

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY]

The Synthesis of D-Glyceraldehyde-3-phosphate

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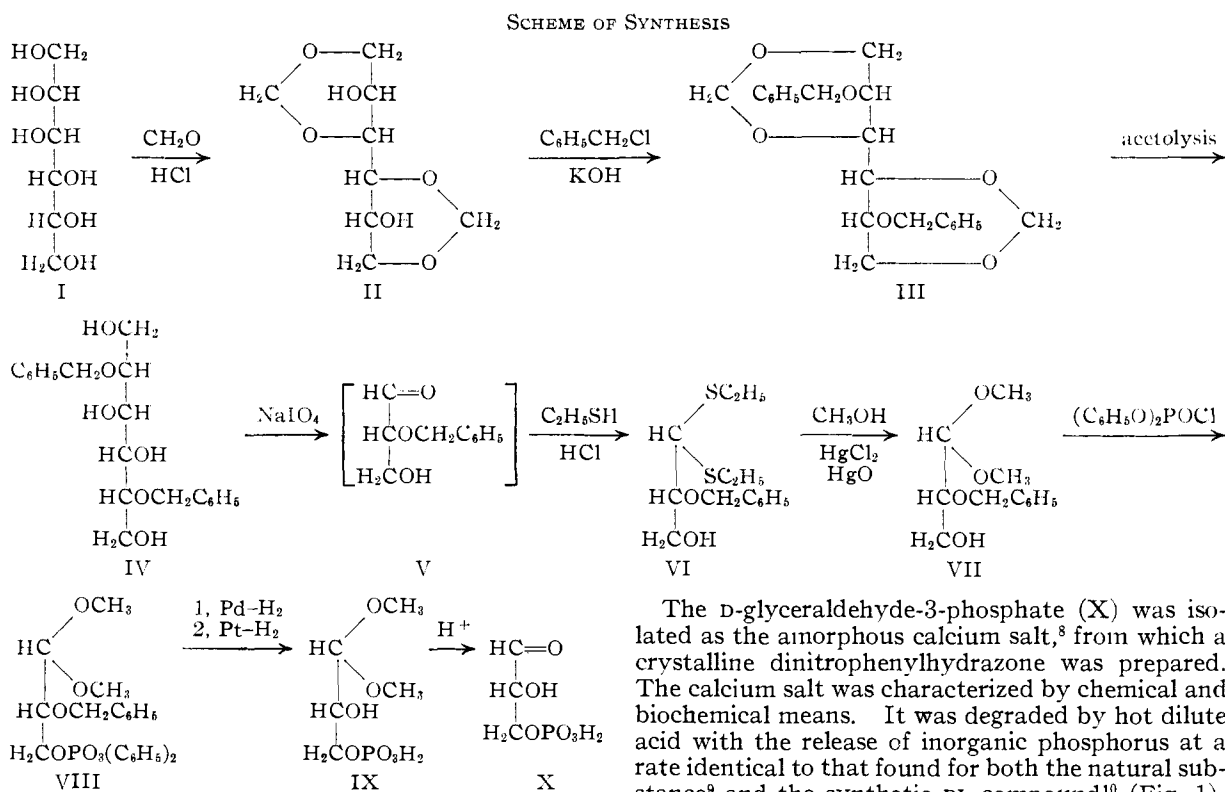
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This paper describes a chemical synthesis of the natural D-glyceraldehyde-3-phosphate, and records, for the first time, the optical rotation of the pure compound. The route of synthesis leads to the dimethyl acetal of D-glyceraldehyde-3-phosphate. The latter, as its free acid, undergoes in aqueous solution a self-catalyzed hydrolysis to give the free aldehyde in good yield.

D-Glyceraldehyde-3-phosphate has long been of interest to biochemists because of the central role it plays in fermentation and the glycolysis of carbohydrates.¹ The synthesis of racemic glyceraldehyde-3-phosphate² in 1932 made possible the first experiments of Smythe and Gerischer³ which indicated the biological importance of this compound. In the years since, biochemical studies of systems in which D-glyceraldehyde-3-phosphate is involved have been greatly facilitated by the availability

phosphate, a strong inhibitor of hexokinase.⁶ For this reason, D-glyceraldehyde-3-phosphate prepared enzymatically⁷ from D-fructose-1,6-diphosphate by the action of aldose and isomerase is occasionally used in preference to the synthetic DL-compound.

