted at 14.5min and two minor peaks at 8.5 and 6.5min. Kiely & Fletcher (1969) found a very similar elution pattern for the g.l.c. of this sugar, the chromatogram differing only by a further minor peak of higher retention time. Direct comparison of the retention times was not possible because no time-scale was given by these authors.

Characterization of 5-keto-D-glucose. 5-Keto-D-glucose (5mg of syrup) was dissolved in 1.0ml of water and NaB^3H_4 (2mg, 50 μCi) in water (0.5ml) was added over 10min at 2°C . Reduction was not complete and the resulting mixture of sugars was deionized by using Amberlite MB 1 resin and separated by paper chromatography on Whatman 3MM paper with ethyl methyl ketone-acetic acid-water (saturated with boric acid) (9:1:1, by vol.) as solvent. Areas corresponding to D-glucose and D-glucitol were eluted. Carrier (10mg each of D-glucose and D-glucitol) was added, the D-glucose was phosphorylated with hexokinase and ATP, purified by paper chromatography and the ^3H localized in the resultant D-glucose 6-phosphate and the D-glucitol by partial degradation by the methods previously described (Barnett & Corina, 1966).

The tritiated D-glucose 6-phosphate had a specific radioactivity of 14300 c.p.m./ μmol . C-5 and C-6 were isolated as the ethanediol 1-phosphate biscyclohexylamine salt (sp.radioactivity 12300 c.p.m./ μmol ; yield 85%). D-Glucitol (2100 c.p.m./ μmol) yielded C-1 and C-6 as the dimedone-formaldehyde complex (410 c.p.m./ μmol ; 21%). Most of the ^3H was located at C-5 of glucose. The low proportion of the label isolated from C-1 suggests a large isotope discrimination in the reduction at this position in comparison with C-5 of 5-keto-D-glucose.

5-Keto-D-glucitol 6-phosphate (L-sorbose 1-phosphate). This was prepared as the barium salt from 2,3:4,6-di-*O*-isopropylidene-L-sorbose (Reichstein & Grüssner, 1934) by the method of Mann & Lardy (1957). Before use, Ba^{2+} ions were removed by Amberlite IR-120 (H^{+} form) resin and the solution was neutralized with 0.1M-NaOH. The organic

phosphate content was determined by hydrolysis with 6M-HCl and assay of P_i by the method of Chen *et al.* (1956).

Preparation of inositol cyclase. Inositol cyclase was prepared from rat testes as previously described (Barnett & Corina, 1968), except that, at all stages of the purification, 0.1 mM-dithiothreitol was added to the buffer. The enzyme purified from six rats (about 8 units) contained 1.5–2.0 units/ml and the specific activity was about 0.35 unit/mg of protein, where 1 unit is the amount of enzyme that cyclizes 1 μ mol of glucose 6-phosphate/h under the standard assay conditions. The enzyme prepared in this way was stable (80%) for 8 days at -10°C and was used for most of the experiments described below.

Inositol cyclase was further purified by passage through a Sephadex G-200 column (52 cm $\times$ 2.5 cm) eluted with 0.05 M-Tris acetate buffer, pH 7.4, containing 0.1 mM-dithiothreitol. Fractions (5 ml) were collected and tested for enzyme activity and protein content. Enzyme activity was in fractions 25–31, and maximum specific activity was 2.8 units/mg of protein. The combined fractions were passed through a Sephadex G-25 column (18 cm $\times$ 1 cm) eluted with 50 mM-Tris acetate buffer, pH 7.4, and freeze-dried. The eluted activity was 0.91 unit/mg and this decreased to 0.42 unit/mg of protein on freeze-drying.

Determination of apparent molecular weight. The apparent molecular weight of inositol cyclase was determined by the method of Andrews (1965) with the Sephadex G-200 column described above and horse heart cytochrome *c*, pig heart malate dehydrogenase, pig heart lactate dehydrogenase, ox liver catalase, pig liver urease and Blue Dextran as markers. The apparent molecular weight was 2.15×10^5 .

Preparation of 'charcoal-treated' enzyme, lacking NAD^+ . Activated charcoal was repeatedly washed with 50 mM-Tris acetate buffer, pH 7.5 (usually about 10 washings), until the washings were free of phosphate. Purified inositol cyclase was stirred with the charcoal (100 mg/ml) for 15 min at 0°C and then centrifuged at 1400g for 5 min. The resulting enzyme preparation was totally inactive if assayed in the absence of added NAD^+ , but had 80% of its original activity when assayed in the presence of NAD^+ .

