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THE PREPARATION OF TRANSKETOLASE FREE FROM D-RIBULOSE-5-PHOSPHATE 3-EPIMERASE

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Summary

A procedure for the purification from *Candida utilis* of transketolase (sedoheptulose-7-phosphate: D-glyceraldehyde-3-phosphate glycolaldehydetransferase, EC 2.2.1.1) free from D-ribulose-5-phosphate 3-epimerase (EC 5.1.3.1) was developed using acetone precipitation, elution from DEAE-cellulose, adsorption of epimerase by thiopropyl-Sepharose, and chromatography on D-ribose 5-phosphate-Sepharose and DEAE-Sephadex. The final product had a specific activity of 43 units/mg, a transketolase/epimerase activity ratio greater than 53 000 to 1, an apparent K_m for D-xylulose 5-phosphate and D-ribose 5-phosphate of 77 and 430 μM, respectively, and ran as a single band using electrophoresis on polyacrylamide gel. It was inhibited by D-arabinose 5-phosphate and D-glucose 6-phosphate. During the purification by column chromatography, multiple forms of the enzyme were detected by gel electrophoresis but these gradually disappeared as the enzyme was further purified.

Introduction

Transketolase (sedoheptulose-7-phosphate: D-glyceraldehyde-3-phosphate glycolaldehydetransferase, EC 2.2.1.1) must be free from contamination by D-ribulose-5-phosphate 3-epimerase (EC 5.1.3.1) if it is to be used for analytical purposes, such as the determination of D-xylulose 5-phosphate and D-ribulose 5-phosphate [1—3] or as an auxiliary enzyme in the assay of D-ribulose-5-phosphate 3-epimerase in either the forward [4] or the reverse [5] direction, or for the enzymic synthesis of D-xylulose 5-phosphate [6].

A previous purification procedure [7] proved unsatisfactory when applied to material from some commercial batches of *Candida utilis*. Contrary to earlier

experience [7] both transketolase and the epimerase eluted from DEAE-Sephadex together. Alternative methods of specifically separating these two enzymes were developed and are described below.

Materials and Methods

Candida utilis (batch 32C-8500), D-glucose 6-phosphate disodium salt, D-arabinose 5-phosphate disodium salt, D-ribose 5-phosphate disodium salt and other biochemicals were obtained from Sigma Chemical Company. DEAE-Sephadex A-50, Sephadex G-25, epoxy-activated Sepharose 6B and thiopropyl-Sepharose 6B were from Pharmacia. DE-32 DEAE-cellulose was from W. and R. Balston Ltd., Maidstone, Kent, U.K. D-Ribose 5-phosphate-Sepharose 6B was prepared from D-ribose 5-phosphate and epoxy-activated Sepharose 6B [8]. Ultrogel AcA-34 was obtained from LKB-Producter AB, Bromma-1, Sweden. Diaflo UM-10 and PM-30 ultrafiltration membranes were from Amicon Corporation, Lexington, MA.

D-Erythrose 4-phosphate was prepared by oxidation of D-glucose 6-phosphate [9], equilibrium mixtures of D-ribose 5-phosphate, D-ribulose 5-phosphate and D-xylulose 5-phosphate for use in routine assays were prepared as described previously [1]. D-Xylulose 5-phosphate for kinetic studies was prepared by reacting D-glyceraldehyde 3-phosphate with hydroxypyruvate in the presence of transketolase and precipitated as the barium salt [6].

Enzyme assays. All enzyme assays were performed at 30°C in a Unicam SP 1800 spectrophotometer attached to a Vitraton 400 recorder. For routine purposes, in order to conserve D-xylulose 5-phosphate, transketolase was assayed at 1 mM D-ribose 5-phosphate and 0.1 mM D-xylulose 5-phosphate as described previously [7]. The final enzyme preparation was also assayed at near saturating concentrations of 2 mM D-ribose 5-phosphate and 0.5 mM D-xylulose 5-phosphate. D-Ribulose-5-phosphate 3-epimerase activity was measured by replacing the D-xylulose 5-phosphate with D-ribulose 5-phosphate and adding 0.2 units transketolase. Transaldolase (EC 2.2.1.2) and D-ribose-5-phosphate ketol-isomerase (EC 5.3.1.6) were assayed as described earlier [7]. Alcohol dehydrogenase (EC 1.1.1.1) was assayed by the method of Bonnichsen [10].

