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Specific Anion Binding to Fructose Diphosphate Aldolase from Rabbit Muscle*

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ABSTRACT: The binding of sulfate, phosphate, and hexitol diphosphate (mixture of D-mannitol and D-sorbitol 1,6-diphosphates) to native fructose 1,6-diphosphate (FDP) aldolase each was measured by equilibrium dialysis against the radioactive anion at pH 7.8 in 0.02 M Tris–0.017 M chloride. In addition, phosphate and hexitol diphosphate ion binding to the inactive β-glycerophosphate aldolase derivative of Horecker and co-workers [Horecker, B. L., Rowley, P. T., Grazi, E., Cheng, T., and Tchola, O. (1963), *Biochem. Z. 338*, 36] was examined under the same conditions. The results indicate that the native protein at this pH has approximately 2.6 highly specific binding sites for phosphate (k_A' = 28,000, where k_A' is the apparent association constant) and about 2.9 additional sites with a lower affinity for phosphate (k_A' = 1100). The enzyme binds sulfate under these conditions to an apparent extent of 5.5 ions/molecule with k_A' = 2100 for all sites.

About 2.7 hexitol diphosphate molecules/molecule of native aldolase are bound to apparently equivalent sites with k_A' = 830,000. (The binding of fructose 1,6-diphosphate at 10^{-4}–10^{-3} M concentrations duplicates that of the hexitol diphosphate analogs.) The inactive derivative, formed here with ~2.6 equiv of dihydroxyacetone phosphate covalently attached (presumably to the active sites of the aldolase molecule), does not exhibit the very specific binding of either phosphate or hexitol diphosphate. The magnitude of the affinity constants measured for the anion binding to aldolase implicates cooperating positive charges clustered at each site as proposed by Velick, Saroff, and Loeb and Saroff [Velick, S. F. (1949), *J. Phys. Colloid Chem. 53*, 135; Saroff, H. A. (1957), *J. Phys. Chem. 61*, 1364; Loeb, G. I., and Saroff, H. A. (1964), *Biochemistry 3*, 1819]. Thus, it appears that native FDP aldolase has *three* highly organized active sites, each of which contains two phosphate binding sites.

Rabbit muscle aldolase is a globular protein with a molecular weight of 142,000 and a hydrodynamic frictional ratio of ~1.26 (Stellwagen and Schachman, 1962). It is composed of three polypeptide chains of about equal size, one of which appears to differ in its carboxyl-terminal sequence (Kowalsky and Boyer, 1960; Stellwagen and Schachman, 1962; Deal *et al.* 1963; Winstead and Wold, 1964). Aldolase is inactive when *reversibly* dissociated into its constituent polypeptide chains under fairly mild conditions (Swenson and Boyer, 1957; Schachman, 1960; Stellwagen and Schachman, 1962; Deal *et al.*, 1963). Thus, the secondary and tertiary structure of this protein, while governed by the primary structure, is an integral part of the configuration of the active site(s).

The question arises as to the actual number of active

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sites possessed by this enzyme. Westhead *et al.* (1963) found one substrate binding site per molecule of aldolase by equilibrium dialysis against either fructose 1,6-diphosphate or dihydroxyacetone phosphate. However, Horecker *et al.* (1963) and Lai *et al.* (1965b) have chemically labeled two combining sites of aldolase with dihydroxyacetone phosphate, and from the correspondence of the loss in aldolase activity with the covalent attachment of dihydroxyacetone phosphate have concluded that two active sites exist in each molecule of enzyme. More recent estimates by this group[1] approach three binding sites for dihydroxyacetone phosphate. The apparent discrepancy in the number of active sites possessed by aldolase has been approached in the present studies by an investigation of equilibrium anion binding to native aldolase and to the inactive β-glycerophosphate derivative of Horecker *et al.* (1963).

Anions have been reported to exert large effects on the properties of aldolase. Velick (1949) showed that the net charge of the aldolase molecule with an alkaline isoionic pH varies greatly with changes in the ionic composition as well as the pH of the medium, and demonstrated that this effect involves specific interactions between the protein and buffer anions. He measured 6–7 moles of bound phosphate ions/mole of aldolase by equilibrium dialysis in phosphate buffer at pH 6.05 and 0.10 ionic strength. Phosphate, as well as other anions, is a competitive inhibitor of aldolase (Mehler, 1963), and the K_m data for aldolase (see Rutter, 1961) are consistent with aldolase having at least a 100-fold greater affinity for fructose 1,6-diphosphate than for fructose 1-phosphate or for the monophosphorylated triose phosphate substrates. Also, the hexitol 1,6-diphosphates obtained by reduction of fructose 1,6-diphosphate act as effective competitive inhibitors of aldolase (Mehler and Viswanatha, 1961), as might be expected from a comparison of their structures with that of the substrate. More recently, Hartman and Barker (1965), from studies of the competitive inhibition of aldolase by other structural analogs of fructose 1,6-diphosphate, concluded that the affinity of aldolase for fructose diphosphate is primarily due to the substrate's phosphate groups at 10- to 12-A separation. The apparent affinity, estimated from K_m data, of aldolase for either fructose 1-phosphate or fructose 1,6-diphosphate (Mehler, 1963) is constant in the pH range of 7.2–8.8, where the substrates are completely ionized and aldolase is stable (Hass and Lewis, 1963). Thus, it appears that the apparent pK values of the amino acid residues of aldolase involved in the substrate association are not within this pH range. The data of this report have been treated accordingly (Saroff, 1957; Saroff and Carroll, 1962; Loeb and Saroff, 1964). In addition, competitive anion effects are considered and, when possible, estimated.

Experimental Section

Materials. ALDOLASE. Three- to six-times-recrystallized aldolase was prepared by the procedure of Taylor *et al.* (1948) as modified by Swenson and Boyer (1957). The crystals were stored at 4° in 0.42 saturated ammonium sulfate and collected by centrifugation as needed. For all the studies reported the crystals were dissolved in 0.02 M Tris–0.017 M chloride buffer at pH 7.4[2] and dialyzed at 4° *vs.* 1000 volumes of this buffer with three dialysate changes occurring over an approximate 50-hr period. The dialyzed solutions were clarified by centrifugation and stored at 4°. At 0.4–1% aldolase concentrations, these solutions are stable for several months at 4°. The specific activity of the aldolase solutions used was 14–20 units/mg. Trace amounts, <0.01% of aldolase by weight (Beisenherz, 1955), of triose phosphate isomerase activity (<0.1 unit/mg) could be detected in all aldolase preparations.

OTHER ENZYMES. α-Glycerophosphate dehydrogenase, free of triose isomerase activity, was purchased from the Sigma Chemical Co., and chromatographically purified bacterial alkaline phosphatase (6164) from the Worthington Biochemical Corp. Glyceraldehyde phosphate dehydrogenase was obtained from Boehringer and Soehne. Triose phosphate isomerase was kindly supplied by Dr. B. Bloom.

