acid it is qualitatively obvious that the undissociated hemerythrin loses its violet-red color (and thus its oxygen-carrying capacity) rapidly. Likewise in experiments with the azomercurial dye, it was found that dye was bound to the protein but that the hemerythrin, standing at about 4°, did not dissociate until several days had passed. The molecular events following attachment of the mercurial to the SH are still obscure.

Since hemerythrin is an oxygen-carrying pigment it is of interest to compare its macromolecular constitution with that of hemoglobin. The native macromolecule in the latter case has four sites for oxygen, and four protein subunits, in contrast to eight for hemerythrin. In hemoglobin, the subunits generally fall into two categories (e.g., α and β); in hemerythrin the identity or nonidentity of merohemerythrins has yet to be established. In both proteins SH groups are present but not S–S. On the other hand, blocking of SH groups disaggregates hemerythrin but does not change the macromolecular size of hemoglobin, although it does affect interactions between oxygen-binding sites. In an evolutionary sense these two pigments seem very far apart, not only in the absence of a heme group in hemerythrin, but even in differences in protein component. The subunit merohemerythrin has a molecular weight near 13,500, whereas that of hemoglobin is near 17,000, although a *paramecium* hemoglobin with weight near 13,000 has been reported recently (Smith *et al.*, 1962). It seems more likely that hemerythrin may be related to other nonheme iron-containing proteins such as ferredoxin (Valentine, *et al.*, 1963) and homogentisate oxidase (Flamm and Crandall, 1963) than to the heme pigments, despite the closer similarity to the latter group in physiological function.

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Binding Sites, Reactivation Phenomena, and Possible —S—S— Groups of Rabbit Muscle Aldolase*

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Equilibrium dialysis and ultracentrifuge experiments with rabbit muscle aldolase in presence of added dihydroxyacetone phosphate or fructose 1,6-diphosphate establish the presence of one substrate binding site per aldolase molecule. The binding of fructose 1,6-diphosphate has a K_d of about 4×10^{-6}, and is considerably stronger than the binding of dihydroxyacetone phosphate. Removal of the terminal tyrosine residues lowers the affinity of the enzyme for fructose diphosphate. Aldolase exposed to *p*H 3 loses activity and shows structural disorganization and chain separation which is nearly completely reversible upon return to *p*H 8. This suggests a dominant role of primary structure in determination of configuration of this multichain enzyme. A rapid initial and much slower secondary rate of reactivation was observed, and addition of chelating agents prevented the second, slower reactivation. No metals which might be suspected of contributing to aldolase activity were found in the native enzyme, however. Upon reduction with mercaptoethanol in 8 M urea, aldolase shows appearance of about one —SH group per enzyme molecule. Thus native aldolase probably has one or less reducible —S—S— group per molecule.

The presence in aldolase of at least three peptide chains has been demonstrated by determinations of carboxy- and amino-terminal groups (Kowalsky and Boyer, 1960; Udenfriend and Velick, 1951). This raised the question of whether one or more substrate binding sites are present on the aldolase molecule, and one purpose of this paper is to report data on determination of the number of binding sites per molecule. Concomitant with these studies, other investigations were made on the regaining of activity of acid-inactivated aldolase. Results obtained led to interest in possible disulfide groups and undetected metals in muscle aldolase, and results of studies in this area are also reported.

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† National Science Foundation predoctoral fellow.

The probability that aldolase undergoes dissociation in acid below a *p*H of about 4 was indicated by the early observations of Gralen (1939) with crystalline "myogen." That such dissociation does indeed occur has been demonstrated by Deal and coworkers (1963) and by Stellwagen and Schachman (1962). Thus the regaining of activity from dissociated aldolase as reported by these investigators and as studied in this paper is important in clarifying relationships between amino acid sequence and configurations essential for catalytic activity.

Experimental

Enzymes.—Aldolase was crystallized from frozen rabbit muscle by the method of Taylor (1955) with the minor modifications of Swenson and Boyer (1957). All preparations were recrystallized at least twice, with washing of crystals twice in cold $(NH_4)_2SO_4$ (0.48 part H_2O and 0.52 part $(NH_4)_2SO_4$ solution saturated at room temperature), and stored as a suspension in $(NH_4)_2SO_4$ solution. When desired, traces of triose phosphate isomerase were removed by passing up to 300 mg of enzyme, in 20–30 ml of 5×10^{-3} M Tris-HCl buffer, *p*H 7.4–7.5, containing 10^{-3} M EDTA, through a 6-g column of DEAE-cellulose. The molar concentration of aldolase was determined from its absorbancy at 280 mμ ($A_{280}/0.91$ equals grams of native aldolase per liter (Taylor, 1955) and $A_{280}/0.75$ equals grams of aldolase per liter in 8 M urea) and from its reported molecular weight of 149,000 (Taylor, 1955).[1]

Mixtures of triose phosphate isomerase and glycerol phosphate dehydrogenase prepared by C. F. Boehringer und Soehne were purchased from the California Corporation for Biochemical Research. Some lots contained appreciable amounts of contaminating aldolase activity and were therefore unsuitable for use in the aldolase assay.

Carboxypeptidase-degraded aldolase was prepared as described by Drechsler *et al.* (1959).

