THE MECHANISM OF ACTION OF 2-KETO-3-DEOXY-6-PHOSPHOGLUCONATE ALDOLASE OF PSEUDOMONAS FLUORESCENS

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Jordan M. Ingram

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W. Wood

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ABSTRACT

THE MECHANISM OF ACTION OF 2- KETO-3-DEOXY-6-PHOSPHOGLUCONATE ALDOLASE OF PSEUDOMONAS FLUORESCENS

by Jordan M. Ingram

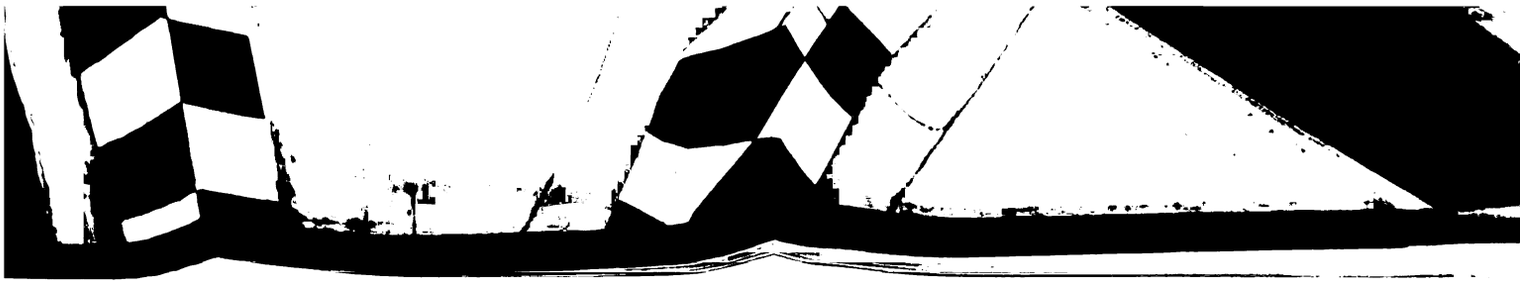
6-Phospho-2-keto-3-deoxy-D-gluconate-D-glyceraldehyde-3-phosphate lyase (KDPG-aldolase) was purified from glucose-grown Pseudomonas fluorescens. In the presence of sodium borohydride and pyruvate-^{14}C an inactive, labeled enzyme derivative was obtained. Upon complete acid hydrolysis of the $LiAlH_4$-reduced derivative, a new component identical to N^6-α- (1-hydroxypropyl) lysine was isolated. This constitutes firm evidence that the aldolase forms an azomethine between the carbonyl carbon of the substrate and an enzyme bound lysine ε-amino group.

Upon treatment with 2, 4-dinitrofluorobenzene, (FDNB), KDPG-aldolase is inactivated by reaction with four moles of FDNB per mole of enzyme. The reaction is prevented by phosphate ion, KDPG, and partially by D, L-glyceraldehyde -3-phosphate. After complete acid hydrolysis of the treated enzyme, only ε-dinitrophenyl lysine was found. These results imply that four lysine ε-amino groups function as a phosphate binding site.

Jordan M. Ingram

The specificity for azomethine formation was studied by incubating various α-carbonyl compounds with the enzyme in the presence of sodium borohydride. Only pyruvate and α-ketobutyrate gave complete inactivation. Monohydroxyacetone, α-ketoisovalerate, α-ketoglutarate, 5-keto-4-deoxyglutarate, 2-keto-4-hydroxyglutarate, and 2-keto-3-deoxygluconate inactivated between 25 and 75 per cent. Hydroxypyruvate, 2-ketogluconate, and dihydroxyacetone gave no inactivation. These results suggested that the only limitation for azomethine formation is the absence of a polar function, i.e. a hydroxyl group, at the C_3-position. α-Ketobutyrate did not bind to the aldolase previously rendered inactive with pyruvate and borohydride, and it competed favorably with pyruvate-^{14}C for inactivation of the aldolase suggesting that both compounds bind at the same lysine ε-amino group.

Consistent with the inactivation of the enzyme in the presence of borohydride and α-ketobutyrate is the aldolase-catalyzed tritium exchange into α-ketobutyrate. The aldolase catalyzed the exchange of one proton from tritiated water, presumably in a stereospecific manner, into α-ketobutyrate. The rate of exchange with α-ketobutyrate was at least 37 times slower than the rate obtained with pyruvate. Neither hydroxypyruvate, nor α-ketoglutarate exchanged to this degree even after prolonged incubation. The results of these



experiments imply that azomethine formation is a reaction distinct from exchange.

The enzyme catalyzed the cleavage of 2-keto-4-hydroxyglutarate and the β-decarboxylation of oxalacetate, but did not cleave 2-keto-3-deoxygluconate to any extent. These results together with the borohydride inactivation studies and the observation that FDNB-treated enzyme binds pyruvate suggests that the phosphate group of KDPG not only increases the binding efficiency of the substrate, but also aids catalysis by providing stress upon the electronically weakened C_3 -C_4 bond. These results suggest that cleavage is not the direct result of azomethine formation. Hence, the complete aldolase reaction may be visualized as proceeding from azomethine formation to cleavage and exchange.

A radioactive peptide containing the lysine-azomethine forming site was isolated and the amino acid composition of the peptide and the native protein was determined.

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VITA

Jordan M. Ingram was born on August 24, 1936 in Ottawa, Ontario, Canada. He graduated from Glebe Collegiate, Ottawa, in May 1956. He received the degree of Bachelor of Science in Agriculture from McGill University, Montreal, in May 1959. He received the Master of Science degree from the same institution in May, 1961. He accepted a graduate research assistantship in the Department of Biochemistry at Michigan State University from September, 1961 until the present time.

Mr. Ingram is a member of the American Chemical Society, the American Association for the Advancement of Science, and the Canadian Society for Microbiologists. He is married and has one child.

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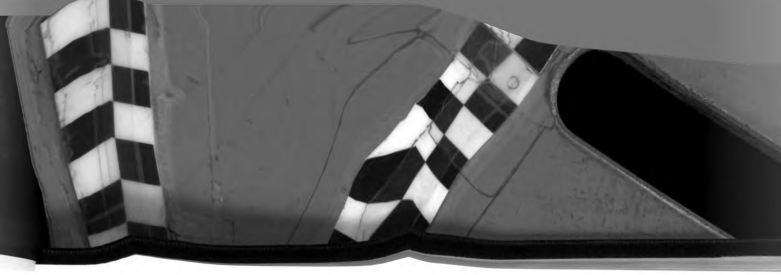
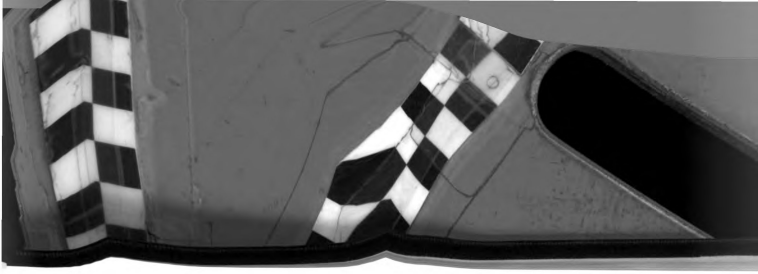


CHAPTER I

INTRODUCTION

Enzymes which catalyze aldol-type reactions are found in mammalian, microbial, and plant systems. The enzyme under present investigation, 2-keto-3-deoxy-6-phosphogluconate (KDPG-aldolase) 6-phospho-2-keto-3-deoxy-D-gluconate-D-glyceraldehyde-3-phosphate lyase (E. C. 4.1.2.14) is characteristic of microorganisms which metabolize substrates, such as glucose, via the Entner-Doudoroff pathway.

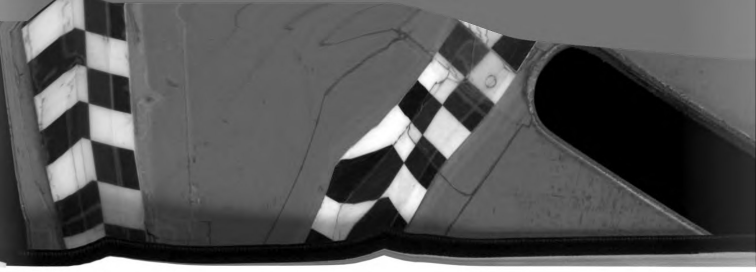
With the crystallization of KDPG-aldolase from Pseudomonas fluorescens by Meloche and Wood (1), an aldolase with unique properties became available for study. Although the mechanism for aldol cleavage by this enzyme is similar to that reported for muscle fructose diphosphate (FDP) aldolase (2), the activity of the KDPG-aldolase is completely preserved under acid conditions which inactivated FDP-aldolase by dissociation into subunits (3). Further, lack of agreement between molecular weights derived from sedimentation constants (assuming an average partial specific volume and spherical shape) and sedimentation equilibrium data indicated a rather large axial dissymmetry. From equilibrium sedimentation data the molecular weight was calculated to be approximately 90,000 (1). As with other aldolases,



incubation with sodium borohydride and the carbonyl substrate, pyruvate, caused inactivation. Based upon the amount of stably bound pyruvate-1-^{14}C, one mole of pyruvate was bound per 43,500 g of aldolase (1). These data reveal the existence of two active sites per aldolase molecule.

Although the detailed reaction mechanism of KDPG-cleavage is known (1), there is little evidence available concerning the residue involved in the reductive binding of pyruvate-^{14}C with borohydride. Also, information regarding other residues which might be important in other phases of the reaction is lacking. The investigations to be discussed were begun with the original intent of elucidating the residue involved in the binding of the carbonyl portion of pyruvate of KDPG. Methods were later developed to obtain a peptide fragment containing the residue responsible for carbonyl binding. In order to pursue the latter study, a modified procedure was developed for the preparation, in good yield, of highly purified KDPG-aldolase. The amino acid composition of the aldolase was determined to facilitate a comparison of the structure of the peptide with that of the native protein.

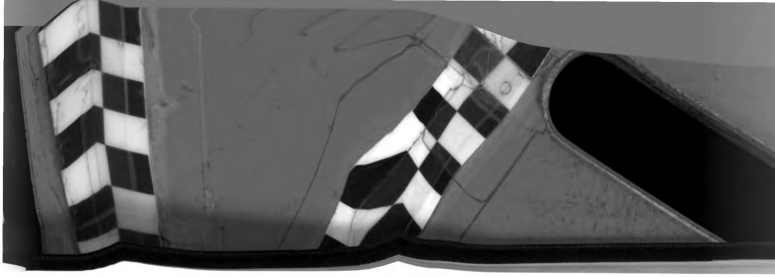
The reaction of a non-specific reagent under specified conditions was used to ascertain the existence and role of additional groups concerned with catalysis. The ability to inhibit enzyme action in the presence of such a reagent, 2, 4-dinitrofluorobenzene, suggested the presence of an anionic



binding site for the substrate phosphate group. Methods were developed to doubly label the enzyme with pyruvate-^{14}C and FDNB to eventually study the spatial relationship of these two important sites.