SUBSTRATES AND REAGENTS. DHAP[3] was purchased from the California Corp. for Biochemical Research as the cyclohexylamine salt of the dimethyl ketal, and was converted to the free ketone by the method of Ballou and Fischer (1956). The tetrasodium salt of FDP, DPN^+, the barium salt of DL-glyceraldehyde 3-phosphate, and reagent grade Trisma Base were supplied by the Sigma Chemical Corp. DPNH was obtained from Boehringer and Soehne. The barium salt of uniformly labeled [^{14}C]FDP was obtained from Schwarz Bio-Research, Inc.[4] Sodium [^{35}S]sulfate (154 μc/μmole) was obtained from the New England Nuclear Corp. and carrier-free inorganic [^{32}P]phosphate was purchased from Oak Ridge or Volk Radiochemical Corp. Sodium borohydride (99% pure) was purchased from Metal Hydrides Inc.

Deionized water with a conductivity $\leqq 1.7 \times 10^{-6}$ ohm^{-1} was obtained from a water deionizing unit (Model DJ-128) of the Crystal Research Laboratory, Inc., and this water was used exclusively.

The pH standards used were obtained from the National Bureau of Standards. All other materials were reagent grade.

[1] Personal communications with Dr. B. L. Horecker.

[2] pH = 7.38 at 25°. The temperature dependence from 6° to 35° of this buffer was determined to be −0.024 pH units/°C.

[3] The abbreviations used are: DHAP, dihydroxyacetone phosphate; FDP, fructose 1,6-diphosphate; TCA, trichloroacetic acid; hexitol diphosphate, a mixture of D-mannitol 1,6-diphosphate and D-sorbitol 1,6-diphosphate from the chemical reduction of FDP; DPNH and DPN^+, reduced and oxidized diphosphopyridine nucleotides.

[4] The authors gratefully acknowledge a gift of 100 μc of this compound, and for information supplied by Schwarz Bio-Research, Inc., on the preparative procedures used.

Methods. ASSAYS. Aldolase was assayed by the method of Racker (1947) using α-glycerophosphate dehydrogenase and DPNH buffered with Tris at pH 7.4. With deionized or dialyzed aldolase a time-dependent activation with phosphate ions is observed. Routinely the aldolase was diluted to about 0.5–1 mg/ml concentration with 0.01 M phosphate buffer at pH 7.6 and the concentration of protein was determined. The diluted enzyme was iced for 3–4 hr for maximum activation, and then an aliquot containing 1–3 μg was used in an aldolase assay mixture containing a high level of FDP (0.01 M) to offset the competitive inhibitory effect of phosphate (Mehler, 1963). Aldolase at <1 mg/ml of concentrations is unstable, and phosphate ions do not sufficiently stabilize the diluted protein to prevent slow inactivation during storage at 4°. Units of aldolase activity are expressed as the number of micromoles of FDP cleaved per minute at 25°. Protein concentration for native aldolase was determined by the absorbance at 280 mμ using $\epsilon_{1cm}^{0.1\%}$ 0.938 (Donovan, 1964) and a molecular weight of 142,000 (Stellwagen and Schachman, 1962). In 0.2 N NaOH (peak at 290 mμ), $\epsilon_{1cm}^{0.1\%}$ 0.983 at 290 mμ was used.

Triose phosphate isomerase was assayed by the procedure of Beisenherz (1955). DHAP was assayed spectrophotometrically with α-glycerophosphate dehydrogenase and DPNH (Racker, 1947) at pH 7.4. For FDP assays, aldolase and triose phosphate isomerase were included in the reaction mixture. Glyceraldehyde 3-phosphate was assayed spectrophotometrically either using the procedure of Horecker *et al.* (1963) or using glyceraldehyde 3-phosphate dehydrogenase, DPN^+, and arsenate at pH 8.0 (Warburg and Christian, 1939). All substrates used were over 95% pure based on a molecular extinction coefficient of 6220 for DPNH (Horecker and Kornberg, 1948). A Cary Model 14 recording spectrophotometer was used throughout. Organic and inorganic phosphate analyses were made by the method of Ames and Dubin (1960). Inorganic sulfate was quantitatively precipitated as the barium salt for sulfate analysis.

PREPARATION OF THE β-GLYCEROPHOSPHATE DERIVATIVE OF ALDOLASE.[5] Uniformly labeled [^{14}C]DHAP was prepared from [^{14}C]FDP by the procedure similar to that of Horecker *et al.* (1963) except that the radioactive [^{14}C]DHAP was isolated from the reaction mixture by chromatography on a thoroughly washed neutral Dowex-1 (Cl^-)×2, 200–400 mesh, 0.8 × 10.3 cm column, eluting the DHAP with 0.03 N HCl after a water wash (Bartlett, 1959). The eluted [^{14}C]DHAP was concentrated on a Rinco evaporator to a small volume. (The yield of DHAP recovered from these steps is about 95%.) Glyceraldehyde 3-phosphate which was 2–5% of the DHAP at this step was oxidized with a small amount of bromine to glyceric acid and the excess bromine was removed by aeration. The [^{14}C]DHAP was stored at 4° at pH ~1. The final molar specific activity of the [^{14}C]DHAP was equal to one-half of that of the [^{14}C]FDP used as the starting material. Preparations I and II had specific activities of 3.8×10^5 and 1.7×10^6 cpm/μmole, respectively. The latter was diluted with unlabeled DHAP prior to use so that the specific activity was 1.84×10^4 cpm/μmole. A modification of the procedures of Horecker *et al.* (1963) and Lai *et al.* (1965b) was used in the preparation of the β-glycerophosphate derivative of aldolase. Dialyzed aldolase was placed in a jacketed pH-Stat cell with circulating ice water maintaining the temperature at 2–4° and with magnetic stirring used for mixing. A 50-fold excess of DHAP was added after pH adjustment to pH 6. Approximately 16 drops of 2-mercaptoethanol and 10 drops of *n*-octyl alcohol were added. (Preparation I = 100 mg of aldolase in ~14 ml; preparation II = 67 mg of aldolase in ~8 ml.) Freshly prepared 3 M $NaBH_4$ was added in 20-μl additions. The pH was maintained at pH 6.0 ± 0.3 by the simultaneous addition of 1.2 N HCl from a micrometer syringe. No further loss of aldolase activity could be detected after a 70-fold excess of $NaBH_4$ to DHAP had been added, and no more than a 100-fold excess of $NaBH_4$ was added. (Recently Lai *et al.* (1965b) have found that a 2.5-fold excess of $NaBH_4$ is actually sufficient.) An aliquot from the reaction mixture was precipitated with 2 volumes of 10% TCA and washed with three to four 3-ml portions of 3% TCA until free of soluble ^{14}C counts. The washed precipitate was dissolved in 0.2 N NaOH, and aliquots from this solution were removed for counting and concentration determinations. The remainder of the β-glycerophosphate aldolase derivative was exhaustively dialyzed against the 0.02 M Tris–0.017 M chloride buffer,[2] clarified by centrifugation, further analyzed, and then used in the equilibrium dialysis experiments (Table I).

TABLE I: Analyses.

Prepn	I	II	Native Aldolase
Final yield of protein (% of initial)	49	30	*a*
Moles of [^{14}C]DHAP/mole of aldolase	2.58	2.54	...
Activity (units/mg)	0.1	0.1	16–19
% of original activity	<1	<1	100
λ_{max} (mμ) in ultraviolet spectra	280	280	280
λ_{min} (mμ) in ultraviolet spectra	252	252	252
(OD) max/(OD) min	2.85	2.8	2.8–3.0
$\eta_{sp/c}$ (ml/g)[b]	4.5	4.6	4.4

[a] See Grazi *et al.* (1962a). [b] Measurement at 10.1° (I), 11.9° (II), and 10.1° (control), with c = 2.86, 2.88, and 4.07 mg/ml, respectively.

