

choline phosphate should prove to be of great interest. It is also being determined whether, upon administration to rats and mice, 2-aminoethylphosphonic acid can be incorporated into the phospholipids in the place of 2-aminoethyl phosphate.

REFERENCES

- Awapara, J., Landua, A. J., and Fuerst, A. (1950), *J. Biol. Chem.* 183, 545.
 Baxter, C. F., and Roberts, E. (1958), *J. Biol. Chem.* 233, 1135.
 Chen, P. S., Toribara, T. Y., and Warner, H. (1956), *Anal. Chem.* 28, 1756.
 Horiguchi, M., and Kandatsu, M. (1959), *Nature* 184, 901.
 Horiguchi, M., and Kandatsu, M. (1960), *Bull. Agr. Chem. Soc. Japan* 24, 565.
 Kittredge, J. S., Simonsen, D. G., Roberts, E., and Jelinek, B. (1962), in *Amino Acid Pools: Composition, Formation and Function*, Holden, J. T., editor, Amsterdam, Elsevier Publishing Company.
 Kosolapoff, G. M. (1947), *J. Am. Chem. Soc.* 69, 2112.
 Kosolapoff, G. M. (1950), *Organophosphorus Compounds*, New York, John Wiley and Sons, p. 156.
 Norman, J. M., and Dawson, R. M. C. (1953), *Biochem. J.* 54, 396.
 Roberts, E., and Lowe, I. P. (1954), *J. Biol. Chem.* 211, 1.
 Roberts, E., and Simonsen, D. G. (1960), in *Amino Acids, Proteins, and Cancer Biochemistry*, Edsall, J. T., editor, New York, Academic Press, Inc., p. 121.
 Smith, R. H. (1954), *Biochem. J.* 57, 140.

The Enzymic Production of 2,5-D-Threo-diketo-hexose*

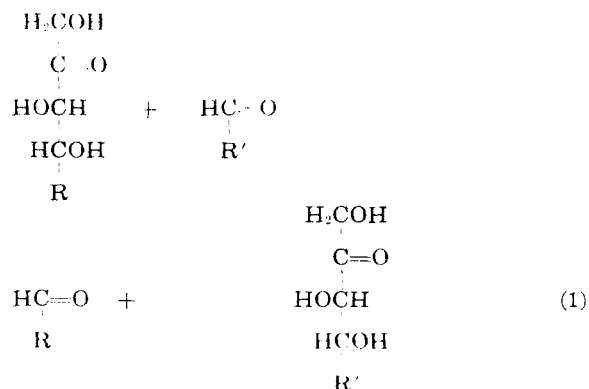
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The enzymic synthesis of 2,5-D-threo-diketo-hexose has been achieved. The compound has been obtained as a product of a transaldolase reaction between fructose 6-phosphate and hydroxypyruvic aldehyde. The nature of the compound has been established by paper chromatography after reduction to the corresponding alcohols and by periodate oxidation of the isolated product before and after NaBH₄ reduction. Further identification was provided by the susceptibility of the reduction product to oxidation by an enzyme of known stereospecificity.

The enzyme transaldolase catalyzes an exchange reaction in which a dihydroxyacetone group is transferred from a ketose donor to an aldehyde acceptor. The reaction may be represented as follows:



As indicated in equation (1), both the substrate and the product which is formed must have hydroxyls in the *trans* configuration in positions 3 and 4 (Bonsignore, 1959). In addition to

erythrose 4-phosphate and glyceraldehyde 3-phosphate, which were the first acceptors described for this reaction (Horecker and Seegmiller, 1953), other aldehydes have been recognized to react, including D-ribose 5-phosphate (Racker, 1955-1956), L-glyceraldehyde 3-phosphate (Venkataraman *et al.*, 1960), D-erythrose (Prandini and Lopes do Rosario, 1960), D-glyceraldehyde (Bonsignore *et al.*, 1959), and formaldehyde (Venkataraman and Racker, 1961). Some of the products of the transaldolase reaction are familiar metabolites. Others, like octulose 8-phosphate, which is formed when ribose 5-phosphate is the acceptor, were first synthesized by this enzymic reaction and only later demonstrated in biological materials (Charlson and Richtmyer, 1960).

