2-Keto-3-deoxy-D-arabo-heptonic Acid 7-Phosphate Synthetase*

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In the preceding paper (1) it was demonstrated that cell-free extracts of Escherichia coli converted n-erythrose 4-phosphate and phosphoenolpyruvate almost quantitatively to 5-dehydroquinic acid. It was postulated that the initial reaction in this conversion is a condensation of phosphoenolpyruvate and n-erythrose 4-phosphate to yield inorganic phosphate and 2-keto-3-deoxy-D-arabo-heptonic acid 7-phosphate. The present paper describes the purification and properties of the enzyme carrying out this reaction, and the identification of the product as KDHP by comparison with the chemically synthesized compound. The name KDHP synthetase is suggested for this enzyme. The later stages of the enzyme purification were greatly aided by the discovery of Weissbach and Hurwitz (3) (communicated to us before publication) that P-formylpyruvic acid, derived from the diacetamide of cyclohexylamine by the action of periodate, reacts with thiobarbituric acid to give an intense pink color with an absorption maximum at 549 mp.

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EXPERIMENTAL

Materials—The dimethylacetal of cyclohexylammonium erythrose-4-P was prepared according to the method of Ballou et al. (5), and used as sodium erythrose-4-P (6). Barium P-enolpyruvate was a generous gift of Mr. W. E. Pricer, Jr., and was converted to the potassium salt. KDHP and 3-deoxy-D-arabo-heptonic acid 7-phosphate (configuration of α-hydroxyl unknown) were prepared according to an unpublished procedure. The name KDHP synthetase is suggested for this enzyme. The later stages of the enzyme purification were greatly aided by the discovery of Weissbach and Hurwitz (3) (communicated to us before publication) that P-formylpyruvic acid, derived from the diacetamide of cyclohexylamine by the action of periodate, reacts with thiobarbituric acid to give an intense pink color with an absorption maximum at 549 mp. A part of these results was published in preliminary form (4).

Experimental—E. coli mutant 83-24 (5) was grown for 18 hours with aeration at 37° in medium A (11) supplemented with 0.2 per cent yeast extract (Difco) and 0.2 per cent casein hydrolysate. The cells were harvested by centrifugation at 2°, washed with cold water, suspended in ~/30 potassium phosphate buffer pH 7.4 and dialyzed against the same buffer (4 changes of 2 liters each at 2-hour intervals). The precipitate was removed by centrifugation and discarded. The procedure was repeated with 21 gm. of (NH4)2S04 on the supernatant solution.

Enzyme Purification—E. coli mutant 83-24 (5) was grown for 18 hours with aeration at 37° in medium A (11) supplemented with 0.2 per cent yeast extract (Difco) and 0.2 per cent casein hydrolysate. The cells were harvested by centrifugation at 2°, washed with cold water, suspended in ~/30 potassium phosphate buffer pH 7.4 (20 ml./5.0 gm. of wet bacteria), and disrupted by means of sonic oscillation for 30 minutes in a g-kc. Raytheon oscillator. The suspension was centrifuged at 2° and, after stirring for another 20 minutes, the precipitate was removed by centrifugation, dissolved in ~/30 phosphate buffer containing 16 mg. of protein per ml. All subsequent operations were carried out at 2°.

Two hundred ml. of the cell-free extract was treated with 28 ml. of 2 per cent protamine sulfate solution, the precipitate was removed by centrifugation. To 218 ml. of the supernatant solution was added slowly with stirring 54 gm. of (NH4)2S04 and, after stirring for another 20 minutes, the precipitate was removed by centrifugation and discarded. The procedure was repeated with 21 gm. of (NH4)2S04 on the supernatant solution. The precipitate was removed by centrifugation, dissolved in 45 ml. of ~/30 potassium phosphate buffer pH 7.4 and dialyzed against the same buffer (4 changes of 2 liters each at 2-hour intervals).

