casei the autolytic activity of log and stationary walls was about the same, although there was a distinct difference between the intact cells from the corresponding growth phases.

The autolysin of *S. faecalis* is highly activable by trypsin and other proteinases (Shockman *et al.*, 1968), whereas our system from *L. fermenti* is not. Also the autolysin of *L. casei* has been reported as not being activable by trypsin (Coyette and Ghuysen, 1970). The autolysin recently described by Kawata *et al.* (1971) represents still another type as it contains an endo-*N*-acetylglucosaminidase instead of a muramidase. It is interesting to note the inhibition of *L. fermenti* autolysis in the presence of lysozyme. Also this contrasts with *S. faecalis* in which autolysis and lysis by lysozyme have been reported to partially overlap each other (Shockman *et al.*, 1968).

Finally, we would like to emphasize the advantages of using a tritium labelled reducing agent, NaB^3H_4 (Warth and Strominger, 1971). It facilitates the positive identification of the reducing groups released during lysis of bacterial cell walls. Ninhydrin is no longer needed for detection. Thus, there is no interference from amino acids and unreduced amino sugars. Because of the great sensitivity of the radioisotope method only minute amounts of samples are required for the identification of the reduced amino sugar(s). This eliminates the interference caused by sugars present in the nonpeptidoglycan wall portion. Employing the NaB^3H_4 method we have obtained best results with paper electrophoretic separation at various pH's, whereas paper chromatograms were more difficult to interpret.

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Complexes of Fructose Diphosphate Aldolase with Dihydroxyacetone Phosphate and Dihydroxyacetone Sulfate†

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ABSTRACT: Dihydroxyacetone sulfate has been tested as a substrate for fructose diphosphate aldolase from rabbit muscle. It has been found that it specifically forms a Schiff base with the enzyme but that it does not act as a substrate. The reactivity with CN^- and with BH_4^- of the dihydroxyacetone phosphate and sulfate complexes with aldolase has been studied and found to be differently affected by changes in the pH. The significance of these differences has been discussed and it has been proposed that the phosphate group of dihydroxyacetone phosphate, by interacting with the imine nitrogen of the protonated Schiff base, could modify the reactivity of the C-2 of the substrate.

The mechanism of action of fructose diphosphate aldolase from muscle involves the specific formation of a Schiff base between dihydroxyacetone phosphate and the lysyl residues of the active site of the enzyme (Grazi *et al.*, 1962; Kobashi *et al.*, 1966) (Scheme I). The protonated Schiff base has a preeminent role since it originates the carbanion intermediate (enamine), thus allowing the condensation to occur (Rose and Rose, 1969).

The factors which influence the specific recognition by dihydroxyacetone phosphate of the lysyl residues of the active site, as well as the formation and the deprotonation of the Schiff base, are not completely understood. They could also be strictly related and one of them could be represented by the phosphate group itself of dihydroxyacetone phosphate.

To test this possibility we have made a comparison of the behavior of dihydroxyacetone phosphate and of its analog dihydroxyacetone sulfate. The latter is capable of forming specifically a Schiff base with the enzyme but it does not act as a substrate. The different effects of pH on the reaction of aldolase with dihydroxyacetone phosphate and dihydroxyacetone sulfate have been discussed and it has been proposed that the phosphate group of dihydroxyacetone phosphate, by

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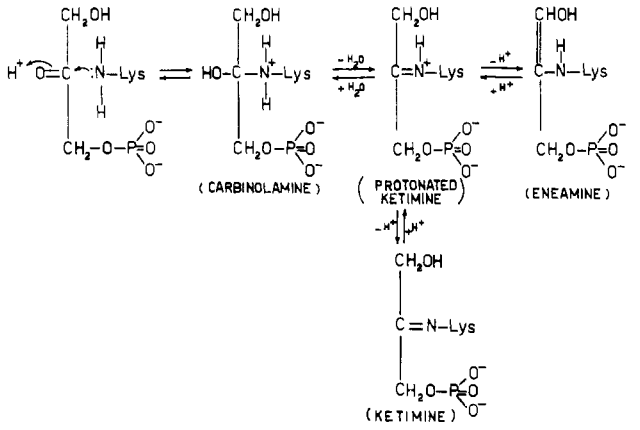

interacting with the imine nitrogen of the protonated Schiff base, could modify the reactivity of C-2 of the substrate.

Experimental Section

Materials. Fructose diphosphate aldolase was prepared from rabbit muscle by the procedure of Taylor *et al.* (1948) and was recrystallized five times. The specific activity of the preparations used in these experiments was 21 units/mg of protein. A unit was defined as the amount which catalyzes the cleavage of 1 μmol of fructose 1,6-diphosphate per minute under standard assay conditions.

Dihydroxyacetone sulfate was prepared as described under Methods. Dihydroxyacetone, crystalline, and dihydroxyacetone phosphate were purchased from Sigma. [^{14}C]Dihydroxyacetone phosphate was prepared from commercial [^{14}C]fructose 1,6-diphosphate following the procedure of Horecker *et al.* (1963) as modified by Ginsburg and Mehler (1966).

The triethylamine–sulfur trioxide complex was prepared as described by Whistler and Spencer (1964).

Dowex 50-X2, 100–200 mesh, was from Sigma Chemical Co. and AG3-X4, 100–200 mesh, was from Bio-Rad.

Methods. Elemental analyses were performed by Bernhardt laboratorium, Elbach über Engelskirchen, West Germany.