Except for formaldehyde all of the known acceptors for transaldolase possess a hydroxyl group in the position adjacent to the aldehyde group. We have now found that hydroxypyruvic aldehyde, which does not possess such a hydroxyl group, may also act as an acceptor for transaldolase. The 2,5-diketo-hexose which is formed has been characterized as 2,5-D-threo-diketo-hexose, possessing the configuration of the hydroxyl groups in positions 3 and 4 which would be expected from the known specificity of trans-

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TABLE I
REACTION OF FRUCTOSE 6-PHOSPHATE AND HYDROXYPYRUVIC ALDEHYDE

Compounds	Minutes				
	0 (μ moles)	30 (μ moles)	90 (μ moles)	120 (μ moles)	210 (μ moles)
Fructose 6-phosphate	40.0	34.2	28.0	26.6	24.8
Hydroxypyruvic aldehyde	40.0	—	—	—	—
Total triose phosphate	—	6.0	11.6	13.0	14.7

The incubation mixture (4.0 ml) contained 1 mg of transaldolase (specific activity 200 units/mg of protein), 0.01 M fructose 6-phosphate, 0.01 M hydroxypyruvic aldehyde, 0.04 M triethanolamine buffer, pH 7.5, 5.10^{-4} M versene, 5 mg egg albumin. This last serves to protect transaldolase from inactivation by hydroxypyruvic aldehyde. The temperature was 37°. The reaction was begun by the addition of the enzyme. Aliquots were assayed at intervals for fructose 6-phosphate and triose phosphate.

aldolase. The compound is therefore identical with the one chemically synthesized by Micheel and Horn (1934) and shown by the same authors to be rapidly fermented by yeast. No compounds of this type have been so far described in biological materials, the only comparable compound reported being the product of oxidation of fructose by *Acetobacter suboxydans*, 6 - aldehydofructose (Weidenhagen and Bernsee, 1960). It is possible that a metabolic relationship may exist between 2,5-diketohexose and 6-aldehydofructose.

METHODS

Materials.—Crystalline transaldolase (Pontremoli *et al.*, 1960 and in press) and polyol dehydrogenase (Chakravorty *et al.*, in press) were prepared as previously described. Glycerophosphate dehydrogenase and a glycerophosphate dehydrogenase preparation containing D-glyceraldehyde 3 - phosphate isomerase were obtained from Böehringer und Soehne, as were D-glucose 6-phosphate dehydrogenase, aldolase, D-fructose 6-phosphate (Ca salt), and sodium pyruvate. TPN, DPNH, DPN, and D-glucose 6-phosphate isomerase were obtained from the Sigma Chemical Corp.

Hydroxypyruvic aldehyde was prepared and characterized according to the procedure of Evans *et al.* (1938).

Anion (AG 3X4) and cation (AG 50 WX4) exchange resins were obtained from California Corp.

Analytical Methods.—D-Fructose 6-phosphate and triose phosphate (D-glyceraldehyde 3-phosphate plus dihydroxyacetone phosphate) were determined enzymatically according to the procedures described by Racker and co-workers (Klybas *et al.*, 1959). For sugar chromatography the solvent systems were phenol-water (1:0.395 w/w) and *n*-butanol-pyridine-water (6:4:3). The samples were generally chromatographed on Whatman No. 1 paper for 24 hours in the descending manner. The spots were visualized with the aniline phthalate (Partridge, 1949) or orcinol (Bevenue and Williams, 1951) spray reagents.

For alcohols the moving solvent was ethylacetate-pyridine-water saturated with boric acid (60:25:20) (Grado and Ballou, 1961). The

spots were visualized with periodate benzidine spray (Viscontini *et al.*, 1955). For good separation the temperature was kept between 28° and 30°.

