Carbanion Intermediates in the Reaction of Yeast and Muscle Aldolase*

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ABSTRACT: Fructose 1,6-diphosphate aldolase from rabbit muscle is a class I aldolase, *i.e.*, it forms a Schiff base ezyme–substrate complex involving an active-center lysyl residue. Its mechanism of action involves a tetranitromethane-reactive intermediate, most likely a carbanion (Christen, P., and Riordan, J. F. (1968), *Biochemistry 7*, 1531). The carbanion reactivity of tetranitromethane has now been confirmed by titration studies of a series of carbon acids. Yeast aldolase is a class II aldolase, *i.e.*, it requires a metal ion for catalysis and does not form a Schiff base complex. The present investigation demonstrates that this enzyme also forms a tetranitromethane-reactive carbanion intermediate. Kinetic data reveal that the reactive carbanion occurs during catalysis and is located on the dihydroxyacetone phosphate moiety of the yeast aldolase–substrate complex. The activating effect of potassium ions on the tetranitromethane reaction closely parallels their known effects on cleavage activity of this enzyme suggesting that potassium ions enhance the rate of carbanion formation. EDTA abolishes the tetranitromethane reaction, consistent with the view that the metal ion of the yeast enzyme serves a function analogous to that of the lysyl residue of the muscle enzyme.

The tetranitromethane reaction traps similar fractional amounts of the total intermediary dihydroxyacetone phosphate carbanion of both the yeast and muscle enzymes. Even though these two enzymes employ different catalytically functional residues their reaction mechanisms are apparently very similar.

Two classes of enzymes which catalyze the aldol cleavage–condensation reactions of fructose 1,6-diphosphate (FDP) and which differ in several distinctive features appear to have evolved independently. Class I aldolases, by definition, form Schiff base complexes with substrate through an active-center lysyl residue and occur in animals, plants, protozoans, and green algae. Class II aldolases, by definition, require divalent metal ions for activity and are found in bacteria, in fungi, and in the blue-green algae. Schiff base complexes have not been observed in class II enzymes (Warburg and Christian, 1943; Horecker *et al.*, 1963; Rutter, 1964; Kobes *et al.*, 1969).

Present concepts of the general mechanism of action of aldolases have been based on aldol condensation reactions (Meyerhof *et al.*, 1936). Tritium- and deuterium-exchange studies of aldolase from rabbit muscle, a class I enzyme, support the postulate that an intermediate carbanion forms at the C-3 carbon atom of dihydroxyacetone phosphate (Rose and Rieder, 1955; Bloom and Topper, 1956; Rutter and Ling, 1958). This is also suggested by both the stereospecificity and the inhibition by FDP or DL-glyceraldehyde 3-phosphate of hydrogen exchange. The exchangeable proton and the C-1 carbon of the aldehyde bind in the same stereospecific position to the C-3 carbon of dihydroxyacetone phosphate (Rose and Rieder, 1958; Rose, 1962). Since yeast aldolase, a class II enzyme containing zinc, also catalyzes tritium exchange with dihydroxyacetone phosphate (Rose and Rieder, 1958), its analogy to muscle aldolase might extend beyond substrate and reaction specificities to the mechanism of action, *i.e.*, both might operate *via* intermediary carbanions.

Recently we have described the reaction of an enzyme–substrate complex of rabbit muscle aldolase with tetranitromethane. Since nitroformate, a product of this reaction, is colored, the chemical and kinetic characteristics of the enzyme–substrate intermediate can be investigated readily (Christen and Riordan, 1968a,b). Experiments with nonenzymatic models and carboxypepidase-treated muscle aldolase suggested that tetranitromethane reacts with an aldolase–substrate carbanion intermediate.

The present study confirms the reactivity of tetranitromethane toward carbanions as evidenced by the reaction between tetranitromethane and carbon acids with various electron-withdrawing substituents and suggests that the reagent is suitable for titrating deprotonated carbon acids in a manner analogous to bromination (Ingold and Wilson, 1934; Bartlett and Stauffer, 1935). Comparison of class I and II aldolases reveals a close similarity between the reaction of their enzyme–substrate complexes with tetranitromethane suggesting that a carbanion intermediate occurs in the catalytic mechanism of both enzymes.