In the preceding paper (1) it was demonstrated that cell-free extracts of Escherichia coli converted n-erythrose 4-phosphate and phosphoenolpyruvate almost quantitatively to 5-dehydroquinic acid. It was postulated that the initial reaction in this conversion is a condensation of phosphoenolpyruvate and n-erythrose 4-phosphate to yield inorganic phosphate and 2-keto-3-deoxy-D-arabo-heptonic acid 7-phosphate. The present paper describes the purification and properties of the enzyme carrying out this reaction, and the identification of the product as KDHP by comparison with the chemically synthesized compound. The name KDHP synthetase is suggested for this enzyme. The later stages of the enzyme purification were greatly aided by the discovery of Weissbach and Hurwitz (3) (communicated to us before publication) that P-formylpyruvic acid, derived from the diacetamide of cyclohexylamine by the action of periodate, reacts with thiobarbituric acid to give an intense pink color with an absorption maximum at 549 mp. A part of these results was published in preliminary form (4).

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The rest of the fractionation procedure was best carried out on a smaller scale. Twenty ml. of the dialysate was adjusted to pH 5.4 with 1 n acetic acid and the precipitate was removed by centrifugation. The supernatant solution (cooled by an ice bath) was treated with 17 ml. of acetone (precooled to \(-15^\circ\)C) slowly with stirring and the precipitate was removed by centrifugation and discarded. The supernatant solution was treated similarly with 12 ml. of acetone, and the precipitate was removed by centrifugation, dissolved in 5 ml. of M/30 potassium phosphate buffer, pH 7.4, and dialyzed against 2 liters of 0.01 M phosphate buffer, pH 6.8, as described previously.

The diethylaminoethyl cellulose column used in the next step was prepared as follows. To 3 gm. of diethylaminoethyl cellulose suspended in 100 ml. of H2O at room temperature, 1.5 ml. of 1.0 M butyric acid in 0.125 M H2SO4 was added with stirring. (The pH of the solution was 6.8.) The cellulose was removed by filtration on a Buchner funnel, washed several times with 0.01 M potassium phosphate buffer, pH 6.8, and suspended in 70 ml. of the same buffer. Ten ml. of this suspension was placed on a chromatographic column (15 mm. diameter) and packed by mild suction (length, 35 mm.). The column was transferred to a cold room at -2° and equilibrated with cold buffer by allowing approximately 50 ml. to pass through the column (2 hours).

One ml. of the dialyzed acetone fraction was placed on the column and eluted successively with 5.0 ml. each of phosphate buffer, pH 6.8, of the following molar concentrations (flow rate 5 mm. in 20 minutes): 0.01, 0.02, 0.04, 0.06, 0.08, 0.10, 0.10, 0.16, and 0.20. The desired enzyme activity was present in the two 0.1 M fractions. Four such fractions (from two column operations) were combined (3.8 mg. of protein), and immediately concentrated by precipitation with (NH4)2SO4 (80 per cent saturation). The precipitate was dissolved in 0.8 ml. M/30 potassium phosphate buffer pH 7.4, dialyzed against the same buffer, and clarified by centrifugation. Recovery of protein was 1.8 mg.

A summary of the enzyme purification is given in Table I. The (NH4)2SO4 and acetone fractions were stable for at least several weeks at \(-15^\circ\). The cellulose fractions were stored at 2°.

Assay — The activity of the enzyme was determined by measuring the amount of KDHP formed from erythrose-4-P and P-enolpyruvate. A unit of enzyme was defined as that amount of enzyme which will form 0.1 pmole of KDHP in 5 minutes under the following conditions.

The incubation mixture contained 100 mmoles of potassium phosphate buffer (pH 6.4), 0.5 mmole each of erythrose-4-P and P-enolpyruvate, and enzyme fraction in a total volume of 1.0 ml. The reaction was started by the addition of enzyme after preincubation of the other constituents at 37° for 10 minutes. After 5 minutes at 37° the reaction was stopped by the addition of 0.4 ml. of 10 per cent trichloroacetic acid and the mixture was centrifuged. Aliquots were removed for the estimation of KDHP by cleavage with periodic acid to \(\delta\)-formylpyruvic acid (butyric acid-2,4-dione), and estimation of the latter by the color produced with thiobarbituric acid.