Paper electrophoresis, for the separation of the products of the sulfurylation reaction, was performed for 30 min at 1500 V in 1% acetate–2% pyridine buffer, pH 5.2, containing 1 mM ethylenediaminetetraacetate. The spots were visualized with the resorcinol–phosphoric acid spray reagent (Brison and Mitchell, 1951). For the location of the sulfate esters the paper was first sprayed with 1 N NaOH, heated 5 min at 90°, sprayed again with 1 N hydrochloric acid in ethanol followed by 0.02 M barium acetate in water, air-dried for 30 min, and finally sprayed with 0.2% sodium rhodizonate in water (modified from Lloyd, 1960).

Inorganic sulfate was determined turbidimetrically as barium sulfate. Organic sulfate was determined as the difference between total and inorganic sulfate by taking advantage of the alkali lability of dihydroxyacetone monosulfate. The procedure was as follows: to a 0.7-ml sample 0.1 ml of 1 N NaOH was added; after 5 min at 20° 0.1 ml of 2 N HCl was added followed by 0.1 ml of 0.2 M barium acetate. Readings were taken at 340 mμ immediately after mixing. For the determination of inorganic sulfate the hydrochloric acid solution was added first, followed by the sodium hydroxide solution. The method was standardized with anhydrous sodium sulfate.

The reduction of periodate was followed spectrophotometrically at 265 mμ in a Beckman cuvet (optical path 1 cm) containing 1 mM sodium periodate and 0.1 M acetate buffer, pH 4.5. Mannitol and glycerol were used as the standards (Dixon and Lipkin, 1954). Formaldehyde formed by periodate oxidation was determined by the procedure of MacFadyen (1945).

Infrared spectra were recorded in KBr with a Perkin-Elmer Model 337 spectrofluorimeter.

Iodination Studies. Reactions were carried out in a medium consisting of 0.1 M sodium phosphate, pH 7.0, containing 1 M KI and KI_3 at various concentrations. KI_3 was generated by suitable additions of 5 mM I_2 in 1 M KI. The reactions, in a final volume of 1 ml with a 1-cm light path, were initiated by suitable addition of dihydroxyacetone, dihydroxyacetone phosphate, and dihydroxyacetone sulfate and were followed at 351 mμ and 20° in a Gilford recording spectrophotometer. The molar extinction coefficient of KI_3, under these conditions, was determined as 23,000.

Fructose diphosphate aldolase activity was measured in the test system described by Racker (1947).

Sephadex G-50 filtration was performed at 20° on columns (1.2 × 37 cm) equilibrated with [^{14}C]dihydroxyacetone phosphate and 0.05 M Tris-HCl buffer at the pH indicated in the single experiments. $\Gamma/2$[1] was adjusted to 0.05 by the addition of NaCl. The flow rate was 4 ml/min. Fractions of 1 ml were collected.

Radioactivity determinations were made in a Packard TriCarb liquid scintillation counter in 10 ml of Bray' solution (Bray, 1960). Protein concentration was measured from the absorbance at 280 mμ considering that the absorbance of 1 mg of pure enzyme/ml (light path 1 cm) is 0.91 (Baranowski and Niederland, 1949). The number of molecules of substrate bound to the enzyme was estimated from the specific activity of the radioactive substrate on the basis of a mol wt of the enzyme of 159,000 (Kawahara and Tanford, 1966).

The formation of the cyanohydrin of dihydroxyacetone phosphate was studied by treating 9.5 mM dihydroxyacetone phosphate with 0.45 M KCN, at the pH indicated in the single experiments. After 10 min of incubation at 20°, 0.005 ml of the mixture was transferred to a spectrophotometric cuvet containing, in a final volume of 1 ml, 0.1 mM DPNH, excess glycerophosphate dehydrogenase, and 0.1 M Tris-HCl buffer. In every single experiment the pH was the same as that of the original mixture containing the cyanohydrin. The rate of cyanohydrin decomposition was determined by following DPNH oxidation. An initial rapid reaction was attributed to free dihydroxyacetone phosphate. The subsequent slower reaction was attributed to the decomposition of cyanohydrin to cyanide and dihydroxyacetone phosphate. The equilibrium constant for cyanohydrin formation was calculated from the concentrations of dihydroxyacetone phosphate, cyanohydrin, and cyanide present after dilution in the cuvet. The first-order rate constant for the decomposition of cyanohydrin was calculated directly from the experimental data while the second-order rate constant for the formation of the cyanohydrin was calculated by dividing the equilibrium constant by the rate constant for the decomposition of cyanohydrin.

Tritium exchange reaction at C-1 of dihydroxyacetone phosphate and sulfate was studied as follows. The incubation mixtures (0.1 ml) contained 0.05 M Tris-HCl buffer, pH 6.0, 7.5, or 9.0, either 2.2 mM dihydroxyacetone phosphate or 5 mM

[1] Abbreviations used are: $\Gamma/2$, ionic strength; DPNH, reduced diphosphopyridine nucleotide.

dihydroxyacetone sulfate, 0.01 ml of tritiated water (sp act. 200 mCi/ml), and aldolase, 0.03–15 μM. The temperature was 22°. After incubation (2–60 min) the reaction was stopped by the addition of 0.01 ml of 70% perchloric acid and the solution was frozen and lyophilized. The residue was dissolved in 0.2 ml of water and the solution lyophilized again. This operation was repeated five times. The residue was finally dissolved in water and the radioactivity determined.