Materials

Yeast aldolase prepared according to Rutter *et al.* (1966) was a gift from Dr. W. J. Rutter and was stored in the cold as a suspension (4 mg/ml) in 70% saturated ammonium sulfate containing 0.025 M mercaptoethanol. For the work with yeast aldolase, buffer solutions were extracted by dithizone to prevent contamination by adventitious metal ions (Thiers, 1957). All other chemicals were of reagent grade and were used without further purification. Tetranitromethane, FDP, F-1-P,

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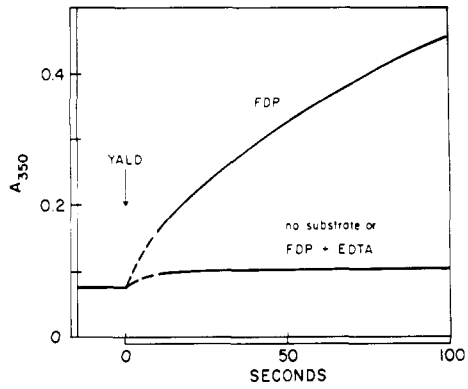


FIGURE 1: Production of nitroformate in the presence of yeast aldolase and substrate. Yeast aldolase (50 μl of a 0.1% solution) was added to 1.95 ml of 0.01 M imidazole, 0.1 M KCl, and 4.2×10^{-4} M tetranitromethane, pH 7.0, 25°, in a 1-cm cuvet containing either 5×10^{-4} M FDP, no substrate, or 5×10^{-4} M FDP and 10^{-3} M EDTA, respectively. The enzyme added to the reaction mixture containing EDTA was incubated, prior to addition, in 10^{-3} M EDTA for 15 min at 0°.

dihydroxyacetone phosphate, and DL-glyceraldehyde 3-phosphate were obtained as previously reported (Christen and Riordan, 1968b). Nitromethane, nitroethane, malononitrile, and methylsulfone were products of Aldrich Chemical Co.; 2,4-pentanedione was obtained from Eastman Organic Chemicals; acetonitrile from J. T. Baker Chemical Co.; acetone from Merck; and EDTA from Allied Chemical Co.

Methods

Yeast aldolase was dialyzed immediately before use for three 90-min periods at 4° *vs.* 500 volumes of 0.01 M imidazole–10^{-5} M zinc acetate (pH 7.0). The specific activity after dialysis was 110 units/mg at 25° (110 μmoles of FDP cleaved per min per mg). All experiments were carried out within 6 hr after dialysis at which time the specific activity was about 90 units/mg. Protein concentrations were measured spectrophotometrically (A_{280} 1.00 for a 0.1% solution; Kobes *et al.*, 1969). Aldolase activity was determined as reported previously (Riordan and Christen, 1968). The rate of production of nitroformate (ϵ_{350} 14,400; Glover and Landsman, 1964) in the reaction of tetranitromethane with the enzyme–substrate complex was measured by the increase in absorbance at 350 nm using a Unicam SP-800 recording spectrophotometer with a cell holder maintained at 25° (Christen and Riordan, 1968b). For determination of reaction rates with carbon acids, the concentrations were chosen according to their rates of ionization (*cf.* Table I); fast-ionizing carbon acids were used at low concentrations with an excess of tetranitromethane and the increase in A_{350} was plotted as a first-order reaction. Slowly ionizing compounds were added in excess of tetranitromethane and treated as zero-order reactions. Correction was made for the spontaneous production of nitroformate from tetranitromethane at pH 11. Zero-order rates were calculated assuming mononitration, no product other than nitroformate absorbing at 350 nm, and 1 mole of nitroformate produced per mole of tetranitromethane reacting.