The reaction mechanism was clarified further by the development of a technique involving the borohydride reduction reaction. The reaction of substrate analogs of pyruvate and KDPG with the aldolase in the presence of borohydride indicated the importance of the presence or absence of certain substrate group substituents in forming the azomethine with the enzyme. During the course of this investigation a novel β-decarboxylase activity of KDPG-aldolase was discovered.

The results of the forementioned experiments have been interpreted with a view towards the importance of the catalytically active centers of the enzyme and the requirements of substrate structure as each relate to the detailed reaction mechanism.



CHAPTER II

LITERATURE REVIEW

Occurrence of KDPG-Aldolase in Metabolism

Early studies on the growth of Pseudomonas saccharophila in the presence of glucose-1- ^{14}C showed that two moles of pyruvic acid were obtained and that the pyruvate carboxyl was labeled to the extent of 50 per cent of that of the glucose-1- ^{14}C. Thus, Entner and Doudoroff (4) postulated a new route for the metabolic utilization of glucose by this organism. The apparent sequence of reactions was believed to be:

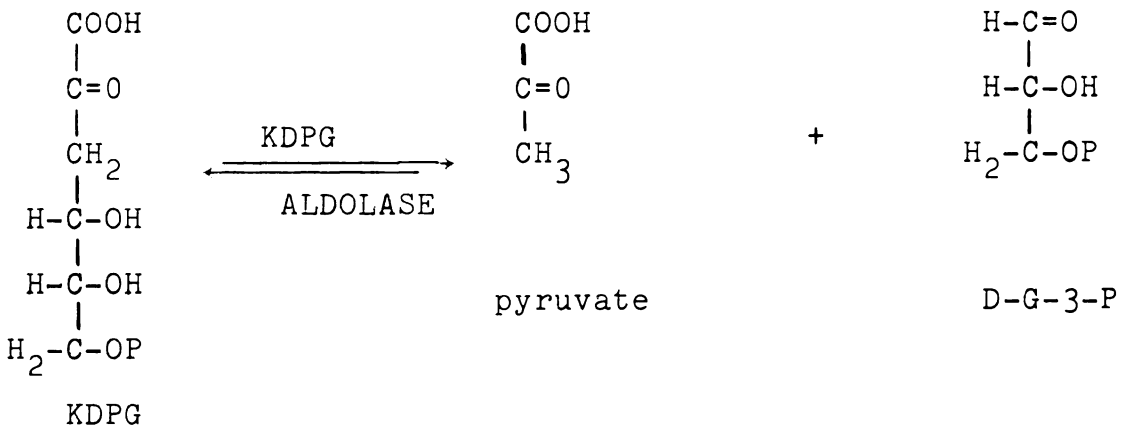
D-glucose → glucose-6-PO_4 → 6-P gluconate

glyceraldehyde-3-PO_4 ← [X]

pyruvic acid

In later reports, which appeared almost simultaneously, Macgee and Doudoroff (5) and Kovachevich and Wood (6) described the occurrence of a new phosphorylated derivative, 2-keto-3-deoxy-6-phosphogluconate (KDPG), in the reaction sequences of P. saccharophila and P. fluorescens, respectively. It was definitively shown by the latter investigators that 6- P gluconate was converted to KDPG by a dehydrase enzyme and subsequently the KDPG was cleaved to D-G-3-P and

pyruvate by the newly discovered KDPG-aldolase. The C_1 to C_3 portion of KDPG thus became pyruvic acid:



In addition to the two organisms just described the aldolase has been postulated, or shown, to occur in other microorganisms as well. Gibbs and DeMoss (7) studying the anaerobic dissimilation of glucose by P. lindnerii concluded that the formation of ethanol and carbon dioxide occurred by a mechanism analogous to the aerobic pathway of P.saccharophila. Later, Ashwell and co-workers (8) studying the metabolism of uronic acids by Escherichia coli showed that both glucuronic and galacturonic acids were dehydrated in the presence of specific dehydrases to yield a common intermediate, 2-keto-3-deoxygluconic acid. This latter intermediate was subsequently phosphorylated by a specific kinase to yield KDPG. The KDPG was then cleaved by an aldolase presumably similar to that already reported to be present in Pseudomonas species. Prior to these studies, DeLey and Doudoroff (9) demonstrated that galactose was metabolized

by P.saccharophila through a route similar to that for glucose. Galactose yielded KD-galactonate which was phosphonylated by a specific kinase and subsequently cleaved by an aldolase to yield D-G-3-P and pyruvic acid.

The general pattern appears to be, therefore, that KDPG-aldolase is found in organisms which metabolize hexose sugars either by an initial phosphorylation, such as the case of glucose, to ultimately yield KDPG; or the enzyme also occurs in organisms which convert the hexose to an "onic" acid which is dehydrated to a 2-keto-3-deoxy-derivative. This compound is phosphorylated and then cleaved by a KDPG-aldolase type enzyme.

Properties of the Purified KDPG-Aldolase

The first attempts to purify KDPG-aldolase were those of Kovachevich and Wood (6) with P. fluorescens as the source of the enzyme. The aldolase was purified about 20-fold by treating a crude extract with protamine sulfate, ammonium sulfate, and calcium phosphate gel absorption and elution. In this manner the aldolase was purified free of the 6-P gluconate dehydrase. In the presence of the aldolase, KDPG, excess NADH, and crystalline lactic acid dehydrogenase a decrease in the absorbancy at 340 mμ was recorded. This assay system was utilized as a measure of KDPG-aldolase activity.

In the presence of the appropriate coupling enzyme systems and the aldolase, KDPG was converted quantitatively to pyruvate and D-G-3-P. No cofactor or metal requirements could be demonstrated for maximal activity. Later studies by Meloche and Wood (1) produced crystalline KDPG-aldolase. With these preparations the reaction was shown to be freely reversible in the presence of imidazole buffer.

Mechanism of the Aldol Reaction

Biological Aldol Reactions

The aldol condensation classically involves the formation of a carbon-carbon bond between a hydroxymethyl or methyl carbon and an aldehyde. Biologically this reaction has been found to occur in a wide variety of microbial and mammalian systems. The types of reactions which occur biologically may be clarified to some extent by summarizing the characteristics of some known aldol enzymes. The characteristics of KDPG-aldolase were described in the previous section.

1. Muscle FDP-Aldolase

$$\text{Fructose-1, 6-di}PO_4 \rightleftarrows \text{D-glyceraldehyde-3-}PO_4 + \text{dihydroxyacetone-3-}PO_4$$

This reaction was demonstrated in yeast and muscle by Warburg and co-workers (10, 11). The yeast enzyme has since been shown to differ with respect to that of muscle

in that it contains one mole of zinc per 60,000 gm of protein and also that the reaction is considerably stimulated by potassium ions. No cofactor requirements have been found for the muscle enzyme.

2. Liver FDP-Aldolase

Fructose-1-PO_4 $\rightleftharpoons$ D-Glyceraldehyde + dihydroxyacetone -3-PO_4

This enzyme was first implicated to play an important role in fructose metabolism by the liver (12). Peanasky and Lardy (13) crystallized the enzyme from bovine liver and showed that the rate of cleavage of F-1-P was 42 per cent the rate of FDP cleavage. The liver enzyme was later prepared by Rutter and co-workers (14) and shown to be unaffected by treatment with carboxypeptidase. On the other hand, muscle aldolase treated with carboxypeptidase yielded a preparation which cleaved F-1-P as well as the liver enzyme, but which cleaved FDP at a much slower rate. Based upon the above observations, these investigators concluded that muscle aldolase was an enzyme distinct from liver aldolase.

3. Transaldolase.

Fructose-6-P + Erythrose-4-P $\rightleftharpoons$ Sedeoheptulose-7-P + glyceraldehyde-3-P

The enzyme transaldolase, which catalyzes the above reaction was discovered by Horecker and co-workers (15)

and shown to use F-6-P or S-7-P as carbonyl donors. The enzyme differs from those mentioned previously because detectable cleavage does not occur in the absence of an aldehyde acceptor. In addition a net synthesis of F-6-P from dihydroxyacetone and glyceraldehyde-3-P occurs with difficulty indicating the inability of this enzyme to bind free dihydroxyacetone (16).

4. 2-Deoxyribose-5-Phosphate aldolase

2-Deoxyribose-5-P $\rightleftarrows$ acetaldehyde + glyceraldehyde -3-P

Deoxyribose-5-P aldolase was purified from crude extracts of Lactobacillus plantarum by Pricer and Horecker (17). An interesting point of this reaction is the strict requirement for a dicarboxylic acid. A recent report by Hoffee, Rosen, and Horecker (18) describes the preparation of a crystalline zinc salt of DR-aldolase.

5. Mono- and Dicarboxylic Acid Aldolases

Blumenthal and Fish (19) described the occurrence of an aldolase, present in E.coli, which cleaves both 2-keto-3-deoxyglucarate and 5-keto-4-deoxy-glucarate to yield pyruvate and tartronic semialdehyde. The enzyme is present in cells grown on D-glucarate or galactarate. A similar enzyme, present in D-arabinose-grown P.saccharophila, was described by Weimberg and Doudoroff (20). The enzyme cleaves D-arabonate to yield pyruvate and glycolaldehyde as

products. Maitra and Dekker (21) isolated a partially purified enzyme from rat liver which catalyzes the cleavage of 2-keto-4-hydroxyglutarate. The enzyme is apparently involved in the metabolic reactions of δ-hydroxyglutamic acid. On the basis of amination of KH-glutarate, both erythro and threo δ-OH glutamic acids resulted suggesting the ability of the aldolase to utilize each of the two possible stereoisomers of KH-glutarate.

6. Aldol Condensation Involving Activated Substrates

Aldolase enzymes which require activated substrates have also been found in biological systems. It is suggested that these enzymes are involved in biosynthetic rather than degradative reactions. An enzyme involved in the synthesis of 2-keto-3-deoxy-D-araboheptonic-7-PO_4, an intermediate in the shikimic acid pathway, was purified and characterized from E.coli by Srinivasan and Sprinsin (22). The enzyme utilized phosphoenol-pyruvic acid and erythrose -4-PO_4 as substrates. The loss of the α-phospho group of PEP renders the reaction irreversible. A similar type enzyme was also described by Levin and Racker (23) which catalyzed an apparent aldol condensation of PEP and D-ribose-5-PO_4 to yield KD-octanoic-8-PO_4. However, further study indicated that D-R-5-PO_4 was first converted to D-arabinose-5-PO_4 which then served as the aldehyde acceptor.

