In words: during the small part of the time in which the molecule is in an aggregate, it relaxes much faster (15 times) than the monomer, and during that time it relaxes toward the little enhanced steady-state magnetization $M_0 \times 1.05$. The effects of the aggregation are on one hand to raise the average relaxation rate by ~30% and on the other to decrease the Overhauser enhancement by ~20%, relative to the respective values for the case of a never-aggregated molecule. But, whereas there are no "never-aggregated" data with which to compare the observed relaxation time, the effect is clearly visible on the Overhauser enhancement, because its value for the never-aggregated case is known to be exactly 0.5.

Reaction of the Carbanionic Aldolase–Substrate Intermediate with Tetranitromethane. Identification of the Products, Hydroxypyruvaldehyde Phosphate and D-5-Ketofructose 1,6-Diphosphate[1]

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Abstract: The reaction of the carbanion–enamine intermediate of the aldolase dihydroxyacetone phosphate complex ($^-CHOHC{=}NH^+RCH_2OPO_3^{2-}$; H_2NR = lysyl residue of aldolase) with tetranitromethane resulted in irreversible modification of the substrate. The nature of the reaction has now been elucidated by isolation and identification of the products. The products were separated by anion exchange chromatography and their structures deduced from their chromatographic and spectral properties and from their specificity as substrates in enzymatic analyses. The effect of tetranitromethane is an oxidation of the carbanion–enamine intermediate of dihydroxyacetone phosphate to hydroxypyruvaldehyde phosphate ($CHOCOCH_2OPO_3^{2-}$), the reagent being reduced to nitrite and nitroformate. A secondary product is generated in an aldolase-catalyzed condensation of hydroxypyruvaldehyde phosphate with dihydroxyacetone phosphate, yielding D-5-ketofructose 1,6-diphosphate. The occurrence of specific oxidation of aldolase-activated dihydroxyacetone phosphate at C-3 thus accounts for the trapping of the carbanion by tetranitromethane. The susceptibility of the carbanion to oxidation may indicate a general applicability of suitable oxidants as mechanistic probes of nonenzymatic and enzymatic reactions involving carbanionic intermediates.

Intermediates of enzymatic reactions can be detected and identified on the basis of the specific reactivity which certain groups of the enzyme–substrate complex acquire transiently in the course of catalysis. Such syncatalytic reactivity changes may involve certain groups both of the enzyme and of the substrate moiety of the enzyme–substrate complex.[2] The latter possibility has been exemplified by an intermediate in the reaction mechanism of fructose 1,6-diphosphate aldolase which was found to be selectively reactive toward tetranitromethane.[3,4]

The aldolase-catalyzed cleavage–condensation reactions are thought to involve an intermediary carbanion on C-3 of dihydroxyacetone phosphate which is evidenced by stereospecific pro-*S* hydrogen isotope exchange on C-3 in the absence of an aldehyde.[5,6] In class I aldolases the intermediary carbanion apparently is stabilized by resonance with a protonated Schiff base of C-2 to an active-site lysyl ε-amino group;[7] in class II aldolases it is stabilized by interaction with a zinc atom at the active site.[8-10] Kinetic examination of the reaction of tetranitromethane with the aldolase–substrate intermediate as well as the selective reactivity of the reagent toward the carbanionic forms of carbon acids[11] indicated a carbanion trapping action of the reagent. Tetranitromethane reacts also with a number of catalytic systems other than aldolase which are thought to involve carbanionic intermediates, *i.e.*, pyridoxal plus glutamate,[3] aspartate aminotransferase plus the substrate analog *erythro*-β-hydroxyaspartate,[12] and the substrate complexes of pyruvate decarboxylase and of 6-phosphogluconate dehydrogenase.[13]

During the reaction of the ternary system of aldolase, substrate, and tetranitromethane the concentration of substrate was found to decrease progressively, thus indicating that the substrate was modified irreversibly.[3] In the present study the substrate derivative produced in the reaction of tetranitromethane with the aldolase–

(1) This work was supported by Schweizerischer Nationalfonds Grant No. 3,220,69.
(2) P. Christen, *Experientia*, **26**, 337 (1970).
(3) P. Christen and J. F. Riordan, *Biochemistry*, **7**, 1531 (1968).
(4) J. F. Riordan and P. Christen, *ibid.*, **8**, 2381 (1969).
(5) I. A. Rose, *Brookhaven Symp. Biol.*, **15**, 293 (1962).
(6) J. F. Biellmann, E. L. O'Connell, and I. A. Rose, *J. Amer. Chem. Soc.*, **91**, 6484 (1969).
(7) B. L. Horecker, P. T. Rowley, E. Grazi, T. Cheng, and O. Tchola, *Biochem. Z.*, **338**, 36 (1963).
(8) W. J. Rutter, *Fed. Proc.*, **23**, 1248 (1964).
(9) R. D. Kobes, R. T. Simpson, B. L. Vallee, and W. J. Rutter, *Biochemistry*, **8**, 585 (1969).
(10) A. S. Mildvan, R. D. Kobes, and W. J. Rutter, *ibid.*, **10**, 1191 (1971).
(11) P. Christen and J. F. Riordan, *Anal. Chim. Acta*, **51**, 47 (1970).
(12) S. V. Shlyapnikov and M. Y. Karpeisky, *Eur. J. Biochem.*, **11**, 424 (1969).
(13) M. J. Healy and P. Christen, *Experientia*, **28**, 736 (1972); *Biochemistry*, in press.

