

# Mechanism of Action of Transketolase

## I. PROPERTIES OF THE CRYSTALLINE YEAST ENZYME\*

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For detailed studies on the mode of action of an enzyme a readily available source and a convenient method of preparation are of distinct advantage. Accessible sources such as bakers' yeast (1) and spinach leaves (2) have, therefore, been used for large-scale preparations of transketolase in spite of the fact that extracts of *Escherichia coli*, in which the cleavage of ribose-5-P to triose-P was first observed (3), or *Torula* yeast (4) are 3 to 5 times as active. With the development of cellulose derivatives for protein fractionation (5), the procurement of transketolase in sufficient quantities (4) for isolation of enzyme-substrate intermediates has become feasible (6, 7). It is the purpose of this paper to report on properties of the crystalline yeast enzyme that have not been recorded previously.

### EXPERIMENTAL PROCEDURE

#### Methods

**Substrates**—An equilibrium mixture of the D-isomers of ribose-5-P, ribulose-5-P, and xylulose-5-P was prepared as described previously (1) except that purified ribose-5-P isomerase and xylulose-5-P epimerase (8) were used instead of the crude yeast preparation. The equilibrium mixture was also prepared with spleen preparations by the method of Ashwell and Hickman (9). DL-Glyceraldehyde-3-P, D-ribose-5-P, and thiamine pyro-P were obtained from Schwarz Laboratories, Inc. The dimethyl acetal of D-erythrose-4-P was kindly donated by Dr. C. Ballou. L-Glyceraldehyde-3-P was prepared from DL-glyceraldehyde-3-P according to Venkataraman *et al.* (10). D-Arabinose-5-P was prepared as described by Levin and Racker (11). Glycolaldehyde was obtained from Aldrich Chemical Company, Inc., and lithium hydroxypyruvate was prepared by the method of Dickens and Williamson (12). Other chemicals were prepared as described previously (4, 8, 13).

**Enzymes**—In all experiments described in this paper, crystalline transketolase prepared from bakers' yeast (4) was used. Transaldolase (4), ribose-5-P isomerase (8), xylulose-5-P epimerase (8), phosphofructokinase (14), and glucose-6-P isomerase (15) were prepared as described. Glyceraldehyde-3-P dehydrogenase, glucose-6-P dehydrogenase, alcohol dehydrogenase, lactic dehydrogenase, and mixed crystals of  $\alpha$ -glycerophosphate dehydrogenase and triose phosphate isomerase were purchased from Boehringer and Sons, Mannheim, Germany.

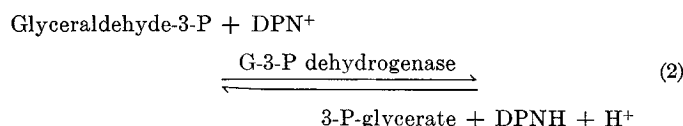
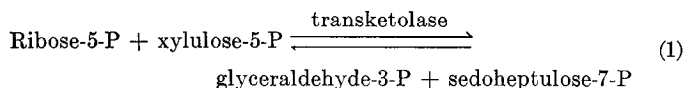
**Definition of Unit**—One unit of enzyme is defined as the

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amount of enzyme which catalyzes the turnover of 1  $\mu$ mole of substrate per minute. Specific activity is defined as units of enzyme per milligram of protein.

**Assays of Enzymes and Substrates**—Activity measurements of transketolase and quantitative determination of the pentose-P cycle intermediates were carried out as described by Cooper *et al.* (13). In the transketolase assay, 20  $\mu$ g of mixed crystals of  $\alpha$ -glycerophosphate dehydrogenase and triose phosphate isomerase were used instead of the crude rabbit muscle fraction.

**Assay of Ribose-5-P**—Ribose-5-P was assayed with transketolase in the presence of an excess of xylulose-5-P as follows:



The reaction mixture contained in a final volume of 1.0 ml, the following reagents: 25  $\mu$ moles of glycylglycine buffer, pH 7.6, 0.47  $\mu$ mole of xylulose-5-P, a sample of ribose-5-P not exceeding 0.05  $\mu$ mole, 0.6  $\mu$ mole of DPN, 0.2  $\mu$ mole of thiamine pyro-P, 2  $\mu$ moles of  $\text{MgCl}_2$ , 4.5  $\mu$ moles of arsenate, 100  $\mu$ g of glyceraldehyde-3-P dehydrogenase, and 0.5 unit of transketolase (10 units/mg). The check cells contained all reagents including the unknown sample except DPN. The total change in optical density was stoichiometric to the amount of ribose-5-P, assuming 1  $\mu$ mole of DPNH had an optical density of 6.22.

**Assay of Glycolaldehyde**—Glycolaldehyde was assayed by measuring the decrease in optical density at 340  $m\mu$  in the presence of DPNH and alcohol dehydrogenase. The reaction mixture contained in a final volume of 1.0 ml: 25  $\mu$ moles of glycylglycine buffer, pH 7.6, 0.12  $\mu$ mole of DPNH, 600  $\mu$ g of alcohol dehydrogenase, and limiting amounts of the glycolaldehyde sample (0.02 to 0.05  $\mu$ mole). The check cell contained all reagents, including the sample, except DPNH. Another control cell that contained all reagents except glycolaldehyde was used to correct for traces of DPNH oxidase present in some preparations of alcohol dehydrogenase. Optical density readings were taken before and after the addition of alcohol dehydrogenase and the reaction was followed until there was no further change in the optical density.

