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L-*myo*-Inositol-1-phosphate Synthase from Bovine Testis: Purification to Homogeneity and Partial Characterization†

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ABSTRACT: L-*myo*-Inositol-1-phosphate synthase has been purified to homogeneity from bovine testis by $(NH_4)_2SO_4$ precipitation on Celite followed by reverse $(NH_4)_2SO_4$ gradient elution, DEAE chromatography, gel filtration, and hydroxylapatite chromatography. The enzyme is then pure by the criteria of elution profile from the hydroxylapatite, electrophoresis, and sedimentation properties. We find no overall (gluconeogenic) reversibility of the enzyme using 6 mM DL-*myo*-inositol-1-P. The first three steps of the reaction are reversible as determined by uptake of isotope from a D_2O incubation medium into the 6 position of D-glucose-6-P. Thus, substrate binding, dehydrogenation, and proton removal prior to the aldol cyclization are all reversible steps. The enzyme is <5% NAD^+ independent and is not inhibited by substrate or product (5 mM D-glucose-6-P or 0.8 mM DL-*myo*-inositol-1-P). The enzyme is twofold stimulated by either 50 mM NH_4^+ or 50 mM K^+; the activation by these ions is not additive. Sodium ions inhibit the enzyme by 78% at 153 mM. The effect of sodium and potassium is not on the K_m of D-glucose-6-P but on V_{max}. We propose that K^+ activates the enzyme by stabilizing a carbanion intermediate. Ethanol stimulates the enzyme 2-fold and 2.5-fold with added K^+. The effect of ethanol appears to be via lowering of the D-glucose-6-P K_m. In the presence of ethanol the effect of salt on V_{max} disappears.

L-*myo*-Inositol-1-phosphate synthase (M1P[1] synthase) (EC 5.5.1.4) and L-*myo*-inositol-1-phosphatase (EC 3.1.3.25), provide the only known biological pathway for the de novo formation of *myo*-inositol. The substrate for the synthase is

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[1] Abbreviations used: DTT, dithiothreitol; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; 5-ketoglucose-6-P, D-*xylo*-hexos-5-ulose 6-phosphate; M1P, L-*myo*-inositol 1-phosphate; synthase, L-*myo*-inositol-1-phosphate synthase; Me_3Si, trimethylsilyl.

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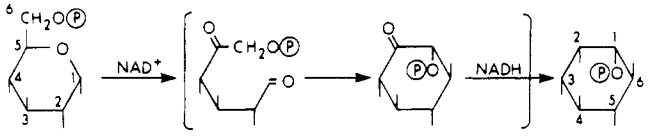
Scheme I



D-glucose-6-P | 5-keto-glucose-6-P | L-*myo*-inosose-2-1-P | L-myo-inositol-1-P

D-glucose 6-phosphate and the mechanism of the transformation seems to be similar, if not identical, regardless of which of the many sources of the enzyme have been studied [for a brief review see Sherman et al. (1977)].

The M1P synthase is a complex enzyme combining the activities of an NAD^+-dependent oxidoreductase with an aldolase. In the oxidoreductase reaction the synthase removes the 5-H of the substrate to form D-*xylo*-hexos-5-ulose-6-P (5-ketoglucose-6-P; Scheme I). The same hydrogen (Sherman et al., 1969) is then returned to another intermediate (L-*myo*-inosose-2-1-P; Scheme I) to give L-M1P which is acted on by a specific phosphatase to give the final product (Eisenberg, 1967). The mammalian M1P synthases which have been studied are unusual aldolases in being neither Schiff base forming enzymes nor divalent metal requiring ones (Sherman et al., 1977). The proposed mechanism of the M1P synthase is outlined in Scheme I.

In this paper we describe the purification to homogeneity of M1P synthase and report on our studies of some properties of this enzyme.

Experimental Procedures

Methods. The general procedures including sources of reagents, methods of preparing isotopically labeled D-glucose-6-P, gas chromatographic and gas chromatography–mass spectrometry techniques, derivatization, and enzyme assay procedures are described in detail in Sherman et al. (1977).

Protein concentration was determined spectrophotometrically either by the method of Warburg & Christian [1941; see Layne (1957)] or by the dye binding technique of Bradford (1976) using protein standards as noted.

L-*myo*-Inositol 1-phosphate was prepared enzymatically from D-glucose-6-P by the method of Burton & Wells (1974) using bovine testis M1P synthase. DL-*myo*-Inositol 1-phosphate dicyclohexylammonium salt was a gift from Dr. D. E. Kiely, University of Alabama, Birmingham, AL.

Standard buffer contained 50 mM Tris-HCl, pH 7.4, with 5% ethanol, 0.2 mM dithiothreitol, and 0.02% NaN_3. For some experiments, which are noted, the ethanol was omitted.