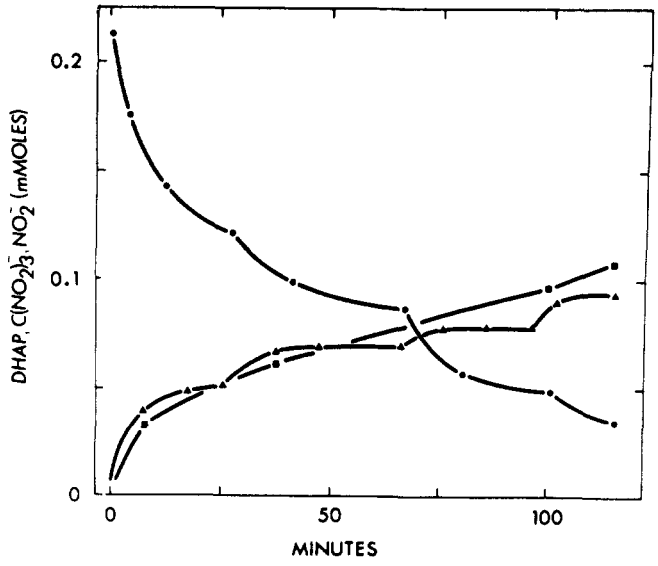


Figure 1. Reaction of the aldolase–dihydroxyacetone phosphate complex with tetranitromethane, disappearance of dihydroxyacetone phosphate (DHAP), and production of nitroformate and nitrite. To 0.215 mmol of dihydroxyacetone phosphate in an initial volume of 8.7 ml at pH 6.0, 2 ml (20 mg) of carboxypeptidase A treated aldolase in 0.01 *M* sodium acetate was added, followed by addition of 0.5 ml of 0.28 *M* tetranitromethane in absolute ethanol. Additions of aldolase and tetranitromethane were repeated after 30, 73, and 100 min. During the reaction the pH of the reaction mixture was kept at 6.0 by adding 1 *N* sodium hydroxide. Dihydroxyacetone phosphate (●), nitroformate (▲), and nitrite (■) were determined as described in the Experimental Section. As the reaction progressed part of the enzyme precipitated due to chemical modification.[3] The precipitate was removed by centrifugation after 60 min and at the termination of the reaction. Although progress to only 85% loss of dihydroxyacetone phosphate is shown, all substrate can be consumed if further large aldolase additions are made.

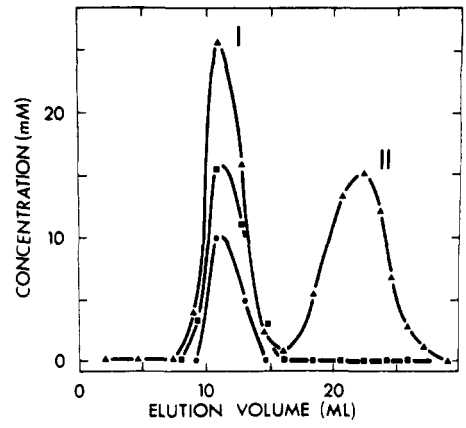


Figure 2. Anion exchange chromatography of dihydroxyacetone phosphate derivatives. The reaction mixture (see Figure 1) was freed from protein by ultrafiltration and then applied to the top of a 0.9 × 21 cm² column of AG1-X2-chloride equilibrated with H_2O. Elution was carried out with 0.2 *N* HCl at a flow rate of 0.4–0.5 ml/min: total phosphate ester (▲), dihydroxyacetone phosphate (●), hydroxypyruvaldehyde phosphate determined with glyoxalase I (■). Nitroformate was retained at the top of the column.

dihydroxyacetone phosphate intermediate has been isolated. The primary product of the reaction has been identified as hydroxypyruvaldehyde phosphate (hydroxymethylglyoxal phosphate). A secondary product is generated in an aldolase-catalyzed condensation of hydroxypyruvaldehyde phosphate with dihydroxyacetone phosphate, yielding D-5-ketofructose 1,6-diphosphate. The present data support the postulate of a reaction of tetranitromethane with the intermediate carbanion of C-3 of aldolase-bound dihydroxyacetone phosphate, the overall effect being an oxidation of an alcohol to an aldehyde group. Recent experiments have demonstrated that the same oxidative reaction is achieved with other oxidants besides tetranitromethane, *e.g.*, by oxidation–reduction indicators.[13] The susceptibility of carbanions to oxidation as evidenced in the present study thus provides the basis for a perhaps generally feasible chemical probing for these intermediates.