**Assay of Erythrulose**—Erythrulose was assayed in the same system measuring glycolaldehyde released by transketolase from erythrulose in the presence of ribose-5-P as acceptor aldehyde. The reaction mixture contained the following reagents in a final

volume of 1.0 ml: 25  $\mu$ moles of glycylglycine buffer, pH 7.6, limiting amount of erythrulose (0.02 to 0.05  $\mu$ mole), 2.0  $\mu$ moles of ribose-5-P, 0.2  $\mu$ mole of thiamine pyro-P, 2.0  $\mu$ moles of  $MgCl_2$ , 0.12  $\mu$ mole of DPNH, 1.61 units of transketolase, and 600  $\mu$ g of crystalline alcohol dehydrogenase. The check cells contained all the reagents except DPNH. The control for DPNH oxidase contained all reagents except the erythrulose sample.

*Assay of Sedoheptulose-7-P*—Sedoheptulose-7-P was measured colorimetrically according to Dische (16), or by the enzymic method (13).

*Assay of Octulose-8-P*—Glucose-6-P released by transketolase from octulose-8-P in the presence of glyceraldehyde-3-P as acceptor aldehyde was determined with glucose-6-P dehydrogenase. In a final volume of 1.0 ml, the following reagents were added: 25  $\mu$ moles of glycylglycine buffer, pH 7.6, limiting amount of octulose-8-P (0.02 to 0.05  $\mu$ mole), 0.63  $\mu$ mole of glyceraldehyde-3-P, 0.2  $\mu$ mole of thiamine pyro-P, 2.0  $\mu$ moles of  $MgCl_2$ , 0.27  $\mu$ mole of TPN, 0.1 unit of glucose-6-P dehydrogenase, and 1.0 unit of transketolase. Optical density readings were taken before and after the addition of transketolase and the reaction was followed until there was no further change in the optical density.

*Assay of Hydroxypyruvate*—Hydroxypyruvate was assayed at 340  $m\mu$  in the presence of DPNH and 15  $\mu$ g of lactic dehydrogenase.

## RESULTS

*Resolution of Crystalline Preparations of Transketolase*—Two different kinds of preparation of transketolase were used which are dependent on thiamine pyro-P for activity. One preparation was obtained by dialysis of the enzyme against 1000 volumes of 1.6 M ammonium sulfate at pH 7.8 for 48 hours. This procedure resulted in a soluble transketolase preparation which was almost completely resolved but was considerably less stable than the crystalline holoenzyme. Simpson (17) observed a similar lower stability of pork liver transketolase in the absence of thiamine pyro-P.

A second type of preparation of resolved transketolase was observed accidentally. Crystalline suspensions of transketolase that had been stored in 2 M ammonium sulfate for 8 to 12 months in the cold room were found to be quite insoluble in water or dilute buffer. Such enzyme suspensions decreased the transmission of light as shown in Fig. 1. In these experiments, 340  $m\mu$

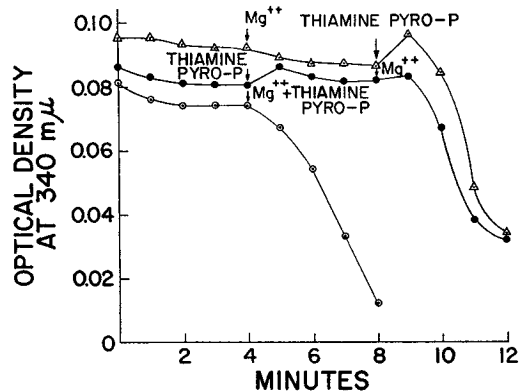


FIG. 1. The effect of  $Mg^{++}$  and thiamine pyro-P on the solubility of transketolase. In a final volume of 1 ml, the cuvettes contained 25  $\mu$ moles of glycylglycine buffer, pH 7.4, and 0.5 unit of transketolase (8.2 units/mg).  $MgCl_2$  (2.0  $\mu$ moles) and 0.2  $\mu$ mole of thiamine pyro-P were added in the order indicated in the figure.

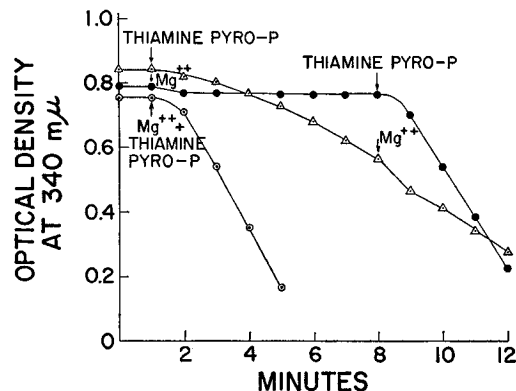


FIG. 2. The effect of the order of addition of cofactors on transketolase. In a final volume of 1.0 ml, the following reagents were added: 0.018 unit of transketolase (8.0 units/mg), 25  $\mu$ moles of glycylglycine buffer, pH 7.4, 0.9  $\mu$ mole of xylulose-5-P, 1.2  $\mu$ moles of ribose-5-P, 20  $\mu$ g of a mixture of  $\alpha$ -glycerophosphate dehydrogenase and triose phosphate isomerase, and 0.12  $\mu$ mole of DPNH.  $MgCl_2$  (2.0  $\mu$ moles) and 0.2  $\mu$ mole of thiamine pyro-P were added in the order indicated in the figure.

was selected for convenience since activity measurements were also carried out at this wave length.