D-Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) was measured by a modification of the procedure described by Taylor [11]. The assay mixture contained 0.20 ml 40 mM D-fructose 1,6-diphosphate at pH 7.6/0.30 ml 170 mM sodium arsenate/0.30 ml 270 mM glycine/0.05 ml freshly prepared 10 mM NAD^+/0.60 ml freshly prepared 100 mM cysteine base/water to a final volume of 3.00 ml/1 unit crystalline rabbit muscle aldolase. The mixture was incubated at 30°C in the cuvette for 5 min to allow saturating concentrations of D-glyceraldehyde 3-phosphate to be formed. The enzyme sample was then added at a dilution sufficient to give a linear change of absorbance over the first 3—4 min and the initial velocity was recorded.

Protein determinations. Protein in the initial extract and the acetone fractions was determined by the biuret method and at later stages of purification from the absorbance at 280 nm [12]. The protein content of the purified enzyme was measured after precipitation with deoxycholate [13] by the

method of Lane [12] using bovine serum albumin as a protein standard.

Electrophoresis. Electrophoresis was carried out at 5°C in a 7% polyacrylamide gel at pH 8.9 [14] and in a 7.5% gel at pH 7.5 [15], after a 10 min prerun of the gel. Gels were stained for protein with 0.5% amido black in 7% (v/v) acetic acid. Gels run at pH 7.5 were stained for enzyme activity (unpublished data). SDS-polyacrylamide gel electrophoresis was carried out as described previously [16].

Inhibition studies. Inhibition of the enzyme by D-glucose 6-phosphate and D-arabinose 5-phosphate was studied at 30°C in a volume of 1.5 ml. Reaction mixtures contained 100 mM glycylglycine buffer pH 7.4/2.7 mM $MgCl_2$/0.2 mM thiamine pyrophosphate/1.3 mM dithiothreitol/0.13 mM NADH/0.3 units α-glycerophosphate dehydrogenase/0.87 units triose phosphate isomerase plus the desired concentrations of D-ribose 5-phosphate, D-xylulose 5-phosphate and inhibitor. The reaction was started by adding transketolase. Inhibition by 2 mM D-arabinose 5-phosphate and 3.3 mM D-glucose 6-phosphate was studied at a saturating concentration of 4 mM D-ribose 5-phosphate over the concentration range 0.02—0.2 mM D-xylulose 5-phosphate. Inhibition by 2 mM D-arabinose 5-phosphate and 6 mM D-glucose 6-phosphate was studied at near-saturating concentrations of 0.41 mM D-xylulose 5-phosphate over the concentration range 0.1—1.0 mM D-ribose 5-phosphate. Reciprocal plots were constructed and the best straight line through the points calculated by the method of least-squares.

Extraction of C. utilis and acetone fractionation. Batches of 50 g commercial dried *C. utilis* were extracted and after adjustment of the pH to 6.5, the extract was fractionated twice with acetone, as described previously [7]. The 25—35% and 35—50% acetone fractions were redissolved in 20 mM triethanolamine-chloride buffer, pH 7.5/1 mM EDTA. MgCl, thiamine pyrophosphate, and dithiothreitol were added to final concentrations of 1 mM, 0.3 mM and 1 mM, respectively, to stabilise the enzyme, the pH was adjusted to 7.5, and the solution stored frozen at −15°C. In this form it was stable for 1—2 months.