Assay of inositol cyclase. Inositol cyclase activity was measured by the method of Barnett *et al.* (1970), which involves assay of P_i released after periodate oxidation of the 1 L-myoinositol 1-phosphate formed by the cyclization. It was noted that the assay was unsuitable for crude extracts, which contain too much P_i , or in the presence of substances containing unstable phosphate, such as 5-keto-D-glucitol 6-phosphate (L-sorbose 1-phosphate), or even D-galactose 6-phosphate, which hydrolysed to the extent of 7% under the conditions of the assay, possibly

owing to rearrangement to galactose 4-phosphate. In alkaline media NAD^+ also gave high blanks.

Enzymic reduction of 5-keto-D-glucitol 6-phosphate (L-sorbose 1-phosphate). Charcoal-treated enzyme (about 1 unit), 10 mM-5-keto-D-glucitol 6-phosphate and 0.25 mM-[4- ^3H]NADH (4.6×10^6 c.p.m./ μ mol) in 50 mM-Tris acetate buffer, pH 7.4 (0.75 ml), containing EDTA (0.1 mM) and ammonium acetate (1 mM), were incubated at 37°C for 1–4 h. The reaction was stopped by addition of 20% (w/v) trichloroacetic acid (0.25 ml) and, after 5 min, the protein was removed by centrifugation (1400g for 5 min). The supernatants were extracted with diethyl ether (2 $\times$ 20 ml) to remove trichloroacetic acid and 4.8 mg of carrier D-glucitol 6-phosphate was added to each. The solutions were treated with charcoal (100 mg) to remove most of the [^3H]NADH (about 80%) and centrifuged again.

The supernatants were hydrolysed by 6M-HCl for 18 h at 105°C and the aqueous HCl was removed *in vacuo* (water-bath temperature $30\text{--}40^\circ\text{C}$). After addition and removal of water several times, the residue was applied to Whatman 3MM paper as a band and eluted by using descending chromatography with ethyl methyl ketone–acetic acid–water (saturated with boric acid) (9:1:1, by vol.) for 48 h.

The band shown by standards (detected with AgNO_3) to correspond to D-glucitol was eluted and evaporated to dryness. The residue was dissolved in dry pyridine (1.0 ml) and cooled in ice. Benzoyl chloride (0.3 ml, about 8 molar equivalents) was added dropwise and the mixture left overnight at room temperature. Water (1.0 ml) was added and the mixture was left for 1 h at room temperature, when it was extracted with chloroform (10.0 ml). The chloroform layer was washed twice with 1.5 M- H_2SO_4 (20.0 ml) and twice with saturated NaHCO_3 , and then dried over Na_2SO_4 . The chloroform was removed and the benzoates were applied to a preparative t.l.c. plate (silica gel G_{254} ; Merck, Darmstadt, Germany) with a glucitol hexabenzoate standard. Usually there was only one band visible in the u.v., corresponding to the hexabenzoate. Occasionally the benzylation was imperfect and traces of other slower-running benzoates were observed. The area corresponding to the hexabenzoate was eluted with diethyl ether, and the E_{232} measured, compared with a standard curve of authentic D-glucitol hexabenzoate (m.p. 128°C), and the concentration was thus calculated. A portion of the solution was also counted for radioactivity. Recovery of D-glucitol hexabenzoate was 3–6 mg, 25–50% of theoretical calculated from the carrier D-glucitol added. The counts were corrected to 100% recovery and the results are shown in Table 2.

Enzymic reduction of 5-keto-D-glucose 6-phosphate. The incubations were as described above for the

reduction of 5-keto-D-glucitol 6-phosphate. Reactions were terminated by addition of 20% trichloroacetic acid and 5mg of glucose 6-phosphate was added as carrier. After removal of protein, trichloroacetic acid and NAD, the solution was chromatographed on Whatman 3MM paper with acetone–water (3:1, v/v) as descending solvent for 24h, and the area corresponding to glucose 6-phosphate was eluted. The solution (0.2ml) was reduced by NaBH_4 (1.0mg), and was then hydrolysed by 6M-HCl at 110°C for 16h. The HCl was removed under vacuum, and the D-glucitol was chromatographed on Whatman 3MM paper as described above for reduction of 5-keto-D-glucitol 6-phosphate. As described above, eluted D-glucitol was converted into the hexabenzozoate, purified on t.l.c., and the concentration was measured by the E_{232} and the radioactivity by scintillation counting. Recovery of carrier was 40–50% of theoretical, and counts were corrected to 100% recovery and the results are shown in Table 1.