Reduction with NaBH_4 .—The reduction of 2,5-diketohexose to the corresponding alcohols was carried out in 0.1 M phosphate buffer, pH 7.5, and NaBH_4 was added gradually up to a concentration of 1 mg/ml. The concentration of 2,5-diketohexose was 10^{-3} M. The reduction was followed by determination of the disappearance of reducing power with the resorcinol-thiourea color reaction (Roe and Papadopoulos, 1954). The reaction was complete in 90–120 minutes; the solution was then deionized by treatment with cationic and anionic resins.

Oxidation with Bromine.—The samples in 0.15 M phosphate buffer, pH 7.5, were shaken for 20 minutes at 35° with 0.05 M Br_2 . This period of time was found to be sufficient to oxidize hydroxypyruvic aldehyde almost completely to hydroxypyruvic acid, which was then removed by treatment with mixed bed resins. This treatment did not produce significant changes in the properties of the diketohexose as shown by paper chromatography before and after bromine oxidation.

Periodate Oxidation.—The reduction of periodate was followed spectrophotometrically at a wave length of 223 m μ (Dixon and Lipkin, 1954) in a Beckman cuvet containing 10^{-4} M sodium periodate and 0.05 M phosphate buffer, pH 7.5.

RESULTS

Synthesis of the Product with Transaldolase.—In a reaction mixture containing fructose 6-phosphate, hydroxypyruvic aldehyde, and transaldolase, triose phosphate appears in amounts stoichiometric to the disappearance of fructose 6-phosphate, in agreement with the general mechanism of a transaldolase reaction (Table I).

Chromatographic Evidence of the Formation of a New Product.—As shown in Table II, the paper chromatograms revealed the appearance of two new compounds, one corresponding to dihydroxyacetone and the other (green with aniline-phthalate spray) to an unknown compound having an R_F different from that of the dephosphorylated

TABLE II
CHROMATOGRAPHIC ANALYSIS OF THE DEPHOSPHORYLATED TRANSALDOLASE REACTION MIXTURE

Solvent	Fructose (R_{DHA})	Hydroxy- pyruvic Aldehyde (R_{DHA})	Di- hydroxy- acetone (R_{DHA})	New Product (R_{DHA})
Phenol-water (1:0.395, w/w)	0.67	0.26	1.00	0.35
<i>n</i> -Butanol- pyridine- water (6:4:3)	0.76	1.01	1.00	0.68

The reaction mixtures were as in the legend to Table I. After incubation for 210 minutes 0.5 ml of the solution was deproteinized with perchloric acid and neutralized with KOH in the cold. $KClO_4$ was removed by centrifugation and the supernatant solution was brought to pH 5 with acetic acid and treated for 3 hours at 37° with 4 mg of acid phosphatase (Sigma). The solution was then deionized as previously described and chromatographed with the two solvent systems shown. After 24 hours the chromatograms were dried and sprayed with aniline-phthalate or with the orcinol reagents (the latter to detect fructose). The values of R_{DHA} for the compounds are the ratios of their mobility to that of dihydroxyacetone on the same chromatogram.

substrates. Only a faint spot corresponding to glyceraldehyde was present. This is not surprising, since glyceraldehyde 3-phosphate isomerase is present in trace amounts in the transaldolase preparation, and under the conditions of our experiment most of the triose phosphate formed is converted to dihydroxyacetone.

Identification of 2,5-Diketohehexose.—In order to remove fructose 6-phosphate and triose phosphate, another aliquot of the incubation mixture was treated with a mixed bed resin. Hydroxypyruvic aldehyde which remained was converted to hydroxypyruvic acid with bromine oxidation and the solution again deionized. The resulting solution contained only the new compound and this was calculated by periodate oxidation to be present in a quantity corresponding to about 90% of the triose phosphate formed, assuming five equivalents of periodate to be consumed per mole of compound (see below). The sample was then treated with $NaBH_4$ until disappearance of reducing groups was complete. After reduction the incubation mixture was deionized by successive treatment with cation (AG 50 W X4) and anion (AG 3 X4) resins, and aliquots were chromatographed for 24 hours as described under Methods. Three spots were present, corresponding to mannitol, sorbitol, and iditol (Table III).

