D-MYO-INOSITOL-1-PHOSPHATE, AN INTERMEDIATE IN THE BIOSYNTHESIS OF INOSITOL IN THE MAMMAL[1]

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Previous communications have reported an enzyme system in homogenate of rat testis (Eisenberg and Bolden, 1963) which catalyzes the cyclization of the glucose chain to myo-inositol (Eisenberg, Bolden and Loewus, 1964). A study of this reaction in yeast (Chen and Charalampous, 1965) has shown that glucose-6-P and myo-inositol-1-P are intermediates in the cyclization. Since myo-inositol-1-P can exist in enantiomeric forms it was of interest in proposing a mechanism of cyclization to determine which optical isomer is involved. Although the L configuration has been observed repeatedly among the inositol phosphates derived from phosphoinositides from many natural sources (Rapport and Norton, 1962), the D isomer has been known only synthetically (Ballou and Pizer, 1959). Evidence that the intermediate in the formation of inositol in the rat is D-myo-inositol-1-P is presented in this paper.

Preparation of the Enzyme - The 100,000 g supernatant from a 33% homogenate of rat testis in 0.154 M KCl was heated for 2 min at 60°. This extract converted glucose-6-P-^{14}C, but not glucose-^{14}C, to inositol-^{14}C. Heating was necessary to destroy a phosphatase, which rapidly hydrolyzed glucose-6-P, precluding the formation of inositol. Further heating at 60° destroyed the synthetic activity.

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[1] Part of this work was presented at the Second Meeting of the Federation of European Biochemical Societies, Vienna, April 21-24, 1965.

TABLE I

Cofactor Requirements for Inositol Synthesis

Incubation mixture: dialyzed supernatant of rat testis; 9 mg protein; DPN, 0.001 M; $MgCl_2$, 0.003 M; Tris buffer, pH 7.4, 0.01 M; glucose-6-P-^{14}C, 0.003 M; total volume, 2.4 ml; 26°. Inositol-^{14}C was assayed by addition of 100 mg carrier, deionization, evaporation, and methanol precipitation of crystalline inositol, counted in suspension (Snyder and Stephens, 1962).

System	μmole Inositol/hr
Complete	0.70
$-Mg^{++}$	0.07
-DPN	0.20
$-Mg^{++}$ -DPN	0.002

Purification of the Enzyme - Dialysis of the heated supernatant against isotonic KCl inactivated the system, which could be restored to full activity by addition of DPN and Mg^{++} (Table I).

Fractionation with ammonium sulfate precipitated the activity at 30-40% saturation, with purification of 220-fold.

Formation of Inositol-1-P - Omission of Mg^{++} from the system inhibited the synthesis of inositol (Table I) but led to the accumulation of a compound which migrated chromatographically with L-myo-inositol-1-P and not with inositol-2-P (Figure 1a). Further evidence of a phosphate intermediate was obtained on addition of Mg^{++} and alkaline phosphatase to the heat inactivated system whereupon the intermediate was converted quantitatively to inositol.

Large Scale Preparation of Inositol-1-P - To determine the configuration of the phosphate a large scale preparation was undertaken to provide sufficient material for polarimetric measurement. Combined testes weighing 76 gm from 24 Sprague-Dawley rats were homogenized with 2 volumes of isotonic KCl. The high speed supernatant was fractionated between 30 and 40% saturation with ammonium sulfate and the pellet was dissolved in KCl and dialyzed against 6 liters of KCl. The solution of enzyme containing 96 mg of protein in a final volume of 150 ml was incubated at 38° for 2-1/2 hr with DPN, 0.001 M; Tris