The condensation reaction of erythrose 4-phosphate with dihydroxyacetone phosphate and sulfate was studied as follows. The incubation mixtures (0.1 ml) contained 0.05 M Tris-HCl buffer, pH 6.0, 7.5, or 9.0, either 2.2 mM dihydroxyacetone phosphate or 5 mM dihydroxyacetone sulfate, 2 mM erythrose 4-phosphate, and 0.03–30 μM aldolase. The temperature was 22°. After incubation (5–60 min) the reaction was stopped by the addition of 0.1 ml of 8% trichloroacetic acid. In the mixture sedoheptulose 1,7-diphosphate formed was determined according to Dische (1953).

Synthesis of Dihydroxyacetone Sulfate. Dihydroxyacetone (5 mmol) (450 mg) was dissolved in 4.5 ml of anhydrous dimethylformamide. The solution was cooled to 0° and 10 mmol (1670 mg) of triethylamine–sulfur trioxide complex was added. After 2 hr at 0°, with stirring, the reaction mixture was diluted to 50 ml with water and poured through an 11 × 1.5 cm column of Dowex 50-X2 (hydrogen form) resin (16 g). The column was washed with 20 ml of water. The effluent and washing were collected and treated for 5 min with 2 g of AG3-X4 resin (chloride form); the pH of the mixture was 1.8. The resin was then discarded and the solution was treated for 5 min with a new aliquot (16 g) of the same resin while the pH was kept at 1.8 by the addition of 1 N perchloric acid. The resin was collected, washed on a glass-sintered funnel with 200 ml of water, and then eluted for 15 min with 200 ml of 0.1 M perchloric acid solution taken to pH 4 with potassium carbonate. The eluate solution (196 ml, pH 3.8), which contained approximately 1 mmol of pure dihydroxyacetone sulfate, was brought to pH 4.1 by the addition of 0.1 M potassium carbonate and was concentrated under reduced pressure.

To the concentrated solution (24 ml, pH 4.1) ethanol (24 ml) and acetone (72 ml) were added. After 2 hr at −14° the crystalline precipitate of potassium perchlorate was discarded. Acetone and ethanol were removed by concentration under reduced pressure of the solution to a syrup. The residue was dissolved in a small amount of water and the pH adjusted to 4.1.

Under these conditions dihydroxyacetone sulfate is stable for weeks at 2°.

By lyophilization of the solution a white powder of the potassium salt of dihydroxyacetone sulfate was obtained which was used for the elemental analysis and for the infrared spectroscopy.

Reduction of Dihydroxyacetone Sulfate with Sodium Borohydride. Dihydroxyacetone sulfate (150 μmol) dissolved in 8 ml of water was brought to pH 6.0 by the addition of 0.5 ml of 0.5 M potassium carbonate. Sodium borohydride (5 mg) was added and the pH was kept at 6.0 by the addition of 5 N acetic acid. After 5 min at 20° a new aliquot (5 mg) of sodium borohydride was now complete as shown by the total disappearance of the alkali labile sulfate. The solution was poured through a 10 × 1 cm column of Dowex 50-X2 (hydrogen form) resin and the column was washed with water. The acidic fractions were pooled (total volume 18 ml) and taken to dryness under reduced pressure.

The residue was dissolved in 3 ml of methanol and the resulting solution was taken to dryness. This procedure was repeated two additional times. The residue was either utilized for the determination of the infrared spectrum or dissolved in water and utilized for the other analyses.

TABLE I: Characterization of Compound II.[a]

Treatment	Periodate Consumed (*a*) (μmol)	Formaldehyde Produced by Periodate Oxidation (*b*) (μmol)	Alkali-Labile Sulfate (*c*) (μmol)	*a*/*b*	*c*/*a*
None	19.5	19.0	19.1	1.02	0.97
Reduction with $NaBH_4$	17.3	17.5	0	0.89	0

[a] Aqueous solutions of compound II (4.2 mg/ml) and of reduced compound II (3.5 mg/ml) were prepared. On aliquots of the two samples alkali-labile sulfate, periodate consumption, and formaldehyde formation, after periodate oxidation, were then determined, following the procedures described under Methods.

Identification of Dihydroxyacetone Sulfate among the Products of the Reaction of Dihydroxyacetone with Triethylamine–Sulfur Trioxide. By treating dihydroxyacetone with the triethylamine–sulfur trioxide complex, three new compounds were formed. These three compounds were resolved by paper electrophoresis at pH 5.2 and visualized with the resorcinol–phosphoric acid spray reagent.

All the three compounds moved toward the anode, compound I (faint spot) by 3.7 cm; compound II (intense spot) by 6.7 cm; and compound III (faint spot) by 10.1 cm. Compounds II and III could also be located with the sodium rhodizonate spray reagent, thus behaving as sulfate esters.

Following the procedure described under Methods, compound II was obtained free of the contaminant compounds I and III. This was clearly shown by the electrophoretical analysis.

Several lines of evidence supported the identification of compound II as dihydroxyacetone monosulfate. (1) *Anal.* Calcd for $C_3H_6O_6S_1$: C, 21.2; H, 3.5; S, 18.8. Found: C, 21.3; H, 3.4; S, 18.3. (2) The ratios moles of alkali-labile sulfate per mole of periodate reduced and moles of periodate reduced per mole of formaldehyde produced were as expected for dihydroxyacetone sulfate and DL-glycerosulfate, respectively (Table I). (3) the aqueous solution of compound II was characterized by a broad absorption band in the ultraviolet region with a maximum at 263 mμ and a molar absorbance coefficient of 10.2 (light path 1 cm, temperature 20°). (4) The infrared spectrum of compound II (Figures 1A and 1B) revealed absorption bands with maxima at 3420 (OH), 2915 (CH_2), 1720 (C=O), 1250 (S=O), and 795 (COS) cm^{-1}. (5) Compound II was a good substrate for α-glycerophosphate dehydrogenase as will be reported in a separate paper. Compound II was therefore identified as dihydroxyacetone monosulfate.