Elution from DEAE-cellulose. The frozen 25—35% and 35—50% acetone fractions were thawed, mixed and dialysed at 5°C for 3 h against 500 ml 20 mM sodium phosphate buffer, pH 7.2/1 mM EDTA/1 mM mercaptoethanol. The enzyme solution was clarified, to it was added one-half the volume of 10 mM sodium-citrate buffer, pH 6.0/1 mM EDTA/1 mM mercaptoethanol and the pH adjusted to 6.0 with 0.1 M HCl. It was then mixed with 100 ml of DEAE-cellulose previously equilibrated with the above 10 mM citrate buffer at pH 6.0, drained at the pump and washed with the 10 mM citrate buffer. To this was added 100 ml ice-cold 10 mM citrate buffer, pH 6.0, and the whole mixture was cooled in ice and stirred periodically. Samples of the supernatant were taken, clarified in a bench centrifuge and the absorbance at 280 nm and the enzyme activity measured. Equilibration was achieved after a few minutes stirring and adsorption of the enzyme was judged to be complete when the absorbance had fallen to a value in the region 0.3—0.6 in a 1 cm cell and the activity in the supernatant to between 2 and 4% of the total. If appreciable amounts of enzyme remained unadsorbed, more asorbent was added together with water to lower the ionic strength of the solution.

The DEAE-cellulose was poured into a short 4 cm diameter column in the

cold room and the liquid drained to the top of the column by mild suction if necessary. The adsorbent was then washed with three batches of 40 ml of the 10 mM citrate buffer and each washing was collected separately. Absorbance measurements at 280 nm indicated whether the washing was complete. The adsorbent was then eluted with batches of 40 ml 40 mM $MgCl_2$ in 20 mM sodium-citrate buffer, pH 6.0/1 mM EDTA/1 mM mercaptoethanol, and the pH of each batch was adjusted to 7.0 with 0.1 M NaOH. Elution was continued until measurements of absorbance and enzyme activity indicated that no more enzyme was being removed. The batches containing useful amounts of enzyme (usually the first two or three) were combined, mercaptoethanol added to 5 mM and concentrated to 4 ml by ultrafiltration at 5°C through a PM-30 membrane in a 50 ml cell.

Treatment with thiopropyl-Sepharose. Dithiothreitol was added to the ultrafiltrate to 10 mM and the solution was left to stand at room temperature for 1 h. The dithiothreitol was removed by gel filtration at 5°C through a 20 × 1.4 cm diameter column of coarse grade Sephadex G-25. The column was washed with 20 mM triethanolamine-chloride buffer, pH 7.4/1 mM EDTA and 4 ml fractions were collected. The enzyme fractions were combined, placed in a tube and 1 g lyophilised thiopropyl-Sepharose 6B that had been freshly swollen and washed, was added. The contents of the tube were gently mixed at room temperature by end-over-end rotation for 1 h. The gel was centrifuged down washed twice with buffer, and the supernatant and washings were combined and assayed, before being concentrated to 4 ml by ultrafiltration through a UM-10 membrane in the presence of 5 mM mercaptoethanol.

Chromatography on ribose 5-phosphate-Sepharose. Phenylmethylsulphonyl fluoride was added to the concentrated enzyme solution to 0.1 mM followed by $MgCl_2$ and thiamine pyrophosphate, to 1 mM and 0.3 mM, respectively. The solution was then dialysed overnight against 100 vol. 10 mM triethanolamine-chloride buffer, pH 7.6/1 mM EDTA/1 mM mercaptoethanol. It was then passed through a 8 × 1.2 cm column of D-ribose 5-phosphate-Sepharose 6B [8] and washed through with the same buffer. The column was eluted first with 50 mM triethanolamine-chloride buffer, pH 7.4/1 mM EDTA/1 mM mercaptoethanol and then with 4 mM D-ribose 5-phosphate dissolved in the same buffer. Finally, the column was eluted with 500 mM KCl dissolved in the 50 mM buffer and readjusted to pH 7.4. Fractions of 5 ml were collected. Active fractions eluted by ribose 5-phosphate were combined, mercaptoethanol added to 5 mM, and concentrated by ultrafiltration through a UM-10 membrane to 2 ml.