Aldolase activity was measured by decrease in absorbancy of DPNH in presence of α-glycerophosphate dehydrogenase and triose phosphate isomerase (Richards and Rutter, 1961) with 0.05 M imidazole, *p*H 7.4, or 0.007 M phosphite (Robertson and Boyer, 1956), *p*H 7.5 as a buffer, or by using the increase in absorbancy at 240 mμ in presence of hydrazine (Dreschler *et al.*, 1959). Specific activities, expressed as ΔA_{240} per minute per mg protein in the hydrazine assay at 25° were about 16, higher than previously reported (Drechsler *et al.*, 1959); the higher activity resulted from use of purer reagents. Activities as measured in the coupled assay at *p*H 7.4–7.5 were similar with imidazole, glycylglycine, or phosphite as a buffer, and have ranged from 14 μmoles of fructose 1,6-diphosphate cleaved at 25° per minute per mg protein to 24 μmoles at 30° during widely different time periods in course of the research. This variation appears to reflect primarily unknown differences in reagents used. In any given series of recovery experiments the same reagents were used. Reagents and enzymes used for substrate binding experiments gave activities close to 24 μmoles per minute per mg protein at 30°.

Special Reagents.–Fructose 1,6-diphosphate solutions were prepared by reacting the calcium salt (Mann Research Laboratories) with Dowex 50 (H^+) and subsequently adding sodium hydroxide to *p*H 7.5, or from the trisodium salt (Sigma; California Biochemical Corp.). Dihydroxyacetone phosphate dimethyl ketal (cyclohexylammonium salt) (California Biochemical Corporation) was hydrolyzed by stirring a solution of 100 mg/5 ml with 2 ml of Dowex 50 (H^+) resin for a minute or two, removing the resin by filtration, and allowing the acid solution to stand at 40° for 4 hours. The solution was then neutralized to *p*H about 5–6. Substrate solutions were stored frozen; they were found to be stable for at least several months.

Urea was recrystallized twice from hot 95% ethanol. Iodoacetamide was purchased from Eastern Chemical Corporation and was recrystallized twice from hot ethanol. β-Mercaptoethanol was obtained from Eastman Organic Chemicals and was redistilled before use. All other chemicals were commercial preparations used without further purification.

Substrate Assays.—Fructose 1,6-diphosphate was assayed by measuring the total oxidation of DPNH, as decrease in A_{340}, in a 0.4-ml cuvet containing DPNH, aldolase, α-glycerophosphate dehydrogenase, and triose phosphate isomerase in 0.05 M imidazole buffer, *p*H 7.4, at 30°. Concentration of dihydroxyacetone phosphate was measured in a similar system without aldolase and isomerase necessarily present. Measurements were initiated by adding 5 μl of the dehydrogenase-isomerase preparation to 200 μl of the otherwise complete assay mixture in a microcuvet and mixing with an L-shaped plastic stirrer. Substrates were over 95% pure, based on a molar extinction coefficient of 6,220 for DPNH (Horecker and Kornberg, 1948).

Techniques of Binding Measurement.—The enzyme samples used were first dialyzed against a solution containing 10^{-6} M EDTA, 0.05 M imidazole buffer, *p*H 7.4, and 0.40 M KCl, at 3°, and then concentrated by centrifuging at 144,000 *g* overnight. A 10-fold concentration increase with quantitative recovery was obtained easily. The above buffer solution was used in all binding measurements.

For measurement of dihydroxyacetone phosphate binding, both ultracentrifugation and equilibrium dialysis were used. In the ultracentrifuge technique, a solution of enzyme and substrate was centrifuged 45 minutes in the Spinco No. 40 rotor at 40,000 rpm in the Spinco Model L preparative ultracentrifuge, using tubes with inner dimensions of 0.4×6.5 cm containing 0.6–0.8 ml of solution. The protein meniscus region was clearly visible. Two or three 70-μl aliquots were removed from the upper layer near the top and assayed for substrate and enzyme. Because of the relatively large size of the aldolase molecule and small size of the substrate molecules, corrections for sedimentation of the substrates (see Schachman, 1959) were not necessary. Control experiments established that no sedimentation of substrate occurred within experimental error.

In the dialysis technique, Visking dialysis tubing was first soaked in water, expanded under 20 lb pressure to make it more porous, and then soaked several days in changes of buffer, for use in binding studies. Dialysis cells used were made by milling U-shaped grooves in 2-in. square plexiglass plates, 0.125 inch thick and pressing squares of cellophane membrane between pairs of such plates. A thin film of silicone grease around the edge of the cellophane prevented capillary loss of solvent. Four or five such pairs of cells were stacked together with 0.125-in. brass plates on each end of the stack, and the whole pile was compressed by thumbscrews through the corners of the stack. The volume of the cells was about 0.4 ml on each side. Enzyme and substrate were put into one side of each cell and buffer in the other. Small magnetic stirring bars were cut from iron or steel wire, sealed in glass or

[1] More recently a value of 142,000 for the mw has been reported by Stellwagen and Schachman (1962). Use of the latter value would not change any of the essential conclusions reached in this paper.