Stability of Dihydroxyacetone Sulfate. As shown in Table II, dihydroxyacetone sulfate, like dihydroxyacetone phosphate, is stable in acid but is unstable in alkali. In 0.1 M NaOH it is completely degraded after 6 min at 20°.

Keto–Enol Tautomerization of Dihydroxyacetone Sulfate. The kinetics of enolization of dihydroxyacetone, dihydroxy-

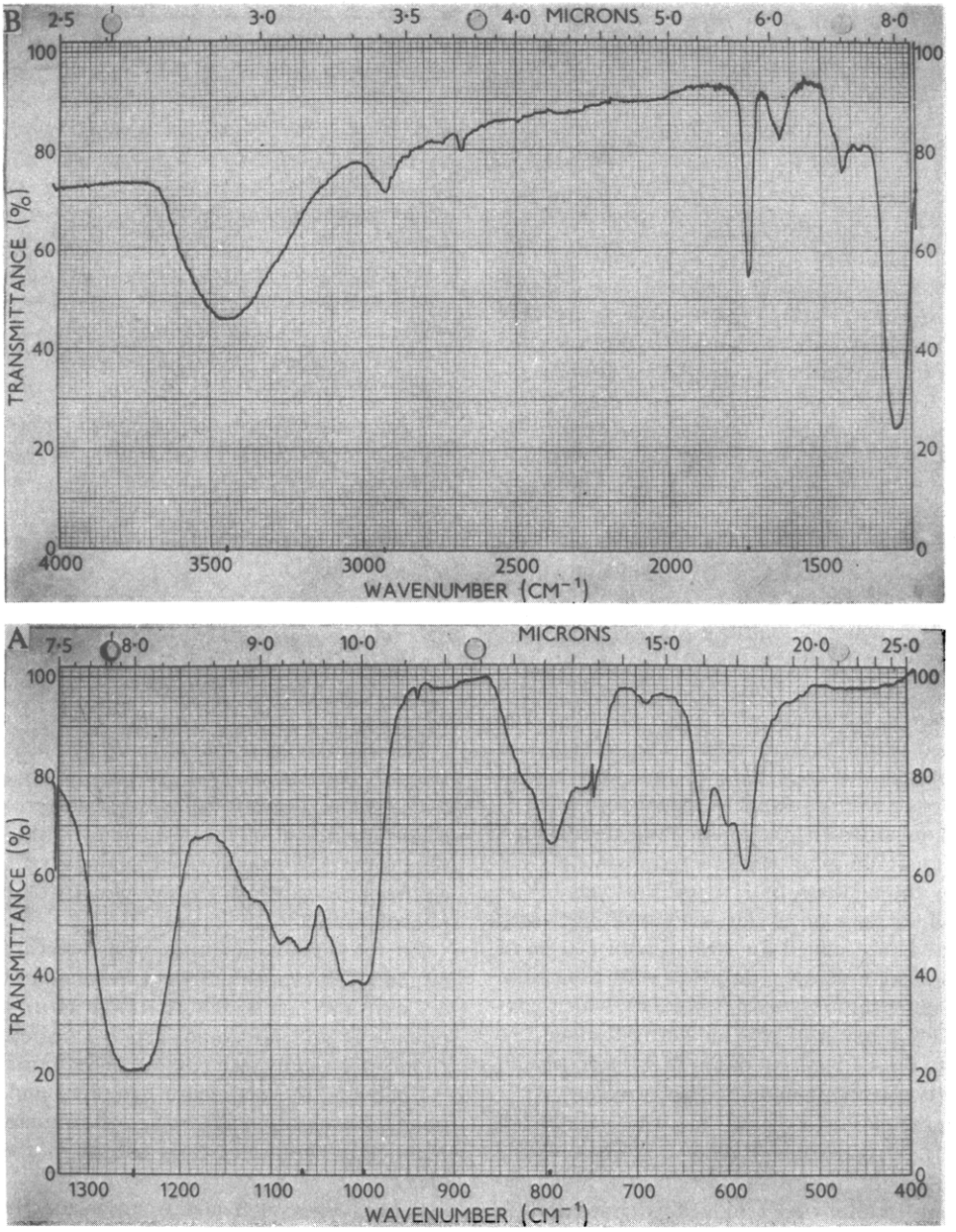

FIGURE 1: Infrared spectra of dihydroxyacetone monosulfate.

acetone phosphate, and dihydroxyacetone sulfate was studied at pH 7.0 and 20° by following the rate of iodine uptake from potassium triodide (Hine, 1956; Bender and Williams, 1966). The progress curves of reactions were similar in all cases. An initial rapid uptake of I_3^- was attributed to the reaction of the free enol present. The subsequent slower I_3^- independent reaction was attributed to formation of enolic species by tautomerization. The total proportions of the enolic species and their rate of formation are given in Table III.

Results

Dissociation Constant of the Enzyme–Substrate Complex. The binding of dihydroxyacetone phosphate to aldolase at different hydrogen ion concentrations was studied by gel filtration on Sephadex G-50 (Table IV). At pH 6.0 and 7.5 about 4 molecules of the substrate were bound per molecule of enzyme with a dissociation constant of 2.4 and 6.4 μM, respectively. By increasing the substrate concentration, additional binding sites were revealed with a larger dissociation constant. At pH 9.0 a single set of approximately eight binding sites was detected with a dissociation constant of 45 μM.

