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Purification and Properties of 3-Hexulose Phosphate Synthase and Phospho-3-hexuloisomerase from *Methylococcus capsulatus*

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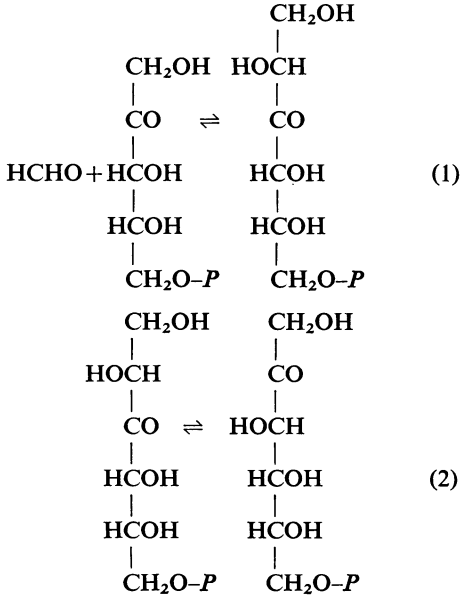
3-Hexulose phosphate synthase and phospho-3-hexuloisomerase were purified 40- and 150-fold respectively from methane-grown *Methylococcus capsulatus*. The molecular weights of the enzymes were approximately 310000 and 67000 respectively, as determined by gel filtration. Dissociation of 3-hexulose phosphate synthase into subunits of molecular weight approx. 49000 under conditions of low pH or low ionic strength was observed. Within the range of compounds tested, 3-hexulose phosphate synthase is specific for formaldehyde and D-ribulose 5-phosphate (forward reaction) and D-*arabino*-3-hexulose 6-phosphate (reverse reaction), and phospho-3-hexuloisomerase is specific for D-*arabino*-3-hexulose 6-phosphate (forward reaction) and D-fructose 6-phosphate (reverse reaction). A bivalent cation is essential for activity and stability of 3-hexulose phosphate synthase; phospho-3-hexuloisomerase is inhibited by many bivalent cations. The pH optima of the two enzymes are 7.0 and 8.3 respectively and the equilibrium constants are 4.0×10^{-5}M and 1.9×10^{2}M respectively. The apparent Michaelis constants for 3-hexulose phosphate synthase are: D-ribulose 5-phosphate, 8.3×10^{-5}M; formaldehyde, 4.9×10^{-4}M; D-*arabino*-3-hexulose 6-phosphate, 7.5×10^{-5}M. The apparent Michaelis constants for phospho-3-hexuloisomerase are: D-*arabino*-3-hexulose 6-phosphate, 1.0×10^{-4}M; D-fructose 6-phosphate, 1.1×10^{-3}M.

The key role of 3-hexulose phosphate synthase and phospho-3-hexuloisomerase in the ribulose monophosphate cycle of formaldehyde fixation, present in many micro-organisms capable of utilizing C_1-compounds, has been established (Kemp, 1972, 1974; Strøm *et al.*, 1974). The reactions catalysed by these enzymes are respectively:



The present paper records a study of the purification and properties of these two enzymes obtained from methane-grown *Methylococcus capsulatus*.

Materials and Methods

Chemicals

D-Ribulose 5-phosphate, D-xylulose 5-phosphate, ovalbumin, bovine serum albumin, cytochrome *c* (horse heart) and alkaline phosphatase (from *Escherichia coli*) were purchased from Sigma (London) Chemical Co. Ltd., Kingston-upon Thames, Surrey, U.K. Phosphoriboisomerase was purchased from Calbiochem Ltd., London W.1, U.K. All other sugar phosphates, coenzymes and enzymes were from Boehringer Corp. (London) Ltd., London W.5, U.K. The preparations of formaldehyde, D-allulose (psicose) 6-phosphate, D-*arabino*-3-hexulose 6-phosphate and buffers have been described (Strøm *et al.*, 1974).

Determination of substrates

The chemical and enzymic determinations of all the substrates used in this study have been previously described (Strøm *et al.*, 1974).

Determination of protein

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Enzyme assays

3-Hexulose phosphate synthase (D-*arabino*-3-*hexulose* 6-*phosphate formaldehyde lyase*). This enzyme was assayed as a routine by measuring the rate of ribulose 5-phosphate-dependent disappearance of formaldehyde. Because of the inhibitory effect of commercial ribulose 5-phosphate (Kemp, 1972), ribulose 5-phosphate was generated *in situ* for each assay from ribose 5-phosphate by the action of phosphoriboisomerase. The assay mixture contained 50mM-sodium–potassium phosphate buffer, pH7.0, 5mM-$MgCl_2$, 0.55mM-formaldehyde, 6μg per ml of phosphoriboisomerase, 5mM-D-ribose 5-phosphate (sodium salt) and was incubated for 15min at 37°C. After this preincubation, extract containing up to 0.04unit of 3-hexulose phosphate synthase activity (per ml of assay mixture) was added and formaldehyde consumption was followed, at 37°C, by taking timed samples from the assay mixture for a period of up to 10min. The samples were added to $HClO_4$ (final concentration 0.5M) and formaldehyde was determined colorimetrically by the method of Nash (1953). Formaldehyde disappearance not dependent on 3-hexulose phosphate synthase activity was corrected for by running parallel assays lacking ribose 5-phosphate. One unit of 3-hexulose phosphate synthase activity is defined as the amount of enzyme that catalyses the removal of 1mM-formaldehyde per min in the above assay.

When defined starting concentrations of ribulose 5-phosphate were required in the assay, 5mM-ribose 5-phosphate was incubated with 6μg per ml of phosphoriboisomerase in 10mM-Tris–HCl, pH7.4, at 37°C for 30min. The phosphoriboisomerase was then inactivated by heating the mixture in a boiling-water bath for 3min. The ribulose 5-phosphate concentration in the mixture was determined by the cysteine–carbazole method (Ashwell & Hickman, 1957), and known amounts were used in the assay for 3-hexulose phosphate synthase, leaving out the preincubation involving D-ribose 5-phosphate and phosphoriboisomerase.