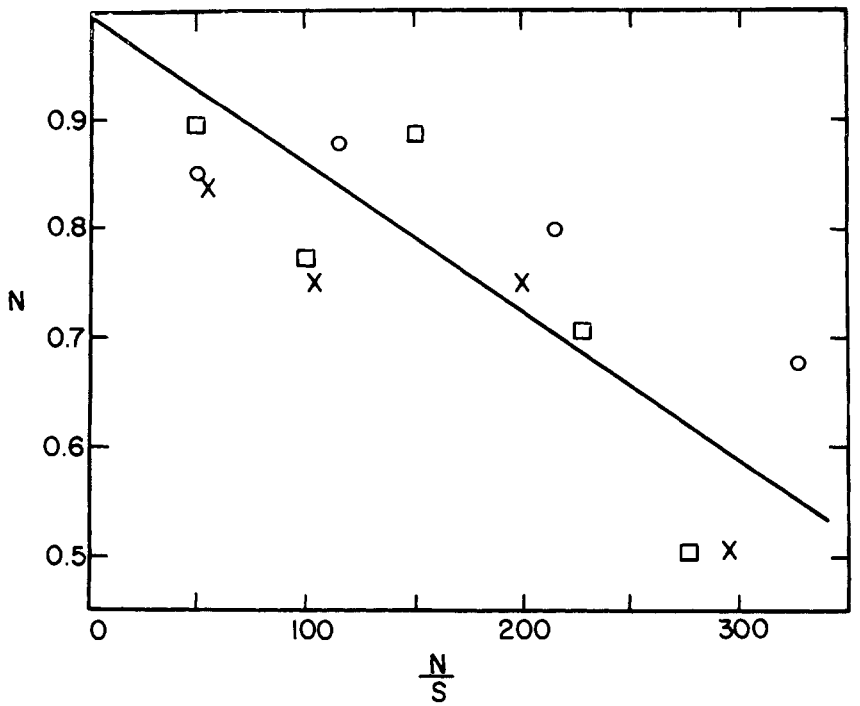

FIG. 1.—Binding of dihydroxyacetone phosphate by aldolase at 3°, in 0.05 M imidazole buffer, *p*H 7.4, 0.40 M in KCl. *N* is the number of moles of substrate bound per mole of enzyme and *S* is the concentration of free substrate. O, binding measured by ultracentrifugal method, E_0 (aldolase concentration) = 1.0×10^{-3} M; □, × binding measured by equilibrium dialysis, $E_0 = 1.3 \times 10^{-3}$ M and 0.95×10^{-3} M, respectively.

polyethylene, and put on the enzyme side of each cell. A magnetic stirrer placed on its side turned the bars very well. Equilibration took place in a few hours, but measurements were usually made after overnight equilibration.

Calculation of Binding Data.—The calculations used were essentially those suggested by Scatchard (1959). The concentration of free substrate, *S*, was subtracted from the concentration of substrate on the enzyme side of the membrane to give the concentration of enzyme substrate complex, *ES*. This value divided by the molarity of enzyme gave *N*, the number of moles of substrate per mole of enzyme. When *N* is plotted as a function of *N*/*S*, a straight line results if all binding sites have the same dissociation constant. The intercept on the *N* axis gives the number of binding sites per molecule and the slope is equal to $-K_d$, the dissociation constant of the complex.

Calculations of binding data for dihydroxyacetone phosphate were made only from measurements of free substrate and the known value of the total substrate concentration, since the very high enzyme concentrations used were found to interfere with the assay for substrate. Although theoretically justifiable, this procedure results in more random error in the data since any error in the determinations of *S* is doubled in the calculation of *ES*. When *ES* is relatively small, errors in *S* approach the value of *ES*.

When protein solutions of the order of 10% are used, a significant correction must be made for the volume occupied by the protein for comparison of the total substrate concentration with the concentration in the protein-free portion of the solution. Such corrections were not necessary for the fructose diphosphate data because much lower enzyme concentrations were used.

The equilibrium dialysis measurements with fructose 1,6-diphosphate showed that this substrate was much more firmly bound than dihydroxyacetone phosphate. Thus in the equilibrium mixtures present, binding other than that of fructose 1,6-diphosphate was probably negligible, and calculations were based on the actual concentration of fructose 1,6-diphosphate. These concentrations were estimated from the equilibrium data of Herbert *et al.* (1940) for aldolase extrapolated to 0°. It was sometimes found that the free dihydroxyacetone phosphate concentration decreased after an initial increase or that the total recovery of fructose diphosphate was low. The former was probably due to the contamination by isomerase; the latter was apparently the result of a contamination by a phosphatase, since free inorganic phosphate was found in a ratio of 2 moles per mole of missing fructose diphosphate. Data from such runs were discarded. Careful recrystallization of the aldolase plus passage through DEAE columns eliminated these problems.

Treatment of Aldolase with Iodoacetamide and β-Mercaptoethanol.—Before the initial iodoacetamide treatment, aldolase was incubated for 1 hour at room temperature with 0.02 M β-mercaptoethanol, 5×10^{-3} M Tris, and 1×10^{-3} M EDTA at *p*H 7.5. This insured that any —S—S— formed from readily reactive —SH groups would be cleaved. The β-mercaptoethanol was then removed by precipitating the protein with saturated $(NH_4)_2SO_4$, *p*H 4.5, or 0.4 M perchloric acid, and the protein dissolved in 4 M urea, *p*H 4. The protein was again precipitated and dissolved in 8 M urea, *p*H 4. The acid conditions were used to minimize oxidative formation of —S—S—. Urea solutions were used soon after adjustment to *p*H 4 with acetic acid; *p*H of acidified urea solutions rises rapidly, presumably because of urea hydrolysis. To a solution containing about 5–8 mg of aldolase per ml was added sufficient iodoacetamide to give a mole ratio of iodoacetamide-aldolase of 150. After several minutes, the mixture was titrated to *p*H 8.5. with 1 N NaOH, and allowed to stand for 2 hours at 40° or overnight at room temperature. Most of the excess iodoacetamide was removed by precipitating the protein with perchloric acid and the last traces were eliminated by passage of the protein dissolved in 8 M urea over a G-50