Chromatography on DEAE-Sephadex. The enzyme solution was diluted twice with water and placed on a 6 × 1 cm diameter column of DEAE-Sephadex A-50 equilibrated with 20 mM sodium-phosphate buffer, pH 7.7/1 mM EDTA/1 mM mercaptoethanol. After washing through any unadsorbed material the column was eluted with a linear gradient of 50 ml of 500 mM KCl in the above buffer running into 50 ml of the 20 mM buffer and 5-ml fractions were collected. After assay, mercaptoethanol was added to 5 mM and the fractions of highest specific activity were concentrated individually by ultrafiltration through a UM-10 membrane. The salt content was reduced by twice diluting the concentrated solution 5-times with 5 mM mercaptoethanol and concentrating again to the original volume. The absorbance at 280 nm of the

concentrated solution was measured and $MgCl_2$, thiamine pyrophosphate and dithiothreitol were added to concentrations of 1 mM, 0.3 mM and 1 mM, respectively. The enzyme was stable for several weeks at 5°C, for several months as a frozen solution at −15°C, and for over a year when lyophilized and stored at −15°C.

Results

A preliminary purification and concentration of the enzyme was achieved by acetone fractionation. Storage of the acetone fractions at −15°C often brought about a useful increase in specific activity (data not shown in Table I) due to the denaturation of some of the contaminating proteins without any significant effect on the transketolase. The insoluble denatured protein was removed when the combined acetone fractions were clarified after dialysis.

At pH 6.0 the enzyme and almost all the accompanying protein was adsorbed to DEAE-cellulose. The enzyme was selectively eluted by $MgCl_2$ as described by Specht [17]. Treatment of the concentrated eluate with thiopropyl-Sepharose [16] reduced the epimerase level to concentrations in the range 0.02—0.2 units/ml. If the preliminary reduction with 10 mM dithiothreitol was omitted, little or no epimerase was bound to the adsorbent. No binding of transketolase was ever observed so this step was extremely useful for the removal of epimerase.

Phenylmethylsulphonyl fluoride (0.1 mM) was added to inhibit proteolysis during overnight dialysis. Transketolase was stable in the presence of this inhibitor for 18 h at 5°C. Almost complete adsorption of transketolase to D-ribose 5-phosphate-Sepharose was obtained. Elution with 50 mM buffer removed considerable amounts of protein and contaminating enzymes such

TABLE I

PURIFICATION OF TRANSKETOLASE FROM 50 g DRIED *CANDIDA UTILIS*

Fraction	Activity (units)	Protein (mg)	Spec. act. (units/mg)	TK/Ep. * ratio	Yield (%)
Initial extract	1200	3600	0.3	0.8	100
30—40% acetone fraction	1050	1540	0.7	1.4	88
25—35% acetone fraction	555	405	1.4	—	46
35—50% acetone fraction	505	1120	0.5	—	42
Thawed and dialysed 25—50% acetone fractions	890	990	0.9	—	74
Conc. $MgCl_2$ eluate	396	92	4.3	3	33
Dialysed thiopropyl-Sepharose supernatant	202	70	2.9	25	17
Fractions 19—25 from the ribose 5-phosphate-Sepharose column.	180	8.6	21	400	15
Fractions 16—19 from the DEAE-Sephadex column	177	8.0	22 **	>53 000	15

* The ratio of transketolase to ribulose-5-phosphate epimerase (Tk/Ep.) activity.

** Spec. act. = 43 units/mg at near saturating substrate concentrations when the protein content was measured by deoxycholate precipitation and the method of Lowry et al. [35] as quoted by Lane [12].