Addition of  $Mg^{++}$  or thiamine pyro-P had no effect on the light scattering but when both cofactors were added, rapid solubilization and increased transmission of light took place. As can be seen from Fig. 1, the order of addition of the two cofactors did not seem to affect the rate of solubilization.

Such insoluble preparations could be washed with water thus removing contaminating proteins such as traces of xylulose-5-P 3'-epimerase. Since this useful procedure of purification was not applicable to freshly prepared crystals of transketolase, various attempts were made to accelerate the "aging process" that resulted in decreased solubility. Neither heating nor repeated fractionations of the enzyme with ethanol were successful. Since thiamine pyro-P and  $Mg^{++}$  were needed for solubilization, transketolase resolved by dialysis against 1.6 M ammonium sulfate was repeatedly precipitated with ammonium sulfate. However, the enzyme remained soluble. The process of aging for 8 to 10 months was successfully repeated three times in the course of this work.

*Effect of Order of Addition of Cofactors to Resolved Transketolase*—In contrast to the above procedure of solubilization, activity measurements of resolved enzyme by addition of thiamine pyro-P and  $Mg^{++}$  were markedly dependent on the order of addition. When thiamine pyro-P was added first and then  $Mg^{++}$ , the enzyme activity, as measured by the oxidation of DPNH, was markedly depressed as shown in Fig. 2. It appears from these experiments that thiamine pyro-P interacted with the enzyme without added  $Mg^{++}$ , resulting in a union which not only was catalytically inactive but which interfered with the proper alignment of thiamine pyro-P after  $Mg^{++}$  addition.

*Effect of pH on Transketolase Activity*—In order to avoid secondary effects of pH on other enzymes used in the spectrophotometric test, transketolase activity was determined colorimetrically by the rate of sedoheptulose-7-P formation. Fig. 3 shows the effect of pH on the formation of sedoheptulose-7-P from pentose phosphates. A pH optimum at about 7.6 was observed.

*Substrate Specificity of Yeast Transketolase*—In the course of experiments on the formation of an active glycolaldehyde enzyme

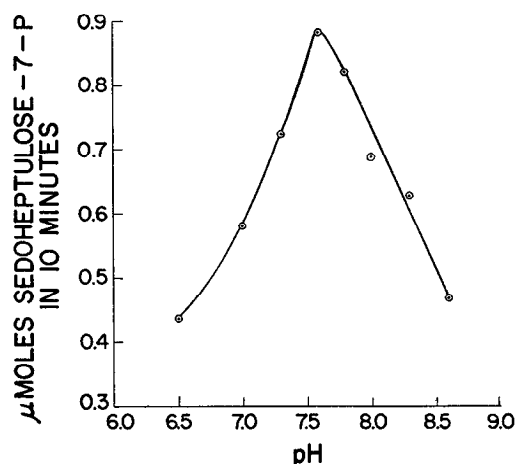


FIG. 3. The effect of pH on transketolase activity. In a final volume of 1.0 ml, 0.1 unit of transketolase (8.5 units/mg) was incubated for 10 minutes at room temperature with the following reagents: 50 μmoles of Tris buffer at different pH, 2.9 μmoles of xylulose-5-P, 3.5 μmoles of ribose-5-P, 0.2 μmole of thiamine pyro-P, and 2.0 μmoles of MgCl<sub>2</sub>. Tris-maleate buffer in the same concentration was used for the pH 6 and pH 6.5 experiments. After 10 minutes, 0.1 ml of 50% trichloroacetic acid solution was added to stop the reaction. Sedoheptulose-7-P was assayed according to the method of Dische (16) with 0.1 ml of the centrifuged solution.

intermediate (18) it was noted that with fructose-6-P as substrate considerably more erythrose-4-P was formed than the stoichiometric equivalent of the transketolase added. Formation of erythrose-4-P was measured in the presence of glyceraldehyde-3-P dehydrogenase by reduction of DPN (19, 20). To eliminate possible side reactions in the spectrophotometric test, fructose-6-P was incubated with transketolase alone and fructose-6-P disappearance was determined as shown in Table I. Since neither glycolaldehyde nor erythrulose accumulated in amounts equivalent to fructose-6-P disappearance, it was considered likely that fructose-6-P contained or gave rise to an acceptor aldehyde such as glucose-6-P. Examination of several commercial preparations of fructose-6-P revealed glucose-6-P contamination. Traces of glucose-6-P isomerase activity were found in the transketolase preparations. That transketolase, indeed, acts on glucose-6-P as acceptor aldehyde was substantiated by further experiments described below. Since the latter has not previously been shown to react, the substrate specificity of transketolase was reexamined with relatively large amounts of the enzyme.

It is already known that several keto sugars can act as donors and several aldehydes can serve as acceptors for transketolase (cf. 21). A convenient assay for the efficiency of various acceptor aldehydes was developed which depends on the rate of disappearance of hydroxypyruvate in the presence of transketolase as shown in Table II. Relatively high concentrations of enzyme were used in these experiments except with D-glyceraldehyde-3-P as acceptor. In addition to the known acceptor aldehydes mentioned above, arabinose-5-P and glucose-6-P were found to serve as acceptors under these conditions. However, little or no reactivity with L-glyceraldehyde-3-P was observed. D-Glyceraldehyde-3-P was included in the table for comparative purposes; however only 1/50 of the enzyme concentration was used. With glucose-6-P and arabinose-5-P as acceptors the product was also analyzed with Dische's cysteine-sulfuric acid reagent (16). With arabinose-5-P, a color with a maximum at 510 mμ was observed

indicating the formation of a heptulose. With glucose-6-P as acceptor, a color with an absorption maximum at 480 mμ was obtained suggesting the production of an octulose. In view of the possible metabolic significance of glucose-6-P as an acceptor, the product of this reaction was isolated and characterized.