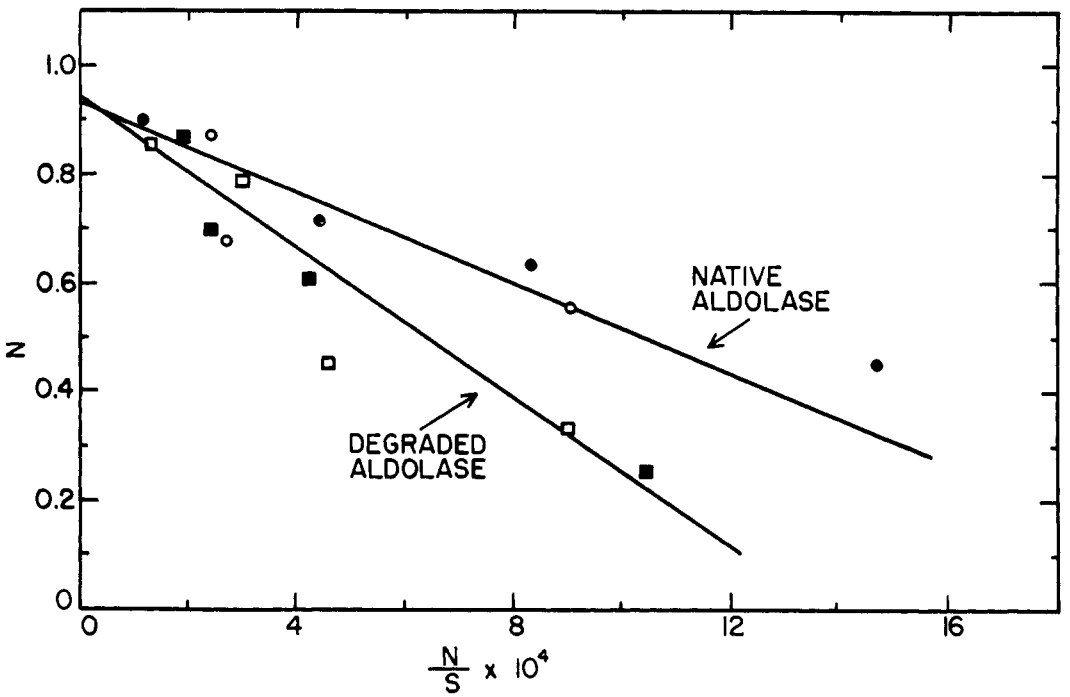

FIG. 2.—Binding of fructose diphosphate by aldolase (circles) and by carboxypeptidase-degraded aldolase (squares); conditions and ordinate symbols as in Figure 1. All measurements were by the dialysis method. Different symbols represent different preparations of enzyme with E_0 approximately as follows: ●, 3.0×10^{-4} M; ■, 2.0×10^{-4} M; ○, 2.7×10^{-4} M; □, 1.1×10^{-4} M. Calculations are based on the concentrations of fructose 1,6-diphosphate in equilibrium mixtures (see text).

Sephadex column which had been equilibrated with 8 M urea.

The iodoacetamide-treated aldolase was treated with 0.05 M β-mercaptoethanol in 8 M urea at *p*H 8.5 for 2 hours at room temperature to reduce any —S—S— present (longer incubations yielded substantially the same results). The β-mercaptoethanol was removed from the protein by perchloric acid precipitation and gel filtration on Sephadex G-50 in 8 M urea, *p*H 4. A portion of the reduced material was immediately assayed for sulfhydryl groups by use of the spectrophotometric assay with *p*-mercuribenzoate (Boyer, 1954; Swenson and Boyer, 1957). The remainder was reacted with iodoacetamide again to block any sulfhydryl groups produced by reduction. The resulting product, after removal of excess iodoacetamide on G-50 Sephadex, was assayed for sulfhydryl groups before and after a subsequent reduction with β-mercaptoethanol.

RESULTS

Binding Site Measurements.—Estimates of the number of binding sites per mole of aldolase were made by equilibrations with dihydroxyacetone phosphate or with fructose 1,6-diphosphate. In the latter instance, an equilibrium mixture of substrates was present, but measurement of total substrate bound gives a measure of number of binding sites with the assumption that a site can be occupied by either fructose 1,6-diphosphate or by two triose phosphates.

Figure 1 shows the results for binding of dihydroxyacetone phosphate. The data lack desired precision, in part because measurements of concentration were possible only on the dialyzate (see experimental section) and in part because of the inherent difficulty in measuring such weak binding constants. The binding constant calculated from the slope drawn is 1.6×10^{-3}. The results appear to show that there is only one instead of multiple binding sites on the aldolase molecule. More important by comparison with Figure 2, the data definitely establish that dihydroxyacetone phosphate is bound much less strongly than fructose diphosphate, thus justifying the assumptions made below.

Figure 2 shows the binding data for equilibration of fructose 1,6-diphosphate with aldolase and with carboxypeptidase-degraded aldolase. From the known equilibrium constant for the aldolase reaction (Herbert *et al.*, 1940), 50–90% of the unbound substrate was present as fructose 1,6-diphosphate at 3° over the range of substrate concentrations used. The binding observed, much tighter than with the dihydroxyacetone phosphate, justifies the assumption that only fructose 1,6-diphosphate binding is being measured within experimental error. The data of Figure 2 were calculated and plotted on the basis of the calculated molarities of fructose 1,6-diphosphate present in the equilibrium mixtures. The results show clearly that about one binding site per mole is present in both aldolase and carboxypeptidase-degraded aldolase. The lines drawn on the figure correspond to dissociation constants of 4×10^{-6} and 7×10^{-6} for aldolase and degraded aldolase, respectively.

Reactivation of Acid-inactivated Aldolase.—Results of most of our experiments on the reactivation of aldolase after exposure to *p*H near 3 will be summarized but not detailed in view of the more extensive similar investigations recently reported from other laboratories (Stellwagen and Schachman, 1962; Deal *et al.*, 1963).[2] In one series of investigations, recovery of activity after exposure to glycine buffer at *p*H 3 was measured by addition to 0.1 M imidazole buffer, *p*H 7.4–7.5, 25°,

[2] While these studies were in progress, information became available from preliminary reports (Deal, W. C., and Van Holde, K. E. [1962], *Fed. Proc. 21*, 254; Stellwagen, E., and Schachman, H. K. [1962], *Fed. Proc. 21*, 409), and private communications that detailed study of physical characteristics of reactivation of acid-treated aldolase was under way in other laboratories. Therefore this aspect of the investigation was not extensively pursued.