7. Amino Acid Aldolases

The isolation of a threonine and allothreonine aldolase from sheep liver has been described by Karasek and Greenberg (24). With threonine as substrate, the reaction rate is some twenty times slower than with allothreonine. The enzyme has a strict dependence upon the presence of pyridoxal phosphate, presumably to facilitate the formation of a Schiff base intermediate between the enzyme and the α-amino group of the substrate. The products of the cleavage reaction were identified as glycine and acetaldehyde.

Biochemical Mechanism of the Aldol Reaction

Mechanistic studies regarding dealdolization in model systems was supplied by Westheimer and Cohen (25). Using diacetone alcohol, these workers showed that catalysis by primary and secondary amines i.e. methylamine and dimethylamine yielded rate equations which differed from those obtained for classical base catalyzed reactions. In addition, it was pointed out that tertiary amines did not catalyze the reaction and the requirement for at least one nitrogen bound proton was implicated. These workers concluded that ketimine formation was most probably involved as an integral part of catalysis.

Later studies by Speck and Forist (26) demonstrated dealdolization catalysis of diacetone alcohol by amino acids. Although glycine was not as effective as methylamine

an appreciable rate increase was observed. Glycine was superior to the other two amino acids, α and β-alanine, which were also tried. This observation lead to a proposal that an amino group present in the aldolase could conceivably assist the dealdolization reaction.

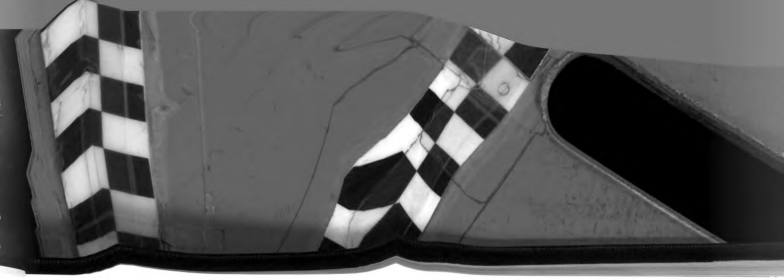
Early work by Rose and Rieder (27) with muscle aldolase showed that the enzyme catalyzed the exchange of tritium from tritiated water into dihydroxyacetone phosphate. The rate of this exchange was decreased in the presence of the substate, FDP. It was postulated, therefore, that the enzyme bound the substrate in some fashion as to cause the displacement of a proton from DHAP. The enzyme-bound DHAP was displaced by neutralization with another proton from the medium or by the acceptor aldehyde, D-G-3-P. Later studies by Topper, Mehler and Bloom (28) showed that in the presence of DHAP, muscle aldolase exhibited a new absorption peak at 235 - 250 mμ. The peak did not appear on the addition of D-G-3-P to the aldolase preparation. These experiments suggested the formation of an enol-like bond between the substrate and the enzyme.

Rose and Rieder (2) in a further study on the mechanism of muscle and yeast aldolases demonstrated that in tritiated water the FDP synthesized from DHAP and D-G-3-P was not labeled. This observation suggested that the proton incorporated, or lost, by DHAP was always in the same location and hence the reaction proceeded in a stereospecific manner.

A mono-tritiated species of DHAP could also be synthesized via the triosephosphate isomerase reaction. The product of this incorporation reaction did not lose radioactivity when incubated with muscle or yeast aldolase. Degradation of the FDP-aldolase labeled DHAP-H^3 and the use of enzymes of known stereospecificity for proton exchange indicated that the triton gained in the aldolase reaction differed from that incorporated in the triosephosphate isomerase reaction. Therefore the absolute stereospecificity for proton incorporation in the aldolase reaction proceeded as shown in the following sequence.

$$H - \overset{|}{\underset{H}{C}} - OH \quad \underset{H_2O}{\overset{T_2O}{\rightleftharpoons}} \quad H - \overset{|}{\underset{T}{C}} - OH$$

The rate of exchange of DHAP-^{14}C into FDP was shown to be more rapid than the rate of proton exchange. However, the intimate association of the exchange reaction with the aldol reaction was established when it was demonstrated that as the levels of DHAP were increased the rate of tritium exchange approximated the rate of cleavage. Therefore, as the concentration of the enzyme-DHAP complex increases the concentration of the free enzyme becomes limiting in the reaction. The results indicated that aldolase forms an enzyme-DHAP complex in the presence of either DHAP or FDP and that the complex must later be dissociated to release free enzyme.



The concept of product dissociation was extended further by the studies of Venkataraman and Racker (16) with transaldolase. In the presence of enzyme and F-6-P a new component, later identified as the tansaldolase-DHA complex, was separated from the native enzyme by carboxymethyl cellulose column chromatography. Upon enzyme denaturation the bound radioactive DHA was released. The net synthesis of F-6-P from DHA and D-G-3-P was also observed indicating that the enzyme-DHA complex dissociated to some extent. Quoting unpublished experiments these investigators state that transaldolase catalyzes a stereospecific exchange of solvent protons into DHA in a manner analogous to the muscle aldolase reaction. The rate of exchange for transaldolase was not given and therefore a comparison with the FDP-aldolase reaction is not possible. In view of later experiments with transaldolase, this reviewer questions whether this exchange is significant.

Pontremoli and associates (29) later described the preparation of crystalline transaldolase from Candida utilis. In the presence of F-6-P^{14}C, a stable transaldolase-DHA-^{14}C complex was isolated by careful ammonium sulfate precipitation. The stabilized enzyme-bound-^{14}C

was transferred to erythrose -4-P or was dissociated from the enzyme by warming to 80°C. A further observation was the fact that in the presence of F-6-P, sodium borohydride and transaldolase, an inactive enzyme preparation was obtained.

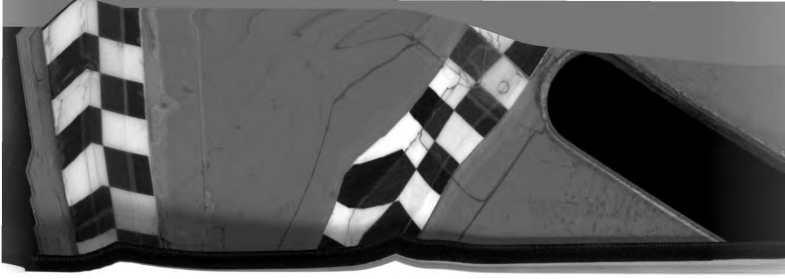
The mechanism of KDPG-aldolase was shown by Meloche and Wood (1) to be similar to muscle aldolase. However, KDPG-aldolase catalyzed a non-stereospecific exchange of solvent protons into all three methyl hydrogens of pyruvate. In addition, chemically synthesized β-ditritio KDPG, in the presence of the aldolase, did not decrease in specific activity indicating that cleavage was more rapid than the exchange reaction. The proposed reaction mechanism envisioned the formation of an enzyme bound pyruvyl anion which was neutralized by a proton from water, to release pyruvate, or neutralized by D-G-3-P to release KDPG.

Nature of the Combining Site of Aldolases

A major clarification concerning the combining site of aldolase enzymes was made by Pontremoli and associates (29), that in the presence of F-6-P and $NaBH_4$, transaldolase was completely inactivated. In a similar study by Grazi, Cheng and Horecker (30) it was demonstrated that in the presence of DHAP-^{32}P and $NaBH_4$ radioactivity was stably bound to muscle aldolase with an accompanied complete loss of enzyme activity. A later communication demonstrated

that $NaBH_4$ probably reduced an azomethine formed between the carbonyl carbon of the substrate and an amino group of the enzyme (31). The product obtained by complete acid hydrolysis of transaldolase or muscle aldolase following substrate inactivation was indistinguishable. When the radioactive derivative was subjected to periodate oxidation at alkaline pH, there was a release of radioactivity indicting the presence of vicinal hydroxyl or hydroxyl and amino groups. Following this treatment, a new compound, identified as lysine, was found. The chemical synthesis of authentic N^6-ß-glyceryl lysine by Speck and co-workers (32) and the comparison of this compound with that obtained following acid hydrolysis of $NaBH_4$ -inactivated transaldolase or muscle aldolase positively identified the enzyme product as the stable secondary amine. The amine could arise only by reduction of an azomethine formed between DHAP or DHA and muscle aldolase or transaldolase respectively:

These experiments indicated that an ε-amino group of an enzyme lysine residue was responsible for azomethine formation with the substrate.



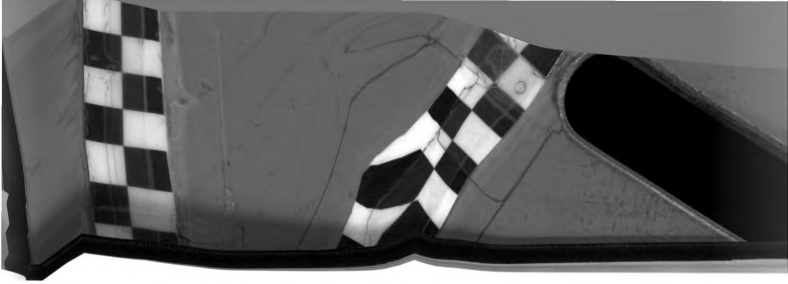
Following the above experiments with muscle aldolase and transaldolase; Grazi, Meloche, Martinez, Wood, and Horecker (33) described the inactivation by $NaBH_4$ of both DR-aldolase and KDPG-aldolase in the presence of the respective substrates, acetaldehyde and pyruvate. These experiments constituted presumptive evidence for azomethine formation between a lysine ε-amino group of the respective enzymes and the carbonyl carbon of the substrate. For KDPG aldolase it was calculated that one mole of ^{14}C-pyruvate was bound per 50,000 gm of protein. The initial report by Grazi et al. (31) indicated that one mole of DHAP was stably bound per mole of FDP-aldolase. An independent study by Westhead, Butler, and Boyer (34) showed by ultracentrifugation and equilibrium dialysis experiments that one mole of DHAP was bound per mole of aldolase. A more recent communication by Lai and collaborators (35), however, indicated that two moles of DHAP per mole of aldolase were stably bound in the presence of $NaBH_4$. Similar studies to those reported above have recently been undertaken with DR-aldolase. N^6-ethyl lysine was synthesized by Rosen, Hoffee, Horecker and Speck (36) and shown to be identical to the product obtained from a complete acid hydrolysis of DR-aldolase inactivated in the presence of acetaldehyde-^{14}C and $NaBH_4$. Results with ^{14}C-acetaldehyde indicate that one mole of substrate is bound per mole of the aldolase (18). It appears that yeast aldolase is not

inactivated by $NaBH_4$ in either the presence or absence of substrate (37). On the basis of these data it was concluded that the mechanism of yeast aldolase differed in a significant manner from that of the other aldolases discussed thus far. Rutter and co-workers (37) speculated that the divalent metal required for activity by yeast aldolase most probably facilitates a true enolization of the carbonyl portion of the substrate. The enolization would, therefore, facilitate the loss of a proton from the β-carbon of the substrate.