*Preparation and Characterization of Octulose-8-P*—Octulose-8-P was prepared by incubating 80.0 μmoles of glucose-6-P at 35° in a final volume of 4.5 ml with the following reagents: 250 μmoles of glycylglycine buffer, pH 7.6, 120 μmoles of lithium hydroxypyruvate, 10 μmoles of thiamine pyro-P, 100 μmoles of MgCl<sub>2</sub>, and 386 units of transketolase (10 units/mg). When 50 μmoles (62%) of the glucose-6-P disappeared (2½ hours), the reaction was stopped and deproteinized by adding 0.5 ml of 50% trichloroacetic acid. From the supernatant solution, octulose-8-P and residual glucose 6-P were isolated as barium salt by addition of excess barium acetate and 4 volumes of ethanol. The barium salts were dissolved in water with a few drops of 1 N HCl and the barium was removed with equivalent amounts of ammonium sulfate. Octulose-8-P was then separated from glucose-6-P on a

TABLE I

*Disappearance of fructose-6-P in presence of transketolase*

In a final volume of 3 ml, 25 μmoles of glycylglycine buffer, pH 7.4, 3.4 μmoles of fructose-6-P, 4 μmoles of MgCl<sub>2</sub>, 0.4 μmole of thiamine pyro-P, and 29 units of crystalline transketolase (9 units/mg) were incubated in duplicate. A third reaction mixture was deproteinized immediately and served as zero time control. All samples were analyzed for fructose-6-P with glucose-6-P isomerase and glucose-6-P dehydrogenase, thus including in the analysis small amounts of glucose-6-P formed during the incubation.

	Fructose-6-P		
	0 hr	1 hr	2 hr
	μmoles		
Complete system.....	3.3	2.7	2.4
Transketolase omitted.....	3.3	3.2	3.25

TABLE II

*Disappearance of hydroxypyruvate in presence of different acceptors in transketolase-catalyzed reaction*

In a final volume of 2.0 ml the following reagents were added: 50 μmoles of glycylglycine buffer, pH 7.6, 2.5 μmoles of hydroxypyruvate, 2.6 μmoles of the acceptor aldehyde, 0.5 μmole of thiamine pyro-P, 5.0 μmoles of MgCl<sub>2</sub>, and 7.1 units of transketolase. In the experiment with D-glyceraldehyde-3-P as acceptor, 0.14 unit of transketolase was used instead of 7.1 units. Hydroxypyruvate was assayed at different intervals of time with lactic dehydrogenase as described under "Experimental Procedure."

Acceptor	Disappearance of hydroxypyruvate	
	1 hr	2 hr
	μmoles*	
L-Glyceraldehyde-3-P.....	0.04	0.12
D-Glyceraldehyde-3-P.....	0.518	0.53
D-Arabinose-5-P.....	0.28	0.39
D-Glucose-6-P.....	0.53	0.574

\* All values were corrected for disappearance without added acceptor aldehyde.

Dowex 1-formate column (22 cm  $\times$  1.2 sq cm) with gradient elution with 150 ml of water in the mixing chamber and 150 ml of buffer (0.5 M ammonium formate + 0.2 N formic acid) in the upper reservoir. Eluate samples of 2.5 ml each were collected and tested for sugar with anthrone (22) and cysteine-sulfuric acid (16). Octulose-8-P was eluted between 100 and 115 ml. This

TABLE III

Concentration of components of transketolase-catalyzed reaction at equilibrium

In a final volume of 2.0 ml, the following reagents were incubated at 25°: 50  $\mu$ moles of glycylglycine buffer, pH 7.6, 1.5  $\mu$ moles of xylulose-5-P, 1.75  $\mu$ moles of ribose-5-P, 0.2  $\mu$ mole of thiamine pyro-P, 2.0  $\mu$ moles of MgCl<sub>2</sub>, and 0.8 unit of transketolase. Small aliquots were removed at 10-minute intervals and glyceraldehyde-3-P production was measured. When the glyceraldehyde-3-P value became constant (usually within 40 minutes under this condition) 0.2 ml of 50% trichloroacetic acid was added and the mixture was centrifuged. The supernatant solution was neutralized and analyzed for the products. Values are expressed as total  $\mu$ moles.

Substrates	0 time	40 min	$\Delta$
Xylulose-5-P.....	1.47	0.649	0.821
Ribose-5-P.....	1.83	1.09	0.74
Sedoheptulose-7-P.....	0.189	0.948	0.759
Glyceraldehyde-3-P.....	0.133	0.883	0.750

TABLE IV

Equilibrium constants with different substrates

System 1a. In a final volume of 2.0 ml, 0.82 unit of transketolase (8 units/mg) was incubated in quadruplicate at 25° with the following reagents: 50  $\mu$ moles of glycylglycine buffer, pH 7.6, 1.55  $\mu$ moles of xylulose-5-P, 1.58  $\mu$ moles of ribose-5-P, 0.2  $\mu$ mole of thiamine pyro-P, and 2.0  $\mu$ moles of MgCl<sub>2</sub>. After incubating for various intervals of time (0, 10, 20, and 40 minutes), 0.2 ml of 50% trichloroacetic acid was added and the mixtures were centrifuged. The supernatant solutions were adjusted to pH 6.8 and analyzed as described under "Experimental Procedure."

