PREPARATION OF D-GLYCERALDEHYDE-3-PHOSPHATE AND DIHYDROXYACETONE PHOSPHATE HYDRAZONES*

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The triose phosphates have been prepared by the enzymatic dismutation of D-fructose-1,6-diphosphate (FDP) in the presence of hydrazine, and the hydrazones decomposed by extraction with benzaldehyde to give a mixture of the two compounds. (Meyerhof and Junowicz-Kocholaty, 1943). The instability of the triose phosphates upon storage in solution or as the corresponding barium salts has limited the usefulness of this procedure for the preparation of these compounds. The chemical synthesis of these compounds has been reported (Ballou and Fisher, 1943; Ballou 1960). This communication describes a method for the preparation of the triose phosphate hydrazones which are stable to alkaline treatment and to storage as the barium salts.

MATERIALS AND METHODS

FDP was purified by a modification of the procedure described by McGilvery (1953). Aldolase, five times recrystallized, was obtained from the Worthington Biochemical Corporation. D-glyceraldehyde-3-phosphate dehydrogenase and glycerol phosphate dehydrogenase were prepared according to Beisenherz, et al. (1953) and were recrystallized until free of undesirable enzymatic activities. Inorganic phosphate was determined by the method of Fiske and SubbaRow (1925), and corrected for interference by N_2H_4 when necessary. Triose phosphate hydrazones eluted from chromatographic columns were determined by heating a suitable 1.0 ml aliquot with 0.40 ml anthrone reagent (15 mg/ml anthrone dissolved in a mixture of 19 volumes glacial acetic acid, 1 volume 88 per cent formic acid) in 6.0 ml $H_2SO_4 \cdot H_2O$. The color formed after one hour of heating at 100° absorbs maximally at 525 mu and is linearly proportional to triose phosphate concentration in the range of 0 to 1 umole.

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EXPERIMENTAL

Alkaline Stability of Triose Phosphate Hydrazones

A mixture of 0.045 M Na_4FDP and 0.72 M N_2H_4 adjusted to pH 8.0 with formic acid, in a final volume of 1.10 ml was incubated with 0.3 mg of aldolase at 25° for 15 minutes and then chilled to 0°. After deproteinizing with $HClO_4$, and immediately neutralizing to pH 8 with KOH, $KClO_4$ was removed by centrifugation. Two 0.50 ml aliquots were withdrawn and treated as follows: (A) Extracted three times with 1.0 ml of benzaldehyde, and 5.0 ml of ether to decompose the hydrazones and remove N_2H_4. After removal of ether *in vacuo*, the solution was made up to volume; (B) Incubated at 25° with 0.50 ml of 2 N NaOH, neutralized with H_2SO_4, extracted as in "A" and made up to volume; and (C) An aliquot from "B" was incubated in 1N NaOH at 20° C for 20 minutes. Inorganic phosphate, and reduction or oxidation of DPN or DPNH in the presence of either glyceraldehyde-3-phosphate dehydrogenase or glycerol phosphate dehydrogenase was determined in each instance (Hohorst, et al, 1959).

As shown in Table I, the triose phosphate hydrazones are remarkably stable to alkaline treatment. However, if an acidified aliquot of the incubation mixture was added to alkali, nearly quantitative liberation of phosphate was obtained. Further investigation has indicated that in acid solution hydrolysis of the hydrazones occurred, and that rapid liberation of phosphate in alkali occurred before the slower reaction to form the hydrazones could take place. (Hall, 1960).

TABLE I

Alkaline Stability of Triose Phosphate Hydrazones

Treatment of neutralized incubation mixture*	Micromoles of Compound Determined		
	Pi	Oxidation of DPNH	Reduction of DPN
None	.083	----	----
A	.085	33.5	33.4
B	.088	33.4	33.2
C	66.6	0.0	0.0

*See text for explanation

Preparation of the Barium Salts of the Triose Phosphate Hydrazones

On the basis of the stability of the triose phosphate hydrazones, a method was developed for the preparation of the barium salts of each. 1.0 mmole of Ba H_2FDP was dissolved in H_2O and passed through a column of Dowex 50, 100-200 mesh (H^+ form, 2.5 meq. exchange capacity) in the cold, neutralized to pH 8.2 to 8.4 with 4.0 M N_2H_4 (6 mmoles), and diluted to 0.05 M. At 25°, 16 mg of crystalline aldolase was added.