**Estimation of KDHP** — 0.01 to 0.05 mmoles of compound in 0.25 ml. of solution was treated with 0.25 ml. of 0.025 M periodic acid in 0.125 N H2SO4. After 45 minutes at room temperature, 0.5 ml. of 2 per cent sodium arsenite in 0.5 N HCl was added to destroy the excess periodate (2 minutes at room temperature). Two ml. of 0.3 per cent thiobarbituric acid solution were added and the tubes were placed in a boiling water bath for 5 minutes. (To prepare the thiobarbituric acid solution, 300 mg. of thiobarbituric acid were dissolved in about 70 ml. of H2O with the aid of 3 ml. of 1 N NaOH; 2.5 ml. of 1 N HCl was added, and the pH adjusted to 2 in a final volume of 100 ml.) After cooling in a water bath at 40° the pink color was measured immediately at 549 m\(\mu\) in a spectrophotometer against a blank run with water under the same conditions. The rate of production of chromogenic material from KDHP by periodic acid is shown in Fig. 1.

**Paper-chromatographic Identification** — A solution equivalent to 2 to 5 \(\mu\)g. of KDHP was spotted on acid washed Whatman No. 1 paper and developed for 24 hours by descending chromatography with a mixture of tertiary amyl alcohol-formic acid (98 per cent)-water (3:3:1). The paper was dried at room temperature and sprayed with 0.1 M periodic acid in 0.125 N H2SO4. After 20 minutes at room temperature the paper was sprayed with 10 per cent sodium arsenite in 0.5 N HCl. The paper became colored by liberated iodine which was removed by further reaction with

![Fig. 1. Development of color with KDHP in the periodate-thiobarbiturate reaction. The reaction time with periodate was varied as indicated, 0.04 \(\mu\)moles of KDHP being treated as described under "Methods."

**Table I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Protein concentration (mg/ml)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>200</td>
<td>16</td>
<td>1080</td>
<td>0.34</td>
</tr>
<tr>
<td>Ammonium sulfate (40-55%)</td>
<td>45</td>
<td>15</td>
<td>790</td>
<td>1.20</td>
</tr>
<tr>
<td>Acetone (47-59%)</td>
<td>11</td>
<td>7.3</td>
<td>440</td>
<td>5.6</td>
</tr>
<tr>
<td>Diethylaminoethyl cellulose (0.1 M phosphate eluates)</td>
<td>110</td>
<td>0.19</td>
<td>420</td>
<td>20</td>
</tr>
</tbody>
</table>
2-Keto-3-deoxy-D-arabohexonic Acid 7-P Synthetase

**TABLE II**

Behavior of various compounds in periodate-thiobarbiturate reaction

<table>
<thead>
<tr>
<th>Compound</th>
<th>λmax</th>
<th>εmax</th>
</tr>
</thead>
<tbody>
<tr>
<td>KDHP*</td>
<td>549</td>
<td>7.2 × 10⁴</td>
</tr>
<tr>
<td>2-Keto-3-deoxygalactonic acid*</td>
<td>549</td>
<td></td>
</tr>
<tr>
<td>2-Keto-3-deoxyglucoce acid 6-P*</td>
<td>549</td>
<td></td>
</tr>
<tr>
<td>1,3,4,5,6-Pentahydroxycyclohexene-carboxylic acid*</td>
<td>549</td>
<td></td>
</tr>
<tr>
<td>Malondialdehyde (1,1,3,3-tetramethoxypropane)†</td>
<td>532</td>
<td>13.0 × 10⁴</td>
</tr>
<tr>
<td>3-Deoxy-β-D-arabohexonic acid 7-P* (none)</td>
<td>542</td>
<td>3000</td>
</tr>
<tr>
<td>β-Ketobutyraldehyde dimethylacetal‡</td>
<td>517</td>
<td>136</td>
</tr>
<tr>
<td>Glyoxal§</td>
<td>546</td>
<td>2560</td>
</tr>
<tr>
<td>Glycolaldehyde§</td>
<td>517</td>
<td></td>
</tr>
<tr>
<td>Acetoacetic acid, acetopyruvic acid (2,4-diketovaleric), dimedon, acetoxylacetonester (none)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Treated with periodate and thiobarbiturate.
† Shikimic acid was hydroxylated with OsO₄ to give the pentahydroxyacid and then treated with periodate to give β-formylpyruvic acid (13).
‡ Treated with thiobarbiturate only.