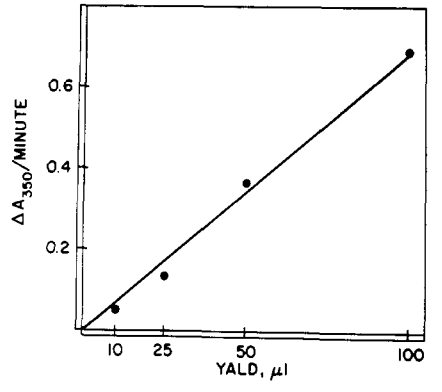


FIGURE 2: Rate of nitroformate production as a function of enzyme concentration. Aliquots of a 0.1% solution of yeast aldolase were added to 0.01 M imidazole, 0.1 M KCl, 5×10^{-4} M FDP, and 4.2×10^{-4} M tetranitromethane, pH 7.0, 25°, in a final volume of 2.0 ml.

Results and Discussion

Addition of yeast aldolase to a mixture of FDP and tetranitromethane instantaneously initiates the appearance of yellow color due to nitroformate production (Figure 1). In contrast, in the absence of substrate, the absorbance at 350 nm increases only slightly, presumably due to oxidation and nitration of the protein and to a nonspecific protein-catalyzed breakdown of tetranitromethane to nitroformate (Riordan and Christen, 1968). In the absence of enzyme, there is no

TABLE I: Carbon Acids. Ionization Rates, k_1, and Rates of Nitration by Tetranitromethane, k_3'.

Carbon Acid	M	k_1, min^{-1} [a]	k_3', min^{-1} [b]
CH_3NO_2	6.2×10^{-5}	2.6×10^{-6}	7×10^{-6}
$C_2H_5NO_2$	4.2×10^{-5}	2.2×10^{-6}	6×10^{-6}
$CH_3SO_2CH_3$	0.133	2×10^{-11}	3×10^{-10}
CH_3CN	0.63	4×10^{-12}	6×10^{-11}
CH_3COCH_3	0.46	2.8×10^{-8}	1×10^{-9}
$CH_2(COCH_3)_2$	4.1×10^{-5}	1.0	4×10^{-5}
$CH_2(CN)_2$	5.0×10^{-5}	0.9	4×10^{-4}

[a] Data of Pearson and Dillon (1953). [b] The carbon acid (50–200 μl of stock solution or an appropriate dilution in water made up immediately before use) and 25 μl of 0.033 M solution of tetranitromethane in 95% ethanol were added simultaneously to a 1-cm cuvet containing 0.1 M glycine–0.1 M NaCl, pH 11.0, 25°, to give a final volume of 3.0 ml. Absorbance was recorded immediately after mixing. The second column of the table gives the final concentration of carbon acid. The concentration of tetranitromethane was 2.8×10^{-4} M in all experiments. At twice that concentration the values of k_3' were virtually unchanged except for pentanedione where it doubled ($k_3' = 7 \times 10^{-5}$) and malononitrile where it became too big to be measured. At pH 11, catalysis of the ionization by cations and by anions other than hydroxide is negligible (Dawson and Carter, 1926). The values of k_3' are corrected for hydroxide ion catalysis of ionization, assuming the same ratio k_{H_2O}/k_{OH} as for acetone, *i.e.*, $k_{H_2O} = k_{pH\ 11}/5.2 \times 10^4$ (Walters and Bonhoeffer, 1938).

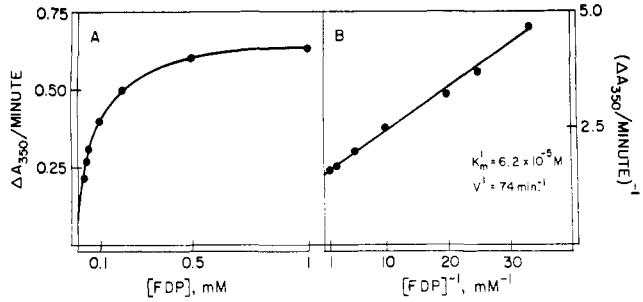


FIGURE 3: Rate of nitroformate production by yeast aldolase as a function of substrate concentration. (A) The reaction mixtures contained FDP at the indicated concentrations; other conditions were the same as those in Figure 1. (B) Double-reciprocal plot of the data of part A. K_m' = Michaelis constant with respect to nitroformate production, V' = maximal molecular activity with respect to nitroformate production calculated assuming a molecular weight for yeast aldolase of 80,000 (W. J. Rutter, personal communication).