Incubations contained 2 mM D-glucose-6-P and 1 mM NAD^+ except as noted.

Affinity Supports. NAD^+–Sepharose prepared by the method of Mosbach et al. (1972) (AGNAD-2) was obtained from P-L Biochemicals, Elkhart, IN.

Enzymes. L-*myo*-Inositol-1-phosphate synthase was prepared from both bovine testis and bovine brain. Fresh or frozen decapsulated testis (100 g) was homogenized in a blender in 200 mL of 0.154 M KCl containing 0.2 mM dithiothreitol and 5% ethanol and then centrifuged at 100000*g* for 1 h. To the resulting supernatant was added 10 g of Celite-545 (Johns Manville Corp.) with stirring at 0 °C, followed by solid $(NH_4)_2SO_4$ to 35% of saturation (at 25 °C). After the mixture was stirred for 1 h, the Celite suspension was filtered on a Buchner funnel and washed with 50 mL of a solution of $(NH_4)_2SO_4$ (40% of saturation in standard buffer). The Celite preparation was resuspended in the same 40% $(NH_4)_2SO_4$ solution by using slow stirring and then poured into a 2.5 × 35 cm column. After settling and decanting of the supernatant, the column was eluted at 15–25 mL/h with a 400-mL linear gradient from 40 to 0% saturation of $(NH_4)_2SO_4$ in standard buffer. Fractions of ~1% of the gradient volume were collected and analyzed colorimetrically. Runs of 10 times this size have been carried out by using a 1000-mL gradient.

After the active Celite fractions were pooled, they were dialyzed against standard buffer. The dialysate was clarified by centrifugation and concentrated to ~6 mg/mL by pressure dialysis against an Amicon XM100A filter (Amicon Corp., Lexington, MA). The Celite concentrate was then pumped onto a column of Whatman DE-52 (6 mg of protein/mL of the DEAE-cellulose, e.g., a 150-mL column for a 1-kg preparation). The DE-52 was eluted with a 6-column-volume linear gradient from 0 to 0.4 M KCl in standard buffer. Active fractions were pooled, concentrated, and clarified by centrifugation, and the enzyme was pumped onto a Bio-Gel A-1.5m column (5 × 100 cm) which had been equilibrated with standard buffer containing 0.2 M KCl. The column was eluted at 40–50 mL/h and 10-mL fractions were collected. At this stage of purification the enzyme had to be stabilized for assay by inclusion of 0.5 mg/mL bovine serum albumin. The active fractions were pooled, concentrated to 5–10 mg/mL by Amicon ultrafiltration, and dialyzed against 5 mM Tris-HCl, pH 7.0, containing 0.2 mM DTT and 0.02% NaN_3. The enzyme was then placed on a column of Bio-Gel HT hydroxylapatite (2.5 mg of protein/mL of the adsorbent) which had been previously equilibrated with the 5 mM, pH 7.0, Tris-HCl buffer. The homogeneous enzyme is eluted from the column with a linear gradient in 5 mM Tris-HCl, pH 7.0, and 0.02% NaN_3 of 0–50 mM potassium phosphate. Each fraction was dialyzed against standard buffer prior to analysis in the presence of bovine serum albumin.

The homogeneity and molecular weight studies were performed on a Spinco Model E ultracentrifuge. Dithiothreitol was dialyzed from the samples immediately prior to analysis.

Bovine brain synthase was purified by the procedure of Eisenberg (1967), i.e., homogenization, centrifugation at 100000*g*, heating (60 °C; 2 min), centrifugation, and precipitation of the activity with 35% $(NH_4)_2SO_4$. Reconstitution in buffer and dialysis gave the enzyme preparation used.

Each of the above enzyme preparations was found to give linear product formation over time (usually 2 h) and with the protein concentrations used.

L-*myo*-Inositol-1-phosphatase was prepared from bovine brain by a method similar to that reported by Naccarato et al. (1974). Tissue was homogenized in 2 volumes of 0.154 M KCl, followed by centrifugation at 10000*g* for 90 min. The supernatant was then treated with $(NH_4)_2SO_4$ (35–60% of saturation), and the resulting precipitate was suspended in a minimal volume of 0.154 M KCl and dialyzed overnight against 0.154 M KCl. After the dialysate was heated at 80 °C for 15 min (Eisenberg, 1967), the precipitated protein was removed by centrifugation at 30000*g* for 30 min. The supernatant was then chromatographed on a 2.5 × 100 cm column of Sephadex G-100 equilibrated with 0.1 M Tris-HCl, pH 7.4, containing 0.2 M KCl and 0.02% NaN_3. The pooled active fractions had 3.3% of nonspecific phosphatase activity as determined by assay using 3 mM D-glucose 6-phosphate and 0.4 mM DL-*myo*-inositol 1-phosphate as substrates.