3-Hexulose phosphate synthase was assayed in the reverse direction, by measuring the rate of formaldehyde formation from D-*arabino*-3-hexulose 6-phosphate. These assays were carried out spectrophotometrically by following continuously the rate of oxidation of NADH in the presence of yeast alcohol dehydrogenase at 340nm. The assay mixtures, at 30°C, in a total volume of 1ml, contained 50mM-sodium–potassium phosphate buffer, pH7.0, 5mM-$MgCl_2$, 0.2mM-NADH, 0.5mM-D-*arabino*-3-hexulose 6-phosphate, 550μg of yeast alcohol dehydrogenase and up to 0.05 unit of 3-hexulose phosphate synthase.

Phospho-3-hexuloisomerase (D-*arabino*-3-*hexulose* 6-*phosphate* 3,2-*ketolisomerase*). This enzyme was assayed discontinuously by following the formation of D-fructose 6-phosphate from D-*arabino*-3-hexulose 6-phosphate. In a total volume of 1ml, the assay mixture contained: 0.1M-Tris–HCl buffer, pH8.3, 1mM-D-*arabino*-3-hexulose 6-phosphate (sodium salt) and 1mM-EDTA. The mixture was incubated at 30°C, and extract containing up to 0.2 unit of phospho-3-hexuloisomerase was added; the reaction was stopped at known times (up to 5min) by the addition of 1ml of 1M-$HClO_4$. Perchlorate was removed from the assay mixture by discarding the precipitate formed after neutralization with 0.5ml of 2M-K_2CO_3 at 0–4°C. The supernatant was assayed for D-fructose 6-phosphate as described by Hohorst (1965). Non-enzymic formation of D-fructose 6-phosphate from D-*arabino*-3-hexulose 6-phosphate (Strøm *et al.*, 1974) was corrected for by running assays in the absence of phospho-3-hexuloisomerase. One unit of phospho-3-hexuloisomerase activity is defined as the amount of enzyme that catalyses the formation of 1μmol of D-fructose 6-phosphate per min from D-*arabino*-3-hexulose 6-phosphate in the above assay.

Phospho-3-hexuloisomerase was assayed in the reverse direction by following the formation of formaldehyde from D-fructose 6-phosphate in a coupled, continuous spectrophotometric assay at 30°C. The assay mixture, in a total volume of 1ml, contained: 50mM-sodium–potassium phosphate buffer, pH7.0, 1mM-$MgCl_2$, 10mM-fructose 6-phosphate, 0.2mM-NADH, 550μg of yeast alcohol dehydrogenase, 1 unit of 3-hexulose phosphate synthase and extract containing up to 1unit of phospho-3-hexuloisomerase activity (enzyme units defined above). The enzyme activity was recorded at 340nm.

Phosphoglucoisomerase, phosphoriboisomerase, ribulose 5-phosphate-3-epimerase. These were all assayed by methods previously described (Strøm *et al.*, 1974).

Growth of organism and preparation of cell-free extract

M. capsulatus, grown on methane as the sole carbon source in a salts medium described by Salem *et al.* (1973), was cultured and harvested as previously described (Lawrence *et al.*, 1970). Harvested cells were stored at −15°C until required.

M. capsulatus (24g wet wt.) was resuspended with 75ml of buffer containing 20mM-sodium–potassium phosphate, pH7.0, and 5mM-$MgCl_2$. The suspension was disrupted by ultrasonication for 6min, at 0–4°C, in an MSE 150W ultrasonic disintegrator and then centrifuged at 6000*g* for 10min, yielding the cell-free extract as supernatant. This extract was further centrifuged, at 38000*g* for 60min, the pellet obtained being used for the purification of 3-hexulose phosphate synthase and the supernatant for the purification of phospho-3-hexuloisomerase as described below.

Table 1. *Purification of 3-hexulose phosphate synthase*

Details of the steps used and the definition of units of activity are in the Materials and Methods section.

Fraction	Total protein (mg)	Volume (ml)	Total activity (units)	Specific activity (units/mg of protein)	Purification (fold)	Recovery (%)
Cell-free extract	1940	91	3310	1.71	1	100
Resuspended particulate fraction	1350	50	2750	2.04	1.2	83
Extract after solubilization with 1 M-NaCl	414	125	1720	4.17	2.4	52
25–50% $(NH_4)_2SO_4$ fraction	194	20.5	1560	8.03	4.7	47
Pooled fractions after DEAE-cellulose chromatography	14.0	5.6*	823	58.9	34	25
Calcium phosphate gel eluate	7.50	6.0	517	69.0	41	15

* Volume after concentration of pooled column fractions by ultrafiltration.

Purification of 3-hexulose phosphate synthase

All steps in the purification were carried out at 0–4°C and are summarized in Table 1.

Step 1. *Solubilization with* 1 M-*NaCl.* The pellet obtained from the centrifugation of the cell-free extract described above was resuspended with the aid of a glass homogenizer in 40ml of buffer containing 20mM-sodium–potassium phosphate, 5mM-$MgCl_2$ and 1M-NaCl, pH7.0. After an hour at 0°C the resuspended particulate fraction was centrifuged at 38000*g* for 60min; the pellet obtained was subjected to two further extractions and centrifugations. The supernatants from the three extractions were combined.

Step 2. $(NH_4)_2SO_4$ *fractionation.* 3-Hexulose phosphate synthase in the combined supernatants was concentrated and further purified by fractionation with $(NH_4)_2SO_4$. The protein that precipitated on the addition of solid $(NH_4)_2SO_4$, between 25 and 50% saturation, calculated from Table 1 of Green & Hughes (1955), was collected by centrifugation at 25000*g* for 15min and dissolved in 10mM-sodium–potassium phosphate–2.5mM-$MgCl_2$ buffer, pH7.4.