System 1b. In a final volume of 2.0 ml, 1.7 units of transketolase (11 units/mg) were incubated at 25° with the following reagents: 50  $\mu$ moles of glycylglycine buffer, pH 7.6, 2.62  $\mu$ moles of sedoheptulose-7-P, 2.12  $\mu$ moles of glyceraldehyde-3-P, 0.2  $\mu$ mole of thiamine pyro-P, and 2.0  $\mu$ moles of MgCl<sub>2</sub>. The rest of the procedure was the same as for system 1a.

System 2. In a final volume of 2.0 ml, 1.6 units of transketolase (10 units/mg) were incubated with 2.02  $\mu$ moles of xylulose-5-P and 1.93  $\mu$ moles of erythrose-4-P, 0.2  $\mu$ mole of thiamine pyro-P, and 2.0  $\mu$ moles of MgCl<sub>2</sub>. Since trichloroacetic acid inhibits glucose-6-P dehydrogenase, metaphosphoric acid was used for deproteinization.

System 3. In a final volume of 2.0 ml, 2.0 units of transketolase (9 units/mg) were incubated at 25° with 2.0  $\mu$ moles of fructose-6-P, 2.0  $\mu$ moles of glycolaldehyde, 0.2  $\mu$ mole of thiamine pyro-P, and 2.0  $\mu$ moles of MgCl<sub>2</sub>. The rest of the procedure was the same as for system 2.

System	Reaction	Equilibrium constants	
		20 min	40 min
1a	Xu-5-P + R-5-P $\rightleftharpoons$ S-7-P + G-3-P..	1.28	1.18
1b	S-7-P + G-3-P $\rightleftharpoons$ Xu-5-P + R-5-P..	0.922	1.04
2	Xu-5-P + E-4-P $\rightleftharpoons$ F-6-P + G-3-P..	8.7	11.9
3	F-6-P + glycolaldehyde $\rightleftharpoons$ eryth- rulose + E-4-P.....	0.0152	0.0150

fraction was free of glucose-6-P as tested with glucose-6-P dehydrogenase and TPN. With Dische's cysteine-sulfuric acid reagent (16) it gave a maximal absorption band at 480 m $\mu$  indicating the presence of an octulose. Barium acetate (300  $\mu$ moles) was added to this fraction and the pH of the solution was adjusted to 7.8. Four volumes of ethanol were then added and the mixture was kept in the refrigerator overnight. The barium salt was collected by centrifugation, washed once with ethanol and once with ether, and dried in a vacuum desiccator; 35 mg of barium salt were obtained. Ten milligrams of this barium salt were converted to the sodium salt in a final volume of 2 ml and assayed for octulose-8-P as described under "Experimental Procedure." The enzymatic assay and total phosphorus gave values of 3.8  $\mu$ moles/ml and 4.2  $\mu$ moles/ml, respectively. Periodate oxidation of this material was measured according to Marinetti and Rouser (23). Octulose-8-P utilized 5.98  $\mu$ moles of periodate per  $\mu$ mole of substrate (as measured by total phosphorus) and 6.46  $\mu$ moles per  $\mu$ mole (as measured by enzymic analysis). The theoretical expected value is 6  $\mu$ moles. Octulose-8-P (1.5  $\mu$ moles) was dephosphorylated with 0.45 unit ( $\mu$ moles of P<sub>i</sub> hydrolyzed per minute) of potato acid phosphatase,<sup>1</sup> deionized, and chromatographed.<sup>2</sup> The material gave the characteristic color changes for octulose in the orcinol spray and traveled with the same R<sub>F</sub> as synthetic D-glycero-D-ido-octulose in two solvent systems which readily separate this octulose from the octuloses of D-glycero-D-manno, D-glycero-D-gulo, and D-glycero-D-altro configuration.

*Equilibrium Constants with Different Substrates*—For equilibrium studies, the substrates were allowed to react in the presence of transketolase, Mg<sup>++</sup>, and thiamine pyro-P until the reaction came to a standstill. The four reactants, *i.e.* two substrates and two products were measured by specific enzymic methods (13). From the data shown in Table III it can be seen that the disappearance of xylulose-5-P and ribose-5-P agrees well with the appearance of sedoheptulose-7-P and glyceraldehyde-3-P. Equilibrium constants obtained with fructose-6-P and glyceraldehyde-3-P as well as with glycolaldehyde as acceptor are recorded in Table IV. In the case of the first reaction listed, the equilibrium was also measured by starting with sedoheptulose-7-P and glyceraldehyde-3-P as substrates and reasonable agreement was obtained with the forward reaction. With xylulose-5-P and erythrose-4-P as substrates, the equilibrium constant was calculated to be 11.9. This value is quite different from the value of unity estimated for the equilibrium constant of this reaction by Horecker *et al.* (2).

*Effect of Concentration of Substrate*—*K<sub>m</sub>* for xylulose-5-P in the presence of 0.005 M ribose-5-P was  $2.1 \times 10^{-4}$ . *K<sub>m</sub>* values for some other substrates are also given in Table V. It can be seen that xylulose-5-P has the greatest affinity for transketolase and that of erythrulose is less than that of fructose-6-P. The Michaelis constant for the acceptor aldehyde, ribose-5-P, in the presence of 0.002 M xylulose-5-P was  $4 \times 10^{-4}$  M.