Results

The reaction of tetranitromethane with the carbanionic aldolase–dihydroxyacetone phosphate complex consumes dihydroxyacetone phosphate on the one hand and produces nitrite and nitroformate on the other (Figure 1). About 1.6 times more substrate disappears than nitrite and nitroformate are produced. In the absence of aldolase, dihydroxyacetone phosphate does not react with tetranitromethane. The enzyme serves as a catalyst and is not implicated in the stoichiometry of the reaction.[14]

The derivatives of dihydroxyacetone phosphate were isolated from the reaction mixture by anion exchange chromatography (Figure 2). The recovery of organic phosphate after chromatography was 84% of the initial dihydroxyacetone phosphate (0.215 mmol). The ester phosphates were equally distributed in two peaks. Peak I (0.09 mmol) proved to contain both unmodified dihydroxyacetone phosphate (0.026 mmol) and a derivative thereof (0.064 mmol). The latter ester phosphate (hereafter referred to as ester phosphate I) reacted quantitatively with reduced glutathione in the presence of glyoxalase I (Figure 2). In view of the known specificity of glyoxalase I for ketoaldehydes[15] this result indicated that this ester phosphate was hydroxypyruvaldehyde phosphate (hydroxymethylglyoxal phosphate), $CHOCOCH_2OPO_3^{2-}$. This was confirmed by alkaline dismutation (see Methods) of ester phosphate I to DL-3-phosphoglycerate and subsequent assay with D-3-phosphoglycerate kinase, which showed the formation of D-3-phosphoglycerate with a yield of 50% from the initial ketoaldehyde.

The identification of ester phosphate I as hydroxypyruvaldehyde phosphate was consistent with the analytical data obtained from its disemicarbazone and 2,4-dinitrophenylosazone. These derivatives were prepared from material of peak I which was free of dihydroxyacetone phosphate and which was obtained by continuing a reaction similar to that of Figure 1 with further aldolase additions until all dihydroxyacetone phosphate had been consumed. The microanalysis of

(14) Though aldolase serves solely as a catalyst in regard to the tetranitromethane–carbanion reaction, it becomes progressively inactivated by modification of side-chain groups (*cf.* legend of Figure 1). Since the rate of enzyme inactivation decreases with decreasing pH (J. F. Riordan and P. Christen, *Biochemistry*, 7, 1525 (1968)), the reaction was carried out at pH 6 rather than at the pH optimum of aldolase. In order to minimize further the consumption of enzyme, carboxypeptidase A treated aldolase instead of native aldolase was used, since the modified enzyme is more active in nitroformate production from tetranitromethane.[3]

(15) (a) E. Knox, *Enzymes*, 2, 253 (1960); (b) H. C. Reeves and S. J. Ajl, *J. Biol. Chem.*, 240, 569 (1965).

the disemicarbazone (mp 218–220°) was consistent with a formulation as hydroxypyruvaldehyde phosphate disemicarbazone. *Anal.* Calcd for $C_5H_{11}N_6O_6P \cdot H_2O$ (mol wt, 300) (disemicarbazone of hydroxypyruvaldehyde phosphate): C, 20.00; H, 3.67; P, 10.30. Found: C, 20.38; H, 3.74; P, 9.12 (determined as phosphate).

The 2,4-dinitrophenylosazone of ester phosphate I melted at 156° as reported for the 2,4-dinitrophenylosazone of hydroxypyruvaldehyde phosphate[16a] and gave the following microanalysis. *Anal.* Calcd for $C_{15}H_{18}O_{12}N_8P$ (mol wt, 528) (2,4-dinitrophenylosazone of hydroxypyruvaldehyde phosphate): C, 34.10; H, 2.46; N, 21.20. Found: C, 33.95; H, 2.78; N, 20.84.

The infrared spectra of the 2,4-dinitrophenylosazone of ester phosphate I and of the 2,4-dinitrophenylosazone of dihydroxyacetone phosphate were identical (major infrared absorption bands: 1614 (s), 1582 (s), 1497 (s), 1425 (m), 1335 (s), 1270 (m), 1220 (m), 1140 (s), 1090 (s), 830 (m), 740 (m) cm^{-1}.[16b]

The ester phosphate of peak II (Figure 2) gave, in contrast to ester phosphate I, a negative glyoxalase I assay, and after alkali treatment assayed less than 5% for D-3-phosphoglycerate. Ester phosphate II had a distinctly higher affinity for the anion exchange resin than the triose monophosphates, dihydroxyacetone phosphate and hydroxypyruvaldehyde phosphate (Figure 2), a property expected of an ester diphosphate.[17] Parallel behavior was observed on thin-layer chromatography of the esters, where ester phosphate II moved similarly to fructose 1,6-diphosphate (Table I). This

Table I. Thin-Layer Chromatography of Dihydroxyacetone Phosphate Derivatives

Compound	R_f	Color
Ester phosphates[a]		
ester phosphate I	0.22	Blue
ester phosphate II	0.05	Blue
fructose 1,6-diphosphate	0.04	Blue
Dephosphorylated sugars[b]		
ester phosphate II[c]	0.1	Orange-brown
ester phosphate II[c,d]	0.1/0.3	Orange-brown–blue-green
sorbose	0.32	Blue-green
fructose	0.33	Blue-green
dihydroxyacetone	0.56	Pink

[a] Solvent system 1, detection with acid molybdate spray. [b] Solvent system 2, detection of ketoses with urea–phosphoric acid spray. [c] After treatment with alkaline phosphatase. [d] Partial reduction with $NaBH_4$ (see text).

suggested that ester phosphate II was the product of a secondary, aldolase-catalyzed condensation of dihydroxyacetone phosphate and hydroxypyruvaldehyde phosphate to D-5-ketofructose 1,6-diphosphate.[18,19]

(16) (a) R. H. Weaver and H. A. Lardy, *ibid.*, **236**, 313 (1961); (b) the infrared spectrum of the 2,4-dinitrophenylosazone derivative of hydroxypyruvaldehyde phosphate will appear following these pages in the microfilm edition of this volume of the journal. Single copies may be obtained from the Business Operations Office, Books and Journals Division, American Chemical Society, 1155 Sixteenth St., N.W., Washington, D. C. 20036, by referring to code number JACS-72-7911. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche.