Studies available on KDPG-aldolase to the present time are restricted to those of Meloche and Wood (1) concerning the tritium exchange reactions into pyruvate and the preliminary report by Grazi et al. (33) that KDPG-aldolase most probably catalyzes azomethine formation between an enzyme bound amino group and the carbonyl carbon of the substrate. Data concerning the positive identification of this group are still lacking.

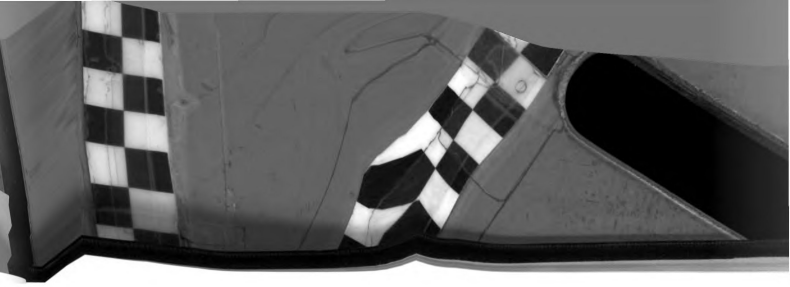
The Role of Active Centers in Aldolase Enzymes

Evidence concerning the participation of other active centers in the aldol reaction is much more limited than that available for the azomethine site, thus making generalizations for aldolase enzymes more difficult. One study performed by Swenson and Boyer (38) showed that muscle aldolase was partially inhibited in the presence of



p-mercuribenzoate. However, the rates of reaction of various thiol groups varied depending upon the conditions employed. For example, up to ten thiol groups reacted without an appreciable loss of activity. However, in 4.8 M urea, 28 thiol groups reacted resulting in an irreversible loss of activity. Reaction of the thiol groups was independent of the presence of substrates. A later report by Westhead, Butler and Boyer (34) showed that reduction of muscle aldolase liberated only one thiol group rather than two as expected if a disulfide bond was cleaved. No experimentally substantiated conclusion was offered for this anomaly.

In a further study Rowley, Tchola and Horecker (39) demonstrated that FDP-aldolase from muscle was inactivated in the presence of 2,4-dinitrofluoro- or 2,4-dinitrochlorobenzene. It appeared 5 to 6 residues were dinitrophenylated before inactivation of the enzyme. In a later report, Cremona, Kowal and Horecker (40) showed that the consumption of one mole of ClDNB resulted in a three-fold stimulation of enzyme activity. The further uptake of two moles of ClDNB diminished the elevated activity to the original level. In all cases thiol groups of cysteine residues were involved in the reaction. Upon single reaction with ClDNB and treatment with mild alkali, a dehydroaldolase derivative was obtained. These workers suggested that the cysteine residue participating in the latter reaction may constitute an allosteric site for FDP-aldolase. Similar studies were

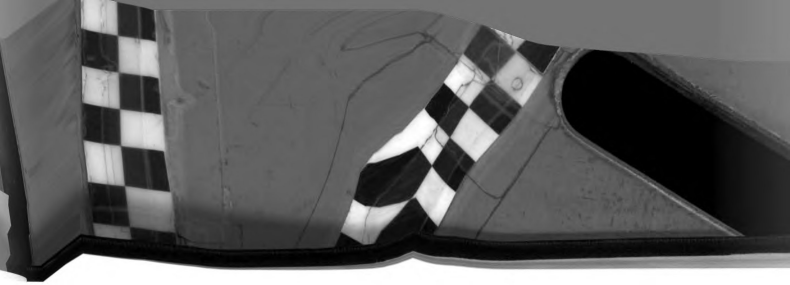


also reported by Rowley et al. for transaldolase. The enzyme was inactivated by the uptake of two moles of ClDNB per mole of enzyme. It was suggested that the reaction occurred at the ε-amino groups of two lysine residues and that these groups possibly functioned as an anionic binding site. The latter conclusion was further substantiated when it was observed that phosphate ion prevented dinitrophenylation. The effect of other inactivating compounds on aldolase enzymes is limited to one report by Rosen, Hoffee, and Horecker (41) with crystalline DR-aldolase. The absence of cysteine residues in this enzyme probably accounts for its extreme stability in the presence of thiol inactivating agents. The enzyme is susceptible, however, to inactivation by methylene blue and light, and diazo-p-nitroaniline suggesting the possible importance of tyrosine and histidine residues for catalysis.

Similar studies to those discussed above are not available for KDPG-aldolase. Therefore generalizations of reaction mechanism and enzyme site structure is difficult because of the lack of data.

Physical Properties of Aldolase Enzymes

The elucidation of physical parameters of aldolase enzymes is limited to a few isolated studies. Reports by Deal, Rutter and van Holde (3) and Stellwagen and Schachman (42) concerning the gross structure of muscle FDP-aldolase appeared simultaneously. Under mildly acidic conditions,



muscle aldolase dissociates into three subunits of approximate equal size. The subunits are reassociated, in vitro, when neutralized to pH 5.5. A complete recovery of activity was not realized; however, physical properties such as $S_{20,w}$, catalytic activity, and immunological response of the recovered enzyme were identical to the native protein. The studies also indicated the existence of an intermediate structure believed to be a trimer of the unfolded units.

Dreschler, Boyer, and Kowalsky (43) found that muscle FDP-aldolase was severely inhibited by treatment with carboxypeptidase. The loss of activity was associated with the loss of three carboxyl terminal tyrosine residues. The degraded aldolase was crystallized and shown to have physical properties identical with the native, unaltered enzyme. In this connection it was noted by Rutter and co-workers (14) that as the aldolase underwent carboxypeptidase degradation the rate of proton exchange into DHAP decreased to a greater extent than the rate of dealdolization, while with the native enzyme the rate of cleavage is more rapid than tritium exchange. Rose, O'Connell, and Mehler (44) demonstrated later that under these conditions of degradation there was an alteration of the rate determining step. The rate determining step now became the proton neutralization of an enzyme-bound DHAP carbanion and the net result was reflected in a decreased rate of proton exchange.

A new concept concerning the physical structure of muscle FDP aldolase was advanced recently by Lai and co-workers (45). These investigators described the isolation of a 28 amino acid peptide containing the "active center" lysine residue which forms the azomethine linkage with the carbonyl carbon of DHAP. After treatment with DHAP and $NaBH_4$, the derivatized protein was treated with trypsin to yield a number of peptide fragments, one of which was labeled. Through a series of purification procedures the peptide was isolated and its primary sequence deduced. The "active center" lysine residue is placed almost equidistant from the N and C-terminal ends of the peptide.

To date the study of the physical characteristics of KDPG-aldolase have been minimal and limited to a few preliminary observations made by Meloche and Wood (1). The molecular weight of the crystalline enzyme is 90,000 as determined by equilibrium ultracentrifugation analysis and the stable reduction of pyruvate-^{14}C. The enzyme displayed a very unique property of extreme stability towards 0.1 N HCl for at least four hours at room temperature. No evidence was found for molecular unfolding under these conditions and refolding upon neutralization. Preliminary data also indicated that upon treatment in guanidine· HCl, the enzyme was split into two subunits. No attempt has yet been made towards the isolation and characterization of the azomethine lysine peptide from KDPG-aldolase.

CHAPTER III

METHODS AND MATERIALS

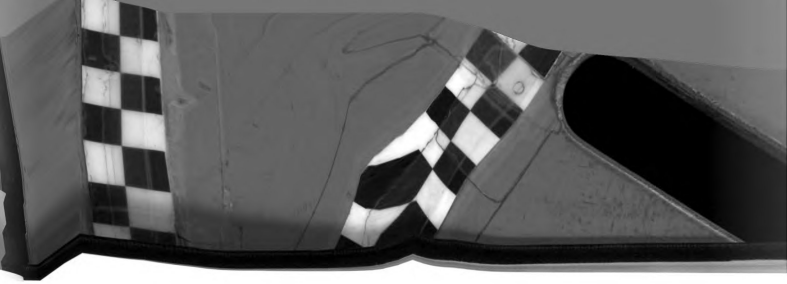
Bacteriological

Pseudomonas fluorescens strain A 3. 12 Stanier was maintained on agar slants composed of 1.2% glucose, 0.6% $(NH_4)_2HPO_4$, 0.3% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.0005% $FeCl_3.6H_2O$, and 0.5% yeast extract. The cultures were incubated at 30°C for growth. Large batches of cells were grown in a 10 gallon fermenter (New Brunswick Scientific) or a similar 30 gallon fermenter, on a medium as described above without the added agar. The glucose and magnesium salts were sterilized separately. Growth was from a 10% inoculum at 30°C until the cells just reached the stationary phase.

Chemical

Materials

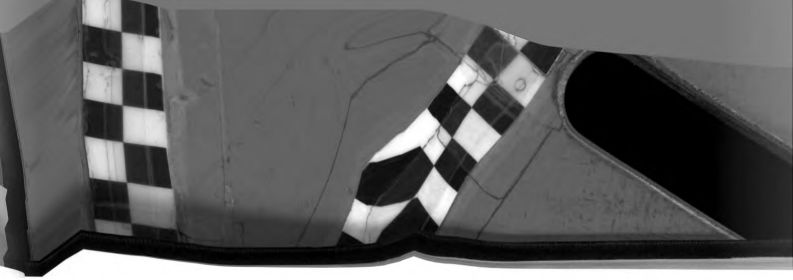
Amino acid preparations, D, L-glyceraldehyde-3-PO_4, protamine sulfate, α-ketoisovalerate, α-ketoglutarate, and dihydroxyacetone were obtained from the California Corp. for Biochemical Research. Sodium borohydride, and α-ketobutyrate were preparations of the Sigma Chemical Co. Hydroxypyruvate was obtained from Nutritional Biochemicals



and monohydroxyacetone from K and K Laboratories. 2,4-Dinitrofluorobenzene and 2,4-dinitrochlorobenzene were obtained from Distillation Products and lithium aluminum hydride from Metal Hydrides Incorporated. N^{ϵ}, $N^{\epsilon'}$-dinitrophenylene-bis-L-lysine was a gift from Dr. Peter Marfey Walker Biochemical Lab. Boston, Mass. 5-Keto-4-deoxygluconate was a gift from Dr. H. Blumenthal, Loyola University (Chicago) and 2-keto-4-hydroxyglutarate was a gift from Dr. E. Dekker, the University of Michigan.

All ion exchange resins were obtained from The California Corporation for Biochemical Research. Sephadex was purchased from Pharmacia, Limited.