*Inhibitors of Transketolase*—There are few compounds that are known to inhibit transketolase. Iodoacetate, *p*-chloromercuribenzoate, or *N*-ethylmaleimide<sup>3</sup> are without effect. Sulfate and phosphate ions were found to inhibit transketolase approximately 50% between 0.01 and 0.02 M concentration. Oxythiamine

<sup>1</sup> A. Kornberg, unpublished procedure.

<sup>2</sup> We wish to thank Dr. H. H. Sephton for carrying out these chromatographic experiments.

<sup>3</sup> G. de la Haba and A. G. Datta, unpublished data.

TABLE V

## Michaelis constants of various substrates

System 1. In a final volume of 1.0 ml, 0.013 unit of transketolase (10 units/mg) and the following reagents were pipetted into a microcuvette with a light path of 10 mm: 25  $\mu$ moles of glycylglycine buffer, pH 7.6, various concentrations of xylulose-5-P (0.033 to 0.52  $\mu$ mole), 5.0  $\mu$ moles of ribose-5-P, 0.2  $\mu$ mole of thiamine pyro-P, 2.0  $\mu$ moles of MgCl<sub>2</sub>, 0.12  $\mu$ mole of DPNH, and 20  $\mu$ g of a mixture of  $\alpha$ -glycerophosphate dehydrogenase and triose phosphate isomerase. The rates of DPNH oxidation were measured at 340 m $\mu$ .

System 2. In a final volume of 1.0 ml, 0.36 unit of transketolase (9 units/mg) was incubated at room temperature with the following reagents: 25  $\mu$ moles of glycylglycine buffer, pH 7.4, various concentrations of fructose-6-P (0.1 to 4.0  $\mu$ moles), 6.0  $\mu$ moles of ribose-5-P, 0.2  $\mu$ mole of thiamine pyro-P, and 2.0  $\mu$ moles of MgCl<sub>2</sub>. After 10 minutes, 0.1 ml of 50% trichloroacetic acid was added. After centrifugation, 0.3 ml of the supernatant solution was used for the colorimetric determination of sedoheptulose-7-P (16).

System 3. In a final volume of 1.0 ml, 0.45 unit of transketolase was incubated at room temperature with the following reagents: 25  $\mu$ moles of glycylglycine buffer, pH 7.4, different concentrations of erythulose (1 to 6  $\mu$ moles), 11.4  $\mu$ moles of glyceraldehyde-3-P, 0.2  $\mu$ mole of thiamine pyro-P, and 2.0  $\mu$ moles of MgCl<sub>2</sub>. After 10 minutes, 0.1 ml of 50% trichloroacetic acid was added. After centrifugation, the supernatant solution was adjusted to pH 7.0 and a 0.2-ml sample was analyzed for glycolaldehyde.

System 4. In a final volume of 1.0 ml, the following reagents were pipetted into a microcuvette with a light path of 10 mm: 25  $\mu$ moles of glycylglycine buffer, pH 7.6, 1.9  $\mu$ moles of xylulose-5-P, various concentrations of ribose-5-P (0.06 to 0.6  $\mu$ mole), 0.6  $\mu$ mole of DPN, 4.5  $\mu$ moles of arsenate, 0.2  $\mu$ mole of thiamine pyro-P, 2.0  $\mu$ moles of MgCl<sub>2</sub>, and 100  $\mu$ g of glyceraldehyde-3-P dehydrogenase. Transketolase (0.015 unit) was added last and the rate of increase in optical density at 340 m $\mu$  was followed.

In all cases, Lineweaver-Burk plots were used to determine the  $K_m$  values.

System	Substrate	Cosubstrate	$K_m$
1	Xylulose-5-P	Ribose-5-P (0.005 M)	$2.1 \times 10^{-4}$
2	Fructose-6-P	Ribose-5-P (0.01 M)	$1.8 \times 10^{-3}$
3	Erythulose	Glyceraldehyde-3-P (0.011 M)	$4.9 \times 10^{-3}$
4	Ribose-5-P	Xylulose-5-P (0.0019 M)	$4.0 \times 10^{-4}$

pyro-P<sup>4</sup> has been found to be a potent inhibitor of resolved transketolase. As shown in Fig. 4, oxythiamine pyro-P in a final concentration of  $3.6 \times 10^{-5}$  M and  $7.2 \times 10^{-5}$  M (Curves 4 and 5) inhibited transketolase approximately 60 and 80%. Addition of 0.5  $\mu$ mole of thiamine pyro-P 4 minutes later (arrow) did not restore the activity. Oxythiamine pyro-P added 2 minutes after addition of thiamine pyro-P had no effect on the initial rate of transketolase (Curve 2). Simultaneous addition of oxythiamine pyro-P and a 100-fold excess of thiamine pyro-P still resulted in partial inhibition (Curve 3). These findings suggested that oxythiamine pyro-P had a considerably greater affinity for the enzyme than thiamine pyro-P. It could therefore be expected that in the course of time, oxythiamine pyro-P should displace thiamine pyro-P from the holoenzyme. When 0.013 unit of holoenzyme was incubated with 0.012  $\mu$ mole of oxythiamine pyro-P and 25.0  $\mu$ moles of glycylglycine buffer, pH 7.6, a

<sup>4</sup> We wish to thank Dr. W. Bartley for a generous gift of oxythiamine pyro-P.