(17) A. A. Benson, *Methods Enzymol.*, **3**, 110 (1957).

(18) W. J. Rutter, *Enzymes*, **5**, 341 (1961).

(19) F. Leuthardt in "Handbuch der Physiologisch- und Pathologisch-Chemischen Analyse," Hoppe-Seyler/Thierfelder, Vol. 6, Part C, Springer-Verlag, West Berlin, 1966, p 607.

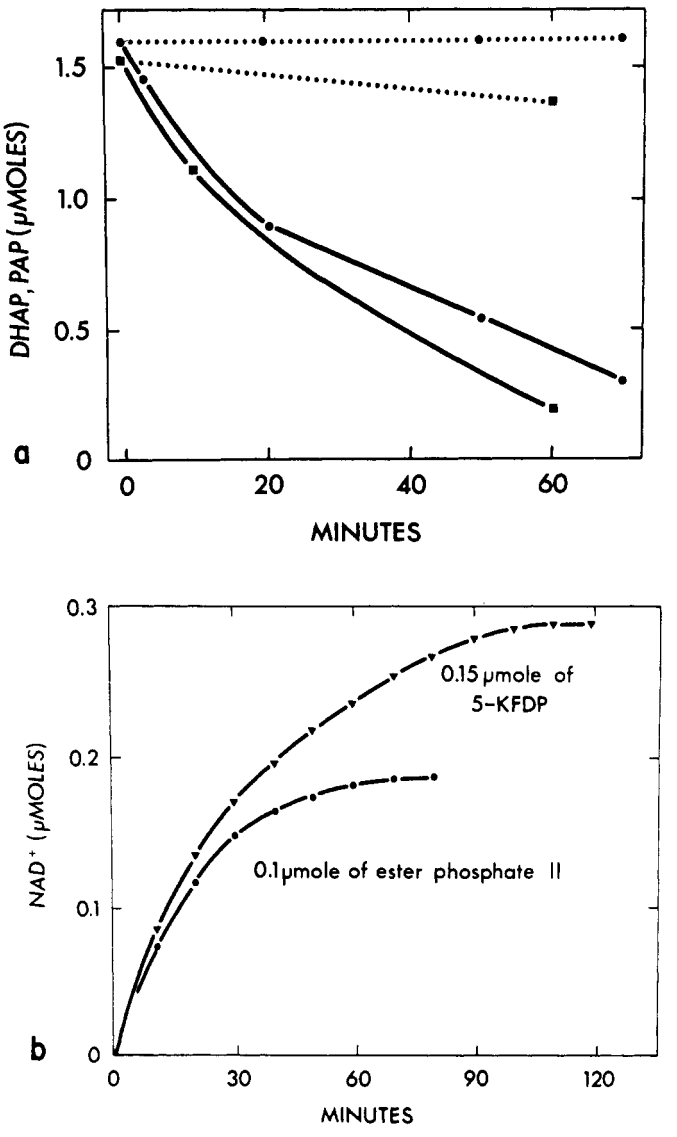


Figure 3. (a) Aldolase-catalyzed condensation of hydroxypyruvaldehyde phosphate (PAP) and dihydroxyacetone phosphate (DHAP). To hydroxypyruvaldehyde phosphate and dihydroxyacetone phosphate, 1.6 μmol each, at room temperature in 0.1 *M* sodium citrate buffer (pH 6.0) 3 mg of carboxypeptidase A treated aldolase was added (final volume 0.9 ml). At timed intervals, aliquots of 20 μl of the reaction mixture were added to 2.7 ml of 0.1 *M* Tris–Cl (pH 7.5) and assayed for dihydroxyacetone phosphate (-●-); 50-μl aliquots were added to 2.7 ml of 0.1 *M* potassium phosphate buffer (pH 6.8) and assayed for hydroxypruvaldehyde phosphate (-■-). In control experiments when aldolase was added either to dihydroxyacetone phosphate (···●···) or to hydroxypruvaldehyde phosphate (···■···), neither one of the triose phosphates was consumed. Further, in the absence of aldolase, no decrease in the concentration of dihydroxyacetone phosphate occurred in a mixture of the two triose phosphates. (b) Aldolase-catalyzed cleavage of ester phosphate II and of D-5-ketofructose 1,6-diphosphate (5-KFDP). The reaction mixtures at 25° contained 250 μmol of Tris–chloride, 0.72 μmol of NADH, 8 units of L-glycerol 3-phosphate dehydrogenase, 1.5 units of triose phosphate isomerase, 1 mg (21 units) of aldolase, and 0.1 μmol (0.2 μmol of organic phosphate) of ester phosphate II (●), *or* 0.15 μmol (0.3 μmol of organic phosphate) of the end product from the reaction of Figure 3a (▲) in a total volume of 2.7 ml (pH 7.6). Hydroxypyruvaldehyde phosphate is slowly reduced, presumably to L-glyceraldehyde 3-phosphate (*cf.* stereospecificity of L-glycerol 3-phosphate dehydrogenase) (T. Baranowski, *J. Biol. Chem.*, **180**, 535 (1949)) which is not a substrate for the D isomer specific triose isomerase (P. Oesper and O. Meyerhof, *Arch. Biochem. Biophys.*, **27**, 223 (1950)) and so is not converted to dihydroxyacetone phosphate.