Reduction of 2,5-D-threo-diketohehexose would be expected to yield D-mannitol, D-sorbitol, and L-iditol. In order to prove that the products corresponded to these compounds rather than to the

TABLE III
CHROMATOGRAPHY OF THE ALCOHOLS WHICH ARE FORMED AFTER 2,5-DIKETOHEXOSE REDUCTION

Compounds	R_{gly}
Iditol	0.09
Dulcitol	0.22
Sorbitol	0.27
Mannitol	0.39
Reduced compound I	0.09
Reduced compound II	0.27
Reduced compound III	0.39

The values of R_{gly} for the compounds are the ratio of their mobility to that of glycerol on the same chromatogram.

optical enantiomorphs, the reduction products obtained with the 2,5-diketohehexose and $NaBH_4$ were analyzed with polyol dehydrogenase purified from *Candida utilis* (Chakravorty *et al.*, in press). This enzyme is active with D-mannitol, D-sorbitol, and L-iditol, but not with L-mannitol, L-sorbitol, or D-iditol. For this purpose the 2,5-diketohehexose product was first separated by chromatography with the solvent system *n*-butanol-pyridine-water, and then reduced with $NaBH_4$ as described under Methods. The hexitols obtained were oxidized with a coupled system (Chakravorty *et al.*, in press) containing polyol dehydrogenase, DPN, pyruvate, and lactic dehydrogenase, and the quantity of ketose formed determined by the resorcinol-thiourea reaction. As shown in Table IV, there is agreement between the quantity of

TABLE IV
OXIDATION WITH POLYOL DEHYDROGENASE OF THE ALCOHOLS FORMED AFTER 2,5-DIKETOHEXOSE REDUCTION

Compounds	μ Moles
Hexitols added	0.28
Ketoses formed	0.27

The incubation mixture (0.19 ml) contained 1.5×10^{-3} M total hexitol, 16×10^{-3} M pyruvate, 1.5×10^{-3} M DPN, 0.18 mg of polyol dehydrogenase (specific activity 6 units/mg of protein), 0.05 mg of lactic dehydrogenase, and 0.15 M glycine-NaOH buffer, pH 8.6. Temperature was 37°. The quantity of hexitol was determined by periodate oxidation.

the polyalcohols added (as measured by periodate consumption) and the quantity of the ketoses formed following oxidation with polyol dehydrogenase. Thus all of the hexitol produced was active as substrate for the polyol dehydrogenase, and carbon atoms 3 and 4 must possess the D-threo configuration.

The formation of only three hexitols, all with the *trans* configuration in positions 3 and 4, proves that the product of the enzymic reaction is a sugar derivative with a six-carbon chain, two carbonyl groups in positions 2 and 5, and the *trans* configuration of the hydroxyl groups in 3 and 4. The absence of aldehyde groups is evident from the

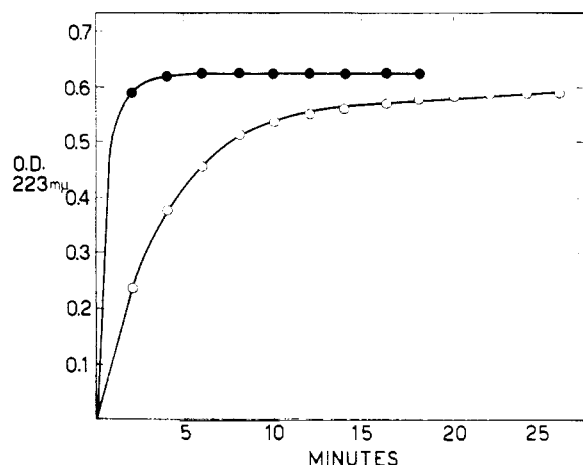


FIG. 1.—Oxidation of 2,5-diketohehexose with sodium periodate before and after reduction with NaBH_4 . Filled circles: 2,5-diketohehexose. Open circles: mixture of the three hexitols derived from 2,5-diketohehexose reduction. The enzymic product prepared as described in Table I was purified by ion-exchange chromatography and bromine oxidation as described under "Identification of 2,5-diketohehexose." The two curves were obtained with amounts of the product equivalent to $1/1000$ of the original incubation mixture (Table I). One aliquot was reduced with NaBH_4 and treated as described in the text; the other had received no further treatment before reaction with periodate. An increase in optical density of 0.5 corresponds to the complete oxidation of $0.01 \mu\text{moles}$ of a mannitol standard.

fact that bromine treatment does not alter the product with respect to its behavior in both paper and ion-exchange chromatography. This structure therefore corresponds to 2,5-D-threo-diketohehexose.