Step 3. *DEAE-cellulose chromatography.* The protein solution from step 2 was applied to a column (19cm × 5cm) of DEAE-cellulose (Whatman DE52) previously equilibrated with 10mM-sodium–potassium phosphate–2.5mM-$MgCl_2$ buffer, pH7.4. 3-Hexulose phosphate synthase activity was eluted from the column with the equilibration buffer, close to the void volume of the column. Fractions containing synthase activity were concentrated by ultrafiltration through a Diaflo PM30 membrane filter (Amicon Corp., Lexington, Mass., U.S.A.).

Step 4. *Calcium phosphate gel treatment.* The concentrated enzyme from step 3 (in 10mM-sodium–potassium phosphate–2.5mM-$MgCl_2$, pH7.4) was further purified by adsorption on to calcium phosphate gel, prepared by the method of Keilin & Hartree (1938). The protein solution from step 3 was treated with the gel, at a protein/dry calcium phosphate ratio of 1:1 (w/w), the gel was washed with 10ml of 10mM-sodium–potassium phosphate–2.5mM-$MgCl_2$, pH7.4, and the 3-hexulose phosphate synthase was extracted by washing the gel twice with 3ml volumes of 0.2M-sodium–potassium phosphate–2.5mM-$MgCl_2$, pH7.4. The two washings were combined. The purified 3-hexulose phosphate synthase was stored frozen, at −15°C in 0.2M-sodium–potassium phosphate–2.5mM-$MgCl_2$, pH7.4.

Purification of phospho-3-hexuloisomerase

All steps in the purification were carried out at 0–4°C and are summarized in Table 2.

Step A. Protamine sulphate precipitation. EDTA was added to the supernatant obtained by centrifugation of the cell-free extract described above to give a final concentration of 1mM. Protamine sulphate (2.0%, w/v) was then added slowly to 40ml of the supernatant, to a final concentration of 1mg of protamine sulphate to 10mg of protein. After 20min, the precipitate was removed by centrifugation at 25000*g* for 15min and discarded.

Step B. $(NH_4)_2SO_4$ *fractionation.* Solid $(NH_4)_2SO_4$ was added to the supernatant from step A to 50% saturation, as calculated from Table 1 of Green & Hughes (1955). The resulting precipitate was removed by centrifugation and discarded. $(NH_4)_2SO_4$ was then added to the supernatant to 95% saturation. The precipitate contained the phospho-3-hexuloisomerase; it was collected by centrifugation and dissolved in 4ml of 10mM-Tris–HCl–1mM-EDTA (pH7.5) buffer.

Step C. Gel filtration. A column (70cm × 2.5cm) of Sephadex G-150 was equilibrated with 10mM-Tris–HCl–1mM-EDTA (pH7.5) buffer. The protein solution from step B was applied to the column and was eluted with the equilibration buffer by ascending chromatography; fractions (10ml) were collected and

Table 2. *Purification of phospho-3-hexuloisomerase*

Details of the purification procedure and the definition of units of phospho-3-hexuloisomerase activity are in the Materials and Methods section. In the second DEAE-cellulose column 30ml of the combined fractions from the first DEAE-cellulose column was used.

Fraction	Total protein (mg)	Volume (ml)	Total activity (units)	Specific activity (units/mg of protein)
Cell-free extract	1015	47.2	3150	3.12
Membrane-free supernatant	312	40	5520*	17.7
Supernatant after protamine sulphate precipitation	304	40	5480	18.0
50–95% $(NH_4)_2SO_4$ fraction	135	5	5720	42.3
Pooled fractions after Sephadex G-150 chromatography	28.8	60	4650	162
Pooled fractions after first DEAE-cellulose chromatography	7.2	105	4450	1060
Pooled fractions after second DEAE-cellulose chromatography	0.55	10†	860	1560

* See text for explanation of apparent increase in total activity.
† Volume after concentration of pooled column fractions by ultrafiltration.

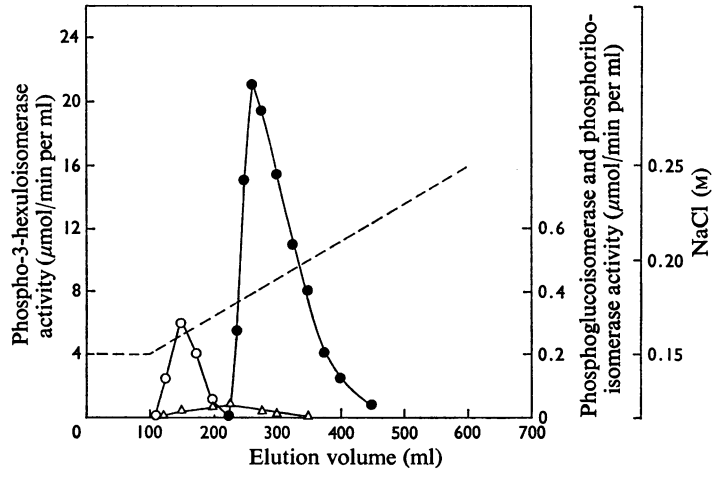


Fig. 1. *Chromatography of phospho-3-hexuloisomerase on DEAE-cellulose*

A column (10cm×1.5cm) of DEAE-cellulose (Whatman DE-52) was equilibrated with 10mM-Tris–HCl–1mM-EDTA–0.15M-NaCl buffer, pH7.5. The enzyme sample, prepared as described in the Materials and Methods section (step E), was applied to the column and eluted with a gradient (----) of 0.15–0.25M-NaCl in the same buffer. The isomerase activities in 10ml fractions were assayed and the elution pattern for phosphoriboisomerase (○), phosphoglucoisomerase (△) and phospho-3-hexuloisomerase (●) are shown.

assayed for the isomerase. Phospho-3-hexuloisomerase was eluted in the effluent volume between 220ml and 280ml (void volume of the column was 135ml). Fractions containing activity were combined.