In fact, addition of carboxypeptidase A treated aldolase to equimolar quantities of dihydroxyacetone phosphate and hydroxypyruvaldehyde phosphate led to virtually complete disappearance of both triose phosphates (Figure 3a). The resulting condensation product, D-5-ketofructose 1,6-diphosphate, and ester phosphate II were cleaved by aldolase at about the same slow rate (Figure 3b), suggesting their identity.

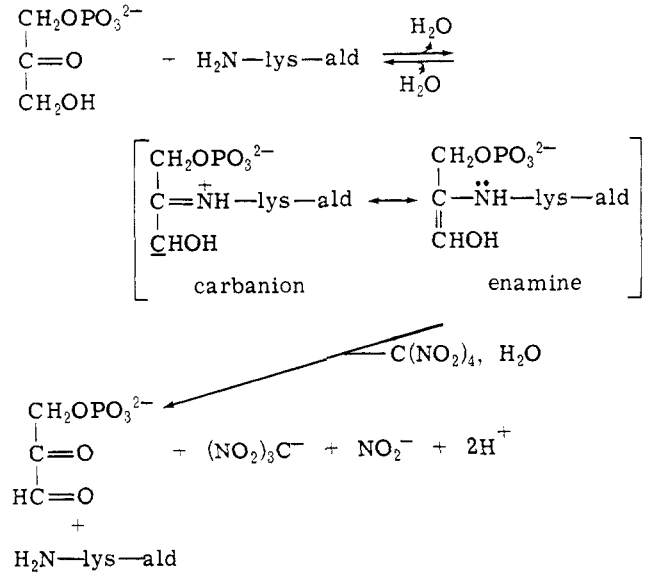
The identification of ester phosphate II as D-5-ketofructose 1,6-diphosphate was further supported by forming its dephosphorylated derivative and comparing its properties with those reported for D-5-ketofructose.[20,21] Employing sorbitol dehydrogenase after the method given for D-5-ketofructose,[21] 0.1 μmol of the dephosphorylated product (0.2 μmol of organic phosphate) oxidized 0.089 μmol of NADH. This value is corrected for the reduction of L-sorbose to sorbitol which occurs simultaneously but at a much slower rate. Dephosphorylated ester II (17 μmol, 2 ml) was also reduced by sodium borohydride (2 ml of 0.1 *M* freshly dissolved sodium borohydride in 0.1 *M* phosphate buffer (pH 7.2)). An aliquot (0.5 ml) of the reaction mixture was taken after 5 min and added to 1 *M* HCl (0.2 ml). On thin-layer chromatography these partially reduced products migrated with R_f values similar to those of fructose or sorbose and stained a similar dark blue-green with the ketose spray (Table I). The totally reduced samples (after 1–2 hr of reduction) were chromatographed on paper.[22] However, the three hexitols D-sorbitol, D-mannitol, and L-iditol, the products of total reduction of D-5-ketofructose, could not be separated using the conditions reported.[20,22] Persistently, the totally reduced samples gave a single spot with a mobility identical with that of sorbitol and mannitol. In the Roe resorcinol test for ketoses[23] phosphate ester II exhibited a single band at 422 nm (= 4 $m^{-1}M^{-1}$ cm^{-1}) similar to those reported for D-5-ketofructose[20] and 5-ketofructose 1-phosphate.[24]

Ester phosphate II gave a disemicarbazone with the same melting point (218–220°) and infrared spectrum as that of hydroxypyruvaldehyde phosphate (major infrared absorption bands: 3460 (s), 3400 (m), 3280 (s), 1715 (s), 1592 (s), 1494 (m), 1470 (m), 1415 (s) cm^{-1}).[25] Similarly, the 2,4-dinitrophenylosazones of hydroxypyruvaldehyde phosphate and of ester phosphate II both exhibited broad absorption bands in alkali, with λ_{max} 560 nm.

Discussion

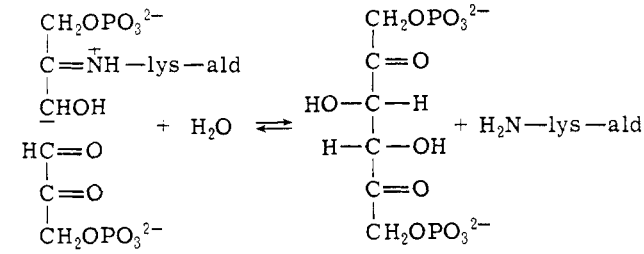
On the basis of the present results the reaction of the carbanionic dihydroxyacetone phosphate–aldolase intermediate[26,27] with tetranitromethane can be described in terms of the mechanism illustrated in Scheme I. The

Scheme I



effect of tetranitromethane is to oxidize the carbanion-enamine intermediate of dihydroxyacetone phosphate to hydroxypyruvaldehyde phosphate (hydroxymethylglyoxal phosphate). Under the present conditions approximately one-half of the hydroxypyruvaldehyde phosphate formed in the reaction of Scheme I is condensed with dihydroxyacetone phosphate in a secondary reaction, also catalyzed by aldolase, to form D-5-ketofructose 1,6-diphosphate (Scheme II). The occurrence of