KDP-gluconate was prepared by incubating a four-fold excess of pyruvic acid with D,L-glyceraldehyde-3-PO_4 and KDPG-aldolase in 0.1 M imidazole buffer, pH 8.0. (Specifically, the quantities used were: 11.2 mmoles of D-G-3-PO_4, 22.4 mmoles of Na pyruvate, and 5,800 units of the aldolase). The reaction was followed to completion by determining the disappearance of pyruvic acid on aliquots with a modified lactic dehydrogenase assay. Eight equivalents of barium acetate were added and the pH of the mixture adjusted to 3.5 with HCl. After the addition of four volumes of ethanol, the mixture was allowed to stand overnight in the cold and then was centrifuged. The precipitated barium salts were redissolved at pH3.5, reprecipitated with four volumes of ethanol, and centrifuged as before. The precipitate was



washed well with absolute ethanol, ether, and dried over P_2O_5 *in vacuo*. After two precipitations, KDP-gluconate was obtained in 90 per cent yield, with 4.5 per cent pyruvic acid content. The purity, without correction for moisture, was 67 per cent as judged by an assay with KDPG aldolase (1) .KD-Gluconate was prepared by incubating KDP-gluconate with alkaline phosphatase (5). The reaction was followed to completion by measuring the disappearance of KDP-gluconate with KDPG-aldolase. The reaction mixture was then chromatographed on Dowex-1-Cl with a 0 to 0.05M HCl linear gradient. The semicarbazide positive (1) fractions were pooled and concentrated on a rotary evaporator *in vacuo*.

DL-Alaninol was synthesized by reducing DL-alanine with lithium aluminum hydride as described by Vogl and Pöhm (46). The alaninol was recovered by distillation at 68 to 70°C and 7 to 8 mm pressure. From 7.0 gm of DL-alanine, 3.03 gm of DL-alaninol was obtained as a colorless oil. The oxalate salt of DL-alaninol had a metling point of 138 to 140°C, uncorrected; the reported value is 141 to 142°C (46).

5-δ-Bromobutylhydantoin was prepared by the method of Gaudry (47). The yields for two separate preparations were 55 per cent and 60 per cent of theory rather than 80 per cent as reported. Authentic N^6-α-(1-hydroxypropyl) lysine was synthesized by reacting a ten-fold excess of DL-alaninol with 5-δ-bromobutylhydantoin under reflux in absolute ethanol for 40 hours as described by Speck *et al*.

(32). The compound was purified in a similar manner except that the reaction mixture was deionzed by passage through Bio-Rad AG-11 A 8 ion retardant resin. After adjusting the pH to 5.8 with HCl, the material crystallized from an ethanol-acetone-water mixture in the cold. The majority of the material was precipitited from solution by the addition of an excess of acetone. Both the crystals and the amorphous material were washed immediately with water-free ether and dried <u>in vacuo</u> over P_2O_5. The compound was very hygroscopic and was stored over P_2O_5. The material was essentially homogeneous on paper chromatography in butanol:acetic acid: water (60:15:25 v/v), but some preparations revealed a second minor component which moved just off the origin. In all likelihood this compound is the disubstituted N^6-lysine derivative.

For the pure N^6-α-(1-hydroxypropyl) lysine

	$C_9 H_{21} O_3 Cl \cdot 1H_2O$
Calculated	C, 41.87%; H, 8.90%; N, 10.84%
Found[1]	C, 41.08%; H, 7.44%; N, 10.94%

The compound had an indistinct melting point range of 185 to 188°C (uncorrected).

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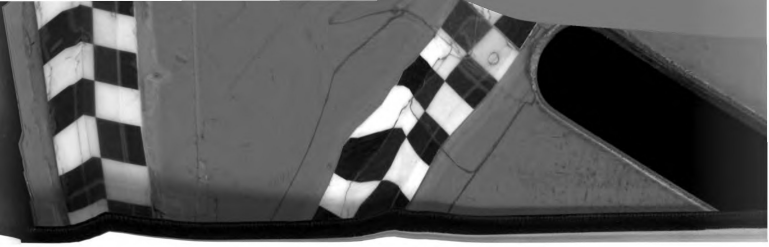
[1]The elemental analysis was performed by Spang Microanalytical Laboratory, Ann Arbor, Michigan.

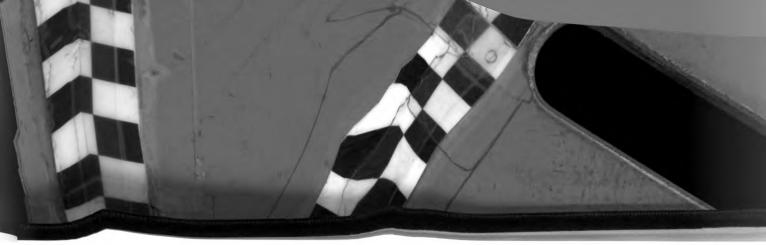


All attempts to synthesize N^6-α-propionyl lysine, the suspected product of borohydride reduction of KDPG-aldolase in the presence of pyruvate, by procedures similar to that described above for N^6-α-(1-hydroxypropyl) lysine have failed. The reaction of alanine with 5-δ-bromobutylhydantoin in ethanol probably failed because of the insolubility of the amino acid. However, no detectable reaction occurred when methyl alaninate was used instead of alanine. Another procedure involving the reaction of α-bromopropionic acid with N-α-carbobenzoxylysine in water yielded only lysine after the removal of the protective carbobenzoxy group.

Authentic ε-DNP lysine was prepared from the copper complex as described by Leggett-Bailey (48). All other DNP-amino acids used as standards were obtained from Mann Research Laboratories.

Enzymes used as reagent chemicals were obtained as follows: crystalline lactic acid dehydrogenase, pepsin, and trypsin from Worthington Biochemical Corporation, and alkaline phosphatase of calf mucosa from the Sigma Chemical Company. The α-glycerophosphate dehydrogenase-triose phosphate isomerase was a product of C. F. Boerhinger and Son.

All radioactive materials were purchased from the Volk Radiochemical Company.

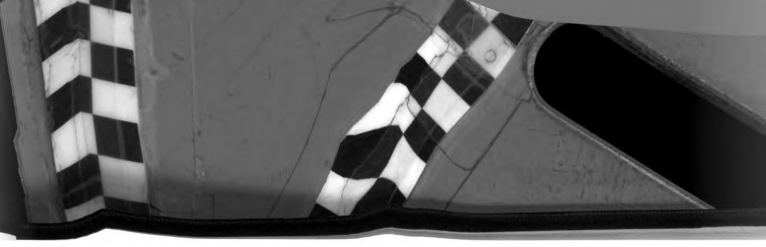


Determinations and Procedures

Alpha-keto acid determinations were performed by the semicarbazide method of Macgee and Doudoroff (5). KDPG was also determined spectrophotometrically by a procedure similar to that used to assay KDPG-aldolase. The separation of α-keto acids from reaction mixtures was achieved by column chromatography on analytical Dowex-1-Cl columns with a linear 0 to 0.05 M HCl gradient as described by Meloche and Wood (1).

Sodium borohydride reductions were performed by the procedure of Grazi et al. (33). The reaction mixture was made to a final volume of 1.0 ml and sodium borohydride (0.01 ml of a 1 M solution) and acetic acid (0.005 ml of a 2 M solution) were added alternately every three minute interval for four additions each. The protein was precipitated by the addition of 600 mg of ammonium sulfate per ml of reaction volume, centrifuged, redissolved and precipitated in a similar manner, this procedure was repeated twice.

Lithium aluminum hydride reduction of the derivatized protein was performed as described by Leggett-Bailey (48). Following reaction of pyruvate-^{14}C and sodium borohydride with the aldolase and the isolation of the inactive protein all of the available carboxyl groups of the enzyme were esterified and reduced with $LiAlH_4$ as follows. The derivatized enzyme, 5.0 mg, was thoroughly dried over P_2O_5 and methanol and methanolic HCl were added to the solid residue



to give 3 ml of 0.1N HCl. The suspension was then shaken gently for 24 hours at 25°C. Eight ml of ether were added and the mixture allowed to stand in the cold for several hours. The precipitate was suspended in 5 ml of tetrahydrofuran (redistilled), 20 mg of $LiAlH_4$ were added and the mixture refluxed gently for 6 hr. Upon cooling, excess methanolic HCl was added to destroy the excess $LiAlH_4$. After one hr, 20 ml of an acetone-ether mixture (1:1) were added and the precipitate which formed was removed by centrifugation. After drying over P_2O_5, the residue was suspended in constant boiling HCl and hydrolyzed at 110°C. The mixture, after hydrolysis, was deionized by passage through a column of Bio-Rad AG-11 A 8 ion retardant resin before further use. The radioactive peak was concentrated prior to chromatography.

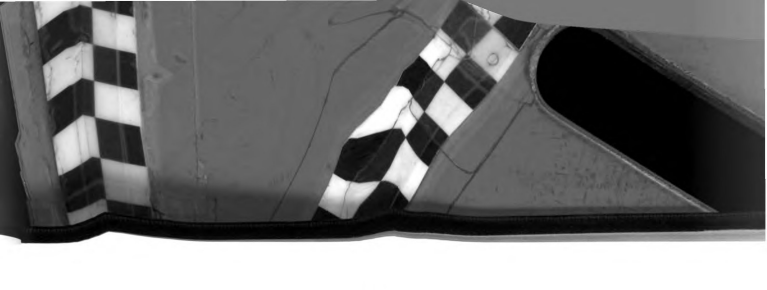
Reactions with 2,4-dinitrofluorobenzene were followed spectrophotometrically at 360 mμ. The reactions, usually in 1.0 ml total volume, were performed with the aid of a Gilford modified Beckman DU spectrophotometer fitted with an auxillary dwell timer. After each time interval the optical density was recorded for a 30 sec period. A control was run in parallel to determine the optical density change in the absence of the enzyme. To determine the degree of dintrophenylation the optical density of the control cuvette was subtracted from the value obtained in the presence of enzyme. The uptake of FDNB was calculated using an extinction coeffecient of 17,700 for ε-DNP lysine (39).

Limited Digest of KDP-gluconate Aldolase

KDP-gluconate aldolase, 40.4 mg of a 62% pure preparation, was treated with pyruvate-1-^{14}C and sodium borohydride as previously described. The protein was precipitated from solution by the addition of one-fifth volume of 50 per cent trichloroacetic acid. The precipitate was removed by centrifugation and washed with 5 per cent trichloroacetic acid. This washing was repeated twice. The mixture was resuspended in 0.02N HCl and 2 mg of pepsin dissolved in 0.02N HCl were added. The mixture was incubated for four hours at 37°C. After 35 minutes the suspension was solubilized.

After completion of the digestion, the pepsin was removed by boiling and centrifugation. The hydrolyzate was adjusted to pH 6.0 and placed on a Sephadex G-25 column (3 x 110 cm) which had previously been equilibrated with 0.05M NH_4OH in deionized water. The hydrolyzate was eluted with 0.05M NH_4OH at the rate of 40 ml per hour. Ten ml fractions were collected and tested for radioactivity and ninhydrin reactive material (48). The tubes containing radioactivity were combined and concentrated for further study.