Degradation of the reduction product of 5-keto-D-glucose 6-phosphate. A portion (0.1 ml) of the glucose 6-phosphate (0.3ml) eluted from the first (acetone–water) chromatogram described above was rechromatographed on Whatman 3MM paper with ethanol–1M-ammonium acetate (7:3, v/v), pH 7.5, for 80h. NADH was located under u.v. and glucose 6-phosphate by alkaline AgNO_3 (Trevelyan *et al.*, 1950). Separation was complete. The glucose 6-phosphate was eluted, concentrated and redissolved in water (0.5ml) and then subjected to the following degradation procedures.

1A: isolation of C-1. The glucose 6-phosphate solution (0.1ml, 700c.p.m.) was added to 100 μmol of glucose 6-phosphate (disodium salt) in water (0.9ml). The solution was reduced with NaBH_4 (8.0mg) and HIO_4 (0.2ml; 50%, w/v) was added. The solution was incubated at 37°C for 1h in the dark and poured into a solution (20ml) containing dimedone (100mg), Na_2HPO_4 (93mg) and NaH_2PO_4 , $2\text{H}_2\text{O}$ (70mg) and left overnight at 2°C. The formaldehyde–dimedone complex was recrystallized from methanol, m.p. 188°C, and 4.3mg (14%) was counted for radioactivity. Background and experimental counts were identical.

1B: isolation of C-5 and C-6. The glucose 6-phosphate solution (0.1ml, 700c.p.m.) was added to 200 μmol of glucose 6-phosphate (disodium salt) in water (0.9ml). Sodium periodate (160mg) in water (1ml) was added, and the solution left at 37°C for 1h. C-5 and C-6 were isolated as the ethanediol dicyclohexylamine salt (m.p. 157°C), 17mg, by the method used by Barnett & Corina (1966) for the biscyclohexylamine salt (Found: N, 4.4; P, 9.2. $\text{C}_{14}\text{H}_{30}\text{NO}_5\text{P}$ requires N, 4.3; P, 9.6%). A 10mg portion was dissolved in 0.15ml of water and counted for radioactivity, giving 47c.p.m. (see Table 3).

1C: isolation of C-1, C-2, C-3 and C-4. Glucose 6-phosphate (0.1ml, 700c.p.m.) was added to HIO_4 (0.15ml; 50%, w/v) and the solution was incubated at 45°C for 30min in the dark. After 10min at room temperature, sodium formate (1mmol) was added and the *p*-bromophenacyl ester was made by the method of Gabriel (1965) (yield 45mg; m.p. 97°C). A portion (30mg) was dissolved in ethyl acetate (0.2ml) and counted for radioactivity, giving 6c.p.m. above background.

2A: isolation of C-6. Glucose 6-phosphate (0.1ml, 700c.p.m.) and carrier glucose 6-phosphate (disodium salt) (100 μmol) were hydrolysed by 6M-HCl at 110°C for 16h. The HCl was removed under vacuum and the residue dissolved in water (1ml). Metaperiodic acid (50%, w/v; 0.6ml) was added and the solution maintained at 37°C for 1h. The formaldehyde–dimedone complex was isolated as described in method 1A (yield 22mg). A 10mg portion was dissolved in 0.5ml of dioxan and counted for radioactivity. The counts were identical with background.

2B: isolation of C-1, C-2, C-3, C-4 and C-5. Syrupy orthophosphoric acid (0.5ml) was added to the mother liquors remaining from the preparation of the dimedone complex in procedure 2A. The solution was distilled and the distillate titrated with 0.1M-NaOH, Methyl Red being used as indicator. After removal of water under vacuum, the sodium formate was converted into the *p*-bromophenacyl ester (yield 90mg). This was dissolved in NE 250 scintillator and counted for radioactivity. After correction for quenching (33%) this gave 303c.p.m. above background.

The results of the degradations are tabulated in Table 3.

Formation of [^3H]myo-inositol 1-phosphate by incubation of 5-keto-D-glucose 6-phosphate with [$4\text{-}^3\text{H}$]NADH and inositol cyclase. For this 0.25mm- [$4\text{-}^3\text{H}$]NADH, 10mm-5-keto-D-glucose 6-phosphate and charcoal-treated inositol cyclase (1.8 units) were incubated as described above for the enzymic reduction of 5-keto-D-glucitol 6-phosphate, and after 3h inositol (10mg) was added as a carrier. The solutions were hydrolysed by 6M-HCl for 18h and, after removal of the HCl, the hydrolysate was chromatographed on Whatman 3MM paper with acetone–water (4:1, v/v) as eluent for 20h. The inositol was eluted and converted into its hexaacetate (Barnett & Corina, 1968) or inositol hexabenzozoate by the method described above for glucitol hexabenzozoate. When the acetate was prepared it was recrystallized and weighed before radioactivity counting, whereas the hexabenzozoate was chromatographed on t.l.c. and estimated by measuring the E_{232} and comparing with a standard. The results are shown in Table 4.