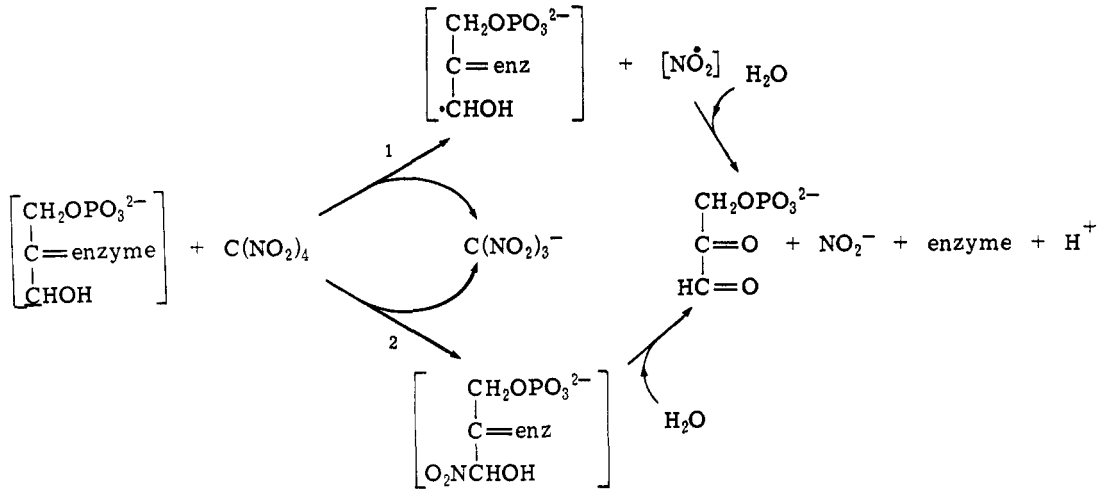
Scheme II



a second dihydroxyacetone phosphate consuming reaction is indicated by a molar ratio of 1.6 times more dihydroxyacetone phosphate disappeared than nitroformate or nitrite produced (Figure 1). The quantities of products, hydroxypyruvaldehyde phosphate, D-5-ketofructose 1,6-diphosphate, nitrite, and nitroformate, generated in the reaction of Figure 1 are in good agreement with the stoichiometry that may be implied from Schemes I and II. Thus, of the total dihydroxyacetone phosphate consumed, 35% was isolated as free hydroxypyruvaldehyde phosphate and 24% each as hydroxypyruvaldehyde phosphate and dihydroxyacetone phosphate condensed to D-5-ketofructose 1,6-diphosphate. Accordingly, the production of both nitroformate and nitrite (Figure 1) was equimolar to the sum of hydroxypyruvaldehyde phosphate and D-5-ketofructose 1,6-diphosphate determined from the reaction mixture (Figure 2).[28]

(20) G. Avigad and S. Englard, *J. Biol. Chem.*, **240**, 2290 (1965).
(21) S. Englard, G. Avigad, and L. Prosky, *ibid.*, **249**, 2302 (1965).
(22) E. Grazi, M. Mangiarotti, and S. Pontremoli, *Biochemistry*, **1**, 628 (1962).
(23) J. H. Roe, J. H. Epstein, and N. P. Goldstein, *J. Biol. Chem.*, **178**, 839 (1949).
(24) G. Avigad and S. Englard, *ibid.*, **243**, 1511 (1968).
(25) Apparently, under the acid conditions employed for the preparation of the semicarbazone derivatives (see Experimental Section), acid-catalyzed aldol cleavage (D. S. Noyce and N. A. Pryor, *J. Amer. Chem. Soc.*, **77**, 1497 (1955); D. S. Noyce and L. R. Snyder, *ibid.*, **81**, 620 (1959)) of the hexose diphosphate 2,5-disemicarbazone to dihydroxyacetone phosphate semicarbazone and hydroxypyruvaldehyde phosphate semicarbazone occurred. Both of these derivatives reacted further to form the same disemicarbazone.
(26) I. A. Rose and Z. Rose in "Comprehensive Biochemistry," M. Florkin and E. H. Stotz, Ed., Vol. 17, Elsevier, Amsterdam, 1969, p 117.
(27) B. L. Horecker, *FEBS (Fed. Eur. Biochem. Soc.) Symp.*, **19**, 181 (1970).
(28) Nitroformate also arises from protein modification but this is estimated to be less than 10% of that observed and shown in Figure 1.[3] Stoichiometric ratios appear to depend on reaction conditions. About 3 mol of nitroformate was produced for every 2 mol of substrate con-