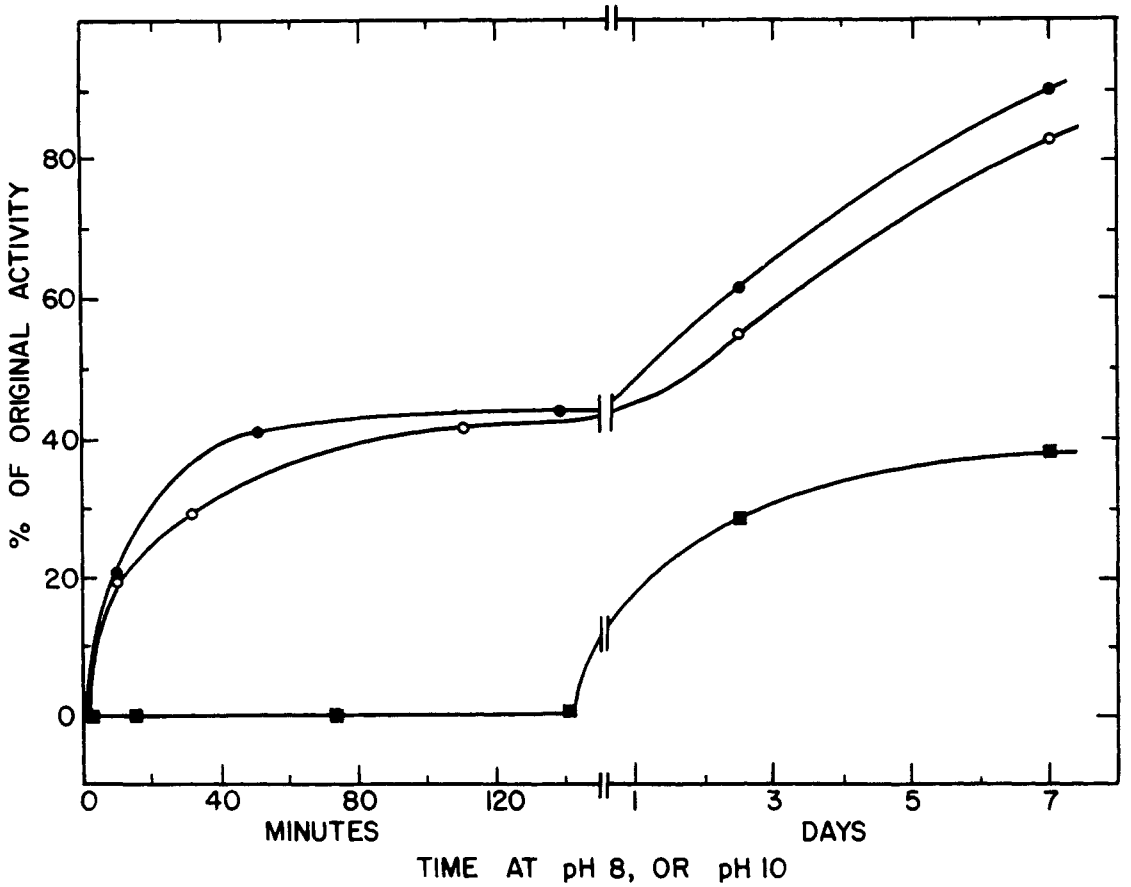

FIG. 3.—Effect of time and *p*H on the reactivation of acid-inactivated aldolase. Aldolase, about 0.45 mg per ml, was inactivated at *p*H 3.3 and 0° in 0.1 M glycine-HCl buffer containing 0.1 M KCl and 10^{-3} M EDTA, and reactivated at 3° by addition to an equal volume of 0.1 M glycine containing 0.1 M KCl and 10^{-3} M EDTA, with sufficient KOH to give the desired final *p*H. —●—●, 10 minutes at *p*H 3.3, *p*H 8 reactivation; —○—○—, 15 minutes at *p*H 3.3, *p*H 8 reactivation; —■—■—, 10 minutes at *p*H 3.3, *p*H 10 reactivation.

containing DPNH, fructose 1,6-diphosphate, α-glycerophosphate dehydrogenase, and triose phosphate isomerase. Recovery of activity decreased from about 40% to 10% with increase in exposure up to 5 hours at *p*H 3, 0.1 M glycine, 0.1 M KCl, and 25°. Recovery was found to be close to first order after various times of exposure although the half time for recovery increased from about 1 to 3 minutes, independent of the enzyme concentration at *p*H 3 from 1.1×10^{-6} to 2.7×10^{-5} M. The same degree of recovery was obtained whether or not substrate was initially present in the *p*H 7.4 buffer, and in an atmosphere of nitrogen compared to air.

Limited ultracentrifugation studies demonstrate that extensive dissociation of aldolase into subunits occurs at *p*H 3 under conditions similar to those used in many of the present experiments. Sedimentation equilibrium measurements were made at *p*H 3.0, 0.1 M glycine buffer, 1.0°, with aldolase concentrations of 5.2, 4.0, and 3.0 mg/ml and at 16,200 rpm in the Spinco analytical ultracentrifuge. With a partial specific volume of 0.742 (Taylor and Lowry, 1956) these measurements indicated a molecular weight of about 30,000. The possibility of dissociation into more than three chains must be considered, and further studies are warranted.[3]

Effects of *p*H, buffer composition, and temperature upon the reactivation process were evaluated. This led to conditions in which up to 90% of the original catalytic activity could be recovered, as shown in Figure 3. The recovery at *p*H 8 occurs in two stages; about 50% of the original activity is regained quite rapidly (half time of roughly 10 minutes) and the balance much more slowly (half time of the order of days). At *p*H 10 only the latter, slow reaction is observed (Fig. 3). Control samples in similar solutions at *p*H 8 and 10 lose less than 10% of their activity over a period of several days.