[5] We are indebted to Dr. Gertrud Szabolcsi for her most generous help with this procedure during a 3-month visit to our laboratory from the Institute of Biochemistry, Hungarian Academy of Sciences, Budapest, Hungary.

PREPARATION OF HEXITOL 1,6-DIPHOSPHATE FROM FDP.[6] FDP and [U-^{14}C]FDP (along with D-fructose and the sodium salt of D-fructose 6-diphosphate as chromatographic controls) were chemically reduced with a 12-fold molar excess of solid sodium borohydride added in the cold, final pH ~10 (see Abdel-Akker *et al.*, 1951; Bragg and Hough, 1957). The reaction mixtures were refrigerated for 20–90 hr. Sodium and other cations were removed by treatment with an excess of Dowex-50 (H^+) resin which was removed by filtration. The acid filtrate containing the product was concentrated under vacuum in a rotatory evaporator; boric acid was removed by alternate addition of methanol and evaporation (four cycles) and the product dissolved in water (Isbell and Frush, 1962). The pH of the product was adjusted to pH 6–7 for storage. If the hexitol diphosphate product, obtained in 95–100% yield, contained any FDP (at most 2%) it was recycled through the above procedure until no FDP remained. The final inorganic phosphate content was ≦2% or about the same as the starting material. Concentrations were determined by organic phosphate analyses (above). Paper chromatography of the phosphate esters (0.1–0.2 μmole), with carrier hexitol diphosphate added to the radioactive product, was carried out by the descending method at room temperature using Whatman No. 1 paper and solvent 1 (methanol–ammonia–water, 7:2:1). Aliquots of the hexitol diphosphates (2.5 μmoles), with and without [U-^{14}C]hexitol 1,6-diphosphate (0.03 μmole), were dephosphorylated with alkaline phosphatase (~25 μg) at pH 8; the release of inorganic phosphate was followed until 99–100% of the theoretical value was observed (~5 hr at 25°). The dephosphorylated compounds (0.05 μmole) were chromatographed overnight in the same way using solvent 2 (ethyl acetate–glacial acetic acid–formic acid (90%)–water, 18:3:1:4). The phosphate esters were detected with the acid molybdate spray of Hanes and Isherwood (1949), and the dephosphorylated compounds by the silver nitrate dip of Anet and Reynolds (1954). Before development, the radioactive chromatograms were scanned in a Vanguard Model 880 strip autoscanner.

In the case of the reduced [U-^{14}C]FDP product (app sp act. ≅ 4.3 × 10^6 μc/μmole) a chromatographic impurity in solvent 1 was detected. Of the total counts applied, 75–80% corresponded to the R_F of FDP or reduced FDP and 20–25% had an R_F about midway between the mono- and diphosphates (R_F 0.50 ± 0.05 and R_F 0.23 ± 0.07, respectively) in solvent 1. The impurity arose from that originally present in the particular batch of [U-^{14}C]FDP used in the reduction (app sp act. = 4.7 × 10^6 μc/μmole before subsequent purification by Schwarz BioResearch, Inc.,[4] using Dowex-1 (Cl^-) chromatography. The specific activity of the further purified, 99% pure, [U-^{14}C]FDP = 3.4 × 10^6 μc/μmole. The [U-^{14}C]hexitol diphosphate preparation was purified before use as follows: 5.9 μmoles (25 μc) of the reduced [U-^{14}C]FDP product in 0.16 ml was streaked onto Whatman No. 1, chromatographed 7 hr in solvent 1, and the radioactive zones were located by a 20-min exposure on X-ray film. The unknown radioactive contaminant with R_F 0.4 was resolved from the major component and stained as a phosphate ester. The major radioactive band, which corresponded to the position of FDP or the hexitol 1,6-diphosphates (R_F 0.2), was cut out and eluted with water. Rechromatography of this compound in solvent 1 indicated that >98% of the radioactivity chromatographed with the diphosphates (FDP and reduced FDP). The yield of the purified hexitol 1,6-diphosphates was 4.92 μmoles with a specific activity of 3.7 × 10^6 μc/μmole; this material was used exclusively.

In 18.5 hr of development with solvent 2 the dephosphorylated hexitol 1,6-diphosphates and the chemically reduced D-fructose moved 15.9 cm from the origin and corresponded exactly with the position of radioactivity from the dephosphorylated [U-^{14}C]hexitol 1,6-diphosphates. The position of D-fructose was 18.1 cm. The hexitols arising from the reduction of D-fructose (Abdel-Akker *et al.*, 1951) are not resolved in solvent 2.

[^{32}P]PHOSPHATE PURIFICATION. When necessary, the method of Bergmann (1962) was used. Carrier-free [^{32}P]H_3PO_4 (3 mc) was chromatographed on a column (0.8 × 10.3 cm) of neutral Dowex-1 (Cl^-) ×2, 200–400 mesh (previously cycled through the (OH^-) and (Cl^-) forms and, after equilibration at pH 1, exhaustively washed). The column was eluted with a solution 0.01 M in HCl and 0.05 M in KCl and fractions of 4 ml were collected. Fractions 7–9 contained 60–100% of the radioactive material applied to the column and represent the [^{32}P]orthophosphate fraction. This fraction was diluted 300–1000 times in preparing the standards for the equilibrium dialysis experiments. A dilution of the carrier-free [^{32}P]phosphate (~2.4 × 10^5 μc/ml) when equilibrated with or without aldolase (see below) was evenly distributed on both sides of the membrane.

PREPARATION OF RADIOACTIVE STANDARDS. [^{35}S]-Sulfate and [^{32}P]phosphate standards were prepared from 0.3099 M Na_2SO_4 and 0.2000 M Na_2HPO_4–KH_2PO_4 solutions, respectively. A micrometer syringe (0.0198 ml/division reading to 0.01 division) was used for the delivery of the stock solution into volumetric flasks. Carrier-free isotope and 1.0 M Tris–0.844 M chloride buffer at pH 7.4[2] were added so that in the final volume the buffer was 50-fold diluted and the phosphate and sulfate specific activities were about 0.2–17 μc/μmole and about 0.1–2 μc/μmole, respectively. [^{14}C]Hexitol diphosphate and [^{14}C]FDP standards were prepared on a smaller scale.

The concentration of all standards was determined to be ≧98% of the calculated value. However, in the organic phosphate determinations on the hexitol diphosphates, values had a maximum deviation of ±10%. The radioactivity of solutions was measured in a Packard Tri-Carb liquid scintillation counter, using the solvent of Bray (1960). The aqueous phase of standards was adjusted to that of the unknown in the different ex-

[6] We are grateful to Dr. Hugo H. Sephton, Laboratory of Chemistry, National Institute of Arthritic and Metabolic Diseases, for his help with parts of the procedures outlined.

periments since with volumes of water >0.1 ml some quenching occurs.

EQUILIBRIUM DIALYSIS METHOD. Techni-Lab Model E-1 (1 ml) dialysis cells were used for all except very low aldolase concentrations, when Model E-5 (5 ml) was used. The membrane was cut from a single layer of ordinary dialysis tubing that had been thoroughly soaked in water (at least 3 weeks with water changes) to rid it of ultraviolet-adsorbing material.