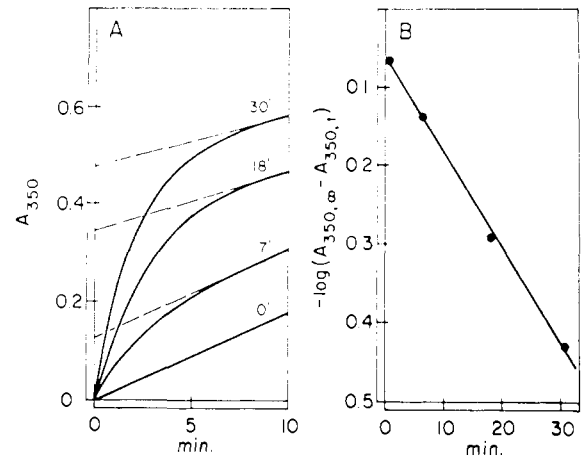


FIGURE 4: Reaction of nitroethane with tetranitromethane after different times of incubation at pH 10. (A) At zero time 50 μl of a 1:5000 dilution of nitroethane in water was added to 3.0 ml of universal buffer, pH 10 (Johnson and Lindsay, 1939), in 1-cm spectrophotometer cuvets at 25°. The final concentration of nitroethane was 4.2×10^{-5} M. At the indicated times 20 μl of a 0.168 M solution of tetranitromethane in 95% ethanol (final concentration 0.00112 M) was added and A_{350} was recorded. (B) First-order rate plot of the extrapolated values taken from the ordinate of part A.

change in absorbance when tetranitromethane is mixed either with substrate, FDP, or with the reaction products, dihydroxyacetone phosphate and DL-glyceraldehyde 3-phosphate. Hence, the increased generation of nitroformate would seem to be attributable to a specific interaction of the yeast enzyme–substrate complex with tetranitromethane.

The rate of nitroformate production in the presence of substrate is proportional to the concentration of the enzyme (Figure 2) and follows saturation kinetics with respect to FDP concentration (Figure 3). The substrate concentration resulting in half-maximal rate of nitroformate production, K_m', agrees within an order of magnitude with the K_m for FDP cleavage; $K_m' = 6.2 \times 10^{-5}$ M *vs.* $K_m = 3.7 \times 10^{-4}$ M (Figure 3 and Rutter, 1964), suggesting that the tetranitromethane-reactive enzyme–substrate complex is probably the one which leads to the cleavage reaction. Since the maximal molecular activity, V', with respect to nitroformate production is much greater than 1 min^{-1} ($V' = 74$ min^{-1}; *cf.* Figure 3), tetranitromethane may be assumed to react with the substrate moiety of the complex rather than with the enzyme itself. The reaction sequence of yeast aldolase thus appears to involve a tetranitromethane-reactive intermediate quite similar to that seen in the mechanism of the muscle enzyme (Christen and Riordan, 1968a,b). Most likely the progressive inactivation of the enzyme as reflected by the nonlinearity of the reaction time course (Figure 1) is due to modification of protein side chains and is independent of the tetranitromethane–carbanion reaction (Christen and Riordan, 1968b).

The nature of the reactive intermediate has been inferred from studies with model systems. Base-catalyzed deuterium exchange between water and acetone (Walters and Bonhoeffer, 1938) suggested that this reaction might serve as a simple prototype for the aldolase-catalyzed proton exchange between water and dihydroxyacetone phosphate. Acetone indeed promotes production of nitroformate from tetranitromethane in alkali as does thiamine and a pyridoxal–glutamate–aluminum ion system, first indications of the carbanion reactivity of tetranitromethane (Christen and Riordan, 1968b).