Complete protein hydrolysis was performed in evacuated, sealed Pyrex tubes in the presence of constant boiling HCl for 20 hours at 110°C in an autoclave. Paper chromatography of all amino acids or peptides was carried out in glass tanks with various solvent systems; butanol: acetic acid:



water (60:15:25 v/v), butanol: pyridine: water (1:1:1 v/v) or phenol:water:ammonia (100:20:1: v/v). Analytical paper chromatography was performed with Whatman 1 mm filter paper and preparative chromatography of peptide digests was done with Whatman 3 mm filter paper.

Amino Acid Composition of KDPG-Aldolase

Crystalline KDPG-aldolase used for amino acid composition determinations was crystallized by the conventional procedure (1). Samples were hydrolysed in sealed, evacuated pyrex tubes in 6N HCl at 110°C for 24, 48, and 108 hours. Upon completion of hydrolysis, the HCl was removed on a rotary evaporator. Water was added and the mixture evaporated again. This procedure was repeated three times. The final material was dissolved in 0.1 N HCl and made to 1.0 ml of 12.5 per cent sucrose.

Amino acid analyses were performed with a modified AutoTechnicon Amino Acid Analyzer. The conventional 1.5 cm flow cell was fitted into a Beckman model DU spectrophotometer fitted with a Gilford log converter. The optical density changes were recorded on a Sargent model MRA recorder. Readings were taken at 570 mμ except in the region where proline eluted in which case the wavelength was changed manually to 440 mμ. For most chromatograms 50 μg of hydrolysed protein sufficed with a recorder setting of 0.4 O.D. full scale. Norleucine was used as an internal standard to correct

for pump tube variations. The citrate buffers were identical to those described except that they were passed through a column of Dowex-50-Na+ after final adjustment of the pH to remove small traces of ammonia. All other aspects of the equipment including the gradient were as described in the AutoTechnicon Manual.

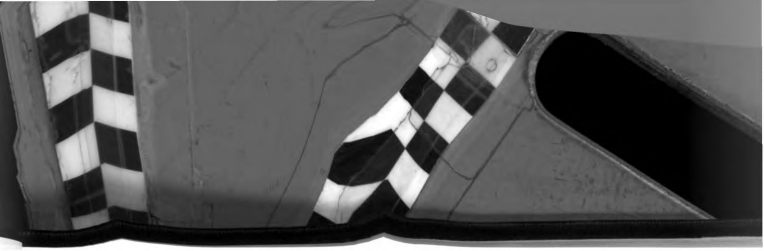
The area of each peak was quantitated by determining the half-height in terms of optical density units and calculating the width at the half-height with the aid of microcalipers. Conversion factors for each amino acid were obtained from chromatograms of standard mixtures.

Cysteic acid determinations were performed as described by Moore (49) after treatment of the enzyme with performic acid. Tryptophan analyses were performed by the spectrophotometric method of Bencze and Schmid (50).

Isotopic

All quantitative radioisotope determinations, either tritium or carbon-14, were performed with the aid of a Packard Tri-Carb liquid scintillometer. The scintillation system of Kinard (51) was used for 1.0 ml of aqueous sample. The efficiency of counting and the degree of quenching was determined by adding standard radioactivity to a previously counted sample.

Radioactivity on paper chromatograms was located with a Nuclear-Chicago strip counter generously supplied by Dr. R. L. Anderson.



Enzymatic

Assay and Preparation of KDP-gluconate aldolase

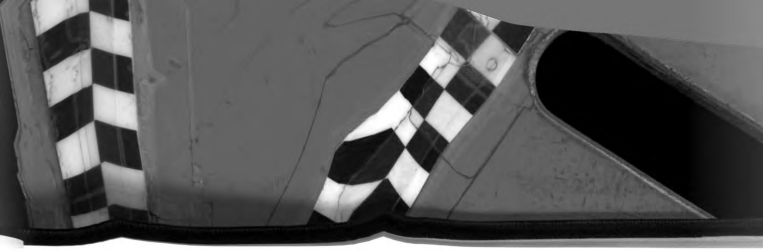
A coupled assay based on the oxidation of NADH as developed by Kovachevich and Wood (6) and modified by Meloche and Wood (1) was used for the estimation of KDP-gluconate aldolase activity. The assay is based on the following reaction sequence:

$$\text{2-keto-3-deoxy-6-phosphogluconate} \xrightarrow[\text{aldolase}]{\text{KDPG}} \text{D-glyceraldehyde-3-PO}_4 + \text{pyruvic acid}$$

$$\text{Pyruvic acid} + \text{NADH.H}^+ \xrightarrow[\text{lactic dehydrogenase}]{\text{excess}} \text{lactate} + \text{NAD}$$

The reaction was followed spectrophotometrically in silica glass cuvettes (b = 1cm) in a Gilford Modified Beckman DU spectrophotometer (52). One unit of activity is described as an absorbance change of 1.0 per minute in a total reaction volume of 0.15 ml. Specific activity is the number of units per mg of protein. Protein was determined by the 280:260 ratio method of Warburg and Christian (53).

Crystalline enzyme was prepared by the method of Meloche and Wood (1). Usually, 200 gm of fermenter-grown cells were suspended in 400 ml of pH 6.0, 0.1M phosphate buffer. The cells were subjected to sonic disruption in a 10-KC oscillator for 15 minutes. All debris was removed by centrifugation at 30,000 x g. The remainder of the purification procedure was similar to that described (1).



A more rapid procedure for enzyme purification was also developed to acquire large amounts of aldolase for exploratory experiments. The efficiency of this procedure is based upon the fact that the enzyme is stable in 0.1 M HCl for at least four hr (1). Fifty ml portions of a cell suspension prepared as above were disrupted in a sonic oscillator as previously described. The cellular debris was removed by centrifugation at 30,000 x g. The supernatant was adjusted to 0.2 N HCl by the slow addition of 4 N HCl, with stirring, at 4°C and the precipitate removed by two successive centrifugations. The subsequent steps were carried out without neutralization of the acidic solution. Solid ammonium sulfate was added successively to 1.0M, 1.5M, 2.0M, and 3.0M concentration and the precipitate which formed at each stage was removed by centrifugation. The precipitate collected between 2.0M and 3.0M ammonium sulfate was dissolved in 20 ml of distilled water. Calcium phosphate gel (8 mg of gel per mg of protein) was added with stirring and collected by centrifugation. The activity was eluted from the gel with an equal volume of 0.2M phosphate buffer, pH 6.0. This rapid and efficient procedure gave 75 to 95 per cent of the specific activity of crystals (1) with a yield of approximately 30 per cent.

Recent preparations have not fractionated with ammonium sulfate as described above. Rather, the enzyme precipitated between 1.3 and 2.0M ammonium sulfate. By the careful addition of ammonium sulfate in a step-wise manner, the

specific activity of the enzyme after this step is maintained. In addition, crystalline enzyme characteristic of KDPG-aldolase crystallized by the procedure of Meloche and Wood (1) was obtained through this rapid procedure.

CHAPTER IV

RESULTS

Purification of KDPG-Aldolase

Crystalline KDPG-aldolase used in critical experiments was prepared by the method of Meloche and Wood (1). The total recovery by this procedure was usually of the order of two or three per cent of the total units present initially. The procedure was slightly modified by concentrating the enzyme solution, after dialysis, on a Swissco evaporator under reduced pressure rather than lyophilizing the dialyzate as described in the procedure of Meloche and Wood. A small amount of Dow Corning antifoam agent was used to prevent excessive foaming while concentrating.

KDPG-aldolase used in exploratory experiments or used for the large scale synthesis of N^6-propionyl-^{14}C peptide was prepared by a modification of the procedure used to obtain crystalline material. The detailed procedure is described under Materials and Methods. It must be pointed out that the steps described must be worked through in a step-wise fashion to achieve the maximum yield and fold purification at each stage. A typical purification is shown in Table 1. This rapid and efficient procedure gave 75 to 95 per cent of the specific activity of crystals with a 30 per cent yield.

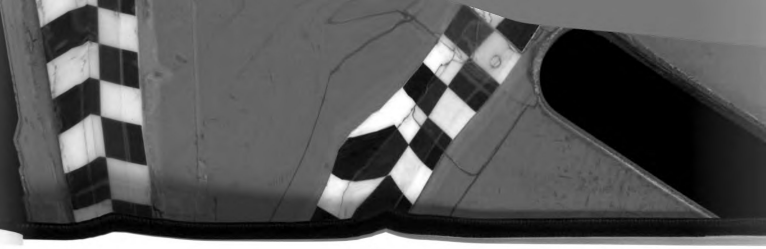


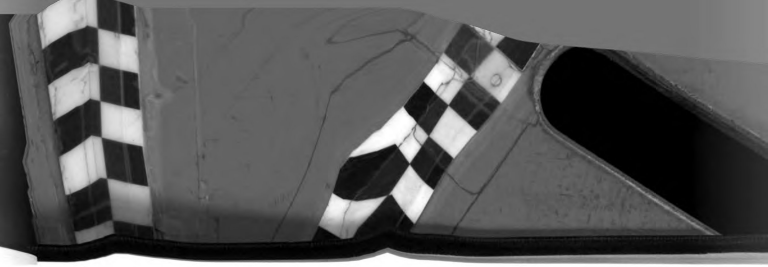
TABLE 1.--Purification of KDPG-aldolase from P. fluorescens.

Step	Total Units ($\times 10^3$)	Specific Activity	Recovery (Per Cent)	Fold
Crude extract	433	23.7	100.0	1
Ammonium sulfate (2.0M to 3.0M)	303.6	4,060	70.0	172
Calcium phosphate gel (elution)	200.0	13,000	46.0	548

Active Sites of KDPG-Aldolase

Amino Acid Involved in Azomethine Formation

Radioactive KDPG-aldolase was prepared by reduction of the pyruvyl-enzyme complex with sodium borohydride in the following manner: KDPG-aldolase, 2.25 mg (specific activity 12,500) was dissolved in 0.05 M phosphate buffer, pH 6.0. Four µmoles of pyruvate-1-^{14}C (specific activity 2.44 µcuries per µmole) were added and the mixture allowed to incubate for 5 minutes at 4°C. Sodium borohydride (0.01 ml of a 1.0M solution) and 0.005 ml of 2 M acetic acid were added alternately at 3 minute intervals. After four additions of each, 99 per cent of the activity was lost. The radioactive derivative of the enzyme was precipitated by the addition of 600 mg of ammonium sulfate per ml of reaction volume. The precipitate was washed by resuspension in three successive portions of 5 per cent tricholoroacetic acid. An aliquot



of the final precipitate was dissolved in 1.0 ml of 0.75 N NaOH and an aliquot placed into scintillation fluid to determine the extent of binding of pyruvate-1-^{14}C. The amount of stably bound ^{14}C was found to be 1.25×10^5 dpm per mg of protein. This corresponded to 1.96 moles of pyruvate-^{14}C bound per mole of aldolase and is in accord with the value obtained by Grazi et al.[1] (33).