Step D. DEAE-cellulose chromatography. NaCl was added to the combined fractions from step C to a final concentration of 0.1M and the pH of the solution was readjusted to pH7.5 with NaOH. This solution was applied to a column (20cm×1.5cm) of DEAE-cellulose (Whatman DE52) that had been previously equilibrated with 10mM-Tris–HCl–1mM-EDTA–0.1M-NaCl, pH7.5. The phospho-3-hexuloisomerase was eluted from the column with 500ml of a linear gradient of 0.1–0.5M-NaCl in 10mM-Tris–HCl–1mM-EDTA, pH7.5; fractions (10ml) were collected; phospho-3-hexuloisomerase was eluted in the effluent volume between 310 and 410ml and selected fractions were pooled.

Step E. Second DEAE-cellulose chromatography. A portion (30ml) of the pooled fractions from step D was diluted to 100ml with 10mM-Tris–HCl–1mM-EDTA buffer, pH7.5, to lower the NaCl concentration in the solution to below 0.15M. This solution was then passed through a column (10cm×1.5cm)

of DEAE-cellulose (Whatman DE52) that had been previously equilibrated with 10mM-Tris–HCl–1mM-EDTA–0.15M-NaCl, pH7.5. The phospho-3-hexuloisomerase was eluted from the column with 500ml of a linear gradient of 0.15–0.25M-NaCl in 10mM-Tris–HCl–1mM-EDTA, pH7.5; fractions (10ml) were collected and those between 290 and 390ml were pooled (see Fig. 1). The protein in the pooled fractions was concentrated by ultrafiltration through a Diaflo PM30 membrane (Amicon Corp.) to a volume of 1.0ml and the residual NaCl concentration was lowered by diluting to 10ml with 10mM-Tris–HCl–1mM-EDTA, pH7.5. The phospho-3-hexuloisomerase was again concentrated by ultrafiltration through the same membrane filter from 10 to 1.0ml and this was again diluted to 10ml with the same buffer.

Molecular-weight determination by gel filtration

The molecular weights of 3-hexulose phosphate synthase and phospho-3-hexuloisomerase were determined by chromatography of the enzymes on calibrated columns of Sephadex G-200 and Sephadex G-100 respectively, by the procedure of Andrews (1965).

Polyacrylamide-gel electrophoresis of proteins

Discontinuous polyacrylamide-gel electrophoresis was carried out at pH8.5 with 7.5% (w/v) polyacrylamide gels as described by Davis (1964). Electrophoresis of protein samples treated with 1% (w/v) sodium dodecyl sulphate, 1% (v/v) 2-mercaptoethanol, on 10% (w/v) polyacrylamide gels containing 0.1% (w/v) sodium dodecyl sulphate, was performed by the procedure of Weber *et al.* (1972). Proteins on the gels were stained with 0.25% (w/v) Coomassie Blue in methanol–acetic acid–water (5:7:88, by vol.) and destained in methanol–acetic acid–water (5:7:88, by vol.) by soaking at 37°C (Weber *et al.*, 1972). The bands on the stained gels were located by scanning the gels, at 540nm, in a Unicam SP1800 spectrophotometer fitted with a scanning densitometer attachment (SP1809).

Results

Purification and properties of 3-hexulose phosphate synthase

Purification. As previously reported (Lawrence *et al.*, 1970), 3-hexulose phosphate synthase activity is associated with the particulate fraction of cell-free extracts of *M. capsulatus*. The results in Table 1 show that over 80% of the 3-hexulose phosphate synthase activity was located in the particulate fraction obtained by centrifugation of cell-free extracts at 38000*g* for 60min and over 60% of this activity was solubilized by repeated extraction of the particulate fraction with 1M-NaCl. After the subsequent steps shown in Table 1, a 40-fold purified preparation of 3-hexulose phosphate synthase was obtained with 15% recovery of activity.

Analysis of 50μg of protein from the purified 3-hexulose phosphate synthase preparation by polyacrylamide-gel electrophoresis either in the absence or in the presence of sodium dodecyl sulphate resulted in the detection of four protein bands, on staining with Coomassie Blue. Although the 40-fold purified enzyme was not homogeneous by the above criteria, the synthase preparation did not contain detectable activities of phosphoriboisomerase, ribulose 5-phosphate 3-epimerase or phospho-3-hexuloisomerase.

Substrate specificity. Studies with the purified synthase by the formaldehyde-disappearance assay (minus added phosphoriboisomerase) showed no detectable activity with the following substrates as acceptors for condensation with formaldehyde: 5mM-ribose 5-phosphate; 1mM-xylulose 5-phosphate; 0.7mM-allulose 6-phosphate; 5mM-fructose 6-phosphate. Glyceraldehyde 3-phosphate and dihydroxyacetone phosphate were also tested simultaneously as substrates by incubating formaldehyde and 3-hexulose phosphate synthase with 2.7mM-glyceraldehyde 3-phosphate in the presence of an excess of triose phosphate isomerase (1μg/ml of assay mixture); no disappearance of formaldehyde was observed. The fructose diphosphate aldolase activity of the preparation of 3-hexulose phosphate synthase was below 0.1% of the 3-hexulose phosphate cleavage activity.

These results confirm the finding of Kemp (1972) that ribulose 5-phosphate is the pentose phosphate acceptor for formaldehyde in the reaction catalysed by the synthase.

Metal ion requirement. 3-Hexulose phosphate synthase had an absolute requirement for a bivalent metal ion, with Mg^{2+} and Mn^{2+} being the most effective for promoting activity, as shown in Table 3. Mg^{2+} and Mn^{2+} were not only required for enzyme activity, but 3-hexulose phosphate synthase was rapidly, and apparently irreversibly, denatured by storage in the absence of one of these metal ions. As shown in Table 3, Zn^{2+} and Co^{2+} (at 1mM) are only partially as effective as Mg^{2+} or Mn^{2+} in promoting synthase activity, whereas Ni^{2+}, Ca^{2+} and Cu^{2+} were inhibitory to the enzyme.