A number of carbon acids containing different electron-withdrawing substituents, mono- or disubstituted by nitro-, cyano-, ketonic carbonyl, or sulfonyl groups (Cram, 1965), have been examined with tetranitromethane in order to test the generality of the carbanion-specific reactivity. All of the carbon acids listed in Table I react readily with tetranitromethane at pH 11 as judged by the production of nitroformate. Assuming that only the anion reacts, reaction 1 may be

$$\mathrm{HA} + \mathrm{H_2O} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{A^-} + \mathrm{H_3O^+}$$
$$k_3 \downarrow + \text{tetranitromethane} \qquad (1)$$
$$[\mathrm{NO_2A}] + \mathrm{C(NO_2)_3^+}$$

employed. In this reaction, analogous to that used in determining the rates of ionization of pseudo acids by bromination (Ingold and Wilson, 1934; Bartlett and Stauffer, 1935), HA denotes the carbon acid, A^- the tetranitromethane-reactive carbanion, and $[NO_2A]$ the uncharacterized product. Furthermore, if $k_2[H_3O^+] \ll k_3[\text{tetranitromethane}] \gg k_1$ then the rate of nitroformate production, k_3', will be equal to k_1, ionization being the rate-limiting step. However, if ionization is not rate limiting the rate of nitroformate production will be determined by $k_3[\text{tetranitromethane}]$. In Table I, when ionization is slow, the rates, k_1, measured by bromination or deuterium exchange, agree within an order of magnitude with those of the reaction with tetranitromethane, k_3'. Malononitrile and pentanedione ionize rapidly, *i.e.*, $k_1 \gg k_3[\text{tetranitromethane}]$, and hence $k_3[\text{tetranitromethane}]$ is rate limiting and as might be expected in these two instances, the rate of nitroformate production is a function of the tetranitromethane concentration. However, for all the other carbon acids examined doubling the concentration of tetranitromethane does not alter the rate of nitroformate production (Table I).

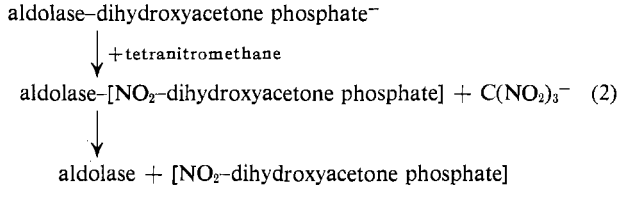
If reaction 1 is valid, as suggested by the data of Table I, the *rate of ionization* should also be obtainable by an indirect assay. Thus the progress of ionization can be measured by incubating the carbon acid at alkaline pH where at equilibrium, the ionization will be complete and by reacting tetranitromethane with aliquots withdrawn at intervals. This approach has been em-

ployed with nitroethane at pH 10 (Figure 4), where the rate of protonation (k_2 [H_3O^+]) is negligible (Pearson and Dillon, 1950), and ionization may be treated as a first-order reaction. The increase in A_{350} upon addition of tetranitromethane reveals a fast reaction (k_3 [tetranitromethane] limiting), superimposed on a much slower reaction (k_1 limiting; Figure 4A). Extrapolation to zero time indicates the amount of nitroethane anion present after the indicated time of incubation at pH 10. The increase with time reflects the progress of ionization of the carbon acid (Figure 4B). The first-order rate constant for this process is 5.3×10^{-6} min^{-1} (corrected for hydroxide ion catalysis; *cf.* legend to Table I) and is in good agreement with the rate of ionization determined by bromination (Table I) thus further supporting reaction 1.

Reaction with tetranitromethane can also be employed to determine the *dissociation constant* of carbon acids. Thus, addition of tetranitromethane to a solution of nitroethane preincubated at various pH values for 48 hr, results in a rapid increase in absorbance at 350 nm followed by a slower increase (insert Figure 5). Extrapolation to zero time indicates the equilibrium concentration of nitroethane anion at each pH. A plot of ΔA_{350} *vs.* pH (Figure 5) coincides with the theoretical titration curve for a group with a pK of 8.6, the value reported for nitroethane (Wheland and Farr, 1943).