During incubation, the pH was maintained by the careful addition of 2 mmoles of N_2H_4. After 45 minutes when the reaction was 90-100 per cent complete, the mixture was chilled to 0°, and 1.0 ml of 70 per cent $HClO_4$ was added. After one minute, the mixture was cautiously neutralized with cold 4 M KOH and $KClO_4$ was removed by centrifugation. The precipitate was washed twice with 2.0 ml of cold H_2O and the combined supernatants were diluted to 100 ml. The solution was transferred to a Dowex 1 x 8, 200-400 mesh (formate form) column, 1.84 x 30 cm, previously equilibrated with 0.01 M hydrazine-formate buffer, pH 7.15 (0.01 M formic acid adjusted to pH 7.1 with N_2H_4). Elution was carried out in the cold using a linearly increasing gradient of hydrazine-formate buffer pH 7.15. Two mixing flasks (McGilvery, 1960) were used to produce a gradient rising from 0.01 M to 0.26 M upon passing 800 ml through the column. 10 ml fractions were collected at a flow rate of 1.2 ml per

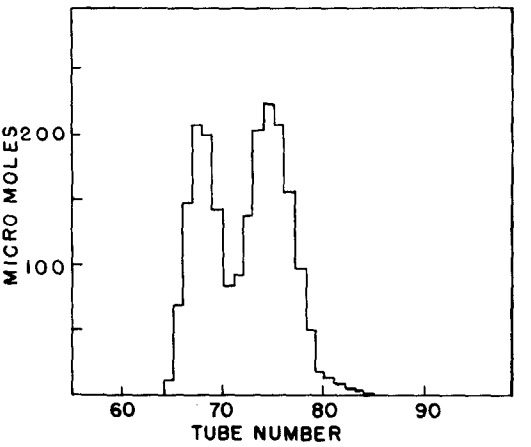

Fig. 1. Chromatographic separation of triose phosphate hydrazones. D-glyceraldehyde-3-phosphate hydrazone is eluted ahead of dihydroxyacetone phosphate hydrazone. Conditions of elution are described in the text.

minute. A typical elution pattern is shown in Figure 1. Attempts to obtain complete separation have not been successful. By appropriate selection of fractions, however, D-glyceraldehyde-3-phosphate hydrazone free of dihydroxyacetone phosphate hydrazone may be obtained in 60-70 per cent yield. Dihydroxyacetone phosphate hydrazone is contaminated with D-glyceraldehyde-3-phosphate hydrazone and must be rechromatographed to obtain a substantially pure compound.

The combined fractions were adjusted to pH 8 with N_2H_4, and 2 mmoles of barium formate were added, followed by the addition of 3 volumes of cold absolute ethanol. After standing at 0° for 3 to 4 hours, the amorphous precipitates were collected by decantation and centrifuga-

tion, washed twice with 70 per cent ethanol, and suspended in 15 ml of cold H_2O. The pH was adjusted to 3.5 to 4.0 by the addition of formic acid to dissolve the compounds, and any insoluble material was removed by filtration. After adjusting the pH to 8.0 to 8.5 with N_2H_4, the barium hydrazones were precipitated with ethanol as before, washed with 70% ethanol, once each with absolute ethanol and ether and then dried *in vacuo*. For purification of dihydroxyacetone phosphate hydrazone, the solution at pH 4 in the above isolation procedure was passed through a column of Dowex 50 (Na^+ form). The eluate was adjusted to pH 8 with N_2H_4, and was rechromatographed as described.

To convert the dry barium salt to the corresponding triose phosphate, the compound was suspended in H_2O, one equivalent of HCl was added, and the solution was passed through a column of Dowex 50 (H^+ form) to remove barium and approximately 90 per cent of the N_2H_4. N_2H_4 was completely removed by extraction of the eluate three times with ½ volume of benzaldehyde, followed by extraction three times with two volumes of ether. Traces of ether were most easily removed from the solution by a gentle stream of H_2. The solution was adjusted to pH 5 to 5.5 with NaOH, and made to volume.

TABLE II

Analysis of the Barium Salts of D-Glyceraldehyde-3-phosphate and Dihydroxyacetone Phosphate Hydrazones

	% Ba	% Total P	% Alkaline Labile P*	umoles of * DPN reduced per mg	umoles of * DPNH oxidized per mg
Ba D-glyceraldehyde-3-phosphate hydrazone	40.69	9.22	9.20	2.98	0.00
Ba dihydroxyacetone phosphate hydrazone	40.74	9.20	9.16	0.00	2.95
Calculated **	40.71	9.19	9.19	2.96	2.96

* Determined after removal of N_2H_4 by extraction with benzaldehyde and ether.

** Calculated as the monohydrate: $C_3H_7N_2O_5PBa \cdot H_2O$

Upon storage for several months at 0°, barium D-glyceraldehyde-3-phosphate hydrazone underwent negligible decomposition. Barium dihydroxyacetone phosphate hydrazone was somewhat less stable, and after six months storage 3 to 5 per cent decomposition was observed.

It is anticipated that the hydrazone of other unstable aldose and ketose phosphates may find wide applications in preparative and analytical procedures. Investigation of this possibility is in progress.

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