In this paper we are reporting a chemical synthesis of D-glyceraldehyde-3-phosphate that yields a product of optical purity and complete biological activity. This scheme of synthesis is outlined below



of the synthetic DL substrate.⁴ Because of the steric specificity of biological systems, however, the use of racemic substrates is undesirable, and may actually give misleading results. For example, the inhibition of fermentation of rat sarcoma by DL-glyceraldehyde⁵ has been shown to result from the aldolase-catalyzed reaction of L-glyceraldehyde with dihydroxyacetone phosphate to give L-sorbose-1-

The D-glyceraldehyde-3-phosphate (X) was isolated as the amorphous calcium salt,⁸ from which a crystalline dinitrophenylhydrazone was prepared. The calcium salt was characterized by chemical and biochemical means. It was degraded by hot dilute acid with the release of inorganic phosphorus at a rate identical to that found for both the natural substance⁹ and the synthetic DL compound¹⁰ (Fig. 1). The aldehyde content estimated by hypiodite oxidation corresponded to the theoretical value. Chemical oxidation of the D-glyceraldehyde-3-phosphate by hypiodite gave, in good yield, 3-phosphoryl-D-glyceric acid, identified by its characteristic optical

(1) B. L. Horecker, "Phosphorus Metabolism," Vol. I, The Johns Hopkins Press, Baltimore, Md., 1951, p. 117.

(2) H. O. L. Fischer and E. Baer, *Ber.*, **65**, 337, 1040 (1932); E. Baer, *Biochem. Preps.*, **1**, 50 (1949).

(3) C. V. Smythe and W. Gerischer, *Biochem. Z.*, **260**, 414 (1933).

(4) O. Warburg and W. Christian, *ibid.*, **303**, 40 (1939); S. F. Velick and J. E. Hayes, Jr., *J. Biol. Chem.*, **203**, 545 (1953).

(5) B. Mendel, *Klin. Wochschr.*, **8**, 169 (1929).

(6) H. A. Lardy, V. D. Wiebelhaus and K. M. Mann, *J. Biol. Chem.*, **187**, 325 (1950).

(7) O. Meyerhof and R. Junowicz-Kochalaty, *Biochem. Z.*, **149**, 71 (1943).

(8) The ease with which the racemic compound crystallizes as the calcium salt may be due to dimerization. This explanation seems to apply to glyceraldehyde itself, in which case DL-glyceraldehyde crystallizes readily as the dimer, whereas D or L-glyceraldehyde do not crystallize.

(9) W. Kiessling, *Ber.*, **67**, 869 (1934).

(10) E. Baer and H. O. L. Fischer, *J. Biol. Chem.*, **150**, 223 (1943).

rotation in the presence of molybdate ion.¹¹ X was further characterized by its enzymatic oxidation with pure glyceraldehyde phosphate dehydrogenase in the presence of excess oxidized diphosphopyridine nucleotide and arsenate,¹² the reduction of the nucleotide corresponding to the D-glyceraldehyde-3-phosphate present.

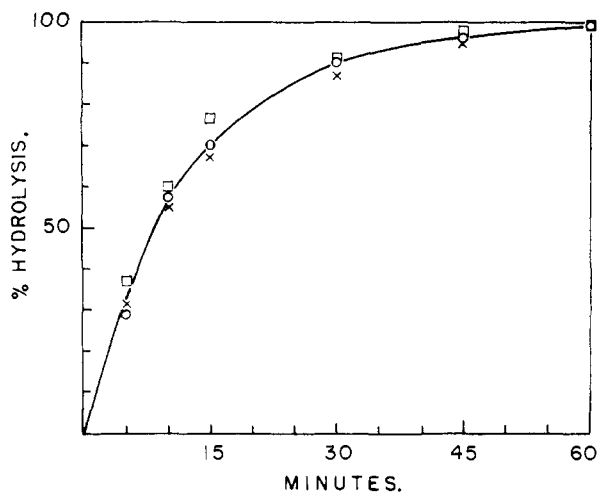


Fig. 1.—Rates of hydrolysis of glyceraldehyde-3-phosphate in 1 *N* acid at 100°; natural substance (O O O)⁹; synthetic DL-compound (X X X)¹⁰; synthetic D-compound (this preparation) (□ □ □).