Scheme III



The identification of hydroxypyruvaldehyde phosphate as the primary derivative of dihydroxyacetone phosphate arising from the reaction of Scheme I is based on the specificity of glyoxalase I for ketoaldehydes[15] and on the alkaline dismutation of the derivative to DL-3-phosphoglycerate followed by enzymatic assay for D-3-phosphoglycerate. No substrate other than D-3-phosphoglycerate is known to undergo the series of transformations in the coupled phosphoglycerate kinase assay employed here.[29] The elementary analyses of both the 2,4-dinitrophenylosazone and the disemicarbazone of the derivative of Scheme I are consistent with its identification as hydroxypyruvaldehyde phosphate; the melting point of the 2,4-dinitrophenylosazone agrees with that of the 2,4-dinitrophenylosazone prepared from chemically synthesized hydroxypyruvaldehyde phosphate.[16a] The structure of the secondary product D-5-ketofructose 1,6-diphosphate (Scheme II) is established from its chromatographic behavior (Table I), from its properties as a substrate of aldolase (Figure 3), and from a comparison of the properties of the dephosphorylated product (Table I) with those reported for 5-ketofructose isolated from *Gluconobacter cerinus*.[20,21] It is proposed that hydroxypyruvaldehyde phosphate condenses with dihydroxyacetone phosphate by binding to the aldehyde site of aldolase. Though aldolase is rather unspecific with regard to the structure of the aldehyde substrate in the condensation reaction, the hexose phosphates produced possess exclusively the threo configuration about the C-3–C-4 bond, specifically 3*S*,4*R*.[18,19]

The oxidative effect of tetranitromethane on enzyme-activated dihydroxyacetone phosphate is in agreement with the chemical properties reported for this reagent. Tetranitromethane forms charge-transfer complexes with olefins and other unsaturated compounds.[30–32] In protein chemistry it has found wide application for nitration of tyrosyl residues.[33] However, tetranitromethane also acts as an electron acceptor,[34–37] *e.g.*, oxidizing sulfhydryl groups of proteins[38,39] and oxidizing the inorganic anions iodide and thiosulfate.[37] For the oxidation of carbanionic dihydroxyacetone phosphate to hydroxypyruvaldehyde phosphate two mechanisms may be considered: (1) oxidation of the carbanion by direct electron transfer to tetranitromethane, possibly involving free-radical intermediates, and (2) nucleophilic attack on tetranitromethane by the carbanion leading to unstable 3-nitrodihydroxyacetone phosphate with subsequent elimination of nitrite (Scheme III).

The scheme is thought to indicate the general features of two likely pathways and is not intended to exclude others such as involvement of radicals of nitroformate or hydroxyl. It is arbitrary in regard to mechanistic details, *e.g.*, no data are available to decide whether the latter steps of the reaction occur on the enzyme–substrate complex or after its dissociation. The present data of equimolar quantities of NO_2^-, of $(NO_2)_3C^-$, and of the sum of hydroxypyruvaldehyde phosphate plus 5-ketofructose diphosphate produced (Figures 1 and 2) are consistent with both mechanisms 1 and 2. Nitration of carbanions by tetranitromethane (mechanism 2) could be observed in cases where a stable nitro derivative is generated, *e.g.*, in the nitration of the anionic form of nitroethane to 1,1-dinitroethane with tetranitromethane.[11] In the present instance the short-lived existence of 3-nitrodihydroxyacetone phosphate cannot be excluded.[40] On the other hand, recent experiments have demonstrated that the dihydroxyacetone phosphate–

sumed in a reaction with 2.0×10^{-5} *M* dihydroxyacetone phosphate, 2.1×10^{-4} *M* tetranitromethane (a concentration about 100-fold lower than in the present experiment), and 0.055 mg/ml of native aldolase (pH 8.0);[3] the same molar ratio pertained also to experiments with these concentrations but at pH 6.0 in citrate buffer.

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(40) The nitrated derivative of acetol phosphate ($CH_3COCH_2OPO_3^{2-}$) would appear to be more stable than nitrated dihydroxyacetone phosphate. Acetol phosphate is a substrate for the proton exchange reaction of aldolase and condenses with glyceraldehyde 3-phosphate at a rate 100 times slower than dihydroxyacetone phosphate (I. A. Rose and E. L. O'Connell, *J. Biol. Chem.*, **244**, 126 (1969)). However, with acetol phosphate (6 m*M*) no discernibly increased production of nitroformate from tetranitromethane (1 m*M*) was observed in the presence of aldolase (0.15 mg/ml) at pH 7.5 and 8.0.

aldolase carbanion is in fact a reducing species capable of reducing a number of oxidants other than tetranitromethane.

The oxidation–reduction indicators phenazine methosulfate, 2,6-dichlorophenolindophenol, hexacyanoferrate(III), porphyrindin, and porphyrexide are reduced to their leuco forms by the aldolase–substrate intermediate.[13] Quite similarly to the experiments reported here for tetranitromethane, hexacyanoferrate(III) oxidizes enzyme-activated dihydroxyacetone phosphate quantitatively to hydroxypyruvaldehyde phosphate. The indicators and tetranitromethane are also reduced by substrate complexes of other enzymes besides aldolase which are thought to involve carbanionic intermediates, *e.g.*, pyruvate decarboxylase, aspartate aminotransferase, and 6-phosphogluconate dehydrogenase. Thus, probing of intermediary carbanions by various oxidants differing in specific properties (*e.g.*, redox potential, polarizability, size) might contribute to the study of mechanistic features of both nonenzymatic and enzymatic reactions involving carbanion intermediates.[13]

Experimental Section

Enzymes. Fructose 1,6-diphosphate aldolase (E.C. 4.1.2.13) from rabbit muscle, L-glycerol 3-phosphate dehydrogenase (E.C. 1.1.1.8), triosephosphate isomerase, 3-phosphoglycerate kinase, glyceraldehydephosphate dehydrogenase, glyoxalase I ((*S*)-lactoylglutathione methylglyoxallyase, E.C. 4.4.1.5), and sorbitol dehydrogenase (L-iditol–NAD oxidoreductase, E.C. 1.1.1.14, from sheep liver) were obtained from Boehringer, and carboxypeptidase A (treated with diisopropyl fluorophosphate) was obtained from Sigma. Alkaline phosphatase was prepared from *E. coli*.[41]