Results

K_m for NAD^+

Charcoal-treated inositol cyclase was incubated under the standard conditions in the presence of 2mM-glucose 6-phosphate and NAD^+ in the concentration range 0.125–1.25mM. The K_m was 5×10^{-5} M.

Inhibition of inositol cyclase by D-glucitol 6-phosphate (L-sorbitol 1-phosphate)

The K_m of D-glucose 6-phosphate for inositol cyclase (0.2 unit/incubation flask, 1mM- NAD^+) was determined over the substrate range 0.5–5.0mM, in the presence and absence of 1.0mM-D-glucitol 6-phosphate (Fig. 1). D-Glucitol 6-phosphate was a good inhibitor with mixed kinetics.

Incubation of inositol cyclase with D-glucitol 6-phosphate in an attempt to detect progressive inactivation

Inositol cyclase (1.5 units) was incubated at 37°C with 1mM-D-glucitol 6-phosphate. Samples (50 μ l) were removed at time-intervals of 1–30min and assayed for enzyme activity. The dilution of the enzyme was 16-fold, into the assay tubes. This was repeated with 5mM and 10mM initial concentrations of the inhibitor. In each case the inhibition of enzyme activity in the final assay, relative to a control lacking inhibitor, was not time-dependent. Further, the inhibition corresponded approximately to that expected if the diluted inhibitor had been added directly to the assay tubes.

Partial reactions of charcoal-treated inositol cyclase

5-Keto-D-glucitol 6-phosphate and 5-keto-D-glucose 6-phosphate were incubated with charcoal-treated inositol cyclase and $[4-^3H]NADH$ and the reduction products, respectively D-glucitol 6-phosphate and D-glucose 6-phosphate or 1L-myoinositol 1-phosphate, were analysed for radioactivity.

Table 1 shows the radioactivity incorporated into D-glucitol 6-phosphate by reduction of 5-keto-D-glucitol 6-phosphate, measured after addition of carrier, hydrolysis and conversion into D-glucitol hexabenzate. In the control incubation trichloroacetic acid was added at zero time. Incorporation of radioactivity into D-glucitol 6-phosphate was progressive with time, and was strongly inhibited by 2-deoxy-D-glucose 6-phosphate, a powerful competitive inhibitor of the overall reaction. In the presence of this inhibitor, the incorporation of radioactivity was less than into the control, and it seems probable that some of the radioactivity incorporated into the control is due to residual enzyme activity, together with a small amount of

Fig. 1. Lineweaver-Burk plot showing the inhibition of inositol cyclase by D-glucitol 6-phosphate

The K_m for D-glucose 6-phosphate was measured in the presence (■) and absence (●) of 1.0mM-D-glucitol 6-phosphate.

non-specific reduction. This is supported (Expt. 2) by the low radioactivity incorporated when enzyme was replaced by bovine serum albumin, or omitted. It is noteworthy that trichloroacetic acid appeared marginally to stimulate non-specific incorporation (Table 1).

Table 2 shows the radioactivity incorporated into D-glucose 6-phosphate by reduction of 5-keto-D-glucose 6-phosphate, measured after addition of carrier and conversion into D-glucitol hexabenzate. The pattern of incorporation of radioactivity was very similar to that found on reduction of 5-keto-D-glucitol, although the extent of the reaction at a similar substrate concentration was higher. In the second experiment, the incorporation into the control treated with trichloroacetic acid at zero time was again higher than that in the presence of 2-deoxy-D-glucose 6-phosphate. In the first experiment, when the control treated with trichloroacetic acid was also boiled for 10min, this was not the case. An additional control was added in Expt. 2 (Table 2). The charcoal-treated enzyme was replaced by 'native' enzyme containing NAD^+ . The incorporation of radioactivity was markedly decreased, suggesting that the NAD^+ of the 'native' enzyme does not readily exchange with $NADH$ in the medium.

The radioactivity incorporated into glucose 6-phosphate in Expt. 2 was localized by selective periodate degradation. The percentage of the recovered activity in atoms or groups of atoms in two degradation procedures is shown in Table 3. Some radioactivity was 'lost' completely. This may be due

Table 1. *Enzymic reduction of 5-keto-D-glucitol 6-phosphate by [4-³H]NADH to D-glucitol 6-phosphate*

Charcoal-treated inositol cyclase (about 1 unit) was incubated with 10mM-5-keto-D-glucitol 6-phosphate and 0.25mM-[4-³H]NADH at 37°C in 50mM-Tris acetate buffer, pH7.4. The reaction was terminated by addition of trichloroacetic acid and addition of carrier D-glucitol 6-phosphate, and the incorporation of radioactivity into D-glucitol 6-phosphate was measured after hydrolysis, chromatographic purification of the D-glucitol formed and conversion into the hexabenzoate. In the control trichloroacetic acid was added at zero time. Counts are corrected to 100% recovery.