Oxidation of 2,5-Diketohehexose with Sodium Periodate Before and After Reduction with NaBH_4 .—Periodate oxidation was carried out in parallel on aliquots of the solution containing the enzymic product alone, before and after reduction with NaBH_4 . To prevent loss of the alcohols, NaBH_4 was not removed with ion-exchange resins but was destroyed by bringing the pH of the solution to 4.5 with acetic acid and then by heating for 3 minutes at 100° . As indicated in Figure 1, the same quantity of periodate is consumed by the nonreduced and reduced samples, suggesting that five periodate equivalents are utilized in both cases. This provides a very simple way to determine the concentration of a pure sample of 2,5-diketohehexose.

DISCUSSION

The results presented in this paper represent a further demonstration of the synthetic activity of the enzyme transaldolase. The synthesis of 2,5-D-threo-diketohehexose, previously chemically obtained (Micheel and Horn, 1934), represents the first report of this compound in a biological system and raises the question of its possible biological significance. It will be of interest to determine whether this compound can be interconverted enzymically with 6-aldehydo-fructose, which has been reported to occur in cultures of *Acetobacter suboxidans* grown on fructose (Weidenhagen and Bernsee, 1960).

REFERENCES

- Bevenue, A., and Williams, K. T. (1951), *Arch. Biochem. Biophys.* 34, 225.
- Bonsignore, A. (1959), V Jornadas Bioquimicas Latinas, Barcelona.
- Bonsignore, A., Pontremoli, S., Grazi, E., and Mangiarotti, M. (1959), *Biochem. Biophys. Res. Commun.* 1, 79.
- Chakravorty, M., Veiga, L. A., Bacila, M., and Horecker, B. L. (1962), *J. Biol. Chem.* (in press).
- Charlson, A. J., and Richtmyer, N. R. (1960), *J. Am. Chem. Soc.* 82, 3428.
- Dixon, J. S., and Lipkin, D. (1954), *Anal. Chem.* 26, 1092.
- Evans, W. E., Jr., Carr, C. J., and Krantz, J. C. (1938), *J. Am. Chem. Soc.* 60, 1628.
- Grado, C., and Ballou, C. E. (1961), *J. Biol. Chem.* 236, 54.
- Horecker, B. L., and Seegmiller, P. Z. (1953), *J. Am. Chem. Soc.* 75, 2021.
- Klybas, V., Shramm, M., and Racker, E. (1959), *Arch. Biochem. Biophys.* 80, 229.
- Micheel, F., and Horn, K. (1934), *Ann.* 515, 1.
- Partridge, S. M. (1949), *Nature* 164, 443.
- Pontremoli, S., Bonsignore, A., Grazi, E., and Horecker, B. L. (1960), *Rev. españ. fisiol.* 16, Suppl. II, 111.
- Pontremoli, S., Prandini, B., Bonsignore, A., and Horecker, B. L. (1962), *Proc. Nat. Acad. Sci. U. S.* (in press).
- Prandini, B., and Lopes do Rosario, J. A. (1960), *Boll. soc. it. biol. sper.* 36, 1224.
- Racker, E. (1955-56), *Harvey Lect.* 143.
- Roe, J. H., and Papadopoulos, N. M. (1954), *J. Biol. Chem.* 210, 703.
- Venkataraman, R., Datta, A. G., and Racker, E. (1960), *Fed. Proc.* 18, 82.
- Venkataraman, R., and Racker, E. (1961), *J. Biol. Chem.* 236, 1876.
- Viscontini, M., Kock, D., and Karrer, P. (1955), *Helv. Chim. Acta* 38, 643.
- Weidenhagen, R., and Bernsee, G. (1960), *Chem. Ber.* 93, 2924.