d-Threonine 2,4-Diphosphate Inhibition of \( \text{d-Glyceraldehyde 3-Phosphate Dehydrogenase} \)^*  

**Arvan L. Fuharty**† and **Clinton E. Balloou**  

*From the Department of Biochemistry, University of California, Berkeley, California*  

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In the course of testing structural analogues of d-glyceraldehyde 3-phosphate as inhibitors for n-glyceraldehyde 3-phosphate dehydrogenase, Racker (1) noted a low level inhibition by glycocolaldehyde phosphate. This phosphate ester is available readily from the periodate cleavage of glycerol 1-phosphate (2, 3). Recently an alternate synthesis of glycolaldehyde phosphate has been reported which involves the diethyl acetate as a stable immediate precursor (4). The compound prepared by the latter method showed almost no inhibitory activity for n-glyceraldehyde phosphate dehydrogenase. Racker (1) noted, however, that when this preparation was stored frozen in solution for several months, it became almost as inhibitory as that produced by periodate treatment of glycerol 1-phosphate. It thus seemed probable that the enzyme inhibition was not due to glycocolaldehyde phosphate, but rather to some contaminant arising from glycocolaldehyde phosphate.

This communication reports studies on the isolation, identification, and synthesis of this inhibitor, as well as some preliminary observations on its action in the enzymatic system. By incubation of glycocolaldehyde phosphate in strong alkali, a strongly inhibitory material was produced. On ion exchange fractionation, a chromatographically homogeneous, highly inhibitory, phosphorylated component was separated. This material has been shown to be a mixture of tetrose diphosphates. Both d- and l-threose 2,4-diphosphate were prepared synthetically and tested for inhibition of d-glyceraldehyde 3-phosphate dehydrogenase. The n-threose 2,4-diphosphate proved to be an unusually strong inhibitor of the enzyme, whereas the l-isomer was much less active. The inhibition was reversible and noncompetitive, and was accompanied by the reduction of a single mole of DPN per mole of enzyme. The results of an independent identification of this inhibitory substance are reported in an accompanying paper (1).

**Experimental and Results**

**Assay Procedure**—Rabbit muscle d-glyceraldehyde 3-phosphate dehydrogenase was prepared by the method of Cori et al. (5). It was active only after incubation in the presence of sulfhydryl compounds. The assay procedure is a modification of that introduced by Warburg and Christian (6). A normal reaction system had the following composition: \( 3 \times 10^{-2} \text{ M sodium pyrophosphate (pH 8.5)}, 6 \times 10^{-3} \text{ M cysteine}, 5 \times 10^{-4} \text{ M sodium arsenate}, 3.3 \times 10^{-4} \text{ M DPN}, 6.6 \times 10^{-4} \text{ M d-glyceraldehyde 3-phosphate, and 6 \times 10^{-4} mg per ml of enzyme protein.} \)

The DPN was purchased from Pabst Laboratories, and d-glyceraldehyde 3-phosphate was prepared from its dimethyl acetal by the procedure previously reported (7). Incubation mixtures, complete except for d-glyceraldehyde 3-phosphate, were allowed at least 10 minutes to come to room temperature. Then the material to be tested for inhibition was added. After exactly 2 minutes, n-glyceraldehyde 3-phosphate was added to start the reaction. The reaction was followed by the increase in 340 \text{ nm} absorbance as a function of time, with the use of a Beckman DU spectrophotometer equipped with a photomultiplier connected to a Brown Recorder with 90 to 100% transmission recorded on full scale. Reaction rates were determined as the linear distance on the recorder chart for an absorbance change of 0.010 unit over the earliest measurable linear portion of the reaction. Inhibitory materials were diluted so that an inhibition of 20 to 80% was obtained.