Stability. The purified enzyme, in the presence of 2.5mM-Mg^{2+}, was stable for 6 months at −15°C, unless repeatedly frozen and thawed. 3-Hexulose phosphate synthase was rapidly inactivated at elevated temperatures; activity was totally lost within 5min at 60°C.

Table 3. *Activation and inhibition of 3-hexulose phosphate synthase by metal ions*

Imidazole–HCl buffer (0.1 M, pH 7.0) was substituted for the phosphate buffer normally used in the standard assay for 3-hexulose phosphate synthase (see the Materials and Methods section). For the measurements of activity of the enzyme in the presence of the metal ions shown, the cations (at a final concentration of 1 mM) were substituted for Mg^{2+} present in the standard assay and the activities obtained are presented as a percentage of the rate measured in the presence of 1 mM-$MgCl_2$. For the investigation of inhibitory effects, the metal ions (at 1 mM concentration) were present in the assay in addition to 1 mM-$MgCl_2$ and the values shown are the percent inhibitions observed, compared with the activity in the presence of Mg^{2+} alone.

Metal ion	Activity (%)	Inhibition (%)
Mg^{2+}	100	0
Mn^{2+}	102	0
Co^{2+}	17	0
Zn^{2+}	10	0
Ni^{2+}	0	64
Ca^{2+}	0	49
Cu^{2+}	0	24

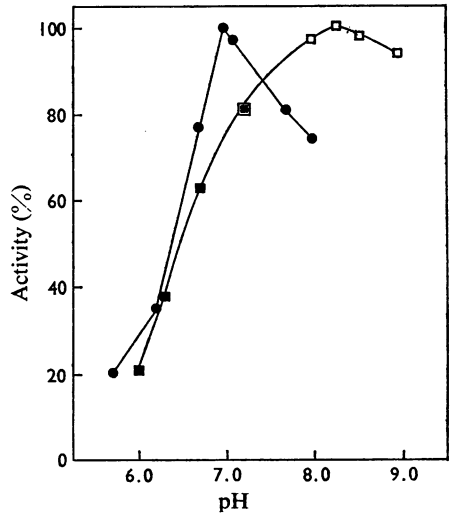


Fig. 2. *pH-dependence of activity of 3-hexulose phosphate synthase and phospho-3-hexuloisomerase*

3-Hexulose phosphate synthase activity (●) was assayed as described in the Materials and Methods section, in the presence of sodium phosphate buffers (Gomori, 1955) in the pH range shown. Phospho-3-hexuloisomerase activity was assayed as described in the Materials and Methods section, in the presence of imidazole–HCl buffers in the pH range 6.0–7.2 (■) and Tris–HCl buffers in the pH range 7.2–9.0 (□). Activities are expressed as a percentage of those at the optimal pH.

Michaelis constants. These constants were determined by double-reciprocal plots (Lineweaver & Burk, 1934) of initial velocity against substrate concentration, for each of the substrates of the synthase. The apparent K_m for ribulose 5-phosphate (determined at 4 mM-formaldehyde concentration) was 8.3×10^{-5} M and for formaldehyde (determined at 0.57 mM-ribulose 5-phosphate concentration) was 4.9×10^{-4} M. In the reverse direction, the apparent K_m for D-*arabino*-3-hexulose 6-phosphate was 7.5×10^{-5} M.

pH optimum. The results shown in Fig. 2 indicate that 3-hexulose phosphate synthase has optimal activity, in the synthetic direction, at pH 7.0.

Inhibition studies. Because 3-hexulose phosphate synthase is a key enzyme in the pathway of formaldehyde assimilation by *M. capsulatus* (Strøm *et al.*, 1974), the possible regulatory effect of a number of metabolites on the synthase activity was investigated. None of the following compounds, at a concentration of 1 mM in the assay mixture, significantly affected 3-hexulose phosphate synthase activity, either with the purified enzyme preparation or with cell-free extracts: ATP, ADP, AMP, NADH, D-fructose 6-phosphate, phosphoenolpyruvate.

Molecular weight and subunit structure. The molecular weight of the 3-hexulose phosphate synthase was measured by the use of a column of Sephadex G-200, by the procedure of Andrews (1965). When a buffer of pH 7.4 (Fig. 3*a*) was used the synthase emerged in a single symmetrical peak at an elution volume of 164 ml. This corresponded to a molecular weight of approx. 310000 (Fig. 3*a*). However, if a buffer of pH 4.6 was used, the enzyme emerged in a single symmetrical peak, but at an elution volume of 252 ml, which corresponded to a molecular weight of approx. 49000 (Fig. 3*b*). These results suggest that 3-hexulose phosphate synthase can dissociate probably into six subunits. Partial dissociation of the enzyme has also been observed; when the synthase was subjected to gel filtration in buffer of low phosphate concentration (10 mM-sodium–potassium phosphate–2.5 mM-$MgCl_2$, pH 7.4) two main peaks of activity were eluted from the column, corresponding to the small and large forms of the enzyme described above. Hence fairly mild changes in pH and ionic strength can result in the dissociation of 3-hexulose phosphate synthase.