The optical rotation of D-glyceraldehyde-3-phosphate has been reported by Meyerhof,¹³ while Smythe and Gerischer measured the rotation of L-glyceraldehyde-3-phosphate remaining after a yeast fermentation of the racemic compound. From the work of Smythe and Gerischer³ a value of $[\alpha]_D +7.6^\circ$ (in dilute acid) was determined, while Meyerhof reported $[\alpha]_D +19^\circ$ (in dilute acid).¹³ The rotation is also reported to vary with the pH,⁷ giving values of $+19.5^\circ$ at pH 5, $+17^\circ$ at pH 6, and $+12^\circ$ at pH 7. Our preparation has a specific rotation of $+14.5^\circ$ (in 0.1 *N* hydrochloric acid). The discrepancy between this value and that found by Meyerhof¹³ may be explained by the fact that his measurements were made on a mixture of products obtained enzymatically in which the concentration of D-glyceraldehyde-3-phosphate was determined indirectly.

For convenience, the D-glyceraldehyde-3-phosphate prepared by this procedure may be stored as the crystalline cyclohexylammonium salt of 3-phosphoryl D-glyceraldehyde dimethyl acetal (IX) a stable substance that is easily converted to D-glyceraldehyde-3-phosphate (X), in a yield of 90% as determined enzymatically. This synthesis makes available to the biochemist, in good quantity, a stable derivative of D-glyceraldehyde-3-phosphate, that can be converted readily to the optically pure substrate. It eliminates both the reason for preparing the aldotriose phosphate enzymatically, and any uncertainties that may be introduced

(11) O. Meyerhof and W. Schulz, *Biochem. Z.*, **297**, 60 (1938); C. E. Ballou and H. O. L. Fischer, *THIS JOURNAL*, **76**, 3188 (1954).

(12) O. Warburg and W. Christian, *Biochem. Z.*, **303**, 40 (1939);

G. Cori, M. Stein and C. Cori, *J. Biol. Chem.*, **173**, 605 (1948).

(13) O. Meyerhof, *Bull. soc. chim. biol.*, **20**, 1345 (1938).

into an experiment by such a procedure. Finally, the availability of the pure D substrate will facilitate the use of polarimetric methods for following the course of any reaction in which this substance is involved.

Experimental

2,5-Di-O-benzyl D-Mannitol.—The synthesis of this compound started with 1,3:4,6-di-O-methylene D-mannitol (II) prepared according to Haskins, Hann and Hudson.¹⁴ One kilogram of D-mannitol gave 95–100 g. of recrystallized product (II) with m.p. 195–205°. The trimethylene D-mannitol formed as a side product (450–500 g.) may be partially hydrolyzed according to Fletcher and Diehl¹⁵ to give an additional yield of the dimethylene compound.

Dimethylene D-mannitol (m.p. 195–205°) was benzylated in the manner described by Allerton and Fletcher.¹⁶ From 100 g. of II was obtained 150–160 g. of 2,5-di-O-benzyl 1,3:4,6-di-O-methylene D-mannitol (III) with b.p. 185–195° at 0.1 mm. pressure (bath temperature 240–250°).

Acetolysis of the dibenzyl dimethylene D-mannitol (150 g.) gave 2,5-di-O-benzyl D-mannitol (IV) (38 g., 27%) with m.p. 117–119°. The poor yield is a result of concomitant debenzylation. The low over-all yield in these first steps is compensated by the fact that the materials are inexpensive and the reactions may be run easily on a large scale.

2-O-Benzyl D-Glyceraldehyde Diethyl Mercaptal.—A solution was prepared from 12 g. of sodium metaperiodate in 240 ml. of water at room temperature (25°). Dibenzyl D-mannitol (12 g.) was then dissolved in 120 ml. of hot 95% ethanol and the solution was cooled rapidly to room temperature. The aqueous periodate solution was immediately added to the alcoholic solution, and the mixture was left at room temperature for 15 minutes during which glycol cleavage occurred.