Results and Discussion

Purification of M1P Synthase. In our initial work in this

Table I: Purification to Homogeneity of Bovine Testis *myo*-Inositol-1-phosphate Synthase[a]

step	total protein (mg) (280/260)	yield of total act. (%)	sp act. [nmol/(min mg of protein)] BSA[b]	γ-globulin[b]	280/260[c]	x-fold purifn (280/260)
pptd on Celite 545 and eluted with 40–0% saturated $(NH_4)SO_4$	261	100	7	22	3	1
DEAE-cellulose (0–0.4 M KCl)	55	85	24	8	12	4
Bio-Gel A-1.5m (0.2 M KCl)	10.3	47	72	23	36	12
hydroxylapatite (0–50 mM P_i)	1.6	37	528	156	181	60

[a] The first three steps are carried out in 50 mM Tris-HCl, pH 7.4, containing 5% ethanol, 0.2 mM dithiothreitol, 0.02% NaN_3, and other additions as noted. The hydroxylapatite step is carried out in 5 mM Tris-HCl, pH 7.0, with NaN_3. [b] Bovine serum albumin or γ-globulin as protein standards with the Bradford (1976) dye binding method of protein determination. [c] The method of Warburg & Christian [1941; see also Layne (1957)].

study we examined bovine brain and testis as potential sources of the M1P synthase. We found that, as reported for rat tissues (Eisenberg, 1967), bovine brain has ~5% of the activity of testis on a tissue weight basis. A few studies reported here are with brain enzyme; however, we concentrated on testis as a more suitable organ for larger scale preparations. In the beginning of this study we used a heating step in the purification (60 °C; 2 min; Eisenberg, 1967) which removes a significant amount of protein and nonspecific phosphatase activity of the preparations without apparent effect on the properties of the M1P synthase. Out of concern that heating might modify the enzyme in an irreversible manner, we later eliminated this step from the purification procedure. Unless otherwise noted, the work presented here has been carried out with unheated preparations.

We have obtained homogeneous preparations of the M1P synthase in four steps by the method shown in Table I. This is a simplification and extension of our previously reported procedure (Sherman et al., 1978). The first step in the purification is the precipitation of the synthase on Celite with $(NH_4)_2SO_4$, followed by elution of the activity with a reverse gradient of $(NH_4)_2SO_4$, a technique first described by King (1972). In subsequent steps the enzyme is chromatographed on DEAE-cellulose and on Bio-Gel A-1.5m. The inclusion of 5% ethanol in the eluting buffers of these columns resulted in reduced trailing and improved the separations and yield over our earlier method. At the Celite or DEAE-cellulose stages of purification the enzyme, after concentration, can be stored at −70 °C in 0.2 M KCl solutions which contain 0.2 mM dithiothreitol and 5% ethanol. Under these conditions several freeze–thaw cycles do not reduce activity substantially. However, following the agarose gel filtration, the M1P synthase is unstable toward freezing. At this and the next stage of purification, incubations at 37 °C require added bovine serum albumin to stabilize the enzyme.

Purification of the Bio-Gel fractions on hydroxylapatite has, in most experiments, given pure synthase. Occasional evidence of heterogeneity which may be due to disaggregation is under study.

Evidence of Homogeneity. The elution profile of the hydroxylapatite column (Figure 1) shows the wide separation of the synthase from contaminating proteins with which it coeluted from the Bio-Gel column. Figure 1 also shows that the protein and the activity appear to elute as a single band from the column. When the dialyzed hydroxylapatite fraction is subjected to discontinuous electrophoresis (Ornstein, 1964; Davis, 1964) in a 7% cross-linked polyacrylamide system, only a single protein band is evident on staining with Coomassie blue. The stained band and the activity coincide. Several hydroxylapatite fractions have been examined by sedimentation equilibrium in an analytical ultracentrifuge. These fractions give linear plots[2] of log C vs. r^2. Linearity of this plot is a necessary condition for homogeneity [e.g., Van Holde & Baldwin (1958)] and, taken together with the hydroxylapatite elution profile and the electrophoresis, indicates that the enzyme is pure. Schlieren patterns from other sedimentation studies have confirmed this; they appear to be symmetrical at all times during analysis, with the exceptions noted.