buffer, 0.05 M, pH 7.4, and glucose-6-P-^{14}C, 0.001 M, 164 μmoles, 19x10^6 cpm. A small sample was assayed for inositol-1-P-^{14}C (as inositol-^{14}C after phosphatase) and showed 37% incorporation of ^{14}C or the formation of 60 μmoles of product. The reaction was terminated by addition of 600 ml of warm ethanol, the suspension chilled and centrifuged, and the supernatant evaporated to a small volume. Cations were exchanged for Li^+ by passage through a lithium charged column of IR-120 cation exchange resin. The eluate was evaporated to dryness and extracted with methanol which removed LiCl. The residue of radioactive organic phosphates was treated in water with Norit, applied in bands to solvent-washed Whatman 3 MM papers and chromatographed descending for 90 hr in 95% EtOH/1 M NH_4OAc, 7:3. The slower moving of the bands revealed by radioautography was cut from each paper, eluted with water, and rechromatographed. Radioautography showed a single band which was eluted first with alcohol to remove NH_4OAc and then with water. A crystalline product was obtained from the aqueous eluates after evaporation to a small volume, addition of cyclohexylamine, followed by the slow addition of several volumes of acetone. After 3 recrystallizations 20 mg (43.6 micromoles) of dicyclohexylammonium salt of inositol-1-P-^{14}C were obtained (26.5% yield).

Purity and Identity of Product - Chromatographically the compound migrated with authentic D^2- and L-myo-inositol-1-P as visualized by $AgNO_3$/NaOH dip (Trevelyan, Procter and Harrison, 1950) and phosphate spray (Hanes and Isherwood, 1949). Scanning showed a radioactive peak coincident with D- and L-myo-inositol-1-P, equal in area to an equivalent amount of added substrate glucose-6-P-^{14}C (Figure 1b).

Incubation of the compound with Mg^{++} and alkaline phosphatase yielded 1 mole of phosphate per mole of inositol phosphate measured by the method of Fiske and Subbarow and a single radioactive product identified chromatographically as inositol (n-PrOH/EtOAc/H_2O, 7:1:2). Incubation with purified testicular extract in the presence of Mg^{++} resulted in 40% conversion to

[2] Generously supplied by Dr. Clinton E. Ballou, Univ. of California, Berkeley.

inositol-^{14}C, measured with the assay, and further identified chromatographically (Figure 1c). In the absence of Mg^{++} there was no hydrolysis.

Final identification of the compound as inositol-1-phosphate was made

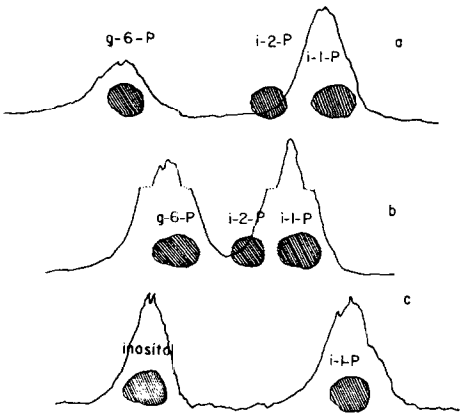

Figure 1. Tracings of ^{14}C scanning records and chromatographic spots.
a. Testicular enzyme incubated with DPN, Tris, and g-6-P-^{14}C. Incubation mixture cochromatographed with g-6-P-Na_2, myo-in-2-P, and dicyclohexylammonium L-myo-in-1-P. 95% EtOH/1M NH_4OAc, 7:3.
b. Crystalline diCHA D-myo-in-1-P-^{14}C produced by testicular enzyme cochromatographed with equimolar amounts of g-6-P-Na_2-^{14}C (substrate), in-2-P, and authentic D- and L-myo-in-1-P diCHA salts.
c. Crystalline diCHA D-myo-in-1-P-^{14}C incubated with testicular enzyme, Tris, and Mg^{++}. Incubation mixture chromatographed in nPrOH/EtOAc/H_2O, 7:1:2.