		Incubation time (h)	Radioactivity in glucitol 6-phosphate (c.p.m.)	Incorporation of ³ H from [4- ³ H]NADH (%)
Expt. 1	Modification or addition			
	Trichloroacetic acid (at zero time)	1	800	0.083
	None	1	2800	0.28
Expt. 2	None	2	7560	0.74
	No enzyme	4	544	0.05
	Bovine serum albumin (in place of enzyme) and trichloroacetic acid added at zero time	4	868	0.086
	Bovine serum albumin (in place of enzyme)	4	575	0.065
	Trichloroacetic acid (at zero time)	4	2390	0.3
	0.2mM-2-Deoxy-D- glucose 6-phosphate	4	1584	0.18
	None	2	9560	1.1
	None	4	12005	1.4

Table 2. *Enzymic reduction of 5-keto-D-glucose 6-phosphate by [4-³H]NADH to D-glucose 6-phosphate*

Charcoal-treated inositol cyclase (about 1 unit) was incubated with 5-keto-D-glucose 6-phosphate and 0.25mM-[4-³H]NADH in 50mM-Tris acetate buffer, pH7.4. The reaction was terminated by addition of trichloroacetic acid and carrier D-glucose 6-phosphate, and the incorporation of radioactivity into D-glucose 6-phosphate was measured after reduction to D-glucitol 6-phosphate, hydrolysis, chromatographic purification of the D-glucitol formed and conversion into the hexabenzoate. In the control, trichloroacetic acid was added at zero time. Counts are corrected to 100% recovery.

		Concn. of 5-keto-D-glucose 6-phosphate (mM)	Incubation time (h)	Radioactivity in glucose 6-phosphate (c.p.m.)	Incorporation of ³ H from [4- ³ H]NADH (%)
Expt. 1	Modification or addition				
	Trichloroacetic acid*	0.4	4	254	0.018
	(at zero time)				
	1.4mM-2-Deoxy-D- glucose 6-phosphate	0.4	4	263	0.019
Expt. 2	None	0.4	2	494	0.035
	None	0.4	4	1190	0.085
	Trichloroacetic acid (at zero time)	15	2	12555	1.2
	0.2mM-2-Deoxy-D- glucose 6-phosphate	15	2	6199	0.6
	'Native' enzyme †	15	2	10971	1.0
	None	15	1	50947	5.0
	None	15	2	65906	8.5

* This control was boiled at zero time and then trichloroacetic acid was added.

† 'Native' enzyme was not pretreated with charcoal.

to inaccuracies inherent in the small amounts of radioactivity finally measured (see the Experimental section), or more probably is due to slight contamination of the glucose 6-phosphate used for the degradations, which was only separated from the very radioactive $[4\text{-}^3\text{H}]\text{NADH}$ by paper chromatography.

Table 4 shows the incorporation of radioactivity into 1L-*myoinositol* 1-phosphate when 5-keto-D-glucose 6-phosphate and $[4\text{-}^3\text{H}]\text{NADH}$ were incubated with charcoal-treated inositol cyclase. Since 1L-*myoinositol* 1-phosphate itself was not available in sufficient quantity, *myoinositol* was used as the carrier, but care was taken that the hydrolysis con-

ditions gave a 100% conversion of the phosphate into *myoinositol*. The incorporations of radioactivity were relatively low, but again a 'boiled' control gave lower results than a trichloroacetic acid control. As previously, the 'native' enzyme containing NAD^+ did not catalyse the reaction.

Reductions of inositol cyclase by NaBH_4

Sodium borohydride (1 mg) in water (0.1 ml) was added to inositol cyclase (1 ml, 2.4 units) and the solution stirred for 30 min at 0°C. A control tube was treated in the same way, but NaBH_4 was omitted.

Table 3. *Degradation of $[^3\text{H}]\text{glucose}$ 6-phosphate isolated after incubation of 5-keto-D-glucose 6-phosphate with $[4\text{-}^3\text{H}]\text{NADH}$ and charcoal-treated inositol cyclase*

D-Glucose 6-phosphate formed by enzymic reduction of 5-keto-D-glucose 6-phosphate was degraded by the procedures described in the text and crystalline derivatives, containing individual and groups of the original hydrogen atoms, were isolated. Counts are corrected for quenching and corrected to 100% yield of the derivative.