At the end of this time the 2-O-benzyl D-glyceraldehyde was extracted from the reaction mixture with 750 ml. of ethyl ether (more ether was used if an emulsion formed). The aqueous layer was extracted twice more with 250-ml. portions of ether, and the combined ether extract was concentrated *in vacuo* at 45° bath temperature to a thick sirup that weighed 12–13 g. This product, redissolved in 200 ml. of ether, was washed once with 50 ml. of water, and again concentrated to a sirup.

The benzyl glyceraldehyde was immediately converted to the mercaptal.¹⁷ The sirup (12 g.) was dissolved in 15 ml. of ethyl mercaptan and the mixture was cooled in ice-water. Concentrated hydrochloric acid (12 ml.) was added and the flask was shaken in the cold for a few minutes. It was then left in ice-water for 30 minutes with occasional shaking to complete the reaction. At the end of this time 100 ml. of cold water was added, and the mercaptal was extracted with chloroform (100 ml.). The chloroform layer was washed three times with water, dried over anhydrous sodium sulfate and concentrated to a sirup *in vacuo*. This was distilled in a high vacuum giving 17.0 g., with b.p. 140–145° at 0.1 mm. (bath 180–190°). Redistillation gave 16.0 g. of a sirup that showed $[\alpha]_D +16.9^\circ$ (in substance).

Anal. Calcd. for $C_{14}H_{22}O_3S_2$ (288): C, 58.7; H, 7.7; S, 22.3. Found: C, 58.7; H, 8.0; S, 22.3.

2-O-Benzyl D-Glyceraldehyde Dimethyl Acetal.¹⁸—A solution of 16 g. of benzyl glyceraldehyde diethyl mercaptal in 200 ml. of dry methanol was efficiently stirred (glass stirring rod) at 55° in a 1-l. three-necked round-bottom flask fitted with a water condenser. Mercuric oxide (15 g.) was added followed by 45 g. of mercuric chloride dissolved in 50 ml. of warm dry methanol. The heat of reaction brought the contents to boiling, and the temperature was maintained at the boiling point for one hour during which stirring was continued.

The reaction mixture was then cooled and filtered by suction, and the filtrate was concentrated to dryness *in vacuo*

(14) W. T. Haskins, R. M. Hann and C. S. Hudson, *THIS JOURNAL*, **65**, 67 (1943).

(15) H. G. Fletcher, Jr., and H. W. Diehl, *ibid.*, **74**, 3797 (1952).

(16) R. Allerton and H. G. Fletcher, Jr., *ibid.*, **76**, 1757 (1954).

(17) H. W. Arnold and W. L. Evans, *ibid.*, **58**, 1950 (1936). Attempts to prepare the acetal directly with ethyl orthoformate gave poor results.

(18) H. A. Campbell and K. P. Luik, *J. Biol. Chem.*, **122**, 635 (1938).

at a bath temperature of 45°. The residue was stirred up with 100 ml. of chloroform, and the insoluble salts were removed by filtration. The chloroform filtrate was washed three times with 100-ml. portions of water, then it was dried over anhydrous sodium sulfate and concentrated to a sirup that was distilled in a high vacuum giving 10.5 g. with b.p. 100–105° at 0.1 mm. After redistillation, the substance showed $[\alpha]_D +25.1^\circ$ (in substance).

Anal. Calcd. for $C_{12}H_{18}O_4$ (226): C, 63.7; H, 8.0; OCH_3 , 27.4. Found: C, 63.3; H, 8.3; OCH_3 , 27.6.

2-O-Benzyl 3-Diphenylphosphoryl D-Glyceraldehyde Dimethyl Acetal.—To a solution of 10.5 g. of the acetal (VII) in 50 ml. of dry pyridine, cooled to 0° in ice and water, was added, under anhydrous conditions, 18 g. of diphenyl phosphorochloridate from a dropping funnel. The reaction flask was continually swirled in the ice during addition of the phosphorylating agent, which took about ten minutes, and was then left in ice for one hour and at 5° overnight.