**TABLE III**

Conversion of P-enolpyruvate-erythrose-4-P condensation product and of synthetic KDHP to 5-dehydroquinicinate*

<table>
<thead>
<tr>
<th>Substrate†</th>
<th>Amount incubated</th>
<th>Dehydroquinicinate formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic KDHP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synthetic KDHP + EDTA‡</td>
<td>0.20</td>
<td>0.17</td>
</tr>
<tr>
<td>Enzymatic product§</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>Enzymatic product§ + EDTA‡</td>
<td>0.27</td>
<td>0</td>
</tr>
</tbody>
</table>

* The enzyme preparation used was a fraction from *E. coli* strain 83-24 capable of converting KDHP to dehydroquinicinate in the presence of Co²⁺ and DPN. This conversion is completely inhibited by EDTA.
† The reaction mixture contained KDHP or enzymatic product, 50 μmole of potassium phosphate buffer pH 7.4, 0.25 μmole of DPN, 1.0 μmole of Co²⁺, and 0.1 ml of enzyme (1.5 mg of protein) in a total volume of 1.0 ml. After incubation at 37°C for 1 hour, 0.02 ml of 6 N HCl was added, proteins were removed by centrifugation, and 0.2 ml of the clear supernatant solution was used for the microbiological assay with *E. coli* mutant A170-143 S₃ (16).
§ 0.1 ml of enzyme, 0.1 ml of buffer, and 0.1 ml of EDTA solution (0.4 μmole) were preincubated at 37°C for 10 minutes before the addition of substrates. In these experiments Co²⁺ was omitted.

With KDHP, the optimal reaction time with periodate was 45 minutes (Fig. 1). However, this could be reduced to 20 minutes by the addition of 10⁻² molar concentrations of glycine or glycylglycine. The function of these compounds is unknown, and is under further investigation.

**RESULTS**

Scope and Sensitivity of Periodate-Thiobarbiturate Reaction

Several compounds structurally related to β-formylpyruvic acid, or expected to give rise to it on periodate oxidation, were tested in the procedure described under “Estimation of KDHP.” The results are summarized in Table II. Simple aliphatic aldehydes, α,β-diketones, β-keto acids, and β-formyl省教育厅 (developed from 3-deoxy-o-arabohexonic acid 7-phosphatene) did not yield colored products under these conditions. Glycolaldehyde and glyoxal gave brown solutions with very small absorptions at 517 and 546 μm, respectively. In addition to the first four compounds in Table II, which would be expected to yield β-formylpyruvic acid with periodate, only β-keto-butyraldehyde dimethylacetal gave a pink color, but it was quite unstable and weak.

With KDHP, the optimal reaction time with periodate was 45 minutes (Fig. 1). However, this could be reduced to 20 minutes by the addition of 10⁻² molar concentrations of glycine or glycylglycine. The function of these compounds is unknown, and is under further investigation.

Identification of Product of Erythrose-4-P and P-enolpyruvate Condensation as KDHP

Chromatographic and Chemical Comparison with Synthetic KDHP—Erythrose-4-P and P-enolpyruvate were incubated with enzyme and aliquots were spotted on acid-washed Whatman No. 1 filter paper along with synthetic KDHP, erythrose-4-P, and P-enolpyruvate. The papers were developed with tertiary amyl alcohol-formic acid-water. KDHP was detected by the periodate-thiobarbiturate reaction as indicated earlier, and also by spraying with semicarbazide (14, 15) (0.1 per cent semicarbazide hydrochloride in 0.15 per cent sodium acetate). The semicarbazide-treated paper was dried in an oven at 95°C for 10 minutes. Under ultraviolet irradiation the semicarbazones were revealed as dark spots. By either method, a strong spot corresponding to KDHP (Rₚ = 0.49) was present in the enzymatic reaction mixture. Identical Rₚ values of 0.26 were also found for synthetic KDHP and enzymatic product with ethanol-water-acetic acid on Whatman No. 50 paper.