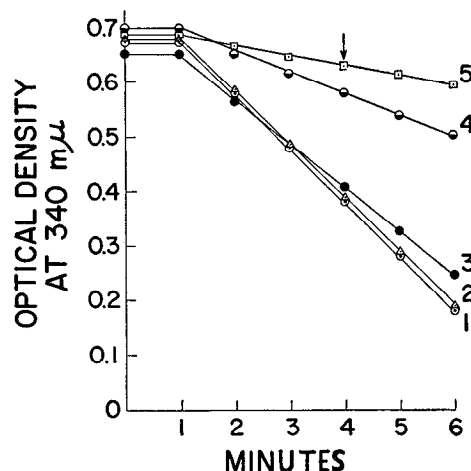


FIG. 4. The effect of oxythiamine pyro-P on transketolase. In a final volume of 0.25 ml, the following reagents were added in the order given below: 0.015 unit of transketolase (8.5 units/mg), 25  $\mu$ moles of glycylglycine buffer, pH 7.6, 2.0  $\mu$ moles of MgCl<sub>2</sub>, and different concentrations of oxythiamine pyro-P and thiamine pyro-P, individually or jointly. After 3 minutes, 0.2  $\mu$ mole of thiamine pyro-P, 0.12  $\mu$ mole of DPNH, 20  $\mu$ g of  $\alpha$ -glycerophosphate dehydrogenase and triose phosphate isomerase mixture, and sufficient water to make a final volume of 0.95 ml were added. A solution (0.05 ml) containing 0.9  $\mu$ mole of xylulose-5-P and 1.2  $\mu$ moles of ribose-5-P was added at 1 minute to start the reaction. Curve 1, control; Curve 2,  $3.6 \times 10^{-5}$   $\mu$ moles of oxythiamine pyro-P were added 2 minutes after the addition of  $3 \times 10^{-2}$   $\mu$ moles of thiamine pyro-P; Curve 3,  $3.6 \times 10^{-5}$   $\mu$ moles of oxythiamine pyro-P and  $3 \times 10^{-2}$   $\mu$ moles of thiamine pyro-P were added simultaneously; Curve 4,  $3.6 \times 10^{-5}$   $\mu$ moles of oxythiamine pyro-P; Curve 5,  $7.2 \times 10^{-5}$   $\mu$ moles of oxythiamine pyro-P. After 4 minutes 0.5  $\mu$ mole of thiamine pyro-P was added as indicated by the arrow in the figure.

progressive inhibition with time was observed (Fig. 5, Curve 2) indicating a slow displacement of enzyme-bound thiamine pyro-P. There was very little inactivation after 4 hours at room temperature in the control containing holoenzyme alone (Curve 1). Attempts to displace oxythiamine pyro-P attached to the enzyme with excess thiamine pyro-P were less successful. Although approximately 1500-fold excess of thiamine pyro-P over oxythiamine pyro-P was used, only 20% of the original activity was restored in 3 hours (Curve 3). In contrast to these findings, the inhibition of wheat germ pyruvic decarboxylase by oxythiamine pyro-P (0.388  $\mu$ M) is reversed by about 30% with the addition of thiamine pyro-P (0.195  $\mu$ M) 20 minutes after the addition of oxythiamine pyro-P (24).

In the course of assays of thiamine pyro-P in transketolase preparations, it was observed that on boiling of a resolved transketolase preparation, a compound was released which was strongly inhibitory to the enzyme. Similar to oxythiamine pyro-P, the inhibitor was most effective when added to resolved enzyme before the addition of thiamine pyro-P. Thiamine monophosphate did not inhibit under these conditions.

## DISCUSSION

In early stages of investigations, enzymes are often considered to be rather specific for a given substrate, usually a metabolic intermediate. Later on when the enzyme becomes available in larger quantities, additional substrates are found to be utilized by the enzyme, although frequently at a much lower rate. In the

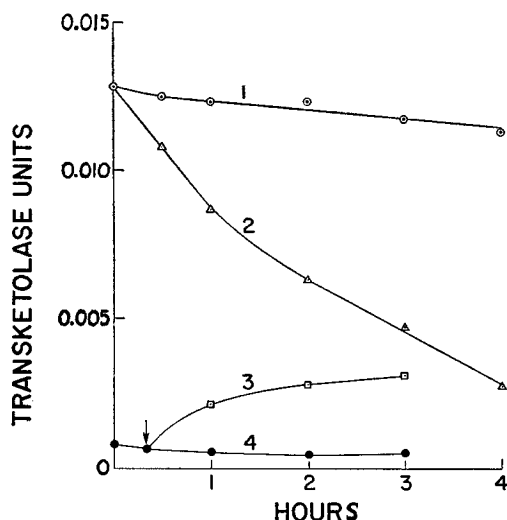


FIG. 5. Curves 1 and 2. Displacement of thiamine pyro-P by oxythiamine pyro-P. Thiamine pyro-P (43  $\mu$ moles) was added to 28.4 units of transketolase (7.5 units/mg) in a final volume of 0.5 ml and incubated at room temperature for 5 minutes. Saturated ammonium sulfate (4.0 ml) was then added and the mixture was centrifuged. The precipitate was washed twice with 4.0 ml of 70% saturated ammonium sulfate and dissolved in 0.4 ml of 0.25 M glycylglycine buffer, pH 7.6. This enzyme was almost fully active without the addition of thiamine pyro-P. This holoenzyme (1.37 units) was incubated in a final volume of 0.5 ml at room temperature with 0.012  $\mu$ mole of oxythiamine pyro-P and 25  $\mu$ moles of glycylglycine buffer, pH 7.6. Samples of the incubation mixture were withdrawn at the intervals indicated in the figure and assayed for transketolase without further addition of thiamine pyro-P. Curve 1, control without oxythiamine pyro-P; Curve 2, oxythiamine pyro-P treated.