The aldolase solution was added to one side of the dialysis cell and the standard to the other by means of syringes fitted with Teflon needles. For equilibration, the closed cells were placed on a gentle shaker at 4°. Overnight was sufficient time to attain equilibrium of solvent ions in the absence of protein, and with protein present on one side of the membrane in the case of the radioactive inorganic ions. However, the equilibrium of the protein with the hexitol diphosphate required much longer times (see Results). After equilibration, both sides of the cell were sampled with micropipets and the radioactivity of each side was determined along with that of the appropriate standard. In all cases, the total radioactivity from the protein and solvent sides after equilibration accounted for >98% of the radioactive anion initially added to the cell. Thus, membrane adsorption of the radioactive anions was negligible in the 0.02 M Tris–0.017 M chloride buffer. Routinely, the concentration of aldolase, its activity against FDP, and the pH of the solutions after equilibration were determined. The solvent side could be replaced with the standard originally used or with the 0.02 M Tris–0.017 M chloride buffer and then the above procedure repeated.

OTHER MEASUREMENTS. Viscosity measurements were made in an Ostwald viscometer with an average shear gradient of 212 sec^{-1} and an outflow time of 55.59 ± 0.05 sec for 1 ml of water at 25.0°. Solution densities were calculated from measured solvent densities and from the weight fraction of protein and the partial specific volume (0.742 ml/g) determined at 20° by Taylor and Lowry (1956). The Leeds and Northrup No. 124138 miniature pH electrode assembly, adapted to a Radiometer TTT-1, equipped with a type PHA-630 scale expander, was used for all pH determinations. The reference electrode of this assembly has a very low leakage of KCl (Ginsburg and Carroll, 1965) and the pH of small volumes (≧0.1 ml) can be determined.

CALCULATIONS. The Donnan effect should not be evident at concentrations of chloride ion ≧2 × 10^{-3} M, estimating that the net charge of the aldolase in the equilibrium dialysis experiments ≦20 from the amino acid composition (Velick, 1949; Lai *et al.*, 1965b; Raftery and Schachman, 1963, unpublished data[7]). The chloride concentration used in the equilibrium dialysis experiments was approximately 10-fold greater than the calculated requirement. Then, under the conditions of our experiments, the difference between the radioactivity measured on the protein and solvent sides of the membrane can be related directly to the number of moles of the radioactive anion bound by the protein and the radioactivity of the solvent related to the concentration of the free anion.

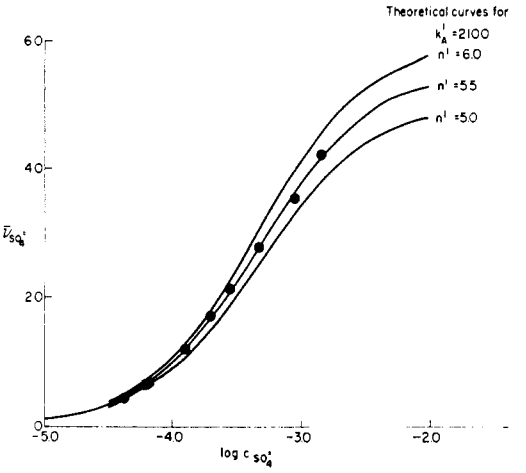

FIGURE 1: Experimental data and calculated curves for the equilibrium binding of sulfate ions to aldolase in 0.02 M Tris–0.017 M chloride buffer at pH 7.86. Aldolase concentration was 3.8 × 10^{-5} M, and equilibration times at 4° were 17–42 hr. Curves calculated from eq 1 and the parameters indicated.

Since the binding measurements are in the presence of relatively high concentrations of chloride ion, it was assumed that the monovalent phosphate ions are bound to a negligible extent. Therefore, the total concentration of free phosphate ions determined from the radioactivity measurements was corrected to that of the divalent species using the experimentally determined pH and the expression of Cohn (1927) for the activity coefficients

$$\mathrm{pH} = \mathrm{p}K_2 - \frac{1.5\sqrt{I}}{1 + 1.5\sqrt{I}} + 0.3I + \log \frac{[HPO_4^{2-}]}{[H_2PO_4^-]}$$

where I is the ionic strength of the solution and pK_2 = 7.20 at 25° (Green, 1933) with a temperature dependence (Bates and Agree, 1943); $pK_2 = 1979.5/T - 5.3541 + 0.01984T$, where T is the absolute temperature.

Results

The results from the measurements of equilibrium anion binding to native aldolase in 0.02 M Tris–0.017 M chloride, buffered at pH 7.8–7.9 at 4°, are shown in Figures 1–3. Tables II and III summarize in detail representative data for the binding of phosphate and hexitol diphosphate, respectively, to native aldolase and the inactive β-glycerophosphate derivative of aldolase. Apparent association constants and apparent number of binding sites may be calculated directly from these data if it is assumed that all nitrogenous groups participating in the binding of anions are protonated

[7] Personal communication from Dr. H. K. Schachman.

TABLE II: Representative Data on the Binding of Phosphate Ion at Equilibrium.

Aldolase Concn (M × 10^5)	pH at 4°	Total Concn Phosphate (M × 10^5)	Sp Act. (cpm/μmole of Phosphate × 10^{-6})	Free Concn of Phosphate (M × 10^5)	Calcd Free Divalent Ion Concn (M × 10^5)	$\bar{\nu}_A$	Equilibration Time at 4° (hr)	Aldolase Act. (Units/mg)
				Native Aldolase				
0.467	7.76	0.500	16.23	0.425	0.347	0.16 ± 0.01	18	13
0.513	7.79	0.513	11.83	0.426	0.352	0.17 ± 0.01	66	15
3.75	7.81	2.67	13.83	0.828	0.689	0.49 ± 0.02	17	13
3.75	7.79	6.30	5.36	2.67	2.21	0.97 ± 0.1	17	15
3.82	7.90	5.66	11.39	1.85	1.59	1.00 ± 0.05	66	11
6.76	7.81	22.1	1.22	9.11	7.58	1.92 ± 0.1	18	14
3.80	7.89	13.76	4.45	6.43	5.51	1.93 ± 0.1	66	12
4.49	7.81	34.6	1.07	22.9	19.1	2.6 ± 0.2	17	15
4.01	7.90	34.1	1.79	23.1	19.9	2.74 ± 0.1	66	12
4.38	7.90	62.5	0.894	48.9	42.0	3.1 ± 0.15	66	13
4.68	7.77	109.3	0.263	91.8	75.1	3.7 ± 0.2	19	18
4.66	7.78	107.6	0.251	89.7	73.8	3.85 ± 0.2	43	18
				β-[^{14}C]Glycerophosphate Aldolase Derivative[a]				
2.16	7.80	2.15	7.13	2.04	1.69	0.05 ± 0.03	23	0.1
2.16	7.80	5.07	2.81	4.90	4.06	0.08 ± 0.04	23	0.1
2.16	7.80	15.75	1.11	15.04	12.47	0.3 ± 0.1	23	0.1
2.16	7.80	30.3	0.572	28.0	23.2	1.1 ± 0.3	23	0.1
2.16	7.82	40.9	0.579	39.8	33.3	0.5 ± 0.3	43	0.1
2.16	7.83	53.2	0.529	52.3	43.8	0.4 ± 0.3	64	0.1

[a] The radioactivity of the ^{14}C bound to the protein (preparation I) made a negligible contribution to the ^{32}P determinations.