The acid-inactivated aldolase is relatively insoluble between *p*H 6 and 7.5, and apparently undergoes a temperature-dependent transition since 0.5% solutions at 0°, *p*H 8, precipitate immediately on warming to 25°.

[3] Such a possibility is also shown by the report of Haas and Lewis, Abstracts, ACS meeting, Cincinnati, January 1963, p. 34A, of the formation of six subunits from aldolase.

TABLE I

SPECIFICITY FOR EDTA IN REACTIVATION OF ALDOLASE

Conditions were similar to those given with Figure 3. Inactivation was for 10 minutes at *p*H 3.3 and 0°, and reactivation at *p*H 10 and 3° for 10 days.

Additions to Alkaline Buffer	Per Cent of Original Activity
None	
5×10^{-3} M *o*-Phenanthroline	0
Dimethylglyoxime	0
Bipyridine	0
Indole	0
Diethyldithiocarbamate	0
EDTA	51
1×10^{-3} M EDTA	36

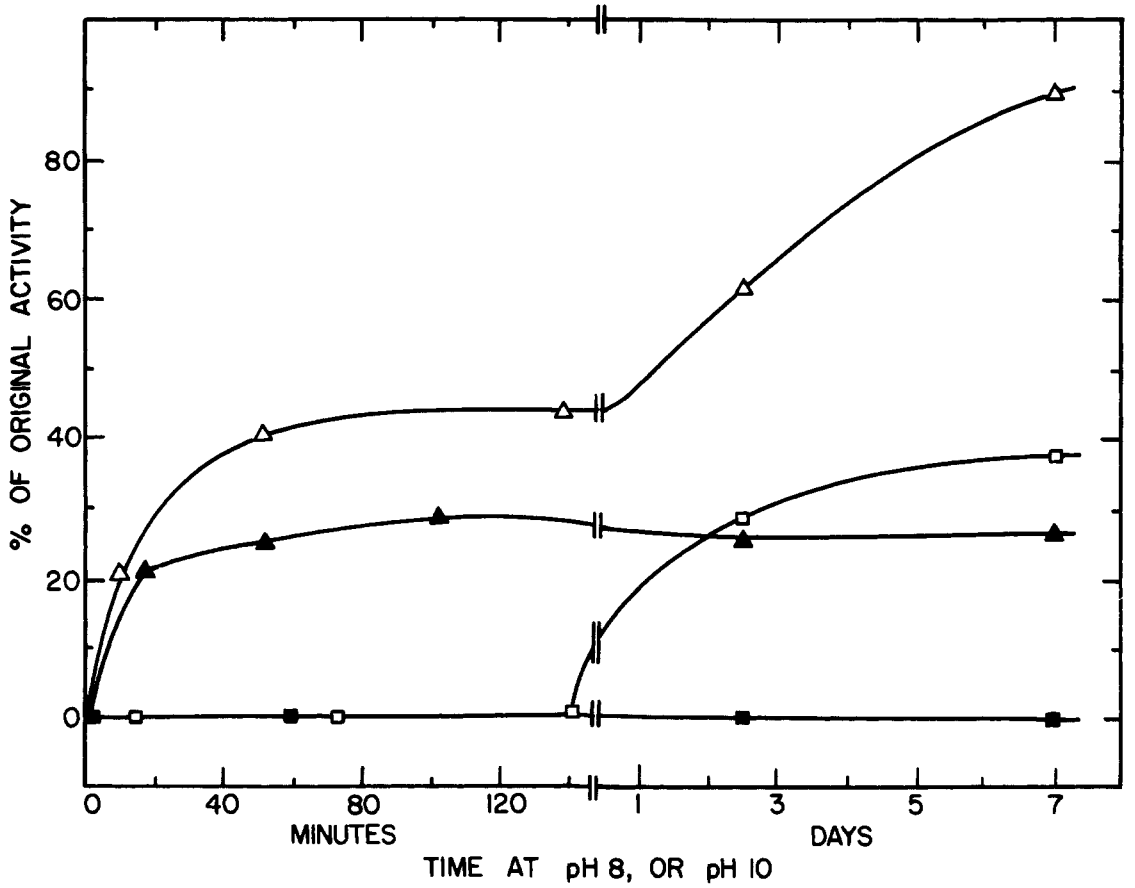

FIG. 4.—Effect of *o*-phenanthroline on the reactivation of acid-inactivated aldolase. Conditions were as given with Figure 3, with a 10-minute exposure to *p*H 3.3, except that where indicated the acid buffer contained 0.02 M *o*-phenanthroline. —△—△—, *p*H 8 reactivation; —▲—▲—, *p*H 8 reactivation with *o*-phenanthroline present. —□—□—, *p*H 10 reactivation; —■—■—, *p*H 10 reactivation with *o*-phenanthroline present.

The reactivation of aldolase exposed to *p*H 3.3 showed a definitive requirement for EDTA when reactivated at *p*H 8 and 10. The data in Table I show that other chelating agents tested will not replace EDTA. Other experiments have shown that reactivation at *p*H 7.4 likewise is increased by presence of EDTA.

A quite unexpected observation was that the presence of *o*-phenanthroline in addition to 10^{-3} M EDTA abolished the slow reactivation process at *p*H 8 and 10, as illustrated by the data in Figure 4. Substantially the same results were obtained whether the *o*-phenanthroline was present in the glycine buffer at *p*H 3.3 or only in the alkaline buffer at *p*H 8 or 10.