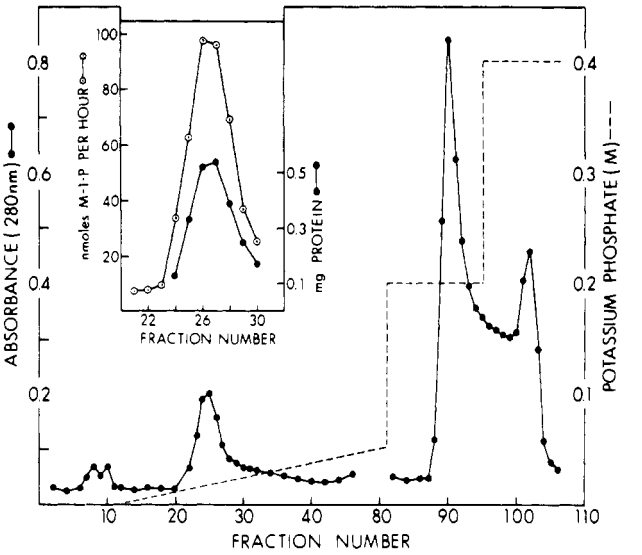


FIGURE 1: (Lower) Final stage in the purification of M1P synthase. Enzyme, at the Bio-Gel A-1.5m stage of purification, is placed on a hydroxylapatite column in 5 mM Tris-HCl, pH 7.0, containing 0.2 mM DTT and 0.02% NaN_3. The activity elutes during a 0–50 mM potassium phosphate gradient. Remaining inactive protein is cleared from the column with higher [P_i]. (Inset) After each fraction is dialyzed, the synthase activity and the protein (280/260 nm) elution profiles coincide.

Recently Maeda & Eisenberg (1978) have reported the complete purification of rat testis M1P synthase using glucose-6-P–Sepharose in the final step following a purification similar to ours up to the hydroxylapatite stage.

NAD^+ Affinity Gels. Pittner & Hoffmann-Ostenhof (1976) have reported the purification of bovine testis M1P synthase to an electrophoretically homogeneous protein in a single step from an $(NH_4)_2SO_4$ fraction by using an NAD^+–Sepharose affinity gel originally described by Mosbach et al. (1972). The enzyme they obtained was reported to have a specific activity of 83 nmol/(min mg of protein) as measured by the Lowry (Lowry et al., 1951) method.

We have been unable to duplicate the procedure for the purification of M1P synthase by the affinity support method described above using either bovine testis or, as described in an earlier paper (Pittner et al., 1974), rat testis enzyme. There

[2] C is the concentration of the solute at each point of distance r from the axis of rotation of the cell in the ultracentrifuge.

appear to be some differences between our enzyme preparations and those of these authors in that our enzyme shows little activity in the absence of added NAD^+ while theirs is largely NAD^+ independent. Pittner & Hoffmann-Ostenhof (1976) found it necessary, prior to affinity chromatography, to remove bound NAD^+ from the bovine testis synthase with activated charcoal, as described for rat testis by Barnett & Corina (1968). We have been unable to obtain any measurable degree of binding to the Mosbach et al. NAD^+–Sepharose of heated, nonheated, charcoal-treated, or noncharcoal-treated rat testis M1P synthase. Similarly, our heated preparations of bovine testis synthase, which are highly NAD^+ dependent for activity, also do not bind to this gel. Out of concern that the NAD^+–Sepharose we were using (a commercial preparation of the Mosbach et al. gel) was inactive, we tested it with lactate dehydrogenase which bound to the support and was eluted by NADH as described by Mosbach et al. We do not have an explanation for these discrepant results.

Molecular Weight. We have obtained the molecular weight of the synthase by sedimentation equilibrium.[2] Using a value of 0.725 for $\bar{\nu}$, the partial specific volume of the enzyme, the molecular weight was found to be 209 000. In this experiment, the sample was centrifuged first for 3 h at 52 000 rpm and then at 10 000 rpm for 24 h, at which time equilibrium had been reached. No further change was observed at 48 h.

The molecular weight we find for the synthase is close to that reported by Pittner & Hoffmann-Ostenhof (1976) for enzyme from the same source. Rat testis synthase is reported to have a similar molecular weight: e.g., 215 000 (Barnett et al., 1973; Pittner & Hoffmann-Ostenhof, 1976); 210 000 (Maeda & Eisenberg, 1978).

NAD^+ Dependence and K_m of M1P Synthase. Unlike the findings of several other laboratories but in agreement with Maeda & Eisenberg (1978), we find no measurable activity of our M1P synthase preparations in the absence of added NAD^+. We have examined the bovine brain enzyme which was purified by heating, followed by ammonium sulfate fractionation, and found no NAD^+-independent activity when analyzed by the colorimetric method (Barnett et al., 1970) or by gas chromatography. In the latter experiment the lower level of activity was ~5%, essentially at the blank level of the experiment (measurable amounts of "bound", i.e., nondialyzable *myo*-inositol accompanies the enzyme at this stage of purification). Bovine testis synthase has repeatedly shown the same NAD^+ dependence regardless of the stage of purification or whether heated in the initial step or not.