The inhibition assay was greatly affected by the order of addition of reaction components. If d-glyceraldehyde 3-phosphate was included in the incubation mixture, and the reaction was started with DPN, the inhibition was almost undetectable during the initial portion of the reaction; however, it increased as the reaction proceeded. A similar behavior was observed if the reaction was started by adding inhibitory material and d-glyceraldehyde 3-phosphate, simultaneously. Only when the inhibitory material was incubated with the reaction mixture, was maximal inhibition observed during the initial portion of the reaction. Maximal inhibition was reached after 2 minutes of incubation, and a slow loss of inhibition with time was noted after the initial rapid increase (Fig. 1). The loss of activity proved to be due to the instability of the inhibitor in the presence of cysteine, similar to that reported for n-glyceraldehyde 3-phosphate (5).

**Preparation of Inhibitor by Alkali Treatment of Glycolaldehyde Phosphate**—Glycolaldehyde phosphate was prepared from the cyclohexylammonium salt of its diethyl acetal (4). When freshly prepared, this material inhibited d-glyceraldehyde 3-phosphate dehydrogenase only at very high concentrations. Overnight treatment in the presence of strong acid, thiosulfate, or cysteine caused no increase in inhibition. Heating it 100\degree for 1 hour was also without effect. However, incubation in the presence of strong alkali produced a solution which strongly inhibited the enzyme system. When followed as a function of time, the inhibition was seen to increase rapidly and then to decrease slowly over a long period of time. During alkali treatment, little inorganic phosphate was released as measured by the
paper and the chromatogram was developed in the above solvent. Moving component seemed to parallel the change in inhibitory activity, (Fig. 2), reaching a maximum after 1 to 2 hours and then decreasing after long periods of treatment. A sample of the enzymatic system.

Preparation of Reduced Inhibitor—The inhibitory material recovered by ion exchange contained large amounts of sodium formate, and attempts to form a solid, formate-free salt resulted in decomposition. However, in the presence of sodium borohydrate the inhibitor was reduced to a stable product. This reduced inhibitor was no longer inhibitory to the n-glyceraldehyde 3-phosphate dehydrogenase reaction. To a sample of inhibitory material recovered from the ion exchange column in the same position as the inhibitory material resulting from alkali treatment (Table I).

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dryness in a vacuum. Methanol was added and then removed under reduced pressure to distill off borate as methyl borate. This was repeated two additional times and followed once with 1% (weight per volume) methanolic hydrogen chloride in the same manner. Water was added and removed in a vacuum twice to remove hydrogen chloride. The resulting sirup was dissolved in a little water and cyclcohexylamine was added to bring the pH to about 9. The solution was taken to dryness and the residue extracted with hot absolute ethanol. An amorphous salt was precipitated by the cautious addition of acetone to the ethanol solution, and was recovered by filtration. On further addition of acetone to the supernatant, crystalline cyclcohexylammonium formate was obtained. When recovered and air dried this material showed a single phosphate component in a number of paper chromatographic systems. It was not possible to obtain a reproducible elemental analysis of this material.

Identification of Reduced Inhibitor—The cyclcohexylammonium salt (20 mg) of the reduced inhibitor was dissolved in 2 ml of a pH 9.1, 0.05 M glycine buffer containing 0.001 M magnesium chloride. Commercial intestinal phosphatase (10 mg) was added, and the solution was incubated for 16 hours at 40°. The mixture was then deionized and deproteinized by passage through Amberlite IR4B (OH-) and Dowex 50 (H+). The resulting solution was evaporated under reduced pressure to a thick sirup and analyzed chromatographically.

In acidic solvent systems the reduced inhibitor moved on paper in the same manner as synthetic samples of D-erythritol and D-threitol 4-phosphate. However, when run in iso-propanol-ammonia-water (70:10:20) (16) for 48 hours at 30° the reduced inhibitor was found to run in a region where only diphosphates are normally encountered. Further, conditions which resulted in a rapid periodate oxidation of D-threitol 4-phosphate, produced no detectable oxidation of the reduced inhibitor as determined at 280 mμ by the spectrophotometric method of Marinetti and Rouser (17).