TABLE II

EFFECT OF OTHER CHELATING AGENTS ON REACTIVATION AT *p*H 10 IN PRESENCE OF EDTA

Conditions were as given with Figure 3 except that the acid buffer contained 2×10^{-3} M EDTA, and reactivation was for 7 days at 3°.

Additions to Alkaline Buffer	Per Cent of Original Activity
None	21, 30
5×10^{-3} M EDTA	31
Bipyridine	10
Dimethylglyoxime	1
Diethyldithiocarbamate	0
8-Hydroxyquinoline	0
o-Phenanthroline	0
o-Phenanthroline + 2.5 $\times 10^{-3}$ M $ZnSO_4$	0

Delayed addition of *o*-phenanthroline essentially stopped further reactivation at the time of addition, but did not decrease the activity already present. Other chelating agents had a similar inhibitory effect, as shown by the data of Table II. Addition of 0.5 mole of Zn^{++} per mole of *o*-phenanthroline did not modify the result (Table II). Addition of pyridine in concentrations up to 0.02 M did not modify the results.

Determination of Metals in Aldolase.—One plausible explanation for the marked inhibitory effects of chelating agents on the reactivation would be the presence in aldolase of an unrecognized metal essential for activity. Exposure to acid could render the metal more susceptible to combination with the chelating agent. Assays for metals were thus made, using aldolase of high specific activity which had been carefully desalted on Sephadex columns and lyophilized. The analyses as reported in Table III demonstrate the absence of significant amounts of metals, in confirmation and extension of earlier reports (Warburg and Christian, 1943; Rutter and Ling, 1958).

Possible —S—S— *Bonds in Aldolase.*—An inhibitory but erratic effect of the presence of —SH compounds on the reactivation of aldolase suggested the possibility that one or more intrachain or interchain —S—S— bonds in aldolase might aid reactivation through stabilization of structure present in the original aldolase. The presence of 27 to 29 —SH groups per mole of aldolase (Swenson and Boyer, 1957; Benesch *et al.*, 1955) far exceeds the reported analytical values for half-cystine residues (Velick and Ronzoni, 1948). This indicates a probable absence of —S—S— groups

TABLE III

METAL CONTENT OF RABBIT MUSCLE ALDOLASE

Analyses were performed by quantitative spectrographic procedures in the laboratory of Dr. B. L. Vallee. Values are the results of triplicate analyses.

Metal	μg/g Protein	Moles/Mole Protein
Ca	110, 98	0.410, 0.360
Mg	19, 14	0.12, 0.09
Al	12, 16	0.07, 0.09
Ba	2.1, 1.1	0.002, 0.001
Cr	11, 8.9	0.03, 0.03
Fe	16, 22	0.04, 0.06
Zn	22, 23	0.05, 0.05
Sr, Ni, Co, Mn, Cd, Mo, Pb were not detected		

in the molecule, but the wide discrepancy permits no definitive conclusions. Determination of any —S—S— present as increase in —SH after reduction of native aldolase was not feasible because of the small difference in total —SH that would be found. Thus all —SH groups were first blocked by treatment with iodoacetamide in urea, excess reagent was removed, and any —S—S— present was cleaved with β-mercaptoethanol. The data in Table IV show that such treatment led to the appearance of roughly 1 —SH per mole of aldolase. That appearance of protein-bound —SH after reduction was not the result of carrying a small amount of extraneous reducing agent (β-mercaptoethanol) through the procedure was shown by the lack of appearance of any measurable —SH when the iodoacetamide and β-mercaptoethanol treatments were repeated upon the product from the first reduction (Table IV). In one experimental series the aldolase was exposed to a third cycle of iodoacetamide and β-mercaptoethanol treatment again without appearance of measurable —SH. The observed —SH resulting from the initial exposure to β-mercaptoethanol clearly was not an artifact of the experimental procedure.

TABLE IV

FORMATION OF —SH GROUPS IN IODOACETAMIDE-TREATED ALDOLASE UPON REDUCTION WITH β-MERCAPTOETHANOL

Conditions of iodoacetamide and β-mercaptoethanol treatment are given in the experimental section.

Experiment and Sample	Aldolase Preparation Assayed	—SH/Mole
1-1	Iodoacetamide-treated aldolase	<0.1[a]
1-2	As 1-1, but reduced with β-mercaptoethanol	1.0
1-3	As 1-2, but after 5 days	0.5
2-1	Iodoacetamide-treated aldolase	<0.1
2-2	As 2-1, but reduced with β-mercaptoethanol	1.2
2-3	As 2-2, but treated again with iodoacetamide	<0.1
2-4	As 2-3, but reduced with β-mercaptoethanol	<0.1

[a] No —SH groups were detected within experimental error, demonstrating the adequacy of the initial iodoacetamide blocking.

DISCUSSION

The presence of one binding site per mole of muscle aldolase from the equilibrium dialysis and ultracentrifuge measurements is in harmony with the data of Grazi *et al.* (1962) showing covalent fixation of one dihydroxyacetone phosphate residue to each aldolase molecule by borohydride reduction. The occurrence of only one binding site per mole is of particular interest in relation to the presence of at least three peptide chains. The possibility indicated by the terminal residue analyses that aldolase consists of three identical subunits with equal catalytic activity is ruled out by the substrate-binding data.

The finding of one binding site per mole in the carboxypeptidase-degraded aldolase substantiates the earlier conclusion (Drechsler *et al.*, 1959) that the activity loss and modification accompanying release of terminal tyrosine residues results from formation of a new species from all the enzyme molecules present, rather than the presence of some residual carboxypeptidase-resistant aldolase with different catalytic properties.