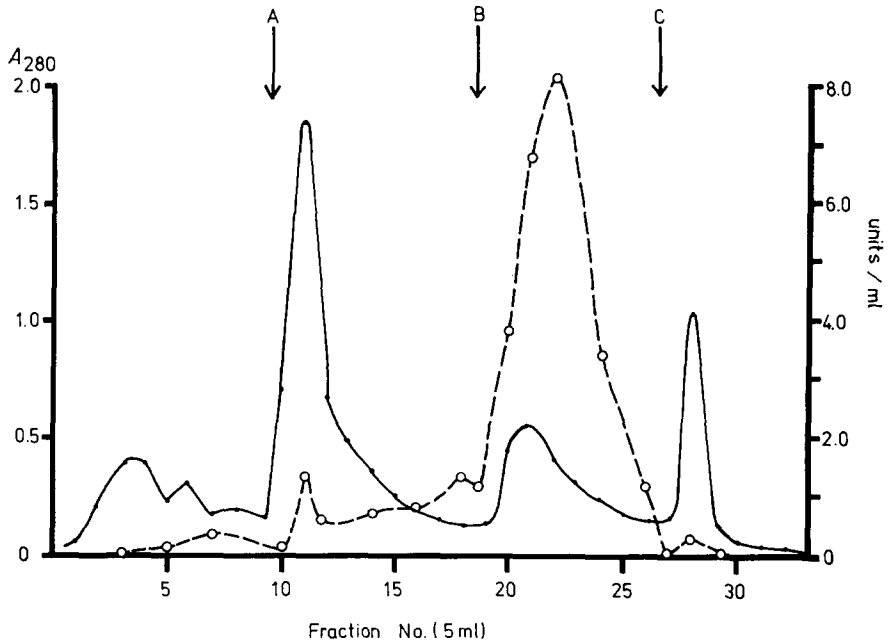

Fig. 1. Affinity chromatography of transketolase on D-ribose 5-phosphate-Sepharose 6B at 5°C and pH 7.4. The concentrated supernatant from the thiopropyl-Sepharose treatment was chromatographed on a 8 X 1.2 cm column of the adsorbent and washed through with 10 mM triethanolamine-chloride/1 mM EDTA/1 mM mercaptoethanol buffer, pH 7.4. The column was eluted at A with 50 mM triethanolamine-chloride/1 mM EDTA/1 mM merceptoethanol buffer, pH 7.4, at B with 4 mM D-ribose 5-phosphate in the 50 mM buffer, and at C with 500 mM KCl dissolved in the 50 mM buffer. Fractions of 5 ml were collected. ———, absorbance at 280 nm (A_{280}); 0- - - - -0, transketolase activity.

Distribution of enzyme activities:	tubes 10—12	tubes 19—25	tube 28
Transketolase	+	+++	+
Transaldolase	+++	trace	—
D-Glyceraldehyde 3-Phosphate dehydrogenase	+	—	+
Alcohol dehydrogenase	+++	—	+
D-Ribulose 5-phosphate 3-epimerase	—	—	+

as alcohol dehydrogenase and some transketolase complexed to glyceraldehyde-3-phosphate dehydrogenase and transaldolase (tubes 10—12, Fig. 1). Elution with 4 mM D-ribose 5-phosphate in buffer specifically eluted uncomplexed transketolase almost free from epimerase (tubes 19—25, Fig. 1 and Table I) which could be eluted later by 500 mM KCl (tube 27, Fig. 1).

The product from the affinity column was adsorbed to a DEAE-Sephadex column at pH 7.7 and eluted as a single symmetrical peak (Fig. 2), in contrast to its behaviour in the impure state when three peaks were obtained each containing the enzyme in association with transaldolase [7]. The specific activity was constant across the peak and only slightly higher than that of the material placed on the column. Gel electrophoresis of individual fractions from the peak