One ml. of water was added to decompose the excess phosphorylating reagent and the reaction mixture was concentrated *in vacuo* (bath 50°) to remove most of the pyridine. The residue, dissolved in 100 ml. of chloroform, was washed with 100 ml. each of water, ice-cold 1 *N* hydrochloric acid, 1 *M* potassium bicarbonate and water. The chloroform layer, dried over anhydrous sodium sulfate, was concentrated *in vacuo* to remove the solvent. The crude benzyl diphenylphosphoryl D-glyceraldehyde dimethyl acetal was obtained in a quantitative yield (21.4 g.). It was used in the subsequent reactions without further purification.

Anal. Calcd. for $C_{22}H_{30}O_7P$ (458): P, 6.75; OCH_3 , 13.5. Found: P, 6.8; OCH_3 , 13.2.

Cyclohexylammonium 3-Phosphoryl D-Glyceraldehyde Dimethyl Acetal.¹⁹—Two and one-half grams of 5% palladium chloride on carbon²⁰ was reduced by shaking with hydrogen in 100 ml. of 95% ethanol at atmospheric pressure, then it was centrifuged down in a 40-ml. tube, and resuspended and washed twice with 95% ethanol and once with absolute ethanol. The catalyst was transferred to a hydrogenation vessel along with 200 ml. of absolute ethanol and 10.0 g. of the compound to be reduced. The mixture was shaken with hydrogen at atmospheric pressure, the benzyl group being removed in 3.5 hours with a hydrogen uptake of 520 ml. (theory requires 500).²¹

The palladium catalyst was removed by centrifugation, and the solution was returned to the hydrogenation chamber along with 1 g. of platinum oxide²² and 1 g. of acid washed Darco G-60. The mixture was shaken with hydrogen at atmospheric pressure, the phenyl groups being cleaved in 1.25 hours with a hydrogen uptake of 5,200 ml. (theory requires 5,500 ml.).

After removal of the platinum catalyst by centrifugation, 50 ml. of water was added to the alcoholic solution and the pH (indicator paper) was brought to about 9 with cyclohexylamine (about 5 ml.). The solution was concentrated to remove the alcohol, then about 50 ml. of water was added and the solution was filtered by suction through filter-cel on #50 Whatman paper to remove a little carbon. The clear filtrate was concentrated to dryness *in vacuo* (bath 45°) to give the crude, crystalline cyclohexylammonium 3-phosphoryl D-glyceraldehyde dimethyl acetal. The solid was recrystallized from 100 ml. of hot isopropyl alcohol by the addition of 50 ml. of absolute ethyl ether. After staying one hour at room temperature, and overnight at 5°, the crystals were filtered by suction and washed on the funnel with absolute ethyl ether. The compound was allowed to dry in air for several hours, then in a vacuum desiccator at 0.1 mm. over sodium hydroxide for two days. The product (7.5–8.5 g.) was obtained in a yield of 70–80% as the di-

(19) Removal of the blocking groups was carried out by reduction first with palladium and hydrogen followed by platinum and hydrogen. Once the phenyl groups are removed, the reaction mixture should be worked up immediately to minimize hydrolysis of the acetal structure. Vigorous shaking during hydrogenation and active catalysts are necessary.

(20) H. Gilman and A. H. Blatt, *Org. Syntheses*, **26**, 77 (1946).

(21) The reduction time may be shortened to about 1.5 hours if the palladium catalyst is removed after the first 30 minutes and replaced by a fresh batch of the reduced and washed palladium catalyst. This should be done if the reduction is very sluggish.

(22) H. Gilman and A. H. Blatt, "Organic Syntheses," Coll. Vol. I, John Wiley and Sons, Inc., New York, N. Y., 1941, p. 463.

amine salt which melted at 155–160° and showed $[\alpha]_D^{25} +8.4^\circ$ (*c* 2, water). The aqueous solution had a pH of about 7.5.

The analysis of this diamine salt showed low carbon and nitrogen values, probably due to partial loss of one of the amine groups during recrystallization or drying. This behavior is characteristic of cyclohexylammonium salts of organic phosphates.²³

Anal. Calcd. for $C_{17}H_{30}O_7PN_2$ (414): C, 49.3; H, 9.4; P, 7.5; N, 6.75; OCH_3 , 15.0. Found: C, 46.1; H, 9.1; P, 7.6; N, 6.4; OCH_3 , 14.9.