Biological Activity of Enzymatic and Synthetic Product—Synthetic KDHP and the product of the P-enolpyruvate-erythrose-4-P condensation were incubated with an enzyme preparation capable of converting KDHP to 5-dehydroquininate, and the latter was estimated microbiologically (16). The results, presented in Table III, show identical behavior for the enzymatically formed product and for synthetic KDHP.

* P. R. Srinivasan and D. B. Sprinson, unpublished results (cf. (12)).
Properties of KDHP Synthetase

**pH Optimum of KDHP Formation**—The effect of pH on the rate of formation of KDHP from P-enolpyruvate and erythrose-4-P is shown in Fig. 2. The pH optimum is at 6.4 in potassium phosphate buffer. With Tris maleate buffer, the H⁺ ion concentration had little effect on the reaction between pH 6.4 and 7.4.

**Specificity**—Erythrose-4-P cannot be replaced by n-erythrose, n-glyceraldehyde a-phosphate, ribose 5-phosphate, glucose 6-phosphate, glucosamine 6-phosphate and N-acetylglucosamine 6-phosphate. Pyruvate or pyruvate plus ATP cannot substitute for P-enolpyruvate. It would have been of interest to test the isomers of erythrose-4-P, but these were not available.

**Effect of Substrate Concentration**—The effect on the reaction rate of varying concentrations of erythrose-4-P (Fig. 3) and P-enolpyruvate (Fig. 4) was determined. These results have been plotted according to Lineweaver and Burk (17) for the determination of Kₘ values. The Kₘ for erythrose-4-P was found to be 1.2 × 10⁻³ M and for P-enolpyruvate 3.5 × 10⁻³ M.

**Stoichiometry**—The disappearance of P-enolpyruvate and the formation of KDHP and inorganic phosphate were measured, and are recorded in Table IV. The stoichiometry agrees with the expected bimolecular condensation of erythrose-4-P and P-enolpyruvate.

**Inhibitors**—With the acetone fraction, EDTA (4 × 10⁻⁴ M) did not inhibit the reaction. Co⁺⁺, Zn⁺⁺, Mg⁺⁺ at a concentration of 2 × 10⁻² M had no effect on the condensation. Fluoride, sodium arsenite, azide, and iodoacetate did not affect the rate of formation of KDHP, p-Chloromercuribenzoate (2 × 10⁻⁴ M) inhibited the reaction completely and this inhibition could be reversed by cysteine.

In crude extracts and in the (NH₄)₂SO₄ fractions KDHP is utilized in the next reaction of aromatic biosynthesis, i.e. its conversion to dehydroquinate. This conversion (which proceeds at a considerably slower rate than the KDHP synthetase reaction) requires DPN and Co⁺⁺ (12). The addition of either DPNase or EDTA, although inhibiting the conversion of KDHP to dehydroquinate (12), does not inhibit KDHP formation in the acetone and cellulose fractions. Nevertheless, the addition of DPNase results in an 81 and 50 per cent inhibition of KDHP formation in the crude and (NH₄)₂SO₄ fractions, respectively, while the addition of EDTA results in a 97 and 44 per cent inhibition. These unexpected inhibitions are being further investigated.

**TABLE IV**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>P-enolpyruvate disappeared</th>
<th>Orthophosphate formed</th>
<th>KDHP formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.53</td>
<td>1.62</td>
<td>1.56</td>
</tr>
<tr>
<td>2</td>
<td>1.68</td>
<td>1.68</td>
<td>1.68</td>
</tr>
</tbody>
</table>

* The reaction mixture (3 ml.) contained 3 μmoles each of erythrose-4-P and P-enolpyruvate, 240 μmoles of Tris-maleate buffer, pH 7.0, and 0.25 ml. of acetone fraction (1.1 mg. of protein) which was dialyzed against 0.2 M Tris-maleate buffer, pH 7.0. The reaction was started by the addition of enzyme after preincubation of the other constituents at 37° for 10 minutes. Before and after an incubation period of 15 minutes aliquots were withdrawn for the determination of P-enolpyruvate, KDHP, and orthophosphate.

Inhibited the reaction completely and this inhibition could be reversed by cysteine.