Purification and properties of phospho-3-hexuloisomerase

Purification. Phospho-3-hexuloisomerase has been purified from the supernatant obtained after removal of the particulate fraction from cell-free extracts of *M. capsulatus*, as summarized in Table 2 and in the Materials and Methods section. An apparent

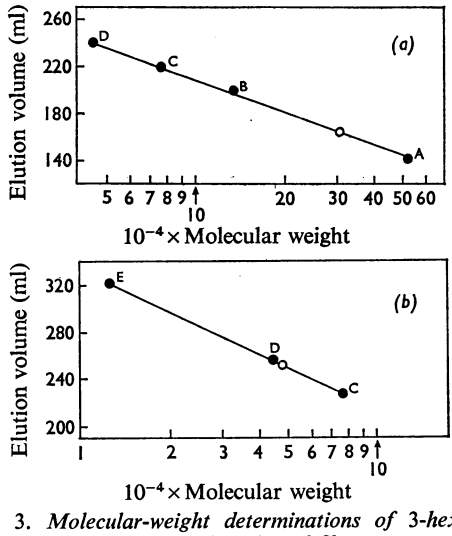


Fig. 3. *Molecular-weight determinations of 3-hexulose phosphate synthase by gel filtration*

(*a*) A column (70cm×2.5cm) of Sephadex G-200 was equilibrated with 50mM-sodium–potassium phosphate–2.5mM-$MgCl_2$ buffer, pH7.4. A sample (3ml) containing 3-hexulose phosphate synthase (○), β-galactosidase (A), lactate dehydrogenase (B), alkaline phosphatase (C) and ovalbumin (D) was applied to the column, which was eluted with the above buffer. Fractions (4ml) were collected and assayed for each of the above enzymes and the protein eluted from the column was detected by following the E_{280} of the eluate. The elution volume (V_e) for each of the above proteins was determined and the data are presented in a plot of V_e against molecular weight as described by Andrews (1965). (*b*) A column (72cm×2.5cm) of Sephadex G-200 was equilibrated with 50mM-KH_2PO_4–2.5mM-$MgCl_2$ buffer, pH4.6. A sample (3ml), containing 3-hexulose phosphate synthase (○), alkaline phosphatase (C), ovalbumin (D) and cytochrome *c* (E) was applied to the column, which was eluted with the same buffer, pH4.6. Fractions were collected and assayed as described for (*a*), and the elution volumes (V_e) of the proteins were plotted against molecular weight.

increase in the total activity of the enzyme was observed in the first steps of the purification up to $(NH_4)_2SO_4$ precipitation. Several observations suggest that this increase in total activity is due to the removal of inhibitory metal ions present in the crude extract. First, the use of buffers containing EDTA was essential for good recoveries of the enzyme during purification; for example, chromatography of the isomerase on DEAE-cellulose in the absence of EDTA resulted in only 10% recovery of activity, compared with 95% during chromatography in the presence of EDTA. Secondly, as shown in Table 4 and discussed below, Mg^{2+}, among a number of metal ions, was found to inhibit phospho-3-hexuloisomerase and was present at 5mM concentration in the cell-free extracts to stabilize the activity of 3-hexulose phosphate synthase, which was purified from the same extracts. Preparation of cell-free extracts in the absence of Mg^{2+} resulted in 45% higher specific activity of the isomerase than found in extracts prepared with 5mM-$MgCl_2$. Further, preparation of cell-free extracts in the absence of Mg^{2+}, but in the presence of 5mM-EDTA, followed by dialysis against buffer containing EDTA, resulted in fourfold higher activity than in cell-free extracts prepared with $MgCl_2$. These results suggest that an inhibitor, besides Mg^{2+} added with the buffer, was present in the cell-free extracts used as the starting material for the purification of the isomerase.

The calculation of the recovery in the purification procedure described in Table 2 is complicated by the observed increase in total activity. However, assuming an initial specific activity of phospho-3-hexuloisomerase of 10μmol/min per mg of protein (found in cell-free extracts prepared under the best conditions tested) the final preparation described in Table 2 would be approximately 150-fold purified with a 24% recovery of activity.

The second DEAE-cellulose column (step E) was found to be necessary to separate phospho-3-hexuloisomerase activity from other isomerases, namely phosphoriboisomerase and phosphoglucoisomerase, present after the first DEAE-chromatography (step D). After step D, the pooled fractions contained phosphoriboisomerase and phosphoglucoisomerase activities at 0.8 and 0.1% respectively of the activity of phospho-3-hexuloisomerase. The resolution of the three isomerase activities by the second DEAE-cellulose chromatography is shown in Fig. 1, and by virtue of the separation of the peaks, it is clear that these isomerase activities are catalysed by different proteins in *M. capsulatus*. By pooling only fractions between 290 and 390ml, the phosphoglucoisomerase activity in the final preparation was 0.01% of phospho-3-hexuloisomerase activity. The final preparation of phospho-3-hexuloisomerase was also free of 3-hexulose phosphate synthase and phosphoriboisomerase activities.

The purity of the final preparation of phospho-3-hexuloisomerase was tested by discontinuous polyacrylamide-gel electrophoresis without added sodium dodecyl sulphate. The electrophoresis of 5μg of protein in the gels resulted in the detection of one major band, after staining with Coomassie Blue, as shown by the densitometer tracing in Fig. 4. As the intensity of staining has been reported to be proportional to the amount of protein present in the range 1–15μg (Weber *et al.*, 1972), it can be estimated, by measuring the areas under the peaks shown in Fig. 4, that the major peak consists of approx. 90% of the protein present. When an unstained gel, electrophoresed under the same conditions, was sliced (1mm thick slices) and the slices were resuspended in 0.1M-Tris–HCl buffer, pH8.3, it was

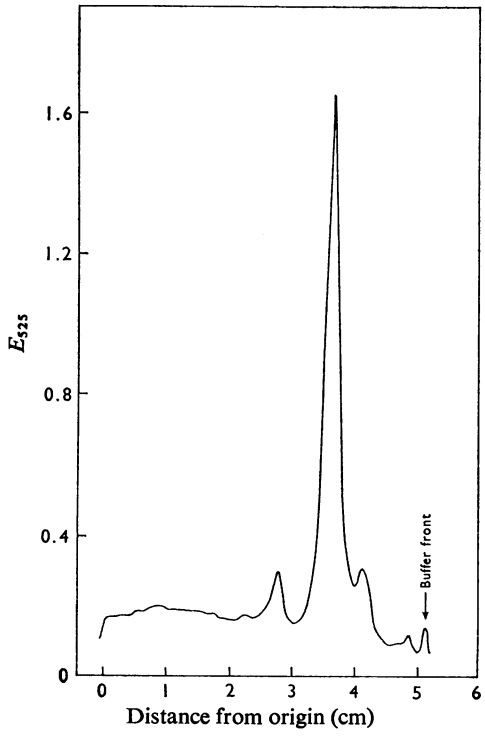


Fig. 4. *Discontinuous gel electrophoresis of phospho-3-hexuloisomerase*

Protein (5μg) of the purified phospho-3-hexuloisomerase was subjected to electrophoresis as described in the Materials and Methods section, and the gel was scanned for protein bands by using a scanning densitometer attached to a Unicam spectrophotometer SP. 1800. The trace shows the E_{525} along the gel.

found that only the major peak shown in Fig. 4 possessed phospho-3-hexuloisomerase activity. In this way, about 15% of the total activity applied to the gel could be recovered in the area corresponding to the major protein peak on the gels.