There are several reports in the literature that M1P synthases from different sources have a variable degree of NAD^+-independent activity. [e.g., Barnett & Corina (1968), Brunner et al. (1972), Naccarato et al. (1974), and Pittner & Hoffmann-Ostenhof (1976)]. Barnett and Corina were the first to report that this bound NAD^+ could be removed by activated charcoal (rat testis). Loewus & Loewus (1973) reported that synthase from *Acer pseudoplatanus* could be prepared which had, in the absence of added NAD^+, 74–100% of the activity found in the presence of 1 mM added NAD^+. These authors had earlier reported that this enzyme had an absolute dependence on added NAD^+ for activity (Loewus & Loewus, 1971). Loewus & Loewus (1973) reported that either 20-h dialysis against EDTA or oxidation with potassium tetrathionate diminished but did not eliminate the NAD^+ dependency of the *Acer* enzyme. Whether or not Loewuses' experiments with *Acer* can be extrapolated to M1P synthase from other sources is not known. However, it seems that these enzymes can be isolated with different amounts of bound NAD^+; thus, the question of which is the native form remains unanswered.

By use of M1P synthase from bovine testis, purified by DEAE chromatography, and with 2 mM glucose-6-P (7 mM Na^+) as the fixed substrate, the K_m of NAD^+ was found to be 0.011 mM. The plot of this data suggests a rate of ~5% of V_{max} in the absence of NAD^+. Maeda & Eisenberg (1978) report that the rat testis synthase has a very similar K_m (0.025 mM). Others have also reported similar values, e.g., Barnett et al. (1973), 0.05 mM (rat testis), and Pittner & Hoffmann-Ostenhof (1976), 0.012 mM (bovine testis).

Reversibility of M1P Synthase. As outlined in the introduction, the pathway of the synthase involves the following steps: the utilization of NAD^+ in the formation of 5-ketoglucose-6-P (intermediate I_1 in Scheme II); the removal of the 6-*pro-S* hydrogen of 5-ketoglucose-6-P to give the carbanion I_1^-; an aldol cyclization to give *myo*-inosose-2-1-P (intermediate I_2); the reduction of that cyclose by NADH to form the product, L-*myo*-inositol-1-P. This sequence, as outlined in Scheme II, does not consider either the binding order of substrate and NAD^+ to the enzyme or the order of disassociation of product and NAD^+ from the enzyme at the end of the reaction.

Scheme II

$$E + S + NAD^+ \overset{1}{\rightleftharpoons} E{\cdot}S{\cdot}NAD^+ \overset{2}{\rightleftharpoons}$$

$$E{\cdot}I_1{\cdot}NADH \underset{(+H^+)}{\overset{3\ (-H^+)}{\rightleftharpoons}} E{\cdot}I_1^-{\cdot}NADH \overset{4}{\rightleftharpoons}$$

$$E{\cdot}I_2{\cdot}NADH \overset{5}{\rightleftharpoons} E{\cdot}P{\cdot}NAD^+ \overset{6}{\rightleftharpoons} E + P + NAD^+$$

Reversibility of the Entire Pathway (Gluconeogenesis). A standard incubation mixture, including 1 mM NAD^+, but with the bis(cyclohexylamine) salt of DL-*myo*-inositol-1-P (6 mM) as the substrate instead of D-glucose-6-P, was incubated for 19 h at 37 °C with bovine testis M1P synthase which had been purified through the Sephadex A-25 stage (Sherman et al., 1978). The enzyme activity was such that 1.2 μmol of *myo*-inositol-1-P would have been generated per h in the presence of 3 mM D-glucose-6-P. Boiled enzyme controls were carried out in duplicate, and five experimental samples were examined. After the incubation period the samples were dephosphorylated with *Escherichia coli* alkaline phosphatase and analyzed for glucose by GC–MS using the acetate–butaneboronate derivative of glucose (Sherman et al., 1977). No glucose formation was observed in any of the samples. Since <15 nmol of glucose per incubation could have been detected, the reverse reaction rate is less than 0.5% of the forward rate. This estimate is based only on 2 h of the 19-h reaction since this is the known period of linearity in the forward direction. Barring the possibility that D-M1P inhibits the reverse reaction, the gluconeogenic reaction seems unlikely.

In a study by Loewus & Loewus (1974) the synthase from *A. pseudoplatanus* has also been found not to be reversible within the limits of experimental error (<5% of the forward reaction).

Reversibility of the 6-pro-S H Bond-Breaking Step. As shown in Scheme II, step 3, the mechanism of the M1P synthase involves the loss of one of the 6-hydrogen atoms of D-glucose-6-P during the formation of the carbanion which undergoes the aldol cyclization. That this is the 6-*pro-S* hydrogen was shown by Byun et al. (1973). While the loss of tritium from the 6 position of G6P to the incubation medium has been observed (Chen & Charalampous, 1967) by using M1P synthase from yeast, the exchange of the medium with the substrate, that is, the experiment to determine the rever-