Another inhibition, discussed in the previous paper (1), is exhibited by sedoheptulose 1,7-diphosphate and several other phosphorylated carbohydrates (Table V). The most inhibitory of these is the α-hydroxy analogue of KDHP which is an intermediate in its chemical synthesis. However, in another experiment, KDHP was not inhibitory, at initial concentrations which were 60 per cent of that of the erythrose-4-P.
0.2 ml. aliquots were used for KDHP analysis. This was followed by the addition of 0.2 ml. of a 10% trichloroacetic acid solution (0.5 ml.) to be incubated at 37°C for 15 minutes. The deproteinization was carried out by adding 0.2 ml. of a 10% trichloroacetic acid solution to the mixture. The reaction mixture was then incubated at 37°C for 15 minutes, and deproteinized by adding 0.2 ml. of a 10% trichloroacetic acid solution. The values reported in the table were corrected accordingly. The titration of KDHP synthetase by phosphorylated compounds is shown in Table V. The titration of KDHP synthetase by phosphorylated compounds is shown in Table V. The titration of KDHP synthetase by phosphorylated compounds is shown in Table V.

### Table V

**Inhibition of KDHP synthetase by phosphorylated compounds**

<table>
<thead>
<tr>
<th>Compound†</th>
<th>Amount added</th>
<th>Yield of KDHP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles</td>
<td>μmoles</td>
</tr>
<tr>
<td>None</td>
<td>0.00</td>
<td>0.14</td>
</tr>
<tr>
<td>Sedoheptulose 1,7-diphosphate</td>
<td>0.25</td>
<td>0.12</td>
</tr>
<tr>
<td>Sedoheptulose 1,7-diphosphate</td>
<td>0.50</td>
<td>0.08</td>
</tr>
<tr>
<td>Sedoheptulose 1,7-diphosphate</td>
<td>1.0</td>
<td>0.06</td>
</tr>
<tr>
<td>Sedoheptulose 1,7-diphosphate</td>
<td>1.5</td>
<td>0.06</td>
</tr>
<tr>
<td>Sedoheptulose 1,7-diphosphate</td>
<td>1.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Fructose 1,6-diphosphate</td>
<td>1.0</td>
<td>0.10</td>
</tr>
<tr>
<td>3-Deoxy-D-arabo-heptonic acid 7-P</td>
<td>1.0</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* 0.25 μmole of erythrose-4-P, 0.30 μmole of P-enolpyruvate, 50 μmoles of potassium phosphate buffer pH 7.0, and inhibitor were preincubated at 37°C for 10 minutes. After the addition of 0.01 ml. of acetone fraction (73 μg. of protein) the reaction mixture (0.5 ml.) was incubated at 37°C for 15 minutes, and deproteinized by the addition of 0.2 ml. of a 10% trichloroacetic acid solution. 0.2 ml. aliquots were used for KDHP analysis.

† Dihydroxyacetone phosphate, D-glyceraldehyde 3-phosphate, and 3-phospho-D-glyceric acid were not inhibitory.

### Table VI

**Extent of reaction in P-enolpyruvate-erythrose-4-P condensation**

<table>
<thead>
<tr>
<th>Additions</th>
<th>KDHP formed at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 minutes</td>
</tr>
<tr>
<td>None</td>
<td>1.2</td>
</tr>
<tr>
<td>Crystalline bovine serum albumin</td>
<td>1.2</td>
</tr>
<tr>
<td>2-Mercaptoethanol†</td>
<td>1.6</td>
</tr>
</tbody>
</table>

* The incubation mixture (2 ml.) contained 2.0 μmoles of erythrose-4-P, 2.5 μmoles of P-enolpyruvate, 200 μmoles of potassium phosphate buffer, pH 6.4, 0.08 ml. of acetone fraction (0.6 mg. of protein), and, when added, 4.0 mg. of serum albumin, or 4 μmoles of 2-mercaptoethanol. The latter was preincubated with the enzyme for 10 minutes at 37°C before addition of the other component.

† At this concentration of 2-mercaptoethanol (2 × 10^-4 M) the thiobarbiturate assay for KDHP was inhibited 27 to 30 percent. Thus KDHP disappearance could not be detected when incubated with enzyme in the presence of an organic phosphate under the following conditions: (a) with ADP, Mg++, and P-enolpyruvate-ATP transphosphorylase; (b) with ADP, Mg++, P-enolpyruvate-ATP transphosphorylase, lactic dehydrogenase and DPNH; (c) with the substrates and enzymes of (b) plus hexokinase and glucose.