The binding data as measured with dihydroxyacetone phosphate or with addition of fructose 1,6-diphosphate to give an equilibrium mixture of substrates shows that the fructose 1,6-diphosphate is much more strongly bound than dihydroxyacetone phosphate. The dissociation constant for fructose 1,6-diphosphate at *p*H 7.4 and 3° as estimated from the data of Figure 2, 4×10^{-6} M, is somewhat smaller than the K_m values $1–1.5 \times 10^{-5}$ M reported by Richards and Rutter (1961) with various assay systems at *p*H 7.5 and 25°, and is considerably smaller than the K_m value of 6.2×10^{-5} M reported by Drechsler *et al.* (1959) at *p*H 7.4 and 30°. The latter value may be less accurate because of impurities present in the fructose 1,6-diphosphate used. The differences between the dissociation constant and reported K_m values may reflect in part, differences in temperature and solution composition, but a contribution to the K_m value of other kinetic constants in addition to those determining the dissociation constant appears likely. The latter view is also favored by the demonstration that the affinity of aldolase for fructose 1,6-diphosphate is decreased by carboxypeptidase digestion (Fig. 2), an effect opposite to that on the Michaelis constant (Dreschlser *et al.*, 1959). If the measured K_m values for aldolase represent dissociation constants, carboxypeptidase digestion would be expected to have given a smaller, rather than an increased dissociation constant. With carboxypeptidase-degraded aldolase, the lowered K_m (2×10^{-5} M according to Dreschsler *et al.*, 1959) might be a close approximation of the dissociation constant. This would appear logical in view of the lowered maximum catalytic rate and the lowered kinetic constant governing product formation for the digested compared to the native aldolase.

The ability of acid-inactivated aldolase to re-form an enzymically active molecule, as demonstrated by the data in this paper, and by studies of Deal *et al.* (1963) and of Stellwagen and Schachman (1962), is, as noted by these investigators, indicative that primary structure of the amino acid chains may govern the secondary and tertiary configuration necessary for catalytic activity. These results thus add to the hypothesis that concomitant with or soon after formation of peptide chains, the chains assume a thermodynamically favored configuration characteristic of the native enzyme. Environment at the site of synthesis and folding as synthesis occurs might be particularly favorable for the rapid attainment of desired configuration.

The almost complete reactivation of acid-inactivated aldolase at *p*H 8 observed in these studies (Fig. 3) appears to be the result of two different reactions which occur to an equal extent but at widely different rates. That these are separate and distinct processes is evidenced by the fact that changes of reactivation conditions do not result in parallel changes in the two reactions. When the reactivation is carried out at *p*H 10 the slow reaction occurs to the same extent as

at *p*H 8 but the fast reaction is abolished. In the presence of 5×10^{-3} M *o*-phenanthroline the slow reaction at both *p*H 8 and *p*H 10 is prevented (Fig. 4), although the fast reaction is relatively unaffected. Longer exposures to acid also appear to inhibit the slow reaction more than the fast. The variable reaction rates could result from formation of two different configurations from one or more of the chains under the conditions of acid exposure.

The mechanism by which chelating agents can prevent reactivation remains obscure. One obvious possibility is that a metal ion may somehow be involved. The chelating agents constitute such a diverse structural group that a nonchelation effect due to a certain structure seems unlikely. Aldolase does not contain stoichiometric amounts of metal (Table III), so any metal involved must come from some other source. Indeed, large variations in the reactivation which have been observed with different enzyme preparations and in the laboratories at Minnesota and Dartmouth might reflect the variable presence of metals.

Since EDTA is required for the slow reactivation (Table I) it seems clear that certain metal ions can inhibit the reaction unless bound by EDTA. The ihibition of reactivation by other chelators in the presence of EDTA may be due to chelation of an ion necessary for reactivation but not bound by EDTA, or perhaps by formation with other compounds of a chelate which can still inhibit reactivation. In this regard, Stellwagen and Schachman (1962) have recently reported that reconstitution of acid-dissociated aldolase is strongly inhibited by pyrophosphate, a metal-binding agent.

The presence of one reducible —S—S— per aldolase molecule would be expected to give rise to two —SH per mole upon reduction. The formation of only about one —SH per mole of aldolase upon reduction thus rules out the presence in each aldolase molecule of more than one reducible disulfide group. The formation of only about one —SH per mole could reflect a heterogeneity of the original aldolase used, i.e., only about half the molecules contain a disulfide. Because prior treatment of native aldolase with β-mercaptoethanol did not increase the amount of protein-bound —SH, any —S—S— present would likely be structurally hindered for reductive cleavage. Another possibility is that a mixed disulfide with a low molecular weight moiety is present in a configuration not readily attacked by RSH in native aldolase. After cleavage of the —S—S— bond in the urea solution, the low molecular weight component would be removed along with the reducing agent, and only one protein-bound —SH group would be detected.

A third possibility is that the appearance of 1 —SH per mole could reflect the presence of an unrecognized structure, not an —S—S—, which forms a free —SH group upon treatment with mercaptoethanol. In this regard, Walsh *et al.* (1962) have shown formation from an unknown structure of an —SH group, in addition to that bound to metal, upon treatment of carboxypeptidase with mercaptoethanol.

Presence of a firmly bound metal could block an —SH group, as demonstrated by the results of Vallee and coworkers (1960) who have described appearance of a titratable —SH group in carboxypeptidase after treatment with *o*-phenanthroline. This —SH group was associated with the Zn prior to *o*-phenanthroline treatment. In analogy, the appearance of only 1 —SH per mole of aldolase could possibly have reflected the involvement of this —SH in a firm combination with a metal. The metal analyses on the native enzyme (Table III), clearly eliminate this possibility.

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