The range of application of tetranitromethane as an analytical reagent for carbon double bonds (Werner, 1909; Ostromisslensky, 1910) and as a mild and versatile reagent for nitration of tyrosyl residues of proteins (Riordan *et al.*, 1966; Sokolovsky *et al.*, 1967) may thus be extended to include the titration of carbanions of carbon acids as well as the detection and examination of carbanion enzyme–substrate intermediates.

The reactions of tetranitromethane with muscle and yeast aldolase substrate complexes can be represented by



Tetranitromethane reacts with the aldolase dihydroxyacetone phosphate carbanion to form a modified enzyme–substrate complex, thereby releasing nitroform. The enzyme is regenerated by the dissociation of the modified substrate. This derivative, shown in brackets, is currently being isolated for structural characterization. In accord with the proposed scheme, dihydroxyacetone phosphate also induces nitroformate production (V' = 90 min^{-1} at a concentration of 3.3×10^{-4} M), but DL-glyceraldehyde 3-phosphate does not.[1] Reaction 2 is not intended to indicate the reaction stoichiometry. With muscle aldolase for every 2 moles of substrate consumed during the tetranitromethane reaction about 3 moles of nitroformate are produced (Christen and Riordan, 1968b). This lack of correspondence may reflect a partial regeneration of the substrate. Nonetheless, since only a small fraction of the reactive intermediate appears to interact with tetranitromethane, the rate of nitroformate production provides a convenient gauge of the relative concentration or reactivity of the carbanion intermediate in the steady state of the cleavage reaction under different conditions.

The ratio of the rate of nitroformate production (V' = 74 min^{-1}) to the rate of FDP cleavage (V = 8800 min^{-1}) indicates the fraction of the intermediary carbanion occurring in the steady state of the cleavage reaction that is trapped by tetranitromethane. Since the experiments with both muscle and yeast aldolase employed the same concentrations of tetranitromethane, this ratio can be compared directly, and indeed proves to be of the same order of magnitude, *i.e.*, 0.08 in the case of yeast aldolase and 0.03 in that of the muscle enzyme (Christen and Riordan, 1968b). Further, the rate of nitroformate production in the presence of FDP measures the steady-state concentration of the reactive intermediate while the rate observed in the presence of dihydroxyacetone phosphate represents the equilibrium concentration. The ratio of these rates is virtually the same for yeast aldolase, 74 min^{-1}/90 min^{-1} = 0.82 (Figure 3 and text), and for muscle aldolase, 60 min^{-1}/71 min^{-1} = 0.85 (Christen and Riordan, 1968b). These identities strongly suggest that the mechanisms of action of the two enzymes proceed through an analogous carbanion intermediate present in both cases in about the same amount and exhibiting almost the same reactivity toward tetranitromethane.

Consistent with the very low cleavage activity of the enzyme toward F-1-P (Richards and Rutter, 1961b) this compound (0.01 M) induces virtually no nitroformate production. Again, similar to muscle aldolase, the rate-limiting step for F-1-P cleavage seems to occur prior to the carbanion intermediate.

Considering the different nature of at least some of the groups which participate in catalysis by the two enzymes, the similarity of the reaction pathway, as implied by the reactivity of a specific intermediate with an extraneous reagent, is remarkable. In the muscle enzyme a Schiff base between the ϵ-amino group of a lysyl residue and the substrate is formed, and the dihydroxyacetone phosphate carbanion is thought to be resonance stabilized. Yeast aldolase apparently does not form a Schiff base. It has been suggested that the metal ion instead acts as an electrophile and inducing carbanion formation, thus serving a function analogous to that of a lysyl residue (Rutter, 1964). Inhibition of the cleavage activity of yeast aldolase by EDTA (Richards and Rutter, 1961a) also abolishes nitroformate production (Figure 1), indicating the dependence of the latter upon the presence of zinc, consistent with the postulated role of the metal ion.