(Saroff and Carroll, 1962; Loeb and Saroff, 1964). Then the expression of Saroff (1957) reduces to the mass law binding expression (see Klotz, 1953)

$$\bar{\nu}_A = \frac{k_A' c_A n'}{1 + k_A' c_A} \quad (1)$$

where $\bar{\nu}_A$ is the average number of anions bound to one mole of protein, k_A' is the apparent intrinsic association constant for the binding of anions at the concentration c_A of the free anion, and n' is the apparent number of available equivalent binding sites on each protein molecule. Activity coefficients of the anions are assumed to be unity.

Equation 1 was applied to the data obtained for the binding of sulfate ions to aldolase and the results are shown in Figure 1. The experimental points are fitted best[8] by a simple binding curve described by k_A' = 2100 and n' = 5.5. Calculated curves with n' set at 5.0 and 6.0 are shown also for qualitatively estimating the effect of variations in this parameter.

For equilibrium dialysis, the ionic strength of the medium is increased so that the Donnan effect is negligible. The anion (A_2) added for this purpose may be considered as a possible competing anion for identical equivalent sites on the aldolase molecule. Then the observed value of $\bar{\nu}_{A_1}$ for the average number of anions (A_1) bound per protein molecule will be a function of the free concentration of the second anion (c_{A_2}), which has an apparent association constant of k_{A_2}''.

$$\bar{\nu}_{A_1} = \frac{k'_{A_1} c_{A_1} n'}{1 + k_{A_1}' c_{A_1} + k_{A_2}'' c_{A_2}} \quad (2a)$$

Conversely, the average number of A_2 anions bound per protein molecule ($\bar{\nu}_{A_2}$), may be estimated from the expression

[8] The best fit is defined as that which minimizes the sum of the squares of the differences between the observed and calculated values of $\bar{\nu}$. We are indebted to Jonathan Schwartz and later James E. Kiefer, Computation and Data Processing Branch, National Institutes of Health, for programing and calculating the curves on a Minneapolis-Honeywell 800 computer.

TABLE III: Representative Data on the Binding at pH 7.8–7.9 of the Diphosphate Esters at Equilibrium.

Aldolase Concn (M × 10^5)	Total Concn of Hexitol Diphosphate (M × 10^5)	Sp Act. (cpm/μmole of Hexitol Diphosphate × 10^{-6})	Free Concn of Hexitol Diphosphate (M × 10^5)	$\bar{\nu}_A$	Equilibration Time at 4° (hr)	Aldolase Act. (Units/mg)
Hexitol 1,6-Diphosphate Binding to Native Aldolase						
0.0750	0.0488	2.0	0.0280	0.3 ± 0.1	66	13
0.0825	0.105	2.05	0.0473	0.7 ± 0.15	66	16
6.23	8.06	3.7	0.108	1.28 ± 0.05	77	17
7.78	10.87	3.47	0.138	1.38 ± 0.05	67	19
0.434	0.840	3.26	0.179	1.5 ± 0.1	66	16
0.47	1.45	3.26	0.464	2.1 ± 0.1	67	14
7.83	16.77	3.7	0.544	2.07 ± 0.05	72	19
4.77	16.4	0.37	3.62	2.7 ± 0.2	64	17
4.66	15.56	0.37	4.14	2.45 ± 0.2	140	19
4.69	15.1	0.37	3.77	2.4 ± 0.2	210	19
4.68	55.9	0.37	43.8	2.6 ± 0.2	64	20
4.77	57.1	0.37	44.8	2.6 ± 0.2	140	20
4.58	55.9	0.37	43.7	2.7 ± 0.2	210	23
Hexitol 1,6-Diphosphate Binding to the β-[^{14}C]Glycerophosphate Aldolase Derivative[a]						
0.388	2.03	3.32	2.03	0	66	0.1
1.94	2.63	3.56	2.43	0.1 ± 0.1	65	0.1
0.932	2.64	3.32	2.43	0.2 ± 0.1	68	0.1
1.94	5.23	3.7	4.84	0.2 ± 0.1	65–89	0.1
1.97	50.4	0.358	48.8	0.8 ± 0.3	65	0.1
1.85	49.2	0.358	46.9	1.2 ± 0.4	65	0.1
Fructose 1,6-Diphosphate Binding to Native Aldolase						
	Calcd Total Concn Fructose Diphosphate[b] (M × 10^5)	Sp Act. (cpm/μmole Fructose Diphosphate × 10^{-6})	Free Concn Fructose Diphosphate[b] (M × 10^5)	$\bar{\nu}_A'$[c] (obsd)	$\bar{\nu}_A'$[c] (calcd)		
4.75	30.3	0.33	18.8	2.7 ± 0.1	2.43	70	15
4.73	18.3	0.33	7.14	2.5 ± 0.15	2.36	70	16
2.36	43.2	0.33	37.3	3.2 ± 0.3	2.50	70	16

[a] Preparation II: The ^{14}C of the preparation contributed ≦1% of the hexitol diphosphate counts. [b] Corrected for dihydroxyacetone phosphate present (the ratio of $c_{FDP}/c_{DHAP} \cong 1$ was determined by assay of the solvent). [c] The observed $\bar{\nu}_A'$ from the number of protein-bound counts includes both [^{14}C]FDP binding and [^{14}C]DHAP binding to aldolase. $\bar{\nu}_A'$ for the moles of FDP bound per mole of aldolase was calculated from eq 2a assuming $k_{A_1}' = 8.26 \times 10^5$ and $k_{A_2}'' = 5 \times 10^4$ (Mehler and Bloom, 1963) for the binding of FDP and DHAP to aldolase, respectively, and $n' = 2.67$ for both substrates.

$$\bar{\nu}_{A_2} = \frac{k_{A_2}'' c_{A_2} n'}{1 + k_{A_1}' c_{A_1} + k_{A_2}'' c_{A_2}} \qquad (2b)$$

The expression 2a may be used to estimate the effect of chloride ion binding ($\bar{\nu}_{A_2}$) on the apparent association constant for sulfate ion binding (k_{A_1}') with the free concentration of chloride ion (c_{A_2}) equal to 0.017 M in all of the equilibrium dialysis experiments. For this

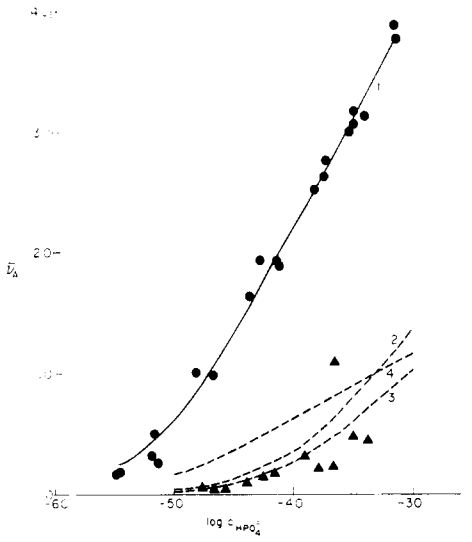

FIGURE 2: Experimental data and calculated curves for the binding of phosphate ions to native aldolase (●) and to the β-glycerophosphate aldolase derivative (▲) in 0.02 M Tris–0.017 M chloride buffer at pH 7.8–7.9. Curve 1 calculated from a two-term form of eq 3 and parameters $n_1' = 2.6$ with $k_1' = 2.8 \times 10^4$, and $n_2' = 2.9$ with $k_2' = 1.05 \times 10^3$. Curves 2 and 3 calculated from eq 1 and parameters $k_A' = 2100$, $n' = 2.0$ and 1.5, respectively. Curve 4 is composite (eq 3) for $n_1' = 0.7$ with $k_1' = 2.8 \times 10^4$, and $n_2' = 0.7$ with $k_2' = 2100$. The data (●, ▲) include that of Table II.