Procedure	Derivative	Carbon atoms of glucose 6-phosphate to which the hydrogen was attached	Radioactivity (c.p.m.)	Percentage of recovered radioactivity
1. Periodate oxidation of D-glucitol 6-phosphate or D-glucose 6-phosphate				
A	Formaldehyde dimedone	1	0	0
B	Ethanediol 1-phosphate dicyclohexylamine salt	5, 6	330	87
C	<i>p</i> -Bromophenacyl formate	1, 2, 3, 4	48	13
2. Periodate oxidation of D-glucose				
A	Formaldehyde dimedone	6	0	0
B	<i>p</i> -Bromophenacyl formate	1, 2, 3, 4, 5	405	100

Table 4. *Enzymic conversion of 5-keto-D-glucose 6-phosphate and $[4\text{-}^3\text{H}]\text{NADH}$ into 1L-*myoinositol* 1-phosphate*

Charcoal-treated inositol cyclase (about 1 unit) was incubated with 5-keto-D-glucose 6-phosphate and $[4\text{-}^3\text{H}]\text{NADH}$ at 37°C in 50 mM-Tris acetate buffer, pH 7.4. The reaction was terminated by addition of *myoinositol* and trichloroacetic acid or (Expt. 2) by adding *myoinositol* and boiling. After hydrolysis and chromatographic separation of the inositol this was converted into the hexabenzoate (Expt. 1) or the hexa-acetate (Expt. 2) and counted for radioactivity. Controls were with trichloroacetic acid added at zero time or with the enzyme boiled for 10 min at zero time. Counts are corrected to 100% recovery.

	Modification or addition	Concn. of 5-keto-D-glucose 6-phosphate (mM)	Incubation time (h)	Radioactivity in inositol 1-phosphate (c.p.m.)	Incorporation of ^3H from $[4\text{-}^3\text{H}]\text{NADH}$ (%)
Expt. 1	Trichloroacetic acid (at zero time)	15	3	344	0.043
	None	15	3	680	0.083
Expt. 2*	Boiled at zero time	2	3	218	0.003
	'Native' enzyme used†	2	3	255	0.003
	None	2	3	1778	0.026
	None	2	3	1756	0.026

* The $[4\text{-}^3\text{H}]\text{NADH}$ added in this experiment was 1 mM and had 6.9×10^7 c.p.m.

† 'Native' enzyme was not pretreated with charcoal.

The solutions were dialysed for 24 h and the enzyme specific activities measured. In the presence of 1 mM- NAD^+ in the assay medium, both activities were identical, showing that NaBH_4 does not cause irreversible changes to the enzyme protein.

The following additions were also made to the reduction vessels and the experiment was repeated. The description of the additions is followed by the percentage inhibition compared with a water control: (a) 1 mM- NAD^+ , 35%; (b) 1 mM- NAD^+ and 2 mM-glucose 6-phosphate, 14%; (c) 1 mM- NAD^+ and 2 mM-pyruvic acid, 50%.

This series of experiments was initiated to test the possibility that a reducible Schiff base might be formed on the enzyme. It was found that the enzyme was inactivated in the presence of NAD^+ , but not in its absence, and that the enzyme was protected against this inactivation by the substrate but not by another carbonyl-containing compound, pyruvic acid. It seems probable that the enzyme-bound NAD^+ can be reduced by NaBH_4 to give an inactive enzyme complex, but there is no evidence for Schiff base formation. In an attempt to regenerate active enzyme, the enzyme inactivated under condition (a) was incubated at 37°C for 20 min with 10 mM-5-keto-D-glucitol 1-phosphate. After dialysis for 4 h, the specific enzyme activity was again determined, and some regeneration was observed (19% of the original activity).

Discussion

Despite the general acceptance of the mechanism shown in Scheme 1 for inositol cyclase, hitherto no direct evidence has been presented for the presence of 5-keto-D-glucose 6-phosphate as an enzyme-bound intermediate. However, model reactions using 6-deoxy-6-nitro-D-glucose (Grosheintz & Fischer, 1948; Lichtenthaler, 1961) and 5-keto-D-glucose (Kiely & Fletcher, 1969) have shown that the proposed cyclization to 1L-*myo*inositol 1-phosphate by way of an aldol reaction is both feasible and favourable.