### Discussion

**Thiobarbiturate Reaction with β-Keto Aldehydes**—It was first observed by Weissbach and Hurwitz (5) that a compound resulting from the action of periodate on 2-keto-3-deoxyaldonic acids reacts with thiobarbiturate to yield an intense pink color with an absorption maximum at 549 μm. From the structure of several compounds giving this test they concluded that the benzaldehyde is responsible for the color reaction. Further support for their conclusion is shown in Table II. Although the benzaldehyde has not been isolated in pure form, it has been previously been isolated as its bis-2,4-dinitrophenylhydrazone from 1,3,4,5,6-pentahydroxycyclohexane-carboxylic acid (13). When the periodate-thiobarbiturate reaction was applied to this compound, an absorption curve identical with that given by KDHP and related compounds was obtained. Since β-ketobutyraldehyde, unlike several other dicarbonyl compounds, also gave a pink color with thiobarbiturate (λ_max = 542 μm), it would appear that the structure of R-CO-CH₂CHO is essential for color formation. The previous application of this test to malondialdehyde (λ_max = 532 μm (21-23)) would exemplify the case where R = H. Since β-formylpyruvic acid reacts slowly with periodate (3), the molar absorbancy index of KDHP recorded in Table II may represent a minimal value.

**Properties of KDHP Synthetase**—It may be seen from Table I that the fraction obtained from the diethylaminoethyl cellulose column represented a 60-fold purification of the KDHP synthetase. In a preliminary investigation this fraction appeared to be homogeneous in the Spinco analytical centrifuge. Most of the work reported in this paper was done with the acetone fraction, which was shown to be free of aldolase (by its inability to cleave sedoheptulose-1,7-diphosphate), P-enolpyruvate-ATP transphosphorylase, the enzyme system for the conversion of KDHP to 5-dehydroquinate, dehydroquinine-dihydride (26), and DPNH oxidase.

As far as is known at present only erythrose-4-P and P-enolpyruvate are condensed by this enzyme. That the product of the condensation is KDHP is shown by comparison of the enzymatic product with synthetic KDHP in chemical and chromatographic behavior, and in enzymatic conversion to dehydroquinine. The enzyme preparation used in the latter test is inactive with 3-deoxy-D-arabo-heptonic acid 7-phosphate, 2-keto-3-deoxy-D-arabo-heptonic acid (dephosphorylated KDHP), 2-keto-3-deoxygluconic acid 6-phosphate, or any related compounds. Furthermore, although erythrose-4-P disappearance was slow, a yield of KDHP of 30 to 35 percent was observed by Weissbach and Hurwitz (5).

β-Ketobutyraldehyde would not be expected to show maximal color development under the conditions of the test, since it condenses in acid solution to 1,3,5-triacetyl benzene (18-20). The fading of the color produced by β-ketobutyraldehyde may be due to a reversal of the reaction with thiobarbiturate favored by the aromatization of the aldehyde.

Presumably this decomposition (3) consists of hydroxylation of the active methylene carbon (24, 25) to yield β-formylhydroxypyruvic acid, followed by periodate cleavage to formate and mesoxalic semialdehyde, decarboxylation to glyoxal, and periodate cleavage to 2 molecules of formate.

We are indebted to Dr. H. C. Lawler for aid with this determination.

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was not measured, the stoichiometry of the reaction (Table IV) is in accord with the concept that erythrose-4-P and P-enolpyruvate are condensed to form KDHP and inorganic phosphate. The specificity of the enzyme for its substrates suggests its use for the assay of either erythrose-4-P or P-enolpyruvate.

KDHP synthetase is apparently a sulfhydryl enzyme, since its inhibition by p-chloromercuribenzoate is reversed by cysteine. Although it is stable during preparation, the purer fractions (starting with the acetone fraction) showed a sharp halt in activity after 10 to 15 minutes, when the yield of KDHP was 50 to 60 per cent of the theoretical. However, when 2-mercaptoethanol was added, complete conversion of the substrates to KDHP occurred. This behavior may be due to a greater tendency of the enzyme to oxidation in the presence of its substrates.