Synthesis of D-Threose 2,4-Diphosphate (See Fig. 4.) 1,3-Benzyldiene-2,4,5-tribenzyol-D-arabitol was prepared by the method of Haskins et al. (18). Six grams of the above compound were dissolved in 500 ml of absolute ethanol and were reduced by hydrogen at atmospheric pressure with 2.0 g of reduced and washed 11% palladium chloride on carbon as catalyst. The reduction required 3 days to come to completion, and it yielded 5.0 g (94% of theory) of a thick colorless sirup after removal of catalyst and evaporation of the solvent. This compound has an \[\alpha\]D = -101° (c 1.6 in chloroform).

\[\text{C}_{17}\text{H}_{22}\text{O}_8\]

Calculated: C 67.28, H 5.17
Found: C 67.48, H 5.48

Of this sirup 2,4,5-tribenzyol-D-arabitol 4 g were dissolved in 20 ml of dry pyridine, and 10 g of diphenylyphosphorochloridate was added. This was followed by a 10% solution of sodium hypochlorite. The reaction mixture was allowed to stand for 15 hours at room temperature and then poured into 50 ml of 1 N sodium hydroxide solution. The precipitate was filtered, washed with water, dried at 50°, and recrystallized from chloroform. A yield of 0.2 g was obtained.

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were added over a 20-minute period while the reaction vessel was cooled in an ice bath. The vessel was stoppered and left at room temperature overnight. Excess phosphorylating reagent was then destroyed by adding water, and the reaction product was extracted into benzene. The benzene layer was washed with water, 1 N hydrochloric acid, 1 M sodium bicarbonate, and then again with water. The benzene solution was dried with sodium sulfate and the solvent was removed under reduced pressure yielding 8.0 g (100% of theory) of a clear sirup. This sirup was hydrogenated at atmospheric pressure in 500 ml of absolute ethanol with 2 g of platinum oxide catalyst. The reduction was complete, with theoretical hydrogen uptake, after 3 days. The catalyst was removed, 30 ml of 3 N potassium hydroxide were added, and the solution was concentrated to dryness under reduced pressure. Water (200 ml) was added, and the resulting solution was left at room temperature for 4 hours for saponification of the esters. The solution was then extracted with ether, and the aqueous layer was passed through IR-120 (H+) to remove the cations. Cyclohexylamine was added to the acid eluate until it was about pH 10, and the mixture was concentrated to dryness under reduced pressure. The residue was extracted with 50 ml of hot absolute ethanol and the solid was collected by filtration. The ethanol-insoluble material was dissolved in hot methanol, and ether was added to turbidity. This was stored at 4° overnight, with small additions of ether until no further precipitate was obtained. The precipitate was filtered off and air dried, yielding 3.8 g (68% of theory) of crude tricyclohexylammonium d-arabitol 1,3-diphosphate monohydrate. The salt was recrystallized by dissolving it in hot methanol, adding ether to turbidity, and allowing the solution to stand overnight at 4°, with cautious further addition of ether after crystallization had started. The crystals, after collection and drying overnight at room temperature under vacuum, melted at 190-192° (decomposes) and had an [α]$_{25}$ = +1.3° (c 2.0 in water).

$$C_{27}H_{39}O_{11}N_5P_2\cdot H_2O$$

Calculated: C 44.07, H 8.83, N 6.69, P 9.87

Found: C 43.51, H 8.72, N 6.54, P 9.70

A sample of 100 mg of this salt was treated with Dowex 50 (H+) to remove the cyclohexylamine, the mixture was filtered, and the filtrate was neutralized with sodium hydroxide. A 2-fold excess of sodium periodate was added and allowed to react at room temperature for 30 minutes. The excess periodate was then destroyed by the addition of 2 drops of ethylene glycol. After an additional 30 minutes, an equal volume of methanol was added and the solution was stored overnight at -10°. Sodium iodate, which had crystallized out of solution, was filtered off and the supernatant solution was purified by the ion exchange procedure used for fractionating the alkali-treated glycolaldehyde phosphate.

Essentially all of the phosphate containing material was eluted in the same region as the inhibitor from glycolaldehyde phosphate. The ratio of phosphate groups per tetrose was 2.0. When assayed in the enzymatic system, this synthetic d-threose 2,4-diphosphate proved to be an exceptionally strong inhibitor. Due to its instability no attempts were made to free this compound from sodium formate or to prepare a solid salt. The presence of formate had no effect on the enzyme assay and the d-threose 2,4-diphosphate was stored frozen in solution as eluted from column. Under this condition of storage the inhibitory activity was stable for several weeks.