The difficulty of establishing the nature of the enzyme-bound intermediates is not peculiar to this enzyme, but is common to all the carbohydrate isomerases that use an oxidation-reduction mechanism, in which the hydrogen removed during the reaction is replaced on the same carbon skeleton. The problem is made more difficult if, as with inositol cyclase, the hydrogen atom abstracted is replaced on the same carbon atom. In this event the only convincing evidence for the nature of the enzyme-bound intermediate must involve partial reactions in which either the oxidation or the reduction alone is observed. This may be illustrated by reference to UDP-glucose 4'-epimerase, for which both UDP-4-

keto-D-glucose (Maxwell, 1957; Wilson & Hogness, 1964) and UDP-3-keto-D-glucose (Davis & Glaser, 1971) have been considered to be possible enzyme-bound intermediates. More recent evidence, in which partial reactions have been employed, favour the former. Maitra & Ankel (1971) reduced the substrate-enzyme complex, containing oxidized sugar with NaB^3H_4 , giving only derivatives of UDP-4-keto-D-glucose, and Nelsestuen & Kirkwood (1971) have observed that inactivated enzyme containing $[4\text{-}^3\text{H}]\text{NADH}$ will transfer ^3H to analogues of UDP-4-keto-D-glucose, becoming reactivated as the enzyme-bound NADH is converted into NAD^+ . We have sought a similar solution to the identification of the oxidized intermediate in the conversion of glucose 6-phosphate into 1L-*myo*inositol 1-phosphate by inositol cyclase by the use of partial reactions.

We had previously observed (Barnett & Corina, 1968) that NAD^+ is tightly bound to inositol cyclase, but treatment with charcoal removed it and gave an inactive enzyme. Addition of NAD^+ restores the activity of the preparation to 80% of the original activity, and the K_m of NAD^+ for the enzyme is $5 \times 10^{-5} \text{ M}$. This charcoal-treated enzyme preparation has been found to catalyse the reduction by $[4\text{-}^3\text{H}]\text{NADH}$ of both 5-keto-D-glucose 6-phosphate (Table 2), the proposed enzyme-bound intermediate, and its analogue 5-keto-D-glucitol 6-phosphate (Table 1). Both reactions were inhibited by low (0.2 mM) concentrations of 2-deoxy-D-glucose 6-phosphate, a powerful competitive inhibitor of the overall reaction (Barnett *et al.*, 1970), and the former reduction was not catalysed to any extent by the 'native' enzyme containing bound NAD^+ . These controls make reduction of the ketose phosphates by a non-specific reductase in the enzyme preparation improbable, because it would be unlikely that such an enzyme would be so powerfully inhibited by 0.2 mM-2-deoxy-D-glucose 6-phosphate or by the trace of NAD^+ remaining after the preparation had been dialysed but not treated with charcoal. Further to eliminate the possibility that a random reductase might be responsible for the reaction, the glucose 6-phosphate obtained from the reduction of 5-keto-D-glucose 6-phosphate was degraded. Almost all the radioactivity was associated with C-5, and none was associated with C-1.

5-Keto-D-glucitol 6-phosphate (L-sorbose 1-phosphate) was used for initial experiments because it was more readily available, and also because it lacks the aldehyde group at C-1 and cannot cyclize to the inositol skeleton. However, we observed that 5-keto-D-glucose 6-phosphate was a better substrate, and that under the conditions used about 1% of the reduced sugar was also converted into cyclitol (Table 4). The relatively poor incorporation into cyclitol would be expected if the rate of cyclization was slower than the rate of reduction.

The reactions did not stop after the first cycle, when NADH on the enzyme had been converted into NAD^+ , but were truly catalytic, proceeding almost linearly for 2–4 h. This implies that the NAD^+ formed can dissociate easily from the charcoal-treated enzyme when the sugar is in the reduced form, and is consistent with the observation that, with the charcoal-treated enzyme, a true K_m can be obtained for NAD^+ in the overall reaction. These partial reactions are consistent with the mechanism for inositol cyclase shown in Scheme 1.

dTDP-D-glucose oxidoreductase is inactivated by dTDP-6-deoxy-D-glucose (Wang & Gabriel, 1970; Zarkowsky *et al.*, 1970). This substrate is oxidized by the enzyme to dTDP-4-keto-6-deoxy-D-glucose with the formation of NADH. Because C-6 is already reduced, the NADH cannot be re-oxidized to give the active NAD^+ -containing form of the enzyme, and the enzyme becomes inactivated. A similar substrate for inositol cyclase might be D-glucitol 6-phosphate (L-sorbitol 1-phosphate), an analogue of the substrate D-glucose 6-phosphate, which cannot cyclize because it lacks the carbonyl group at C-1, but which might still undergo the initial oxidation. D-Glucitol 6-phosphate was a good inhibitor of inositol cyclase, with mixed kinetics (Fig. 1), but attempts to demonstrate progressive inactivation of the type predicted were unsuccessful. A potential inactivator of this type is successful only if the oxidized form of the sugar can diffuse away from the oxidized sugar–enzyme–NADH complex, leaving the inactive enzyme–NADH complex. It must be concluded that either D-glucitol 6-phosphate does not undergo the initial oxidation, or, more probably, that the resulting ternary complex is very stable.