Schiff Base Formation between Aldolase and Dihydroxyacetone Sulfate. The capacity of dihydroxyacetone sulfate to form a Schiff base with aldolase was tested by treating with sodium borohydride a solution containing the enzyme and dihydroxyacetone sulfate. As is shown in Table V, the treatment with the reducing agent at pH 6.0 and 8.0 was followed by 20 and 40% inactivation, respectively. The same treatment performed in the presence of dihydroxyacetone phosphate led to 90% inactivation at pH 6.0 and to 30% inactivation at pH 8.0.

To show that inactivation was related to the reduction of a Schiff base, aldolase (37 nmol), dissolved in 1 ml of 0.05 M Tris-HCl buffer, pH 8.0, containing 16 mM dihydroxyacetone sulfate, was treated with 8 μmol of NaB^3H (sp act. 20×10^6 cpm/μatom). The resulting 40% inactivated protein was precipitated with 1 ml of 10% trichloroacetic acid, washed with 2

TABLE II: Stability of Dihydroxyacetone Sulfate.[a]

	Recovery		
	0.1 hr	3 hr	24 hr
0.1 N HCl		100	100
pH 4.0		100	100
pH 10.0			70
pH 11.0		0	0
0.1 N NaOH	0		

[a] The incubation mixtures (4 ml) contained 1 mM dihydroxyacetone sulfate. The pH was taken to 10.0 and 11.0 by careful addition of 1 M NaOH. The temperature was 20°. At time intervals, samples were taken, and dihydroxyacetone sulfate was determined as alkali-labile sulfate following the procedure described under Methods.

ml of 5% trichloroacetic acid, dried with ether, and dissolved in 2 ml of 2.5 N KOH (820,000 cpm). The protein was then hydrolyzed for 24 hr at 110° in evacuated, sealed tubes.

The hydrolysate was neutralized with perchloric acid, the precipitate of potassium perchlorate was removed by centrifugation, and the supernatant solution was taken to dryness under reduced pressure. The residue was dissolved in 1 ml of water (280,000 cpm). Samples (0.3 ml) (84,000 cpm) of the solution were submitted to paper electrophoresis for 60 min at 3600 V either in 25 mM bicarbonate–carbonate buffer, pH 9.7 (Horecker *et al.*, 1963), or in 4% acetic acid–4% formic acid, pH 1.8 (Speck *et al.*, 1963). A single radioactive spot, migrating to the same position of an authentic sample of β-glyceryllysine, was detected in both cases, thus providing the proof of the formation of the Schiff base.

The inactivation process, promoted by reduction in the presence of dihydroxyacetone sulfate, involved the modification of a number of lysyl residues comparable to that involved in the reaction with dihydroxyacetone phosphate. This is shown in the experiment reported in Table VI where the reduction was performed with tritiated sodium borohydride. In the presence of dihydroxyacetone phosphate inactivation was 45% and 2.8 atoms of tritium were bound per molecule of enzyme inactivated. In the presence of dihydroxyacetone sulfate inactivation was 48% and 2.4 atoms of tritium were bound per molecule of enzyme inactivated. The radioactive background in the control sample was quite high; a number of different procedures to decrease it were tested but without success. In all the experiments performed, however, the figures for the equivalence between tritium labeling and aldolase inactivation were similar. The inactivation of aldolase by sodium

TABLE III: Proportion of Enolic Forms of Dihydroxyacetone and Dihydroxyacetone Phosphate and Sulfate in Aqueous Solutions.[a]

	Enol (%)	Rate Constant of Enolization Reaction (sec^{-1})
Dihydroxyacetone	0.016	0.83×10^{-5}
Dihydroxyacetone phosphate	0.034	1.1×10^{-5}
Dihydroxyacetone sulfate	0.045	5.6×10^{-5}

[a] Reactions with KI_3 were followed spectrophotometrically at 351 mμ and 20°. Other conditions were as described under Methods.

TABLE IV: Effect of the pH on the Formation of the Aldolase–Dihydroxyacetone Phosphate Complex.[a]

pH	Total Aldolase (nmol)	[^{14}C]Dihydroxyacetone Phosphate Equil Concn ($\times 10^{-6}$ M)	[^{14}C]Dihydroxyacetone Phosphate Bound to Enzyme		$\bar{\nu}$
			cpm	nmol	
6.0	19	3.6	21,086	47.6	2.50
6.0	19	8.6	26,092	58.9	3.10
6.0	19	23.0	31,098	70.2	3.70
6.0	19	110.0	55,507	125.3	6.60
7.5	19	5.6	17,720	40.0	2.10
7.5	19	9.1	21,884	49.4	2.60
7.5	19	27.5	29,415	66.4	3.50
7.5	19	110.0	48,021	108.4	5.70
9.0	19	4.95	6,202	14.0	0.73
9.0	19	10.0	11,783	26.6	1.40
9.0	19	27.5	24,054	54.3	2.86
9.0	19	110.0	42,085	95.0	5.00

[a] The incubation mixtures (1 ml) contained aldolase (19 nmol) and 0.05 M Tris-HCl buffer at the pH indicated. Γ/2 was adjusted to 0.05 by the addition of NaCl. The mixtures were applied to Sephadex G-50 columns equilibrated with a solution containing [^{14}C]dihydroxyacetone phosphate (sp act. 443 cpm/nmol) at the concentration indicated, and 0.05 M Tris-HCl buffer at the pH indicated. Γ/2 was adjusted to 0.05 by the addition of NaCl. The temperature was 20°. The elution of the columns and the determination of protein and of radioactivity were performed as described under Methods. $\bar{\nu}$ represents the number of molecules of substrate bound per molecule of enzyme.