When dried at 80° and 0.1 mm. over phosphorus pentoxide for several hours, the diamine salt was converted to monocyclohexylammonium 3-phosphoryl D-glyceraldehyde dimethylacetal.

Anal. Calcd. for $C_{11}H_{20}O_7PN$ (315): C, 41.9; H, 8.3; N, 4.5; P, 9.8; OCH_3 , 19.7. Found: C, 42.2; H, 8.5; N, 4.6; P, 9.7; OCH_3 , 20.2.

This substance melted from 100–130°, and when dissolved in water gave a solution of about pH 5 that showed $[\alpha]_D^{25} +10.2^\circ$ (*c* 1.5, water). It was converted to D-glyceraldehyde-3-phosphate in approximately the same yield as the diamine salt.

D-Glyceraldehyde-3-phosphate.—One hundred mg. of the dicyclohexylammonium salt of the acetal, dissolved in 5 ml. of water, was swirled with 2 ml. of Dowex 50 (200–400 mesh) in the acid form to remove the amine. After a few minutes, the solution of the free acid was filtered from the exchange resin and stored in a glass-stoppered container for three days at 38–40° to undergo hydrolysis. The acetal was 80% hydrolyzed in 24 hours, as determined enzymatically¹²; and the solution reached a maximum D-glyceraldehyde-3-phosphate content after 48 hours, remaining unchanged during an additional hydrolysis period of seven days. The resulting solution showed $[\alpha]_D +14^\circ$ (*c* 1, water), was free of inorganic phosphate, and analyzed 0.048 *M* in easily hydrolyzable phosphate (1 *N* hydrochloric acid at 100° for one hour).

The solution was assayed for D-glyceraldehyde-3-phosphate enzymatically as described by Cori, Slein and Cori.¹² One-tenth ml. of a 1:51 dilution resulted in the rapid reduction of 0.82×10^{-4} mole of diphosphopyridine nucleotide, indicating a D-glyceraldehyde-3-phosphate concentration of 0.042 *M* in the original solution.²⁴

The aldehyde content of an aliquot of the solution was determined by the Willstätter-Schudel alkaline iodine method.^{25,10} A value of 0.96 mole of oxidant per mole of compound was obtained.

Calcium Salt of D-Glyceraldehyde-3-phosphate.—This salt was prepared according to the directions used in making the corresponding derivative of the racemic compound.¹⁰ An amorphous product was obtained in about a 50% yield.

Anal. Calcd. for $C_3H_5O_6PCa \cdot 2H_2O$ (244.2): P, 12.7; Ca, 16.4. Found: P, 12.7; Ca, 16.4.

When dissolved in 0.1 *N* hydrochloric acid the substance showed $[\alpha]_D^{25} +14.5^\circ$ (*c* free acid 1.2) the calculation being based on the free acid. An aliquot of this solution was oxidized with alkaline iodine to give 3-phosphoryl-D-glyceric acid, the specific rotation of which was found to be -660° (*c* 0.06, neutral molybdate). The recorded value is -745° .¹¹

Enzymatic assay of the salt¹² indicated a biological purity of 90% based on a molecular weight of 244.2 (dihydrate), while an estimation of the aldehyde content was 95% of the calculated value.²⁵

Dinitrophenylhydrazone.—This derivative was prepared according to Baer and Fischer.¹⁰

Anal. Calcd. for $C_9H_{11}O_9PN_4$ (350.3): N, 16.0. Found: N, 16.7.

Acknowledgment.—This work was supported by a grant from the Nutrition Foundation, and by a generous gift of D-mannitol from the Atlas Powder Company.

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(23) R. W. McGilvery, *J. Biol. Chem.*, **200**, 835 (1953).

(24) That the yield is not quantitative may indicate some phosphate migration during the acid-catalyzed hydrolysis to give D-glyceraldehyde-2-phosphate. This substance, synthesized by an independent route, is under study in this Laboratory.

(25) R. Willstätter and G. Schudel, *Ber.*, **61**, 780 (1918).