An attempt was made to determine whether a Schiff base with the enzyme is formed by the 5-keto-D-glucose 6-phosphate before cyclization. The 'native' enzyme, NAD^+ and glucose 6-phosphate were reduced by NaBH_4 , the products dialysed and the specific activity of the enzyme was assayed. No significant decrease in activity was observed, but in the absence of substrate the enzyme was 35% inactivated, whereas in the absence of both NAD^+ and substrate the enzyme was not inactivated. No conclusions can therefore be drawn about the involvement of a Schiff base, but it appears that, in the presence of NAD^+ , NaBH_4 can reduce enzyme-bound NAD^+ to give an inactive reduced NAD-containing complex, and that the presence of the substrate will partially protect against the inactivation. Pyruvic acid did not so protect, indicating that protection was not merely due to removal of NaBH_4 .

The NADH–enzyme complex was partially regenerated by incubation with 5-keto-D-glucitol 6-phosphate.

The results presented in the present paper strongly support the hypothesis that the enzyme-catalysed cyclization of glucose 6-phosphate to 1L-myoinositol 1-phosphate takes place by a mechanism similar to that shown in Scheme 1, and that the intermediary ternary complex of oxidized sugar, NADH and enzyme is extremely stable.

References

- Andrews, P. (1965) *Biochem. J.* **96**, 595–606
- Barnett, J. E. G. & Corina, D. L. (1966) *Carbohydr. Res.* **3**, 134–137
- Barnett, J. E. G. & Corina, D. L. (1968) *Biochem. J.* **108**, 125–129
- Barnett, J. E. G., Brice, R. E. & Corina, D. L. (1970) *Biochem. J.* **119**, 183–186
- Chen, I. W. & Charalampous, F. C. (1967) *Biochim. Biophys. Acta* **136**, 568–570
- Chen, P. S., Toribara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28**, 1756–1758
- Davis, L. & Glaser, L. (1971) *Biochem. Biophys. Res. Commun.* **43**, 1429–1435
- Eisenberg, F. (1967) *J. Biol. Chem.* **242**, 1375–1382
- Gabriel, O. (1965) *Arch. Biochem.* **10**, 143–147
- Grosheintz, J. M. & Fischer, H. O. L. (1948) *J. Amer. Chem. Soc.* **70**, 1479–1484
- Hanessian, S. (1966) *Carbohydr. Res.* **2**, 86–88
- Kiely, D. E. & Fletcher, H. G. (1969) *J. Org. Chem.* **34**, 1386–1390
- Lichtenthaler, F. W. (1961) *Chem. Ber.* **94**, 3071–3085
- Loewus, F. A. & Kelly, S. (1962) *Biochem. Biophys. Res. Commun.* **7**, 204–208
- Maitra, U. S. & Ankel, H. (1971) *Proc. Nat. Acad. Sci. U.S.A.* **68**, 2660–2663
- Mann, K. M. & Lardy, H. A. (1957) *Methods Enzymol.* **3**, 177–179
- Maxwell, E. S. (1957) *J. Biol. Chem.* **229**, 139–151
- Nelsestuen, G. L. & Kirkwood, S. (1971) *J. Biol. Chem.* **246**, 7533–7543
- Rasheed, A., Corina, D. L. & Barnett, J. E. G. (1972) *Biochem. J.* **126**, 15P
- Reichstein, T. & Grüssner, A. (1934) *Helv. Chim. Acta* **17**, 311–328
- Sherman, W. R., Stewart, M. A. & Zinbo, M. (1969) *J. Biol. Chem.* **244**, 5703–5708
- Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950) *Nature (London)* **166**, 444–445
- Wang, S. F. & Gabriel, O. (1970) *J. Biol. Chem.* **245**, 8–14
- Wilson, D. B. & Hogness, D. S. (1964) *J. Biol. Chem.* **239**, 2469–2481
- Wilton, D. C., Munday, K. A., Skinner, S. J. M. & Akhtar, M. (1968) *Biochem. J.* **106**, 803–810
- Zarkowsky, H., Lipkin, E. & Glaser, L. (1970) *J. Biol. Chem.* **245**, 6599–6606