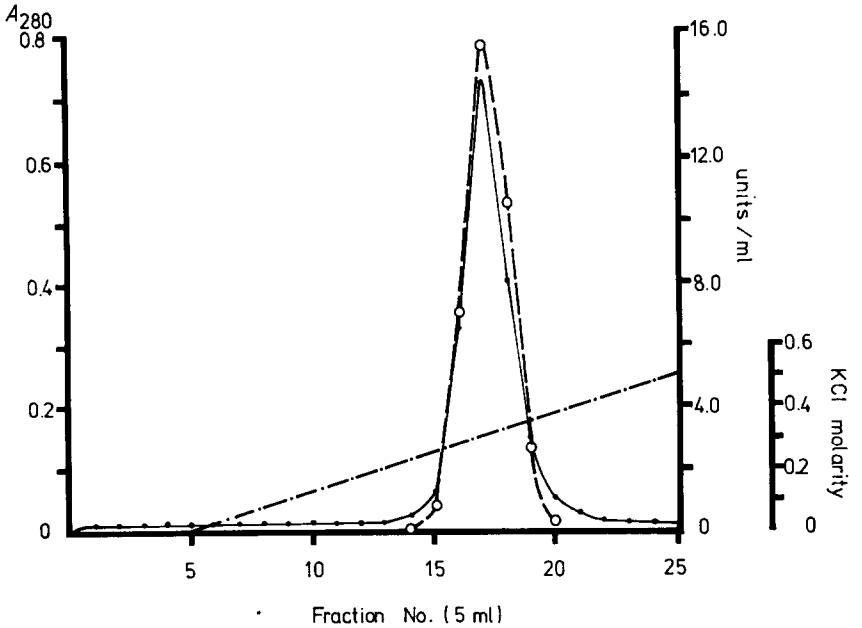

Fig. 2. Chromatography on DEAE-Sephadex at 5°C and pH 7.7. Active material from the affinity column was placed on a 6 × 1 cm column of DEAE-Sephadex A-50 previously equilibrated with 20 mM sodium-phosphate/1 mM EDTA/1 mM mercaptoethanol buffer, pH 7.7. The enzyme was eluted with a linear gradient of 50 ml of 500 mM KCl in the above buffer, running into 50 ml of the 20 mM buffer ●———●, absorbance at 280 nm (A_{280}); ○ - - - - - - ○, transketolase activity; · — · — ·, KCl gradient.

showed that the enzyme in tube 16 was homogeneous at both pH 8.9 and 7.5, while tubes 17 and 18 and the starting material were contaminated with small amounts of other proteins (Fig. 3). After staining for enzyme activity the single activity band was shown to correspond to the major protein band. Electrophoresis in sodium dodecyl sulphate (SDS)-polyacrylamide gels of the pure

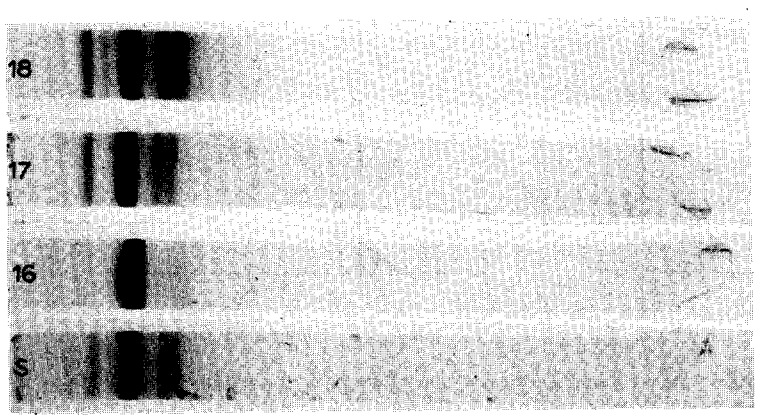

Fig. 3. Electrophoresis on polyacrylamide gel at pH 8.9 of the starting material placed on the DEAE-Sephadex column and the contents of peak tubes eluted from the column. Gels stained for protein. S = starting material (fractions 19—25 from the affinity column); 16, 17 and 18, = contents of tubes 16, 17 and 18, respectively, from the DEAE-Sephadex column.

enzyme from tube 16 gave only a single band corresponding to a molecular weight of 81 000. This confirmed the conclusion of other workers that the enzyme from *C. utilis* is made up of two identical sub-units and the sub-unit weight was closer to Klein and Brand's value of 78 000 [18] than the 70 000 reported by Specht [17].