This material was oxidized with sodium periodate and the reaction mixture purified by ion exchange as reported for its enantiomorph. The resulting d-threose 2,4-diphosphate was found not to inhibit the n-glyceraldehyde 3-phosphate reaction except at much higher concentrations than was required for the d-isomer. A concentration of 2 × 10$^{-5}$ M of the l-isomer was required for 50% inhibition as compared to 2 × 10$^{-7}$ M for the d-isomer.

**Nature of d-Threose 2,4-Diphosphate Inhibition**—The reversibility of the d-threose 2,4-diphosphate inhibition was tested by the criterion of Ackerman and Potter (19). The relative inhibition caused by this diphosphate was independent of enzyme concentration over a threefold change in enzyme concentration (Fig. 5). The effects of changes of n-glyceraldehyde 3-phosphate and DPN concentrations were determined, and the data examined by the classical double reciprocal plots (Fig. 6). Concentrations of DPN and n-glyceraldehyde 3-phosphate were determined enzymatically. d-Threose 2,4-diphosphate concentration was determined by assaying for organic phosphate in the undiluted solution prepared as previously described. The inhibition is completely noncompetitive with respect to both the natural substrate and the cofactor. The Michaelis constants ($K_m$) determined from these plots, 8.7 × 10$^{-4}$ M for n-glyceraldehyde 3-phosphate and 5.4 × 10$^{-8}$ M for DPN, are similar to the values previously recorded for this enzyme (5). The inhibitor constant ($K_i$) for d-threose 2,4-diphosphate from the above data had an average value of 2.1 × 10$^{-7}$ M, and values varying between 1 and 3 × 10$^{-7}$ M were obtained from other data. The presence of 3 × 10$^{-5}$ M zinc chloride in the reaction mixture had no effect on the inhibition. Both muscle and yeast n-glyceraldehyde 3-phosphate dehydrogenases were inhibited, but neither heart lactic dehydrogenase nor yeast alcohol dehydrogenase was affected.

**d-Threose 2,4-Diphosphate Reduction of Enzyme Bound DPN**—Rabbit muscle n-glyceraldehyde 3-phosphate dehydrogenase has been shown to bind tightly 3 moles of DPN per mole of enzyme, about 70% of which can be reduced by n-glyceraldehyde 3-phosphate in the presence of excess DPN and the absence of phosphate and arsenate (20). When d-threose 2,4-diphosphate was added to the enzyme the reduction of 1 mole of DPN per mole of enzyme was observed in the presence or absence of added DPN. The presence of arsenate or phosphate, did not result in any further DPNH formation. When n-glyceraldehyde 3-phosphate was added to enzyme which had already been allowed to react with d-threose 2,4-diphosphate, an additional one-third mole of DPN per mole of enzyme was reduced (Table I). Enzyme concentration was determined with the use of a molecular extinction coefficient of 14.7 × 10$^{4}$ at 276 m$^u$, which was obtained by correction of the value given by Velick (21) to the molecular weight.
Glycolaldehyde phosphate does not interact with glyceraldehyde 3-phosphate dehydrogenase either as a substrate or as an inhibitor. The inhibitory activity of certain preparations of this compound for the enzyme is due to tetrose diphosphate contamination. The presence of a second component in glycolaldehyde phosphate prepared from glycerol 1-phosphate by periodate cleavage had been indicated by the chromatographic observations of Loring et al. (3). Although this second component has not been observed in preparations of glycolaldehyde phosphate prepared in the course of the work reported here, a comparison of relative \( R_P \) values indicates that it was probably due to the presence of tetrose diphosphate contamination. The accidental use of a small excess of barium hydroxide in the preparation of the barium glycolaldehyde phosphate by the procedure of Loring would lead to alkaline conditions, and thus could account for the variable presence of tetrose diphosphates.