The remainder of the radioactive protein was suspended in constant boiling HCl and hydrolyzed in a sealed tube for 22 hours at 110°C. After hydrolysis the excess HCl was removed by repeated lyophilization and the residue dissolved in a minimal amount of water. Samples of the hydrolyzate were chromatographed on paper in various solvent systems and chromatographed on Dowex-50-Na^+ at pH 5.28 (48) (Table 2). As shown in column three of Table 2, the radioactive product behaved differently than lysine in all tests.

By analogy with the studies of Horecker et al. (54) the sodium borohydride-reduced adduct should be N^6 - α - propionyl lysine. However, because of the inability to snythesize this derivative of lysine, a direct comparison of the radioactive compound with authentic N^6 - α - propionyl lysine was not possible. Accordingly, steps were taken to

---

[1] Specific activity of pyruvate = 2.44 μcuries/μmole = 5.5×10^6 dpm/μmole. Amount of aldolase treated = 2.25 mg = 0.026 μmoles (based upon a m.w. of 87,000)

Total dpm bound to aldolase = 2.82×10^5
Dpm per μmole of aldolase = 10.8×10^6
μMoles of pyruvate bound per μmole of aldolase = $\frac{10.8 \times 10^6}{5.5 \times 10^6} = 1.96$

TABLE 2.--Characteristics of radioactive derivatives obtained by hydrolysis of KDPG-aldolase.

	Standards		Hydrolyzate of Aldolase	
Chromatography	L-Lysine[a]	N^6-α-(1-Hydroxypropyl) lysine[a]	Before $LiAlH_4$[b]	After $LiAlH_4$[b]
Paper (Rf)				
Butanol:acetic acid: water (60:15:25)	0.14	0.19	0.087	0.19
Phenol:water:ammonia (80:20:1)	0.85	0.92	0.73	0.93
Dowex-50-Na^+, pH 5.28 (ml of eluate)	66.0	60.0	12.0	60.0

[a]Located by ninhydrin spray.

[b]Located by radioactivity.

prepare the primary alcoholic derivative of the enzyme-pyruvate adduct (48) and to compare the fragment from hydrolysis with authentic N^6 - α - (1-hydroxypropyl) lysine. Following reaction of pyruvate-1-^{14}C and borohydride with 5.0 mg of aldolase and isolation of the inactive protein as described above, all of the available carboxyl groups of the enzyme were esterified and reduced with $LiAlH_4$ as described under Methods. The residue was hydrolyzed in constant boiling HCl and concentrated before further use. As shown in columns three and four of Table 2, the $LiAlH_4$ - reduced derivative migrated more rapidly on paper and more slowly on Dowex-50-Na^+ than did the radioactive derivative (presumably N^6-α-propionyl lysine) isolated before treatment with $LiAlH_4$. Further, the mobility did not correspond to that of authentic lysine. By comparison with column two of Table 2, it can be seen that the new derivative behaved identically to authentic N^6 - α - (1-hydroxypropyl) lysine.

The chemically synthesized lysine derivative, when treated with periodic acid at pH 8.6, as described by Horecker et al. (54), gave a positive test for formaldehyde in the chromotropic acid procedure (55). The lysine derivative obtained by hydrolysis of the $LiAlH_4$-reduced aldolase was treated with periodic acid and then with dimedon, as described by Horecker et al. (54). The dimedon derivative was isolated by filtration and counted. Fifty per cent of the

radioactivity present in the lysine derivative after acid hydrolysis was recovered in the dimedon derivative.

The radioactive derivative isolated after treating the aldolase with $NaBH_4$ (before $LiAlH_4$ reduction) did not adhere to Dowex-50-Na^+ at pH 5.28 as did the derivative obtained after treatment with $LiAlH_4$. This indicated an acidic or neutral character and a liklihood that the acidity of the carboxyl group of N^6-α-propionyl lysine counteracted the basicity of the secondary ε-amino group of this lysine derivative. However, after reduction with $LiAlH_4$, the primary alcoholic function did not offset the basicity of the substituted ε-amino group of lysine with the result that this more reduced and more basic derivative adhered to the resin. It continued to elute ahead of lysine, however. The identification of this derivative is considered definitive proof that KDPG-aldolase forms an azomethine between an ε-amino group of an enzyme-lysine residue and the carbonyl carbon of the substrates pyruvate, or KDPG.

Inactivation of KDPG-Aldolase by 2,4-Dinitrofluorobenzene

KDPG-aldolase was inactivated by FDNB or ClDNB when the reaction was carried out under strictly specified conditions. The enzyme preparation studied had been stored in 0.2 M phosphate buffer, pH 6.0, and was subsequently dialyzed against 0.025 M bicarbonate buffer, pH 9.2, prior to treatment with FDNB or ClDNB. Figure 1 shows the course

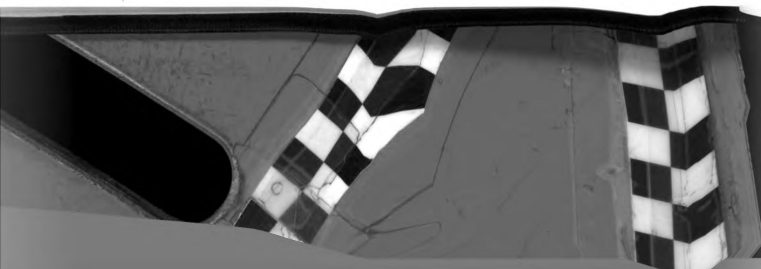
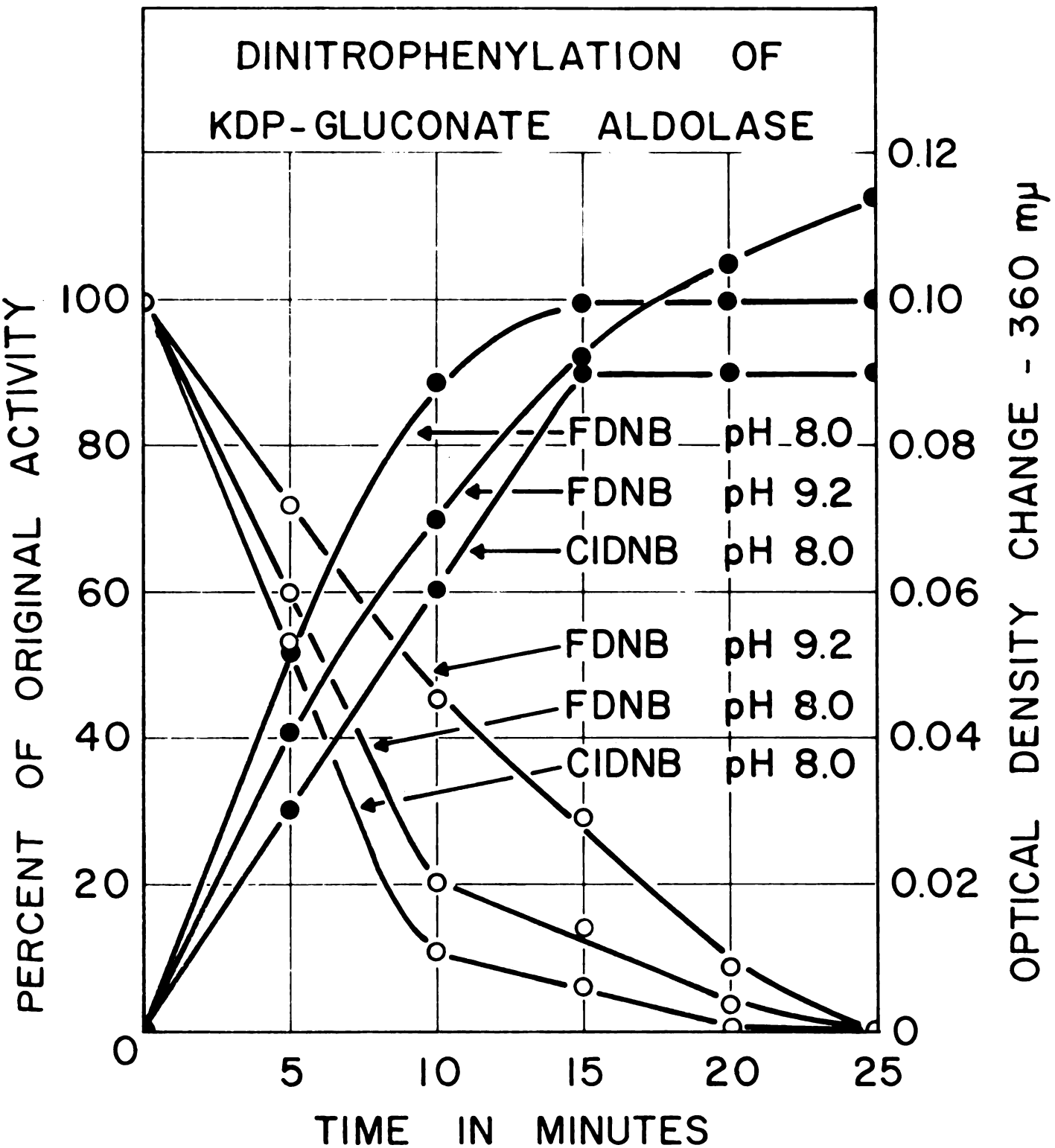


FIGURE 1.--Reaction of KDPG-aldolase with fluorodinitrobenzene and chlorodinitrobenzene. The time scale is expanded two-fold for ClDNB at pH 8.0 and the optical density scale was compressed ten-fold for FDNB at pH 9.2. The open circles relate to loss of enzyme activity and the closed circles to the change in optical density. Each cuvette contained in 1.0 ml: 200 µmoles of bicarbonate buffer (pH 9.2) or imidazole buffer (pH 8.0), about 2.5×10^{-2} µmoles of dialyzed enzyme for FDNB at pH 9.2 or 2.5×10^{-3} µmoles for the other reactions, and 0.5 µmoles of FDNB or ClDNB as indicated. Samples were withdrawn prior to each reading at 360 mµ for the determination of KDPG-aldolase activity.


DINITROPHENYLATION OF
KDP-GLUCONATE ALDOLASE
PERCENT OF ORIGINAL ACTIVITY
OPTICAL DENSITY CHANGE - 360 mμ
TIME IN MINUTES
FDNB pH 8.0
FDNB pH 9.2
CIDNB pH 8.0
FDNB pH 9.2
FDNB pH 8.0
CIDNB pH 8.0
0
20
40
60
80
100
0
0.02
0.04
0.06
0.08
0.10
0.12
5
10
15
20
25

of dinitrophenylation and the parallel loss of enzyme activity. At pH 8.0, and in the presence of a 10-fold molar excess of FDNB, dinitrophenylation, as measured as increased absorbancy at 360 mμ, proceeded until the time of complete inactivation and then ceased. These results suggested that only a few groups of the enzyme were dinitrophenylated with a corresponding inactivation of the enzyme.