When the protein content was based upon the absorbance at 280 nm (assuming $A^{1\%}_{280}$ equal to 10), the purified enzyme had a specific activity of 22 units/mg in the routine assay (Table I) and 30 units/mg at near saturating concentrations of 0.5 mM D-xylulose 5-phosphate and 2 mM D-ribose 5-phosphate. Measurement of the protein content by deoxycholate precipitation [13] and the procedure of Lowry et al. [35] according to Lane [12], gave an $A^{1\%}_{280}$ value of 14.4 close to the 14.5 value reported for the baker's yeast enzyme [19]. The specific activity at 30°C was therefore 43 units/mg and may be compared to values at 25°C of 15 and 25 units/mg reported for the pure enzyme from *C. utilis* [17,18] and 14—18 units/mg for the enzyme from baker's yeast [20—23].

The results of the inhibition studies are shown in Figs. 4 and 5. Double-reciprocal plots characteristic of mixed inhibition were obtained when the concentrations of either D-xylulose 5-phosphate or D-ribose 5-phosphate were varied in the presence of a saturating or near-saturating concentration of the other substrate. Values of the apparent inhibitor constants K_i and K'_i were calculated from the slopes and intercepts of the reciprocal plots and these values together with values of the apparent Michaelis constants are given in Table II. The apparent K_m values of 77 μM for D-xylulose 5-phosphate at 4 mM D-ribose 5-phosphate and of 430 μM D-ribose 5-phosphate when the D-xylulose 5-phosphate concentration was 0.4 mM were comparable to the figures of 70 μM and 300 μM reported by Specht [17]. The inhibition constants listed in Table II indicate that D-arabinose 5-phosphate is a more power-

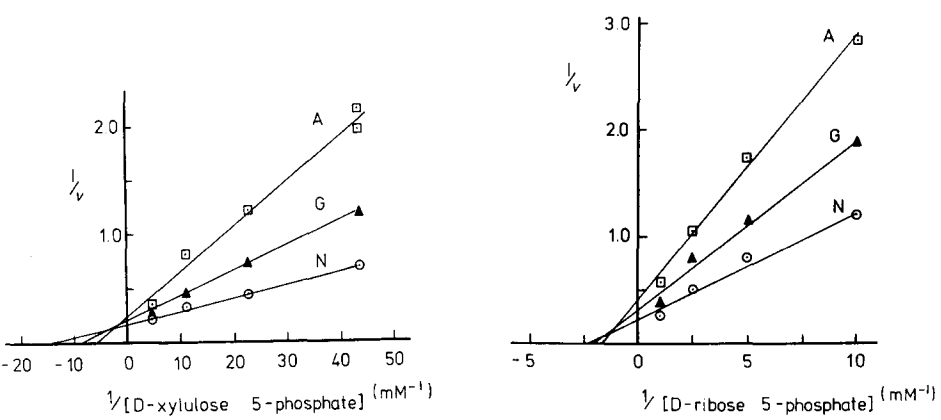

Fig. 4. Inhibition of transketolase at 4 mM D-ribose 5-phosphate with D-xylulose 5-phosphate as the variable substrate. Reaction mixtures contained 0.15 μg transketolase. v = initial velocity in nmol/min; N, no inhibitor; G, 3.3 mM D-glucose 6-phosphate; A, 2 mM D-arabinose 5-phosphate.

Fig. 5. Inhibition of transketolase at 0.41 mM D-xylulose 5-phosphate with D-ribose 5-phosphate as the variable substrate. Reaction mixtures contained 0.1 μg transketolase. v = initial velocity in nmol/min; N, no inhibitor, G, 6 mM D-glucose 6-phosphate; A, 2 mM D-arabinose 5-phosphate.

TABLE II

APPARENT MICHAELIS AND INHIBITION CONSTANTS OF *CANDIDA UTILIS* TRANSKETOLASE

Equation for mixed inhibition: $v = V[S]/K_m(1 + [I]/K_i) + [S](1 + [I]/K'_i)$. Measurements are in mM.