Substrates, Chemicals. NADH and ATP were obtained from Boehringer; reduced glutathione, tetranitromethane, tri-*n*-butyl phosphate, dihydroxyacetone, semicarbazide hydrochloride, 2,4-dinitrophenylhydrazine, 1-naphthylamine, and sulfanilic acid were obtained from Fluka. Acetol phosphate[42] and dihydroxyacetone phosphate[43] (the dicyclohexylammonium salt of the dimethyl ketal) were chemically synthesized; the latter product after acid hydrolysis for 4 hr at 40° analyzed 90% for both ester phosphate and enzymically for dihydroxyacetone phosphate.

Chromatography. AG1-X2-Cl ion exchange resin, 200–400 mesh, was obtained from Bio-Rad and precoated thin-layer chromatographic cellulose F plates were obtained from Merck. Centriflo ultrafiltration membrane cones CF50A were obtained from Amicon.

Enzymatic Analyses. Dihydroxyacetone phosphate was determined with glycerolphosphate dehydrogenase and NADH.[44] Hydroxypyruvaldehyde phosphate (hydroxymethylglyoxal phosphate) was determined both with glyoxalase I using reduced glutathione as cosubstrate and by alkaline dismutation to DL-3-phosphoglycerate (0.1 N NaOH, 30°, 30 min) and subsequent enzymatic assay for D-3-phosphoglycerate.[16a,29] D-5-Ketofructose 1,6-diphosphate (D-*threo*-2,5-hexodiulose 1,6-diphosphate) was assayed with aldolase (for details see Figure 3); after dephosphorylation with alkaline phosphatase the resultant D-5-ketofructose was assayed by reduction with sorbitol dehydrogenase.[21] The conditions for this assay were: triethanolamine hydrochloride, 113 μmol; D-5-ketofructose 1,6-diphosphate, 0.1 μmol; NADH, 0.5 μmol; *E. coli* alkaline phosphatase, 20 μg; sorbitol dehydrogenase, 3 units (80 μg); in a total volume of 1.2 ml (pH 7.6).

Other Quantitative Analyses. Nitroformate was determined spectrophotometrically ($\epsilon_{350} = 14.4\ mM^{-1}\ cm^{-1}$).[45] Nitrite was determined with the diazotization method[46] after extraction of tetranitromethane and nitroformate with tributyl phosphate[47] from 100-μl aliquots of the reaction mixture. Inorganic phosphate was determined as phosphomolybdate blue,[48] organic phosphate was determined as inorganic phosphate after treatment of the esters with alkaline phosphatase (30 μg/ml) in 0.05 *M* Tris–Cl (pH 8.0).

Spectroscopic and Kinetic Measurements. Infrared spectra were recorded with KBr disks on a Perkin-Elmer Model 257 instrument. Kinetic measurements and ultraviolet and visible absorption spectra were obtained at 25° with a Unicam SP-1800 recording spectrophotometer.

Thin-Layer Chromatography. Cellulose F precoated plates were used at room temperature with the following solvent systems: 1, 1-propanol–formic acid–water (5:2:1, v/v); 2, 1-propanol–ethyl acetate–water (7:1:2, v/v).[20] Phosphoesters were visualized with acid molybdate spray,[49] ketoses with urea–phosphoric acid.[50]

Syntheses of Sugar Derivatives. (1) 2,4-Dinitrophenylosazones. To cold 0.02 *M* ester phosphate (1 ml) was added 0.005 *M* 2,4-dinitrophenylhydrazine in 2 *M* HCl (10 ml) chilled in crushed ice. After 1–2 hr at 0° the orange, flocculent precipitate was centrifuged, washed twice with cold 2 *M* HCl (2 ml), once with cold water (0.5 ml), and dried *in vacuo* over P_2O_5–NaOH.

(2) Semicarbazones. To 0.02 *M* ester phosphate (2 ml) was added 1 *M* semicarbazide hydrochloride in 1 *M* sodium acetate buffer, pH 5.0 (0.5 ml). After standing 4–5 hr at room temperature the pH was adjusted to 1.0 with 2 *M* HCl and the solution was left at 4° for 24 hr. A white sediment formed which was centrifuged, washed twice with cold 2 *M* HCl (2 ml) and once with water (0.5 ml), and dried *in vacuo* over P_2O_5–NaOH.

Digestion of Aldolase with Carboxypeptidase A.[51] The crystal suspension of aldolase (100 mg) in ammonium sulfate was centrifuged and the sediment was dissolved in 10 ml of 0.5 *M* Tris–Cl–0.5 *M* NaCl (pH 7.5). To this solution 1 mg of carboxypeptidase A was added and after 3 hr of hydrolysis at 4° the solution was dialyzed overnight against 0.01 *M* sodium acetate (pH 7–8). The specific activity fell from 21 to 2.1 μmol of fructose 1,6-diphosphate cleaved per minute per milligram when assayed spectrophotometrically.[52] Aldolase concentration was measured spectrophotometrically, $A_{280}^{1\ mg/ml} = 0.91$.[53]

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