TABLE V: Aldolase Inactivation by Sodium Borohydride in the Presence of Either Dihydroxyacetone Phosphate or Sulfate.[a]

	pH	Inactivation (%)
Dihydroxyacetone phosphate	6.0	90
	8.0	30
Dihydroxyacetone sulfate	6.0	20
	8.0	40

[a] The incubation mixtures (0.2 ml) contained aldolase (8 nmol), either 2 mM dihydroxyacetone phosphate or 16 mM dihydroxyacetone sulfate, and 0.07 M Tris-HCl buffer at the pH indicated. The reduction was started by the addition of 0.005 ml of 0.25 M $NaBH_4$ in 1 mM NaOH. The temperature was 20°. Before and 7 min after the addition of $NaBH_4$, samples were taken for determination of the catalytic activity. In control samples where the addition of either dihydroxyacetone phosphate or sulfate was omitted no inactivation occurred after the treatment with sodium borohydride.

TABLE VI: Equivalence of Tritium Labeling and Loss of Aldolase Activity.[a]

Addition	Aldolase: Total (nmol) (1)	Aldolase: Inactive (nmol) (2)	^{3}H Bound to Protein: cpm	^{3}H Bound to Protein: natoms (3)	^{3}H Bound to Protein: natoms (4)	Equivalence (4)/(2)
Aldolase + NaB^{3}H	6.2	0.0	4,900	4.0		
Aldolase + NaB^{3}H + dihydroxyacetone phosphate	6.2	2.8	14,400	11.8	7.8	2.8
Aldolase + NaB^{3}H + dihydroxyacetone sulfate	6.2	3.0	13,450	11.2	7.2	2.4

[a] (1) based on a mol wt of 159,000; (4) corrected for the radioactivity present in the control experiment. The incubation mixtures (0.5 ml) contained aldolase (6.2 nmol), either 1.9 mM dihydroxyacetone phosphate or 13 mM dihydroxyacetone sulfate, and 60 mM Tris-HCl buffer, pH 8.0. A control sample without dihydroxyacetone phosphate or sulfate was also prepared. The reduction was started by the addition of 0.01 ml of 0.25 M NaB^{3}H (sp act. 1200 cpm/natom) in 1 mM NaOH. The temperature was 20°. Before and 7 min after the addition of reducing agent samples were taken for the determination of the catalytic activity. The protein was then precipitated with ammonium sulfate, the precipitates were dissolved with 1 ml of water, and the resulting solutions were dialyzed for 24 hr against distilled water, for 24 hr against 6 M urea, and again for 24 hr against water. The radioactivity and the protein content were then determined.

borohydride in the presence of dihydroxyacetone sulfate was also utilized to estimate the affinity of this compound for the enzyme. This was done by performing the reduction in the presence of decreasing concentrations of dihydroxyacetone sulfate (Figure 2). From these data a dissociation constant of 3 mM was estimated for the enzyme–dihydroxyacetone sulfate complex.

Aldolase Inactivation by Cyanide in the Presence of Dihydroxyacetone Sulfate. The reversible inactivation of aldolase by cyanide in the presence of dihydroxyacetone phosphate was reported by Cash and Wilson (1966) and it was explained by the formation of a cyanide adduct of the aldolase–dihydroxyacetone phosphate–ketimine. Inactivation was also obtained in the presence of dihydroxyacetone sulfate but the process showed a different pH dependence (Figure 3).

While at pH 6.0 the rate of inactivation in the presence of dihydroxyacetone phosphate was much higher than the rate of inactivation in the presence of dihydroxyacetone sulfate, at pH 9.0 the rate of inactivation was similar in the presence of either one of the two compounds (Table VII). To get a better understanding of the factors influencing the reactivity of the aldolase–dihydroxyacetone phosphate–ketimine, the effect of pH on the reaction of decomposition of the cyanohydrin of dihydroxyacetone phosphate was studied (Figure 4). The equilibrium and the rate constants were estimated and are presented in Table VIII. Comparison of the values reported in Table VII and VIII reveals two phenomena. First, the rate constant for the reactions of cyanide with the enzyme intermediates are 50–700 times lower than those with free dihydroxyacetone phosphate. Second, the pH dependence is similar for

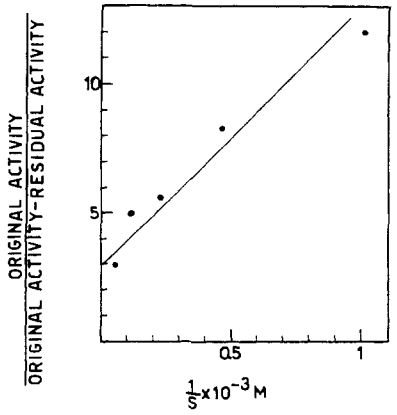

FIGURE 2: Aldolase inactivation by sodium borohydride as a function of dihydroxyacetone sulfate concentration. The experimental conditions were as in Table V. The concentration of dihydroxyacetone sulfate was as indicated in the figure; the pH was 8.0.