Variable substrate	No inhibitor K_m	D-Arabinose 5-phosphate inhibitor		D-glucose 6-phosphate inhibition	
		K_i	K'_i	K_i	K'_i
D-Ribose 5-phosphate	0.430	1.4	2.6	9.8	17
D-Xylulose 5-phosphate	0.077	0.82	3.6	3.6	13

ful inhibitor than D-glucose 6-phosphate and is effective at quite low concentrations. Incubation of 0.4 mM D-xylulose 5-phosphate with 2 mM D-arabinose 5-phosphate in the presence of 0.4 unit of pure transketolase failed to reveal any formation of D-glyceraldehyde 3-phosphate, thus confirming the previous conclusion that D-arabinose 5-phosphate did not act as an acceptor for the *C. utilis* enzyme [34].

Discussion

With a few exceptions [7,24,25] most published procedures for the purification of transketolase [17,18,26—29] pay little attention to the need to remove contaminating D-ribose-5-phosphate 3-epimerase and D-ribose-5-phosphate ketol-isomerase. In the present work, isomerase activity was removed during the acetone fractionation and was almost absent from the DEAE-cellulose eluate. Attention was therefore directed to the removal of the epimerase and the final procedure (Table I) employs two new techniques; the removal of contaminating epimerase by adsorption to thiopropyl-Sepharose and chromatography of transketolase on the affinity adsorbent D-ribose 5-phosphate-Sepharose [8]. Transketolase of a high specific activity was obtained in 15% yield with a greater than 66 000-fold increase in the transketolase/epimerase activity ratio. The final product contained approx. 0.08% transaldolase activity, 0.01% ribose-5-phosphate isomerase activity and 0.03% D-glyceraldehyde-3-phosphate dehydrogenase activity and has been routinely employed for the purposes outlined in the Introduction.

The enzyme purified by Specht from *C. utilis* [17] chromatographed as a single peak on Ultrogel AcA-44 at pH 6.0 and gave a single band on polyacrylamide gel electrophoresis. Likewise the enzyme of Klein and Brand [18] gave a single band on gel electrophoresis and they suggested that the multiple transketolase peaks observed previously [7] were elution artefacts. During the present investigation, multiple peaks have consistently been observed with impure preparations of transketolase eluted from DEAE-Sephadex, cellulose phosphate, and D-ribose 5-phosphate-Sepharose, and multiple bands have been obtained on electrophoresis gels stained for enzyme activity. Multiple peaks have also been observed after gel filtration on Sephadex G-200 and Ultrogel AcA-34. As the enzyme approached purity the multiple peaks on DEAE-Sephadex, Sephadex G-200 and D-ribose 5-phosphate-Sepharose and the multiple bands on electrophoresis disappeared. This was not due to the disap-

pearance of labile isomers of the enzyme as in many cases nearly 100% recovery of activity was obtained in a particular purification step, but appeared to be a consequence of the removal of proteins able to bind transketolase. The existence of three transaldolase-transketolase complexes was reported previously [7] and the occurence of a transketolase-D-glyceraldehyde-3-phosphate dehydrogenase complex is well documented [20,30—33]. The nature and properties of these complexes is under investigation.

The enzyme, isolated as described above, generally had the properties noted earlier by Specht [17] and by Klein and Brand [18]. As observed previously in this laboratory [34], D-glucose 6-phosphate and D-arabinose 5-phosphate inhibited the yeast enzymes and D-glucose 6-phosphate behaved as a very feeble acceptor for the baker's yeast enzyme while D-arabinose 5-phosphate did not. The evidence of mixed inhibition reported here suggests that each of these two aldose phosphates competes at both the donor and acceptor sites on the enzyme for D-xylulose 5-phosphate and D-ribose 5-phosphate, respectively. If competition had been at only one site, reciprocal plots characteristic of competitive inhibition with one substrate and of uncompetitive inhibition with the other would have been expected. As reported by Klein and Brand [18], arsenate, phosphate and sulphate ion were found to be inhibitors of the *C. utilis* enzyme, as also were oxythiamine and neopyrithiamine [8], and *p*-chloromercuribenzoate [16].

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