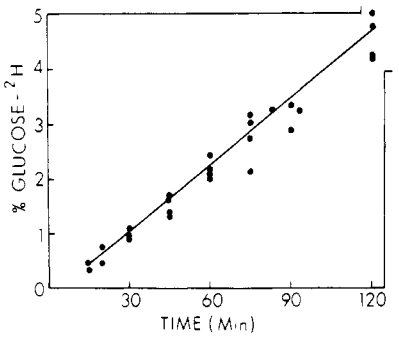


FIGURE 2: Evidence that M1P synthase is reversible from step 3 (Scheme II). The plot shows the uptake of deuterium by D-glucose-6-P (measured as glucose) which results from the incubation of G6P with the synthase in a 95% D_2O buffer. Each time point is the result of two experiments in duplicate.[4] Deuterium incorporated by nonenzymatic exchange was washed out prior to analysis by GC–MS. Uptake is into the 6 position of glucose.

sibility of steps 1–3 of Scheme II, has not previously been performed. We have examined this partial reaction of the M1P synthase by incubating glucose-6-P-d_0 with DEAE-purified enzyme in D_2O-containing buffers. Two experiments were performed.

D-Glucose-6-P was incubated with the synthase for 2 h in a buffer containing 95% D_2O. The reaction was terminated by boiling and *E. coli* alkaline phosphatase was added in order to obtain D-glucose which was then analyzed. The glucose from the three enzyme-treated samples contained an average of 22.6 ± 0.23 atom percent excess d_1 while the two controls contained 11.1% deuterium on the same basis. There was thus a net uptake of 11.5% deuterium. The incorporation of deuterium into the control samples results from enolization of glucose during which the C1 and C2 hydrogen atoms exchange with the medium (Sowden & Schaffer, 1952).

To determine that the enzyme-catalyzed uptake of deuterium was into C6 of the substrate, following alkaline phosphatase treatment, a portion of each sample was reduced with $NaBH_4$. The sorbitol thus formed was acetylated and analyzed for deuterium by GC–MS (Melo et al., 1968). Under electron ionization, sorbitol acetate gives a fragment ion, *m/e* 361, in which the molecule has lost one of the (identical) terminal $-CH_2OAc$ moieties, thus retaining half of the C6 deuterium present in the original glucose-6-P. While the precision of the measurement of *m/e* 362/361 (the d_1/d_0 ratio) was poor (enzyme, 12.2 ± 2.3%; control, 8.1 ± 2.6%), the difference,[3] 4.1%, approaches half the amount of deuterium measured in the unreduced glucose. This supports the C6 locus of the enzyme-catalyzed exchange.

In a second experiment M1P synthase, at the DEAE stage, was incubated in buffer containing 98% D_2O and 2 mM D-glucose-6-P.[4] Samples were removed at various time points and quickly frozen. The samples were lyophilized and then incubated with *E. coli* alkaline phosphatase in H_2O buffer for 3 h in order to form free glucose and to back-exchange the labile deuterium incorporated by non-enzyme-catalyzed exchange. Figure 2 shows that the reverse reaction is linear over 2 h and that it proceeds at a rate of ~2.2% deuterium enrichment/h. The forward rate of the reaction was ~20% substrate conversion/h or ~2 nmol of inositol-P formed/min in the reaction mixture. The rate of deuterium uptake by the substrate, i.e., the reverse reaction rate, was thus ~40 pmol/min, 2% of the forward rate.

These experiments give evidence for the reversibility of the 6-*pro-S* H bond-breaking step by demonstrating enzyme-catalyzed deuterium uptake into the 6 position of glucose. The question may be asked whether or not the exchangeable proton in the 6 position is removed subsequent to the transfer of the C5–H to NAD^+, in the formation of 5-keto-D-glucose-6-P. While there is no direct evidence on this point, based on an indirect experiment and on mechanistic grounds, it would be surprising if it were otherwise. The experiment supporting the prior formation of the ketoglucose is the facile base-catalyzed aldol cyclization of synthetic 5-keto-D-glucose 6-phosphate to a mixture of cyclose phosphates which, on reduction, give a mixture of inositol phosphates, one of which is L-M1P (Kiely & Sherman, 1975). The driving force of that reaction must, in part, result from the increase in acidity of hydrogen at the 6 position by the 5-carbonyl which probably also serves to stabilize the carbanion intermediate once it is formed. The prior formation of the carbonyl group in the enzymatic reaction could have the same function. Through the work of Barnett et al. (1973), it has been known that 5-keto-D-glucose-6-P could be reduced by NADH to D-glucose-6-P using NAD^+-depleted rat testis M1P synthase. Their experiment supported the intermediacy of the ketoglucose and also showed the potential reversibility of the oxidation step. Our work shows that the first three steps (Scheme II) are reversible in the steady-state reaction. Since we were unable to detect any overall reversibility, the irreversible (or slightly reversible) reaction must be either step 4 of Scheme II, the aldol cyclization, step 5, the NADH reduction of the ketoinositol-P (I_2), or step 6, the disassociation of NAD^+ and product from the enzyme.