Activation by monovalent cations is another distinctive property of the class II aldolases (Richards and Rutter, 1961a). The activating effects of potassium ions on cleavage activity and on nitroformate production are closely parallel (Figure 6) and this allows localization of the potassium ion effect to a particular segment of the enzymic reaction sequence. As the rate of nitroformate production is a measure of the steady-state concentration of the carbanion intermediate, its enhancement by potassium ions must be considered to be due to an increase in the rate of carbanion formation rather than to an acceleration of the steps between carbanion formation and the liberation of dihydroxyacetone phosphate from the en-

[1] Although DL-glyceraldehyde does not induce nitroformate production immediately on addition, production increases progressively after the first few seconds due to conversion of the aldehyde into dihydroxyacetone phosphate by contaminating amounts of triose phosphate isomerase activity demonstrated to be present in the yeast aldolase preparation employed in these studies.

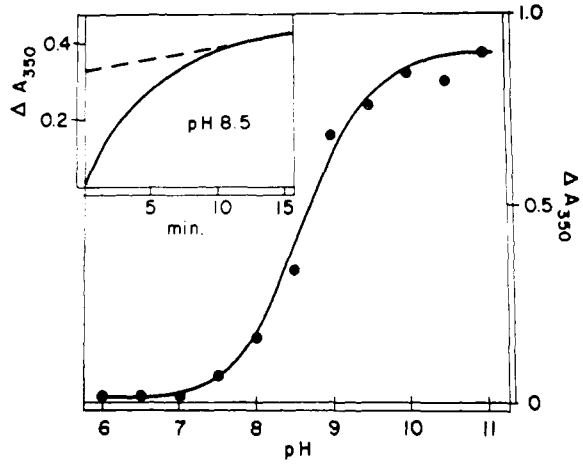


FIGURE 5: Reaction of tetranitromethane with nitroethane at different pH values. Nitroethane (4.2×10^{-5} M) was brought to ionization equilibrium by incubation in universal buffer (Johnson and Lindsay, 1939) at the indicated values of pH for 48 hr, 20°. Then 20 μl of a 0.168 M solution of tetranitromethane in 95% ethanol was added and A_{350} was recorded. The insert shows a typical tracing obtained at pH 8.5. The magnitude of the 350-nm burst (obtained by extrapolation to zero time as indicated by the dashed line in the insert) is plotted *vs.* pH.

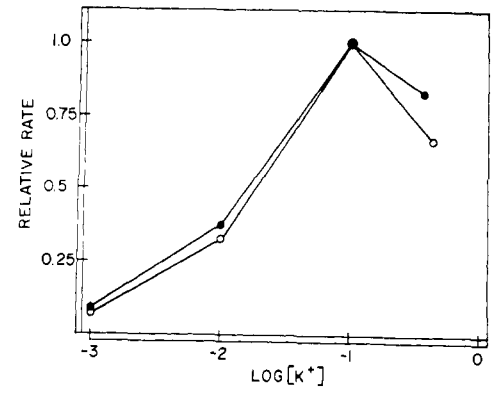


FIGURE 6: Effect of potassium ions on the rates of nitroformate production and FDP cleavage. The reaction mixture contained KCl at the indicated concentrations and 50 μl of a 0.1% solution of yeast aldolase. Other conditions were the same as those of Figure 4. (●) Nitroformate production and (○) FDP cleavage data from Rutter (1964).

zyme. This being the case, potassium ions should not alter appreciably the rate of hydrogen exchange between dihydroxyacetone phosphate and water. Thus, the twofold increase in the rate of tritium exchange observed in the presence of potassium ions can be contrasted with the more than tenfold increase in the rate of FDP cleavage (Richards and Rutter, 1961a,b) and of nitroformate production.

The two classes of aldolases are thought to represent analogous proteins generated independently by a process of convergent evolution (Rutter, 1964, 1965). The experimental data reported here verify the close similarity between the carbanionic dihydroxyacetone phosphate–enzyme intermediates of muscle and yeast aldolase. Although different groups are involved at the active site of these enzymes these affect the transient chemistry of the substrate moiety of the complex in a closely related fashion. Thus, the analogy between the enzymes drawn from their substrate and reaction specificities extends to their mechanisms of action. Similar analogies exist in enzymatic decarboxylation and other aldol-type reactions (Rutter, 1965) and, in fact, there are a number of examples of reactions catalyzed by a metalloenzyme in one species and by a nonmetalloenzyme in another (Vallee and Wacker, 1969).