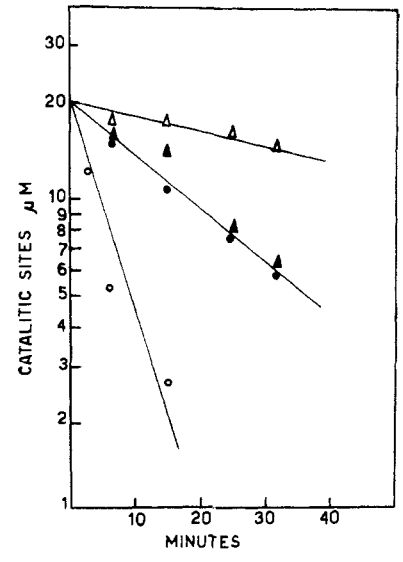

FIGURE 3: Semilogarithmic plot of the reaction of inactivation of aldolase by cyanide in the presence of either dihydroxyacetone phosphate or sulfate. The incubation mixtures (0.5 ml) contained aldolase (2.55 nmol), 0.05 M KCN, and either 3.2 mM dihydroxyacetone phosphate ((○) pH 6.0; (●) pH 9.0) or 18 mM dihydroxyacetone sulfate ((△) pH 6.0; (▲) pH 9.0). The temperature was 20°. At the times indicated samples were taken for the determination of the catalytic activity. In control samples where the addition of either dihydroxyacetone phosphate or sulfate was omitted, the catalytic activity was completely retained after 30 min of incubation. On the ordinate the concentrations of the catalytic sites are plotted. This concentration was calculated by assuming four catalytic sites per molecule of enzyme.

TABLE VII: Rate Constants for the Reaction of Inactivation of the Aldolase–Dihydroxyacetone Phosphate and Aldolase–Dihydroxyacetone Sulfate Complexes by Cyanide.[a]

	pH	K (M^{-1} sec^{-1})
Dihydroxyacetone phosphate	6.0	50×10^{-3}
	9.0	12.6×10^{-3}
Dihydroxyacetone sulfate	6.0	3.3×10^{-3}
	9.0	12.6×10^{-3}

[a] Pseudo-first-order rate constants were determined from the data of Figure 3 and were converted to second-order rate constants by dividing them by the concentration of cyanide (protonated plus unprotonated forms). The values reported were not corrected for the influence of the pH on the concentration of the ionic species.

the reactions of cyanide with both dihydroxyacetone phosphate and the aldolase–dihydroxyacetone phosphate–ketimine but it is completely different for the aldolase–dihydroxyacetone sulfate–ketimine that reacts faster at alkaline pH.

Dihydroxyacetone Sulfate Does Not Work as a Substrate in the Aldolase Reaction. In the previous sections it was shown that dihydroxyacetone sulfate forms a Schiff base with aldolase. Reduction of the Schiff base was accompanied by the irreversible modification of 2.4 lysyl residues and by the inactivation of the enzyme. Inactivation was also obtained by treating the Schiff base with cyanide. Dihydroxyacetone sulfate, however, was inactive as a substrate. This was shown by the lack of both tritium exchange at C-1 of dihydroxyacetone sulfate and of the condensation reaction between dihydroxyacetone sulfate and erythrose 4-phosphate.

Tritium exchange was studied in the presence of 5 mM dihydroxyacetone sulfate, tritiated water, and up to 15 μM aldolase. The condensation reaction was studied in the presence of 5 mM dihydroxyacetone sulfate, 2 mM erythrose 4-phosphate, and aldolase up to 30 μM. The range of pH between 6.0 and 9.0 was tested. The corresponding control experiments with dihydroxyacetone phosphate were also performed.

Discussion

The direct measurement of the binding of dihydroxyacetone phosphate to aldolase, as a function of pH, has shown that a single set of about eight binding sites for dihydroxyacetone phosphate is available at pH 9.0. At lower pH values two sets of about four sites each are available. Of these sites those showing the largest affinity are probably those forming the Schiff base with dihydroxyacetone phosphate. This is in agreement with previous data of Ginsburg and Mehler (1966) showing the presence, at pH 7.8, of two different sets of sites for the binding of the inorganic phosphate. We are perfectly aware of the limitations of the gel filtration technique and we do not claim, therefore, to be able to discriminate, on these grounds, between three and four sites. As a matter of fact all the previous reports (Ginsburg and Mehler, 1966; Castellino and Barker, 1966) present evidence for the existence of only three binding sites.

Our analysis has revealed a clear influence of the pH of the medium on the dissociation constant of the dihydroxyacetone phosphate–aldolase complex. These data are in good agreement with the results of previous indirect, spectrofluorimetric

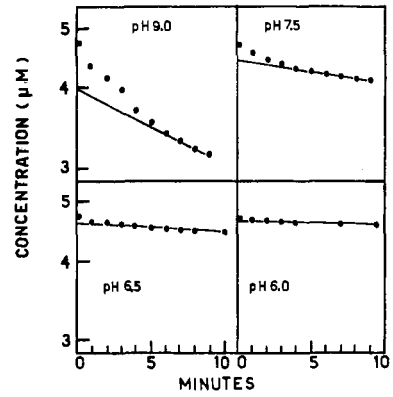

FIGURE 4: Semilogarithmic plot of the reaction of decomposition of the cyanohydrin of dihydroxyacetone phosphate; effect of pH. The experimental conditions were as described under Methods. The initial concentration (dihydroxyacetone phosphate + cyanohydrin of dihydroxyacetone phosphate) in the cuvet was 47.5 μM. The rate of the reaction was studied by following DPNH oxidation in the presence of excess glycerophosphate dehydrogenase.

measurements performed by Rose and O'Connel (1969). The only difference is that we do definitely show a decrease of the dissociation constant not only from pH 9.0 to 7.5 but also from pH 7.5 to 6.0.

From the consideration of the chemical mechanism for Schiff base formation (Scheme I) it is not immediately evident how the dissociation constant of the enzyme–dihydroxyacetone phosphate complex changes with the pH. This is because the enzyme–dihydroxyacetone phosphate complex is not a single chemical species but results from the sum of the different intermediates of the reaction.