Tetrose diphosphates could appear in preparations of glycolaldehyde phosphate as the result of an aldol condensation of glycolaldehyde phosphate molecules. Such an explanation is supported by the finding that basic conditions, which are known to favor such condensations, greatly accelerate the appearance of these diphosphates. The direct aldol condensation product of two glycolaldehyde phosphate units would be a tetrose 2,4-diphosphate. The diphosphate obtained from alkaline treatment, as well as its reduction product, have been found to be unreactive with sodium periodate as would be expected for the direct condensation product. The tetrose diphosphate with the phosphates on the 2,3-positions would also fail to react with sodium periodate, but it could arise only by migration of phosphate from the 4- to the 3-position after condensation. Such a migration from primary to secondary hydroxyl would not be likely. The tetrose diphosphate obtained from alkaline treatment of glycolaldehyde phosphate is thus believed to be a mixture of the four isomeric tetrose 2,4-diphosphates.

The synthetic approach used for the preparation of the two threose 2,4-diphosphates takes advantage of the lack of adjacent free hydroxyl groups in these compounds. After preparation of a polyol diphosphate with appropriate distribution of phosphate groups, the aldotetrose derivative is easily obtained by the action of sodium periodate. The preparation of a pure solid salt of the synthetic threose 2,4-diphosphates is complicated by the instability of these compounds. It has proved best to carry the synthesis only as far as the arabinol diphosphate in large scale. This compound forms a stable salt which is easily stored without decomposition. The threose diphosphate can then be prepared in small quantities as needed.

Due to the structural similarities between the highly inhibitory \( n \)-threose 2,4-diphosphate and the natural substrate of \( n \)-glyceraldehyde 3-phosphate dehydrogenase, the noncompetitive nature of the inhibition was unexpected. The magnitude of the inhibitor constant and the observed stereospecificity indicate that the inhibition is due to some specific binding. The involvement of a particular catalytic locus on the enzyme is demonstrated by the reduction of 1 mole of DPN per mole of enzyme by \( n \)-threose 2,4-diphosphate.\(^2\) The possibility that this postulated site is different from that involved in oxidation of the natural substrate arises from the noncompetitive nature of the inhibition and from

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\(^2\) In private communication Dr. Racker has indicated that he has been able to show reduction of more than one mole of DPN per mole of enzyme with the use of the tetrose diphosphate mixture.
the failure of the inhibitor to bring about the reduction of 2 moles of DPN per mole of enzyme as does n-glyceraldehyde 3-phosphate. A site of secondary activity has previously been implicated for n-glyceraldehyde 3-phosphate dehydrogenase (24), and it will be of interest to determine the nature of the effect of D-threose 2,4-diphosphate upon the reaction involving this site. Complete understanding of the stereochemical requirements for the inhibition awaits the preparation of the two erythrose 2,4-diphosphates.

SUMMARY

1. The inhibition of glyceraldehyde 3-phosphate dehydrogenase by certain preparations of glycolaldehyde phosphate has been shown to be due to a contaminant separable by paper chromatography and ion exchange techniques.

2. This inhibitory material has been isolated as the cyclohexylammonium salt of its reduction product, and this reduction product was identified as a mixture of tetritol diphosphates.

3. Both D- and L-threose 2,4-diphosphates have been synthesized and tested for inhibition of glyceraldehyde 3-phosphate dehydrogenase. The n-isomer was found to be a very strong inhibitor of the enzyme reaction, whereas the L-isomer was much less active.

4. D-Threose 2,4-diphosphate inhibition of glyceraldehyde 3-phosphate dehydrogenase has been shown to be reversible and noncompetitive with both n-glyceraldehyde 3-phosphate and DPN. The \( K_i \) for this inhibition is approximately \( 2 \times 10^{-7} \) M. Under the particular conditions used, this inhibitor reacts with the enzyme resulting in the reduction of 1 mole of DPN per mole of enzyme.

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d-Threose 2,4-Diphosphate Inhibition of d-Glyceraldehyde 3-Phosphate Dehydrogenase
Arvan L. Fluharty and Clinton E. Ballou


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