Stability. Phospho-3-hexuloisomerase has been stored in 10mM-Tris–HCl–1mM-EDTA buffer, pH 7.5, for 4 months at 0–4°C and at −15°C without detectable loss of activity. The enzyme is irreversibly denatured at higher temperatures, all activity being lost within 10min at 60°C.

Inhibition studies. The effect of bivalent cations on the activity of phospho-3-hexuloisomerase was tested, and the results are shown in Table 4. Cu^{2+} was the most effective inhibitor.

The effect of various sugar phosphates on the activity of phospho-3-hexuloisomerase was investigated. At 1mM sugar phosphate concentration, D-allulose 6-phosphate, D-fructose 6-phosphate, 6-phospho-D-gluconate, D-ribulose 5-phosphate, D-xylulose 5-phosphate, D-erythrose 4-phosphate and

Table 4. *Inhibition of phospho-3-hexuloisomerase activity by bivalent cations*

A sample (5ml) of the purified phospho-3-hexuloisomerase was dialysed for 20h at 0–4°C against 2 litres of 10mM-Tris–HCl buffer, pH 7.5. The activity of phospho-3-hexuloisomerase in this preparation was measured as described in the Materials and Methods section, in the absence of EDTA and in the presence of various bivalent cations. The activities obtained at two different cation concentrations are presented as the percentage of the activity measured in the absence of cations.

Cation	Concentration (mM)	Activity (%)
None		100
Ca^{2+}	1	80
	10	75
Mg^{2+}	1	75
	10	65
Zn^{2+}	1	40
Co^{2+}	1	35
	10	32
Ni^{2+}	1	14
	10	7.5
Cu^{2+}	1	8
	10	5.6

glyceraldehyde 3-phosphate did not affect the isomerase activity. However, D-ribose 5-phosphate did inhibit the isomerase slightly, with 5% inhibition observed at 1mM and 15% inhibition at 10mM-D-ribose 5-phosphate. ATP, ADP, AMP, NAD, NADH, NADPH (all at 1mM concentration) had no effect on the activity of phospho-3-hexuloisomerase.

Substrate specificity. The incubation of purified phospho-3-hexuloisomerase with 2mM-D-ribulose 5-phosphate, 2mM-D-xylulose 5-phosphate or 2mM-D-allulose 6-phosphate in the assay mixture for the isomerase described in the Materials and Methods section (but with D-*arabino*-3-hexulose 6-phosphate absent) did not result in disappearance of ketulose or appearance of aldose or D-fructose 6-phosphate, when assayed by methods previously described (Strøm *et al.*, 1974). The finding that D-allulose 6-phosphate was not a substrate for the isomerase is consistent with the findings that D-allulose 6-phosphate was not isomerized by crude cell-free extracts of *M. capsulatus* (Strøm *et al.*, 1974).

pH optimum. The results in Fig. 2 indicate that, after correction for the non-enzymic rate of isomerization of D-*arabino*-3-hexulose 6-phosphate (Strøm *et al.*, 1974), maximum activity of phospho-3-hexuloisomerase was at pH 8.3.

Michaelis constants. The apparent K_m of phospho-3-hexuloisomerase for D-*arabino*-3-hexulose 6-phosphate at pH 8.3 was 1.0×10^{-4}M, as determined by means of double-reciprocal plots of rates of activity against substrate concentration (Lineweaver & Burk, 1934). In the reverse reaction, the apparent K_m of the enzyme for D-fructose 6-phosphate, determined at pH 7.0, was 1.1×10^{-3}M.

Molecular weight. The molecular weight of phospho-3-hexuloisomerase was approx. 67000, as determined by the gel-filtration method of Andrews (1965). In this measurement, a column (70cm × 2.5cm) of Sephadex G-100 was equilibrated at 0–4°C with 50mM-Tris–HCl–1mM-EDTA buffer, pH7.5; phosphohexuloisomerase and the following markers were chromatographed: cytochrome *c* (12400), chymotrypsinogen A (25000), ovalbumin (45000), bovine serum albumin (67000) and *E. coli* alkaline phosphatase (77000). The numbers in parentheses indicate the values taken for the molecular weights.

Equilibrium of the reactions catalysed by 3-hexulose phosphate synthase and phospho-3-hexuloisomerase. The two enzymes, 3-hexulose phosphate synthase and phospho-3-hexuloisomerase, catalyse the first two reactions in the assimilation of formaldehyde by *M. capsulatus* (Strøm *et al.*, 1974). It was of interest, therefore, to determine the position of equilibrium in the reactions catalysed by these enzymes, from a metabolic, as well as an enzymological viewpoint.