Substrate and Product Inhibition. When we avoided the use of the sodium salt of D-glucose-6-P and used instead the free acid neutralized with Tris, we found no evidence of inhibition up to substrate concentrations of 5 mM.

In the experiment shown in Figure 2, the two sets of reactions,[4] one with bovine brain L-M1P phosphatase and the other with no phosphatase, were indistinguishable. The activity of this enzyme was such that, after the 2 h of incubation, the non-phosphatase-containing samples had reached an M1P concentration of 0.8 mM and the phosphatase-containing samples had become 0.8 mM in *myo*-inositol. The linearity of the time course and the identical nature of the two experimental sets suggest that the reaction proceeded without inhibition by M1P or by *myo*-inositol. This interpretation depends on the premise that the D-glucose-6-P exchange process cannot take place if the M1P synthase is inhibited, an argument consistent with our earlier observation that the intermediates in this reaction are tightly enzyme bound (Sherman et al., 1969).

In a separate experiment 0.1 and 0.2 mM concentrations of L-*myo*-inositol-1-P were incubated for 1 h with DEAE-purified synthase in a standard reaction mixture which contained 50 mM K^+ and 5% ethanol. No inhibition of the forward reaction occurred at either concentration of L-M1P by colorimetric analysis.

With respect to the biological significance of these levels of L-*myo*-inositol-1-P, Allison et al. (1976) reported that, in the cerebral cortex of rat, a tissue with high levels of *myo*-

[3] Because the fragment ion contains C1–C5 and C2–C6 in equal amounts, the relative amounts of deuterium in C1 and C2 due to non-enzyme-catalyzed exchange need not be known; the simple difference experiment – control gives the C6 enrichment.

[4] In early stages of this work we believed that the M1P synthase was product inhibited and that the enzyme activity would be enhanced by hydrolyzing the product as rapidly as it was synthesized. To achieve this, we added bovine brain L-M1P phosphatase and 1 mM Mg^{2+} to this experiment (Figure 3). The amount of enzyme used was sufficient to hydrolyze the product at a rate 7 times its rate of formation.

Table II: Effect of Potassium, Ammonium, and Sodium Ions on L-M1P Synthase Activity

synthase source	K^+ (mM)	NH_4^+ (mM)	Na^+ (mM)[b]	rel act.[c]
brain[a]	0	0	7	1.0 ± 0.05
	0	1	7	1.0 ± 0.05
	0	15	7	1.3 ± 0.02
	100	1	7	1.4 ± 0.05
	100	15	7	1.4 ± 0.01
	150	1	7	1.5 ± 0.13
testis[d]	0	0	7	1.0 ± 0.05
	0	50	7	2.0 ± 0.02
	0	100	7	1.9 ± 0.06
	10	0	7	1.8 ± 0.02
	20	0	7	1.9 ± 0.11
	50	0	7	2.0 ± 0.06
	100	0	7	2.1 ± 0.05
	200	0	7	1.9 ± 0.01
	50	10	7	2.1 ± 0.08
	50	50	7	2.1 ± 0.06
	8	0	13	0.82 ± 0.00
	8	0	28	0.67 ± 0.05
	8	0	53	0.53 ± 0.01
	8	0	103	0.29 ± 0.01
	8	0	153	0.22 ± 0.02

[a] A heated ammonium sulfate fraction was incubated with 3 mM D-glucose 6-phosphate in standard buffer without ethanol. K^+, NH_4^+, and Na^+ were added as the chloride salts. The products of the reactions were analyzed by gas chromatography. [b] The 7 mM Na^+ concentrations are due to the use of NaN_3 (3 mM) and Na_2G6P (2 mM) in the incubations. [c] All data are from triplicate experiments expressed as averages ± SD. [d] The dialyzed DEAE-enzyme incubations were assayed colorimetrically by using 2 mM G6P in standard buffer without ethanol. The 8 mM K^+ contribution in the last five experiments resulted from the use of K_2G6P and from the use of undialyzed enzyme from DEAE chromatography.

Table III: Effects of Ions and Ethanol on the Kinetics of Bovine Testis L-M1P Synthase[a]

additions	K_m(D-G6P) (mM)	(V_{max} with salt)/(V_{max} without salt)[b]
none	2.7	2.2
50 mM KCl	2.1	
none	4.5	0.6
50 mM NaCl	4.4	
5% ethanol	0.9	1.0
5% ethanol plus 50 mM KCl	0.24	
5% ethanol	1.6	0.91
5% ethanol plus 100 mM NaCl	2.8	

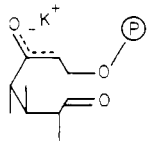
[a] K_m and V_{max} values were determined in paired experiments without ethanol and paired experiments with 5% ethanol. The enzyme used was purified through the DEAE stage. D-Glucose-6-P, dihydrogen form, was neutralized with Tris base and used as the substrate with additions as shown. [b] For L-M1P formation.

inositol, the *myo*-inositol-1-P concentration (of unknown chirality) is 0.34 mmol/kg dry weight or ~70 μmol/kg on a wet weight basis. Since we have found no inhibition of M1P synthase at concentrations of L-M1P from 100 to 800 μM, product inhibition is probably not a significant controlling factor for this enzyme.