**Mechanism of Condensation Reaction**—The condensation of erythrose-4-P and P-enolpyruvate may be tentatively regarded (as shown in Scheme I) as a nucleophilic attack on P-enolpyruvate, since its protonation would create an insurmountable energy barrier to the reformation of P-enolpyruvate. The concerted mechanism is, therefore, more reasonable. For this reason the name originally given (4) to the enzyme described here is changed from "P-enolpyruvate-erythrose-4-P aldolase" to KDHP synthetase. Further work is now in progress to elucidate the mechanism of the P-enolpyruvate-erythrose-4-P condensation.

On the assumption that the free energy change in the formation of the C-3 to C-4 linkage of KDHP is small, the free energy change of the KDHP synthetase reaction, P-enolpyruvate$^-^+$ erythrose-4-P$^-$ $\rightarrow$ H$_2$O $\rightarrow$ HPO$_4^-^+$ $\rightarrow$ KDHP$^-^+$, may be considered to be approximately the same as that of the hydrolysis of P-enolpyruvate (33): P-enolpyruvate$^-^+$ + H$_2$O $\rightarrow$ pyruvate$^-^+$ + HPO$_4^-^+$; $\Delta F^o$ = 13.6 kilocalories. The apparent irreversibility of the KDHP synthetase reaction may be due not only to the large negative value of $\Delta F^o$ for the conversion of P-enolpyruvate to "pyruvate" and orthophosphate, but also to the stability of the pyranose form. In the cleavage of fructose 1-phosphate derivatives by aldolase (34) the pyranoside fructose 1-phosphate can be calculated to be more stable by 3.6 kilocalories than the open chain 5,6-dideoxyfructose 1-phosphate. The participation of P-enolpyruvate rather than of pyruvate in the condensation with erythrose-4-P may be of advantage in a biosynthetic reaction requiring KDHP, since 2-keto-3-deoxy-D-arabonic acids, e.g. 2-keto-3-deoxygluconic acid 6-P (15, 35) and N-acetylneuraminic acid (31), are readily degraded to pyruvate and the corresponding aldohydes (glyceraldehyde 3-phosphate, and N-acetyl-D-mannosamine, respectively).

**Summary**

An enzyme which catalyzes the condensation of d-erythrose 4-phosphate and phosphoenolpyruvate to form 2-keto 3-deoxy D-arabo-heptonic acid 7-phosphate and inorganic phosphate was purified from extracts of Escherichia coli. After a 60-fold purification it appeared homogeneous in the ultracentrifuge. The name 2-keto-3-deoxy-D-arabo-heptonic acid 7-phosphate synthetase is suggested for this enzyme.

The yields of 2-keto-3-deoxy-D-arabo-heptonic acid 7-phosphate were quantitative, and attempts to reverse the reaction were unsuccessful. Only phosphoenolpyruvate and d-erythrose 4-phosphate were activated by the enzyme. It had the properties of a sulfhydryl protein and showed maximal activity in the presence of 2-mercaptoethanol. No cofactor requirements could be demonstrated. The reaction was inhibited by fructose 1,6-diphosphate, sedoheptulose 1,7-diphosphate, and 3-deoxy-D-arabo-heptonic acid 7-phosphate.

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$^{10}$ A $\Delta F^o$ value of approximately $-1000$ calories can be calculated from the published data (32) for the reaction: pyruvate$^-^+$ + N-acetyl-D-mannosamine $\rightarrow$ N-acetylneuraminic$^-^+$. 

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**Scheme I**

A diagram illustrating the condensation reaction of P-enolpyruvate and erythrose-4-P to form KDHP. The reaction is pictured as a nucleophilic attack on P-enolpyruvate, leading to the formation of KDHP and inorganic phosphate. The diagram includes atomic labels for carbon, hydrogen, oxygen, and phosphorus atoms, with arrows indicating the flow of reactants and products.
2-Keto-3-deoxy-d-arabino-heptonic Acid 7-Phosphate Synthetase
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