The equilibrium constant of reaction (1) was determined with the purified 3-hexulose phosphate synthase. The enzyme was incubated at 30°C in an assay mixture (0.5ml total volume) containing 50mM-sodium–potassium phosphate buffer, pH7.0, 5mM-$MgCl_2$ and 0–2.5mM-D-*arabino*-3-hexulose 6-phosphate. To minimize the non-enzymic breakdown of D-*arabino*-3-hexulose 6-phosphate, high enzyme concentrations (24units/ml) and short times of incubation were used to achieve equilibrium, which was reached in less than 10min. The incubations were stopped after 10min by the addition of 0.1 ml of 2.5M-$HClO_4$, perchlorate was removed, at 0–4°C, by neutralization with 0.24ml of 1M-K_2CO_3 and discarding the precipitate formed. The supernatant was assayed for D-*arabino*-3-hexulose 6-phosphate and for formaldehyde by the methods previously described (Strøm *et al.*, 1974). The equilibrium constant, defined as

$$\frac{[\text{formaldehyde}]\,[\text{D-ribulose 5-phosphate}]}{[\text{D-}arabino\text{-3-hexulose 6-phosphate}]}$$

was $4.0\times10^{-5}\pm0.7\times10^{-5}$M (mean of six determinations ± S.D.). Hence the equilibrium of the reaction catalysed by the synthase is in favour of synthesis.

The equilibrium of reaction (2) was found to be so far in favour of fructose 6-phosphate as to preclude accurate direct determination of the equilibrium constant. For a one-substrate enzyme-catalysed reaction, it is, however, possible to calculate the equilibrium constant by means of the Haldane relationship (Haldane, 1930): $K_{eq.} = V_1 \cdot K_2 / V_2 \cdot K_1$, where $K_{eq.}$ is the equilibrium constant and V_1, V_2, K_1 and K_2 are the $V_{max.}$ and K_m values respectively for the forward and reverse reactions. All these values were obtained experimentally for phospho-3-hexuloisomerase, at pH7.0 and 30°C. To assay the forward and reverse reactions under the same conditions, rates of forward reaction (i.e. D-*arabino*-3-hexulose 6-phosphate → D-fructose 6-phosphate) were measured in the assay described in the Materials and Methods section, except that 50mM-sodium–potassium phosphate–1mM-$MgCl_2$ buffer, pH7.0, was substituted for the Tris–HCl buffer normally used. The reverse reaction was followed in the enzyme-coupled assay described in the Materials and Methods section. The Michaelis constants were determined from double-reciprocal plots (Lineweaver & Burk, 1934) and it was found that $V_1 = 524\,\mu$mol/min per mg of protein, $V_2 = 12.8\,\mu$mol/min per mg of protein, $K_1 = 2.4\times10^{-4}$M and $K_2 = 1.1\times10^{-3}$M. From these values $K_{eq.}$ is calculated to be 188. Fructose 6-phosphate exists in solution as a mixture of the acyclic and the α- and β-fructofuranose forms, the last being the predominant form (Benkovic *et al.*, 1972). It is not known which of these forms is the preferred substrate for phospho-3-hexuloisomerase and the value of the Michaelis constant, K_2, is based on the total concentration of fructose 6-phosphate.

Discussion

The purified 3-hexulose phosphate synthase is specific with respect to condensation of formaldehyde with D-ribulose 5-phosphate (reaction 1); no reaction was observed with D-xylulose 5-phosphate, D-ribose 5-phosphate, D-allulose 6-phosphate, D-fructose 6-phosphate, glyceraldehyde 3-phosphate or dihydroxyacetone phosphate in place of D-ribulose 5-phosphate. Reaction (1) may be compared with the analogous condensation of formaldehyde and dihydroxyacetone phosphate to form L-erythrulose 1-phosphate catalysed by fructose diphosphate aldolase from muscle (Meyerhof *et al.*, 1936; Dische & Landsberg, 1960).

$$\text{HCHO} + \text{HOH}_2\text{C–CO–CH}_2\text{OP} \rightarrow \text{HOH}_2\text{C–CH(OH)–CO–CH}_2\text{OP} \qquad (3)$$

Evidence has been obtained for the occurrence of reaction (3) in rat liver (Charalampous, 1954), Swiss chard (Mueller *et al.*, 1955) and erythritol-grown *Propionibacterium pentosaceum* (Wawszkiewicz & Barker, 1968).

3-Hexulose phosphate synthase shows an absolute requirement for a bivalent metal ion and thus resembles a Class II aldolase (Horecker *et al.*, 1972). Not only is the metal ion required for enzyme activity but also for stability of the enzyme. In this respect it is similar to L-rhamnulose 1-phosphate aldolase which contains bivalent zinc (Schwartz *et al.*, 1974). The latter enzyme has a molecular weight of 130000–140000 and can be dissociated into four inactive

subunits of molecular weight 35000. The metal ion is required not only for enzyme activity but also for the formation of active tetramer from the subunits. 3-Hexulose phosphate synthase has a molecular weight of approx. 310000 and on lowering the pH or the ionic strength of the buffer, can dissociate into subunits of molecular weight of approx. 49000. The instability of the enzyme in the absence of Mg^{2+} may well be connected with metal ion stabilization of a hexameric enzyme molecule.

Phospho-3-hexuloisomerase is present in extracts of *M. capsulatus* at high activity and is sensitive to inhibition by many bivalent metal ions, Cu^{2+} in particular; hence it is essential to purify the enzyme in the presence of EDTA. The enzyme appears to be specific with respect to isomerization of 3-hexulose 6-phosphate, no reaction being observed with D-ribulose 5-phosphate, D-xylulose 5-phosphate or D-allulose 6-phosphate. To our knowledge, it represents the first known example of an isomerase which catalyses the isomerization of a 3-ketulose grouping to a 2-ketulose grouping.

No appreciable inhibition of either 3-hexulose phosphate synthase or phospho-3-hexuloisomerase by nicotinamide nucleotides, adenosine nucleotides or certain sugar phosphates was observed. The mechanism of regulation of the ribulose monophosphate cycle is therefore at present unknown. The most likely targets for control may lie in regulation of the availability of formaldehyde coming from the C_1-oxidation sequence or in the generation of glyceraldehyde 3-phosphate, necessary for the regeneration of ribulose 5-phosphate, through phosphofructokinase–fructose diphosphate aldolase or glucose 6-phosphate dehydrogenase–2-keto-3-deoxyphosphogluconate aldolase (Strøm *et al.*, 1974).

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