Thus, to represent correctly the effect of the pH on its dissociation constant, the pK_a of the relevant chemical species (ϵ-amino groups of the active lysyl residues, Schiff base and carbanion) and the relative proportions of the principal intermediates should be known.

In this paper we have tried to evaluate the possible influence on the reaction of the phosphate group of the substrate, by comparing the behavior of dihydroxyacetone phosphate with its analog dihydroxyacetone sulfate. Both compounds react specifically at the active site of the enzyme with the formation of a Schiff base. The complexes formed, however, react differently with nucleophiles. Inactivation by sodium borohydride is faster at pH 6.0 than at pH 8.0 in the

TABLE VIII: Equilibrium and Rate Constants for the Reaction of Formation of the Cyanohydrin of Dihydroxyacetone Phosphate.[a]

HCN + dihydroxyacetone phosphate $\underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}}$ cyanohydrin

pH	K_{eq} (M^{-1})	K_1 (M^{-1} sec^{-1})	K_{-1} (sec^{-1})
6.0	43×10^3	2.15	0.5×10^{-4}
6.5	43×10^3	2.15	0.5×10^{-4}
7.5	11×10^3	1.83	1.66×10^{-4}
9.0	2.2×10^3	0.82	3.8×10^{-4}

[a] The values reported were calculated, as described under Methods, from the data of Figure 4. No correction was made for the influence of the pH on the concentration of the ionic species.

presence of dihydroxyacetone phosphate, while the reverse is true in the presence of dihydroxyacetone sulfate. Furthermore, the second-order rate constant of the reaction of inactivation by cyanide decreases from pH 6.0 to 9.0 in the presence of dihydroxyacetone phosphate, while, in the presence of dihydroxyacetone sulfate, it increases in the same range of pH. Here also the effect of the pH is the opposite in the two cases.

When the behavior of nonenzymatic systems is studied, the results obtained are very similar to those obtained for the aldolase–dihydroxyacetone phosphate–ketimine. The rate of the reaction of cyanide with dihydroxyacetone phosphate increases about fourfold from pH 9.0 to 6.0 and that with the aldolase–dihydroxyacetone phosphate–ketimine increases about threefold in the same range of pH. Also, the initial rate of reduction, with sodium borohydride, of dihydroxyacetone, dihydroxyacetone phosphate, and dihydroxyacetone sulfate is similar for the three compounds but is much larger at pH 7.0 than at pH 9.0.

From these data two points are offered to the discussion. (1) Dihydroxyacetone sulfate displays a much lower affinity for aldolase than dihydroxyacetone phosphate. It is unlikely that this is due to differences in steric hindrance. The charge difference between the two compounds could be considered. It seems, however, that this is not the main reason for the different behavior because dihydroxyacetone phosphate is more tightly bound to the enzyme at pH 6.0, where the monoanionic form is prevalent (Kiessling, 1934). A more important differing factor could be the formation of the carbanion in the aldolase–dihydroxyacetone phosphate complex that, by increasing the number of the intermediates of the reaction, could promote a tighter binding of the substrate to the enzyme. This possibility is lacking with dihydroxyacetone sulfate. (2) pH affects differently the reactivity of the aldolase–dihydroxyacetone phosphate and sulfate complexes toward nucleophiles.

The rate of the reaction with cyanide is a measure of both the concentration of the protonated Schiff base and of the electrophilicity of its C-2 (formerly C-2 of the substrate). In aldolase–dihydroxyacetone phosphate–ketimine, the dianion of the phosphate group, by interacting with the protonated imine nitrogen, could partially neutralize the positive charge and thus decrease the electrophilicity of C-2. The effect would be much smaller with the monoanion. The consequences of a decrease of the pH of the medium should, therefore, be the following: first, an increase in the rate of the reaction with cyanide (this has been found) and second, an easier formation of the carbanion at C-1 of the ketimine. Data supporting this have been provided by Rose *et al.* (1965).

Changes in pH from 6.0 to 9.0 are not expected to affect the anionic form of dihydroxyacetone sulfate. The increase, with pH, of the reactivity toward nucleophiles with dihydroxyacetone sulfate as substrate must be, therefore, dependent on changes of the enzyme. These changes can be, tentatively, assigned to a larger deprotonation of the active lysyl residues. This, in turn, will allow a larger concentration of the Schiff base to be formed. The same reasoning holds also for the aldolase–dihydroxyacetone phosphate–ketimine. The difference, however, is that, in this case, the influence of the phosphate group on the electrophilicity of C-2 of the ketimine could overcome the concentration effect.

The simple procedure we have used for the synthesis of dihydroxyacetone sulfate also deserves some comments. Dihydroxyacetone monosulfate has been found to be the major product of the sulfurylation reaction. This is probably explained by the fact that anhydrous dihydroxyacetone exists as a dimer in which one of the two primary hydroxyl groups would be protected from sulfation (Gardiner, 1966). The compound is stable in acid but is rapidly degraded in alkali. In this respect it is similar to dihydroxyacetone phosphate. The amount of the enolic form in equilibrium with the keto and the hydrated forms has been determined and found to represent 0.045% of the total. In dihydroxyacetone and dihydroxyacetone phosphate it represents 0.016 and 0.034%, respectively. The enol proportion in dihydroxyacetone phosphate solutions is therefore much lower than that (1%) found by Reynolds *et al.* (1971). The rate of enolization of dihydroxyacetone sulfate is about five times larger than that of dihydroxyacetone phosphate and seven times larger than that of dihydroxyacetone.

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