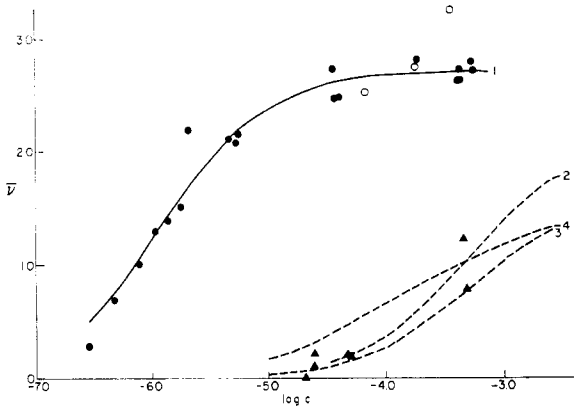

FIGURE 3: Experimental data and calculated curves for the binding of hexitol diphosphate to native aldolase (●) and to the β-glycerophosphate derivative (▲) in 0.02 M Tris–0.017 M chloride at pH 7.8–7.9. Curve 1 calculated from eq 1 and parameters $n' = 2.7$ and $k_A' = 8.26 \times 10^5$. Curves 2–4 calculated as indicated in the legend to Figure 2. (○), data from the binding of fructose diphosphate to aldolase (Table III). The data (●, ▲) include that of Table III.

purpose, the competition between sulfate and chloride anions is assumed to occur at all binding sites. Table IV shows the effect of chloride ion binding, with association constants for Cl^- (k_{Cl^-}'') of 0–1000 assumed, on the apparent association constant for the binding of sulfate ions ($k_{SO_4^{2-}}'$). In the absence of direct measurements, chloride ion binding is unknown. However, it can be estimated that $k_{Cl^-}'' \ll k_{SO_4^{2-}}'$ from the value of the apparent $k_{SO_4^{2-}}'$ measured for the binding of sulfate to aldolase, and from the difference in magnitude of these constants for ribonuclease (Saroff and Carroll, 1962). Thus, in Table IV reasonable values for $k_{SO_4^{2-}}'$ are associated with assumed values of 0 to 100 for k_{Cl^-}''. Using expressions 2a and 2b, $c_{Cl^-} = 0.017$ M, and $k_{Cl^-}'' = 100$, the number of protein sites with sulfate ion competitively displaced by chloride ion is shown not to alter significantly the sulfate binding curve. The binding results (Figure 1) at concentrations $>10^{-3}$ M sulfate ion experimentally are the least precise, and deviations between experimental and calculated values of $\bar{\nu}_A$ in this concentration range are to be expected. For example, at 4.4×10^{-3} M free sulfate and 3.95×10^{-5} M aldolase, experimental $\bar{\nu}_{SO_4^{2-}}$ values were determined to be 6.8 and 5.8 in different successive samplings of the same dialysis cells; consequently, these data were omitted from those used for calculations.[8] The precision at $<10^{-3}$ M concentrations of sulfate was at least ±3%.

TABLE IV: Anion Competition for $c_{Cl^-} = 0.017$ M.

k_{Cl^-}''[a]	$k_{SO_4^{2-}}'$[a]
0	2,100
10	2,500
100	5,700
1000	38,000

$c_{SO_4^{2-}}$ (M)	$\bar{\nu}_{SO_4}$[b]	Assume $n' = 6.0$; $k_{SO_4^{2-}}' = 5700$; $k_{Cl^-}'' = 100$ $\bar{\nu}_{SO_4^{2-}}$	$\bar{\nu}_{Cl^-}$	$\bar{\nu}_{SO_4^{2-}}$[c]
10^{-1}	6.0	6.0	0.02	5.5
10^{-2}	5.9	5.7	0.17	5.2
10^{-3}	5.1	4.1	1.2	3.7
10^{-4}	2.2	1.05	3.1	1.0
10^{-5}	0.3	0.1	3.7	0.1

[a] For $n' = 5.5$. [b] Equation 2a with $\bar{\nu}_{Cl^-} = 0$; $n' = 6.0$; $k_{SO_4^{2-}}' = 5700$. [c] Equation 1 for $n' = 5.5$ and $k_A' = 2100$ used to approximate experimental curve.

Figure 2 shows the experimental data for the binding of phosphate ions to native aldolase and the inactive β-glycerophosphate derivative with the concentration of free phosphate expressed as that of the divalent anion. The correction of the free phosphate ion concentration to that of the divalent species uniformly shifts the binding curve and does not change its shape (Table II). It is immediately apparent that the phosphate ion binding to the native protein is complex in that it cannot be expressed by eq 1. A fit for the data was obtained using the expression

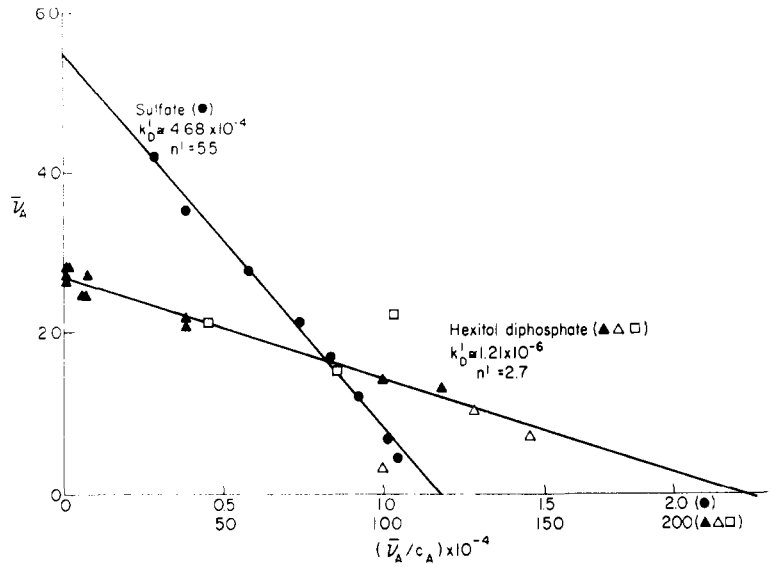

FIGURE 4: Experimental points from Figures 1 and 3 for the binding of sulfate (●) and hexitol diphosphate (▲, △, □) to aldolase plotted in the form of eq 4. Aldolase concentration ranges are 4–9 × 10^{-5} M (●, ▲), 2–6 × 10^{-6} M (□), and 4–9 × 10^{-7} M (△). The slopes of the straight lines are indicated by the apparent dissociation constants (k_D') and the intercept by the apparent number of available sites (n') at infinite anion concentration.