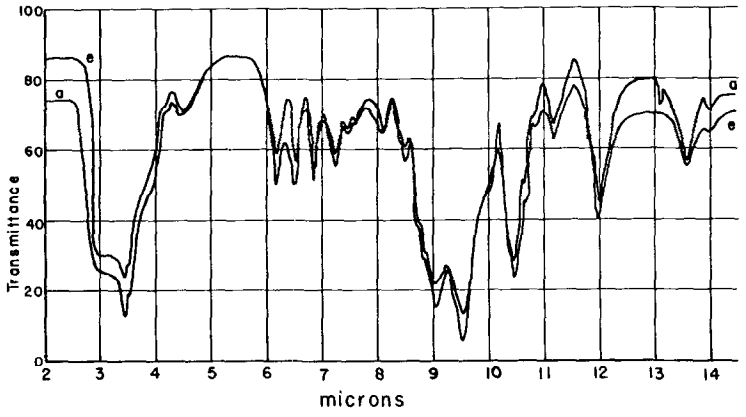

Figure 2. Infrared spectra (KBr) (a) authentic diCHA L-myo-in-1-P; (e) enzymatic product.

by comparison of its infrared spectrum[3] with that of authentic inositol-1-phosphate. The spectra are essentially identical (Figure 2).

Configuration was established by optical rotatory dispersion[4]. Both the D-isomer and the enzymatic product showed a positive Cotton effect in the region of 190 mμ, and the L-isomer a negative effect at the same wavelength (Figure 3). The enzymatic product is clearly D-myo-inositol-1-P-^{14}C. The shift in peak absorption seen in the synthetic D-isomer is probably due to a samll impurity observed previously.

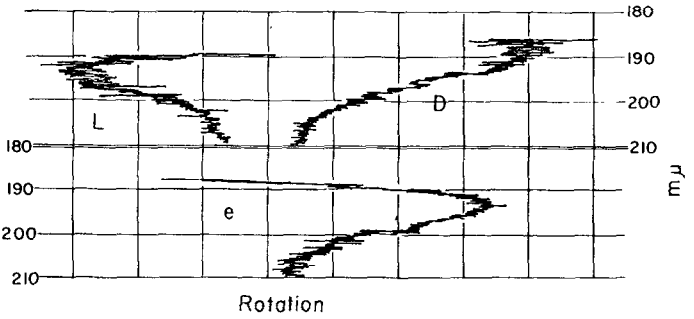

Figure 3. Optical rotatory dispersion, Cary Model 60 Spectropolarimeter. Range 0.04°; Conc., 0.1% in H_2O. (D) authentic D-isomer; (L) authentic L-isomer; (e) enzymatic product.

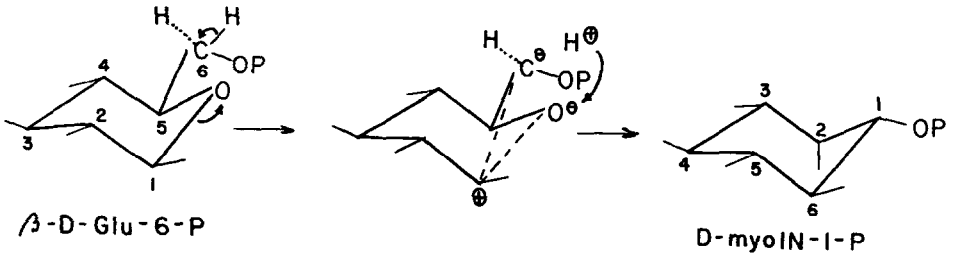

Figure 4. Stereospecific cyclization of D-glucose-6-P to D-myo-inositol-1-P.

[3]Kindly determined by Mrs. Katherine Warren, National Heart Institute.

[4]Kindly determined by Mr. Murray Brandes, Applied Physics Corp.

Discussion - This is the first demonstration of the natural occurrence of the D configuration among the inositol phosphates. From mechanistic considerations D-myo-inositol-1-P is the expected product of cyclization of D-glucose-6-P since the reaction must proceed through stereospecific activation of a C_6-bound H and cleavage of the $O\text{-}C_1$ bond. DPN is probably involved in this activation. The $C_6\text{-}C_1$ bond then closes to produce the myo-inositol configuration (Figure 4). Studies of this mechanism are in progress.

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