Chemical modifications of enzymes are often carried out in the presence of substrates or inhibitors in order to demonstrate selective blocking of reactive groups by the ligand. Alternatively, binding of substrate or the catalytic process itself can generate complexes with a new specific reactivity, detectable by a suitable reagent. This possibility has been investigated with only a few enzymes and reagents. Thus far tetranitromethane seems to be the only reagent whose interactions with enzyme–substrate intermediates is readily accessible to kinetic analysis. Its use as a reagent for detecting enzyme–substrate carbanion intermediates may ultimately not be limited to the aldolases. Related carbon–carbon lyases could be likely candidates for study. Yet other possibilities are suggested by the results with coenzyme systems, *e.g.*, thiamine and pyridoxal (Christen and Riordan, 1968b). However, not all enzymes with carbanion intermediates will necessarily be susceptible to such kinetic studies. Short-lived or relatively unreactive intermediates, steric hindrance, and rapid inactivation of the enzyme by modification of its side chains, among other factors, may preclude the tetranitromethane reaction. In other cases, a reactive carbanion might be generated on the enzyme or coenzyme rather than on the substrate moiety of the enzyme–substrate complex, and the addition of tetranitromethane could then nitrate and thereby inactivate the enzyme rather than lead to a marked production of nitroformate. This situation has been encountered with aspartate aminotransferase, where one essential tyrosyl residue becomes susceptible to nitration during catalysis. In such instances, a burst of nitroformate production, stoichiometric with the enzyme or coenzyme, permits detection of the reaction (Christen and Riordan, 1969).

Acknowledgments

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Purification and Properties of Asparaginase from *Escherichia coli* B*

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ABSTRACT: *Escherichia coli* B asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) has been purified *ca.* 2000-fold by a combination of heat denaturation, gel filtration, chromatography on DEAE-cellulose, and calcium hydroxylapatite, and polyacrylamide gel electrophoresis. The enzyme appears to be homogeneous, as judged by several criteria, including sedimentation equilibrium ultracentrifugation, disc electrophoresis, and immunoelectrophoresis. At 0.1% protein concentration, the molecular weight by equilibrium sedimentation was found to be *ca.* 139,000. In 8 M urea or 5 M guanidinium hydrochloride, dissociation to an inactive species with an apparent molecular weight of 19,000–24,000 was observed. The amino acid composition has been determined, as well as certain other properties of the enzyme.

In 1961, Broome proposed that the enzyme asparaginase is responsible for the antilymphoma activity of guinea pig serum, an activity first noted by Kidd (1953). This has been confirmed by further work from several laboratories (Mashburn and Wriston, 1963; Broome, 1963; Old *et al.*, 1963; Yellin and Wriston, 1966a,b) and is now generally accepted. Several microorganisms have also been found to contain asparaginase activity (*e.g.*, Varner, 1960) and at least three of these bacterial asparaginases possess antilymphoma activity. An *Escherichia coli* asparaginase was described in 1957 (Tsuji, 1957) and subsequently Mashburn and Wriston (1964) showed that partially purified *E. coli* B asparaginase had antilymphoma activity in mice. The *E. coli* enzyme is currently undergoing extensive clinical trials in human beings (Oettgen *et al.*, 1967; Hill *et al.*, 1967). An asparaginase from *Serratia marcescens* gave results in mice similar to those obtained with the *E. coli* asparaginase (Rowley and Wriston, 1967). Also, Wade *et al.* (1968) recently reported the isolation from *Erwinia carotovora* of an asparaginase with antilymphoma activity in mice. Here we wish to report the isolation from *E. coli* B of a 2000-fold purified asparaginase which is essentially homogeneous by sedimentation equilibrium ultracentrifugation and disc electrophoresis, and to describe certain properties of this enzyme. A preliminary account of this work has appeared (Whelan and Wriston, 1968).

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