$$\bar{\nu}_A = \sum_{n'=i} \frac{k_i' c_A n_i'}{1 + k_i' c_A} \qquad (3)$$

for nonequivalent independent binding sites, with the restrictions: $\sum_i n_i' = 5.5$ and $k_1'/k_{2,3\ldots i}' > 4$. The first restriction arises from the reasonable assumption that the divalent phosphate ion would bind to the native protein at the same sites as does the divalent sulfate ion. Thus, the total number of available phosphate ion binding sites should approach 5.5 (Figure 1). The second restriction was introduced because binding sites which only differ slightly in k' cannot be distinguished. Thus, mathematical solutions including small differences in k' are excluded so that the final solutions give only a gross division of the sites. A good fit[8] of the data ($\bar{\nu}_A^{obsd} = \bar{\nu}_A^{calcd} \pm 0.1$) was obtained with a two-term form of eq 3, or with the binding sites roughly divided into two equal sets of sites described by $k_1' = 2.8 \times 10^4$ and $k_2' = 1.05 \times 10^3$. The covalent attachment of ~2.6 equiv of dihydroxyacetone phosphate to aldolase, forming the inactive β-glycerophosphate derivative, eliminates the very specific binding of phosphate ions. However, some binding of phosphate ions to the inactive derivative occurs at the higher concentrations of phosphate. The data for the binding of phosphate to the inactive derivative (Figure 2) are fitted by a curve constructed from eq 1 with $k_A' \cong 2.1 \times 10^3$ and $n' = 1.5$–2. These results obtained at the high concentrations of phosphate ions and low concentration of the derivative are approximate.

Table II is supplementary to Figure 2 in that it gives the experimental details of representative data shown only in Figure 2 in the form of expression 1. Equilibrium of the protein and solvent sides of the membrane was obtained in at most 17 hr. Specific activities of the radioactive [^{32}P]phosphate solutions are shown to indicate the sensitivity of the binding measurements. After equilibration, the measured pH, aldolase concentration, and aldolase activity are indicated.

Figure 3 shows the binding of hexitol 1,6-diphosphate to native aldolase and to the inactive β-glycerophosphate derivative. At three relatively high concentrations of fructose 1,6-diphosphate, at which FDP is the favored form (Herbert *et al.*, 1940), the binding of this compound to native aldolase could be measured also. The experimental points with the native protein are fitted smoothly ($\bar{\nu}_A^{obsd} = \bar{\nu}_A^{calcd} \pm 0.16$) by eq 1 with the parameters $k_A' = 8.26 \times 10^5$ and $n' = 2.7$. In this case, the competition of chloride ions at the 0.017 M concentration (see Table IV) of these experiments would have a negligible effect on the calculated parameters for the binding of hexitol diphosphate. The binding of the diphosphate substrate analog to the β-glycerophosphate derivative is similar to that found for the binding of phosphate ions to this protein.

Representative experimental data for some of the results presented in Figure 3 are given in Table III. Longer equilibration times were required in the binding measurements with hexitol diphosphate than with the inorganic anions, as seen by comparing Tables II and III. The membranes were not treated to increase permeability, but overnight at 4° was sufficient time for complete equilibration of the solvent (see Methods). The longer times required for equilibration in the hexitol diphosphate case, as contrasted with the phosphate case (Table II), suggest the possibility that slow conformational changes are induced by the diphosphate

substrate analog. There does appear to be a stabilization and/or activation produced by the hexitol diphosphate. The time-dependent activation of aldolase by phosphate ions has been mentioned (see Methods).

It is well known that aldolase cocrystallizes to some degree with triose phosphate isomerase and that this makes it difficult to free aldolase preparations completely from the isomerase. The extremely specific binding of hexitol diphosphate to aldolase was used in an ammonium sulfate recrystallization of aldolase in the hopes that the substrate analog would stabilize a particular crystalline form (see Czok and Bücher, 1960). Aldolase, three-times recrystallized with triose phosphate isomerase activity = 0.0058 u/mg of aldolase, was preequilibrated with 5×10^{-3} M hexitol diphosphate at 4°. A somewhat lower concentration of ammonium sulfate (0.37 saturation) then was required for crystal formation. Subsequent harvesting and solubilizing these crystals yielded aldolase with triose phosphate isomerase activity = 0.00048 u/mg of aldolase or 8.3% of the initial activity. Ordinarily, several careful recrystallizations would be necessary to produce a comparable reduction in isomerase activity. However, the results were disappointing in that some isomerase activity persists in all cases. We were equally unsuccessful in removing traces of triose phosphate isomerase by passage of the aldolase through a DEAE-cellulose column either as described by Richards and Rutter (1961) or by Westhead *et al.* (1963). Thus, the equilibrium binding of DHAP to aldolase could not be measured by the method used here.

For equivalent, noninteracting binding sites with k_A' a constant, the binding data can be treated in the manner suggested by Scatchard (1949) in which eq 1 is rearranged to

$$\bar{\nu}_A = -(k_D')\frac{\bar{\nu}_A}{c_A} + n' \qquad (4)$$

where k_D' is the apparent dissociation constant (or $1/k_A'$). A plot of $\bar{\nu}_A$ *vs.* $\bar{\nu}_A/c_A$ has a slope and intercept on the $\bar{\nu}$ axis equal to $-k_D'$ and n', respectively. This treatment of the data from Figures 1 and 3 for the binding of sulfate ions and the hexitol diphosphates to native aldolase is shown in Figure 4. Either set of data gives a linear plot and the different values for k_D' calculated from the slopes are shown in Figure 4 for the diphosphate substrate analog and for sulfate ions. Then, in the binding of one or the other of these specific anions, the apparent association constant or independent probabilities (Scatchard, 1949) are the same at each binding site of the native aldolase molecule. In this respect, the binding to native aldolase of sulfate or hexitol diphosphate anions is quite different from that of inorganic phosphate. In the phosphate case (Figure 2), the binding data cannot be expressed simply by the mass law eq 1 and 4. It is significant that the K_i value determined by the method of Dixon (1953) for competitive inhibition of the hexitol 1,6-diphosphates at two concentrations of fructose 1,6-diphosphate [with assay solutions buffered at pH 7.4 with 0.02 M Tris (see Methods)] is the same within the experimental error of that measurement as the k_D' value shown in Figure 4 for the substrate analog. The K_m value for fructose 1,6-diphosphate in a similar assay system free of inorganic divalent anions is comparable also to the k_D' value for the hexitol diphosphates.

Discussion

The most direct interpretation of the data in this paper is that each molecule of aldolase at pH 7.8 ± 0.1 has six sites to which polyvalent anions are tightly bound. The difference between the measured value of 5.5 and 6 may be attributed to a partial denaturation of the enzyme during isolation or to an electrostatic effect. The latter could involve modification of the enzyme by the anions in the medium or the net-charge change of the protein molecule produced during the successive binding of divalent ions to the specific sites (Linderstrøm-Lang, 1924; Saroff, 1957). These figures are obviously a function of the molecular weight. We have used the more recently published value of 142,000, which gives a smaller value for the number of binding sites than the molecular weight of 149,000 (Taylor and Lowry, 1956) used in earlier studies. The binding data were obtained at pH 7.8 ± 0.1, but it can be anticipated that the binding of anions to aldolase at this pH is fairly representative of at least the pH range 7.2 to ~8.8. The K_m–pH profile of the enzyme is constant in this pH range [the decrease in K_m, pH 7.2–6.0, even being associable with the protonation of the phosphate groups of the FDP substrate (Mehler, 1963)], and in this report a definite relationship between highly specific anion binding and catalytic activity is established. Further, although under somewhat different conditions, Velick (1949) found ~6–7 equiv of phosphate bound to aldolase at pH 6.05 and 0.10 ionic strength in potassium phosphate buffer.