Curves 3 and 4. Displacement of oxythiamine pyro-P by thiamine pyro-P. In a final volume of 1.0 ml, 0.26 unit of resolved transketolase was incubated with 25  $\mu$ moles of glycylglycine buffer, pH 7.6, 2.0  $\mu$ moles of  $MgCl_2$ , and 0.0012  $\mu$ mole of oxythiamine pyro-P in duplicate. Thiamine pyro-P (2.0  $\mu$ moles) was added to one tube as indicated by the arrow. An 0.05 ml aliquot was used for measuring transketolase activity. All assays in this experiment were carried out in the presence of the complete assay system including the usual amount of thiamine pyro-P. Curve 3, thiamine pyro-P added; Curve 4, no thiamine pyro-P added.

case of transketolase the number of compounds known to serve either as ketol group donor or as aldehyde acceptor is slowly increasing. The demonstration in this paper of glucose-6-P as acceptor aldehyde for crystalline transketolase is of particular interest for two reasons. The demonstration of octuloses in plants (25) and the presence of what appears to be an octulose-P in red blood cells<sup>5</sup> may find an explanation in the enzymic formation of octulose-8-P either via transaldolase or via transketolase. It should be emphasized that the octulose-8-P described in this paper is distinct from that formed from ribose-5-P and fructose-6-P by transaldolase (26). Although both octulose-8-P preparations were shown to serve as ketol group donors for transketolase, the octulose-8-P prepared with transaldolase gave rise to allose-6-P, whereas the octulose-8-P described in this paper gave rise to glucose-6-P. These observations indicate that the two preparations of octulose-8-P differ only in the OH position at C-5, but insufficient material was available for structural studies. The configuration of the D-glycero-D-manno-octulose found in avocado

<sup>5</sup> G. R. Bartlett, personal communication.

fruit does not suggest a direct pathway via either transketolase or transaldolase. However, the presence of a second octulose<sup>6</sup> in avocado which has not been identified as yet makes it likely that epimerizations and transformations take place at the octulose level. Similar considerations apply to the utilization of arabinose-5-P by transketolase. Mannoheptulose (27) and mannoheptulose-P which are readily demonstrated in extracts of avocado fruits<sup>7</sup> may be visualized to arise by epimerization of sedoheptulose-7-P. Alternatively, ribose-5-P may be converted to arabinose-5-P (11) which may be channelled into heptulose synthesis.

A second aspect of the function of glucose-6-P as acceptor aldehyde concerns a puzzling finding made independently in various laboratories (28, 29).<sup>8</sup> When fructose-6-P or glucose-6-P was added to enzyme preparations that contained transketolase and transaldolase, the formation of heptulose-P took place at a relatively rapid rate in spite of the fact that no acceptor aldehyde such as glyceraldehyde-3-P or erythrose-4-P was added. With the demonstration of glucose-6-P as acceptor aldehyde for transketolase, these findings may be partially explained by the formation of sufficient erythrose-4-P to initiate the transketolase-transaldolase-catalyzed transformation of glucose-6-P to heptulose-P.

*Interaction of Thiamine Pyro-P and Oxythiamine Pyro-P with Transketolase*—Oxythiamine pyro-P is inhibitory to carboxylase (24) but the affinity of the natural coenzyme to the apoenzyme is greater than that of the inhibitor. In the case of transketolase, the inhibitor has an affinity of several orders greater than thiamine pyro-P. This suggests the possible use of oxythiamine pyro-P as specific metabolic inhibitor in an analysis of transketolase participation in multienzyme systems in cell-free preparations.

That thiamine pyro-P may become attached to transketolase in more than one way is indicated by the experiments revealing an inhibitory effect of the thiamine pyro-P when added to the enzyme before  $Mg^{++}$ . In order to obtain a fully active catalytic site,  $Mg^{++}$  may have to act as a ligand between the enzyme and the coenzyme.

Multiple attachments of thiamine pyro-P are suggested by experiments in which transketolase was treated with large amounts of the coenzyme and reprecipitated several times to remove the excess. Direct analysis indicated as many as 9 moles of thiamine pyro-P per mole of enzyme. The role of thiamine pyro-P as a carrier of active glycolaldehyde will be discussed in the accompanying paper.

#### SUMMARY

1. Transketolase from yeast becomes insoluble in water after storage for several months at 0° as a crystalline suspension in 2 M ammonium sulfate. The enzyme dissolves readily on addition of  $Mg^{++}$  and thiamine pyrophosphate.

2. Arabinose 5-phosphate and glucose 6-phosphate serve as acceptor aldehydes for yeast transketolase. With the latter, an octulose 8-phosphate accumulated which was isolated as a barium salt.

3. Equilibrium constants and  $K_m$  values with different substrate pairs are reported.

4. Oxythiamine pyrophosphate is a potent inhibitor of trans-

<sup>6</sup> N. K. Richtmyer, personal communication.

<sup>7</sup> M. Tabachnick, unpublished observation.

<sup>8</sup> G. de la Haba, unpublished observation.

ketolase. Its affinity to the apoenzyme is several orders of magnitude greater than that of thiamine pyrophosphate.

5. The significance of glucose 6-phosphate as a substrate for transketolase, the formation of octulose 8-phosphates, and the interaction between transketolase and thiamine pyrophosphate are discussed.

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