Effects of Cations on M1P Synthase Activity. Table II shows the effects of K^+, NH_4^+, and Na^+ on bovine brain and testis enzyme preparations. Enzyme activity from both tissues is stimulated by NH_4^+ and K^+. Sodium ion is clearly inhibitory to testis synthase; thus, these effects do not simply result from changes in ionic strength. The effects of K^+ and NH_4^+ are not additive and, at least in testis, the degree of stimulation by each of the ions is comparable for a given concentration. Maximal stimulation occurs at concentrations of K^+ and NH_4^+ of 50 mM or less. In the case of K^+, stimulation decreases between 100 and 200 mM, but at 200 mM the reaction is still well above that of the K^+-free level of activity. Sodium inhibition is found at concentrations comparable to those of K^+ and NH_4^+ which are stimulatory. In the case of Na^+, inhibition continues to increase with the ion concentration throughout the range which we examined.

Effects of Cations on K_m and V_{max}. In the upper half of Table III are given the effects of added sodium and potassium chloride on the K_m of D-glucose-6-P and on the V_{max} of L-*myo*-inositol-1-P formation. In paired experiments with testis enzyme, the K_m values calculated from the incubations with and without added Na^+ or K^+ were the same. However, the V_{max} ratio of the Na^+-containing incubation to its paired salt-free experiment was significantly less than 1 while that ratio in experiments with and without potassium chloride was greater than 1. It thus appears that binding of G6P to the synthase is not affected by these ions. Rather, the effect must be at a later step in the reaction.

A possible locus for monovalent cation stimulation is following step 3 of Scheme II, in the stabilization of the carbanion formed prior to the aldol cyclization. Suelter (1970) suggested that monovalent cation activation of certain enzymes might be through interaction of the ion with a keto–enol tautomer intermediate in the enzyme reaction. Suelter included the M1P synthase among those enzymes which might have such a tautomer. If the function of the ketone carbonyl in 5-ketoglucose-6-P is to delocalize and stabilize the carbanion formed in step 3, then a counterion from the reaction medium might be an activating element:



Synthases from several sources have been found to be stimulated by monovalent cations: Chen & Charalampous (1965), *Candida utilis*, K^+ and NH_4^+; Ruis et al. (1967), *Phaseolus vulgaris* and *Sinapsis alba*, NH_4^+; Piña et al. (1969), *Neurospora crassa*, NH_4^+; Loewus & Loewus (1971), *A. pseudoplatanus*, NH_4^+; Naccarato et al. (1974), rat mammary, NH_4^+, K^+, and Na^+. Inhibition by sodium ions has not been previously reported.

Effects of Ethanol and Cations. After finding the effect of ethanol on the chromatographic behavior of the testis enzyme, we examined its effect on activity. Addition of 5% ethanol to the incubation results in a twofold increase in activity. Further addition of KCl (50 mM) gives an overall stimulation of 2.5-fold. As with KCl alone, increasing the ion concentration in ethanol increases the activity to a maximum which occurs at ~50 mM, with further increase resulting in lesser stimulation. The effect of Na^+ is still inhibitory in 5% ethanol; however, the degree of inhibition is half that found in ethanol-free media.

The lower portion of Table III shows that ethanol appears to lower the K_m of G6P; however, it should be noted that these are not paired experiments. When 50 mM K^+ is added, there is a further reduction in K_m while Na^+ does not have this effect. The V_{max} is not affected by either K^+ or Na^+ in the presence of ethanol. Thus, ethanol eliminates the effect of Na^+ and K^+ on V_{max} and, particularly with K^+, causes a large decrease

in the K_m of G6P, i.e., an increase in its binding to the enzyme.

We do not know the cause of the ethanol effect on the synthase nor do we have any evidence that explains the action of potassium and sodium. The considerable change that these agents bring about on the kinetics of the enzyme suggests that the use of K_m and V_{max} data obtained in vitro could lead to errors in the interpretation of the properties of the synthase in the cell. The large effect of potassium on the bovine testis synthase would seem to favor a cellular location for the synthase. The effect of ethanol on the enzyme may suggest that there are cellular effectors which play an analogous role in modifying the kinetics of this enzyme.

Acknowledgments

We thank Walter Nulty of the Department of Biological Chemistry for carrying out the sedimentation studies and W. H. Holland for his help in the mass spectrometry experiments.

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