It is apparent that only a small fraction of the approximately 202 ionizable nitrogen sites of aldolase[7] participate in the very specific anion binding at neutral pH values, although all may be clustered in an arrangement similar to that proposed by Loeb and Saroff (1964) for ribonuclease (where three charged basic groups are centered between a pair of carboxyl groups). The conditions employed here do selectively measure very specific anion binding of the divalent anions since a competitive (Mehler, 1963) monovalent anion at 0.017 M concentration was in the buffer solvent. However, it may be necessary to discharge the carboxyl groups (numbering about 156)[7] to promote more anion binding than that observed here for the divalent anions at pH 7.8 (Loeb and Saroff, 1964). A number of charged groups obviously participate in the maintenance of structure as shown by the dissociation of aldolase at low pH values (Stellwagen and Schachman, 1962; Deal *et al.*, 1963; Westhead *et al.*, 1963) and in slightly alkaline solutions (Hass and Lewis, 1963).

The six anion binding sites appear to be associated as three pairs. Aldolase binds hexitol diphosphate or fructose diphosphate to about one-half the extent of

inorganic orthophosphate.[9] The binding of the potent competitive inhibitor, hexitol 1,6-diphosphate, appears to involve only one set of equivalent sites, whereas about the same number of sites appear to exhibit an unusually large binding constant for phosphate ions. Thus, the two anion binding sites in each pair are characterized by having different apparent association constants for phosphate ions. The apparent association constant for the binding of hexitol diphosphate to aldolase is about 30 times greater than that for the most specific binding of phosphate ions. This value can be expected to reflect structural, steric, and electrostatic factors. The association of the two binding sites in each pair must be in close physical approximation to permit cooperating protein groups of the pair to bind the two phosphate groups of hexitol diphosphate. Hartman and Barker (1965) estimated a distance of 10–12 A for the optimum separation of the phosphate groups from studies of K_i values for a series of analogs of fructose diphosphate. Extending this interpretation of the experimental results, the phosphate group of the β-glycerophosphate derivative of aldolase occupies the binding site with the greater affinity for phosphate and only the three weaker sites are left for binding either inorganic phosphate or an organic diphosphate. It is implicit in this analysis that 3 eq of dihydroxyacetone phosphate is firmly bound to aldolase on reduction with borohydride. Our measurements of the number of molecules of dihydroxyacetone phosphate combined with the aldolase molecule by this procedure (Horecker *et al.*, 1963; Lai *et al.*, 1965b) have consistently been more than 2. Failure to obtain 3 equiv (full) may be caused by use of enzyme preparations that are not fully active or by denaturation during the preparation of the inactive derivative. We have observed a blue shift in the ultraviolet absorption maximum on storage of the derivatized aldolase, which is consistent with a decreased stability of the protein.

It is not obvious why one set of binding sites should bind phosphate much more strongly than sulfate. The apparent association constant for the binding of sulfate to apparently equivalent sites of native aldolase is approximately equal to that of the less specific phosphate ion binding and also to that exhibited by the inactive β-glycerophosphate derivative toward phosphate or hexitol diphosphate. The phosphate and sulfate ions are very similar in size with each bearing two negative charges. A specific interaction, perhaps a hydrogen bond, may account for the characteristics of the binding of phosphate. A possibility to be considered is that the group that supplies the extra attraction for the phosphate ion is the ϵ-amino group of the lysyl residue located along the polypeptide chain (Lai *et al.*, 1965a) that subsequently forms the Schiff's base with the substrate (Grazi *et al.*, 1962b; Horecker *et al.*, 1963). It might be considered, also, that the entire structure includes a pair of binding sites and that the first divalent phosphate ion to be bound interacts with a centrally located group that can participate in either of the two sites that comprise the pair. If this is so, the two sites are potentially equivalent, but act as strong and weak sites as a result of interaction with the first phosphate group to be bound. Alternatively, the two sites may be distinct and only those enzyme–substrate combinations in which the dihydroxyacetone phosphate component is associated with the stronger site are catalytically active.

The difference between our results, showing approximately three molecules of substrate bound per molecule of aldolase, and those of Westhead *et al.* (1963) showing only a single molecule bound, cannot be explained with certainty. The use of radioactive substrates in the present case allowed the experiments to be conducted with 10- to 100-fold lower enzyme concentrations than were used by Westhead *et al.*, who also used a relatively high ionic strength. The effects of these high concentrations on enzyme conformation, substrate activity coefficient, and the precision of the measurements may have contributed to results different from ours.

The interpretation of the binding studies given above is consistent with independent analyses of aldolase. Kinetic studies (Mehler, 1963) indicated that the primary interaction of aldolase and its substrates is electrostatic. The binding of dihydroxyacetone phosphate measured spectrophotometrically (Mehler and Bloom, 1963) indicated a binding constant similar to that measured for the strong binding of phosphate, while the K_m for glyceraldehyde 3-phosphate and that for fructose diphosphate correspond roughly to the reciprocal of the binding of phosphate to the weaker site and to the apparent dissociation constant of hexitol diphosphate, respectively. The participation of both phosphate groups of fructose diphosphate in binding to aldolase has long been suggested by the low K_m for this substrate compared with that for fructose 1-phosphate (see review by Rutter, 1961). These K_m values probably are not complicated by kinetic factors because they are not affected by modification of aldolase by carboxypeptidase (Drechsler *et al.*, 1959), which decreases the rate of fructose diphosphate cleavage. Finally, the inertness of fructose 6-phosphate in the aldolase-catalyzed aldol cleavage reaction, the slow rate of reaction with fructose 1-phosphate, and the kinetics of the aldolase reaction (Rutter, 1961; Rose *et al.*, 1965) have shown that the combination of aldolase with fructose diphosphate or with the cleavage products is a stereospecific process, of which an important feature is the orientation of the substrate(s) for effective catalysis at the active site through a cooperative binding of the phosphate groups.

The number of active sites can be related to the finding of several groups that aldolase dissociates into thirds under denaturing conditions, if each of the three polypeptide chains of aldolase contains one active ϵ-amino group capable of forming the Schiff's base intermediate in the aldol reaction (Lai *et al.*, 1965a). The subunits have similar molecular weights (Stellwagen and Schach-

[9] Since this paper was submitted for publication, a communication from Castellino and Barker (1966) has presented evidence that three molecules of another effective inhibitor of aldolase, D-arabinitol 1,5-diphosphate, are bound to each molecule of aldolase.

man, 1962; Deal *et al.*, 1963) and apparently identical N- and C-terminal residues (Rutter, 1964; Kowalsky and Boyer, 1960). Although the rate of liberation of amino acids by carboxypeptidase (Kowalsky and Boyer, 1960; Winstead and Wold, 1964) suggests that one chain is not identical with the others, until more direct evidence is obtained about the structure of aldolase, it must be considered that all three chains are very similar, if not identical. That is, not only must the active lysyl ϵ-amino group be a core of each site, but the highly organized tertiary structure of the protein must maintain the correct configuration for each of the two adjacent phosphate binding sites.

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