The stoichiometry of dinitrophenylation at pH 9.2 was investigated using the conditions described in Table 3. The reaction was allowed to proceed until 86.7% of the enzyme activity was lost and the absorbancy change at 360 mμ was recorded. The absorbancy change corresponded to 3.50 moles of FDNB added per mole of aldolase. From this, it was calculated that for 100% inactivation, 4.08 moles of FDNB were required per mole of aldolase. This corresponds to 2.04 moles of FDNB consumed per mole of pyruvate-^{14}C stably bound to the enzyme. At pH 8.0, the stoichiometry of dinitrophenylation was similar to that observed at pH 9.2.

Preliminary experiments indicated that the dinitrophenylation reaction did not occur if the aldolase was dissolved in phosphate buffer. With a dialyzed (phosphate-free) preparation, which was sensitive to FDNB as above, the addition of potassium phosphate to 0.01M concentration preserved enzyme activity (Figure 2); arylation was also inhibited to almost the same degree. Although in this

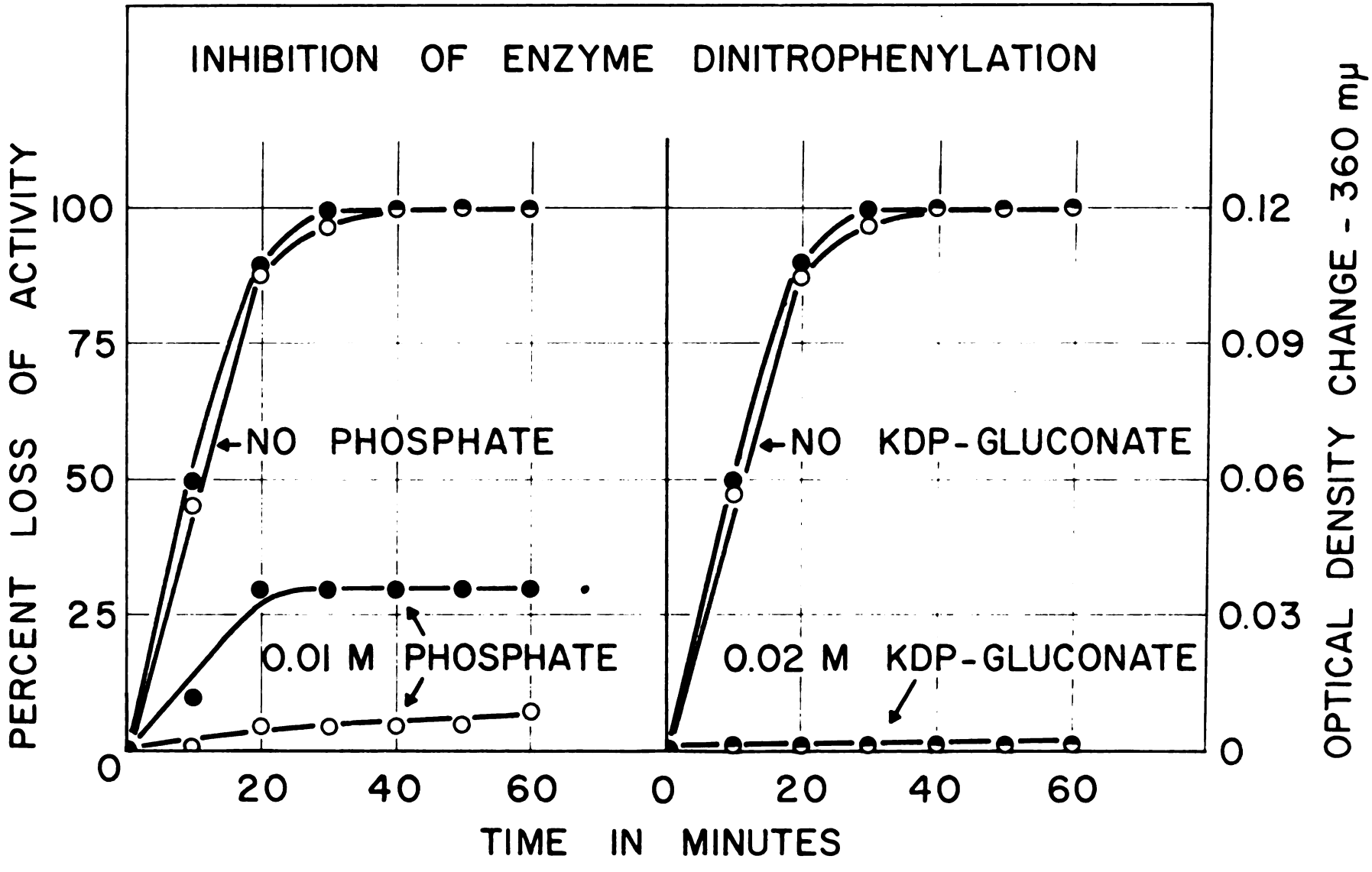
TABLE 3.--Stoichiometry of fluoriodinitrobenzene inactivation of KDPG-aldolase. The reaction mixture contained: 4m μmoles of crystalline aldolase, prepared by Dr. H. P. Meloche (1); 200 μmoles of bicarbonate buffer, pH 9.2; 0.5 μmoles of FDNB and water to 1.0 ml. The uptake of FDNB was followed at 360 mμ and the enzyme activity was assayed prior to each reading. All optical density changes were corrected for changes in a control without enzyme. At 86.7% inactivation, the optical density was determined and the optical density corresponding to 100% inactivation was calculated.

Inactivation	Change in Optical Density at 360 mμ	Moles of DNP Bound per Mole of Aldolase[a]
86.7%	0.250	3.50
100. %	0.288	4.08

[a]Based upon a molecular weight of KDPG-aldolase of 87,000 (1).

particular experiment with a low concentration of aldolase the consumption of FDNB appears not to have been completely inhibited by phosphate, subsequent experiments with higher phosphate concentrations have shown complete inhibition of arylation. As shown in the right-hand portion of Figure 2, at pH 8.0 (the optimum for activity), 0.02M KDPG preserved activity and inhibited arylation completely. 0.02M DL-Glyceraldehyde-3-PO_4 also afforded about 75% protection. Under similar conditions at pH 8.0, pyruvate did not prevent dinitrophenylation or protect against loss in activity. In a control at pH 8.0, the dinitrophenylation of lysine was not inhibited by the same concentration of phosphate ion or of

FIGURE 2.--Protection of KDPG-aldolase against dinitrophenylation by potassium phosphate and KDPG. The open circles relate to the loss of enzyme activity and the closed circles to the change in optical density at 360 mμ. Each reaction cuvette contained in 1.0 ml: 200 μmoles of imidazole buffer (pH 8.0), about 2.5×10^{-3} μmoles of dialyzed enzyme, potassium phosphate, 0.01M, or KDPG, 0.02M as labeled, and 0.5 μmoles of FDNB. Samples were withdrawn prior to each reading at 360 mμ for the determination of KDPG-aldolase activity.


INHIBITION OF ENZYME DINITROPHENYLATION
PERCENT LOSS OF ACTIVITY
OPTICAL DENSITY CHANGE - 360 mμ
100
75
50
25
0
0.12
0.09
0.06
0.03
0
NO PHOSPHATE
0.01 M PHOSPHATE
NO KDP-GLUCONATE
0.02 M KDP-GLUCONATE
20
40
60
0
20
40
60
TIME IN MINUTES

KDPG. At pH 9.2, under the same reaction conditions, KDPG did not prevent the arylation of KDPG-aldolase.

The preceding experiments suggested strongly that FDNB reacted with groups specifically required for binding of the phosphate moiety of KDPG and that it did not react with the lysine residue involved in azomethine formation. The validity of this assumption was tested by following the dinitrophenylation of an aldolase preparation which had been treated with pyruvate-^{14}C and $NaBH_4$ to render unavailable the lysine residue involved in azomethine formation. In the first experiment, aldolase was fully inactivated by reaction with pyruvate-^{14}C and borohydride as described above. After precipitation and dialysis, the aldolase was incubated at pH 9.2 with FDNB, as described. The results in Table 4 show that N^6-α-propionyl aldolase was capable of reacting with FDNB in the same manner and the same extent (4.30 moles of FDNB added per mole of enzyme) as the normal enzyme. Based upon the virtually complete dinitrophenylation of N^6-α-propionyl aldolase, it is apparent that the amino acid residues involved in dinitrophenylation are intact after reduction of the azomethine, and hence, distinct from those involved in azomethine formation.

In the second experiment, native aldolase was fully dinitrophenylated at pH 9.2 (4.08 moles of DNP per mole of aldolase) and then incubated with pyruvate-1-^{14}C and

TABLE 4.--Stoichiometry of pyruvic acid-1-^{14}C and fluorodinitrobenzene treatments to yield doubly labeled aldolase. In the first experiment, 4 mμmoles of enzyme, 200 μmoles bicarbonate buffer, pH 9.2, and 0.5 μmoles of FDNB in a volume of 1.0 ml were incubated at 30°C for 20 minutes. The protein was precipitated with ammonium sulfate, redissolved, and precipitated a second time. The DNP-protein was dissolved in 100 μmoles of phosphate buffer, pH 6.0, and treated with 3 μmoles of pyruvate-1-^{14}C (specific activity 2.44 μcuries per μmole) and sodium borohydride. The protein was precipitated with ammonium sulfate and washed three times with 5% trichloroacetic acid. The pellet was dissolved in 0.75N NaOH and a portion dissolved in scintillation fluid and counted. In the second experiment, the treatments were similar except that 7.23 mμmoles of enzyme were treated with pyruvate-1-^{14}C (specific activity, 5 μcuries per μmole) and sodium borohydride. The N^6-propionyl enzyme was dialyzed overnight against 0.025 M bicarbonate buffer, pH 9.2, and then incubated with FDNB as above.

Order of Treatment	Total Radioactivity x 10^3	Moles of Pyruvate Bound per Mole of Enzyme	Total Optical Density Change 360 mμ	Moles of DNP Bound per Mole of Enzyme
Dinitrophenylation followed by pyruvate-1-^{14}C plus borohydride	10.1[a]	0.98	0.288	4.08
Pyruvate-1-^{14}C plus borohydride followed by dinitrophenylation	166.0	2.06	0.560	4.30

[a]Radioactivity expressed as disintegations per minute.

borohydride. Based upon the amount of radioactivity stably bound to the precipitated and dialyzed aldolase, approximately one mole of pyruvate was found per mole of aldolase. This value corresponds to about 50 per cent of that normally bound. In other experiments somewhat lower values were found. The decreased binding of pyruvate bound in this experiment may be due to the fact that the dinitrophenyl groups sterically hinder the azomethine site thereby decreasing the binding capacity for pyruvate. An important observation, however, is that after dinitrophenylation the cleavage of KDPG is not apparent, while azomethine formation with pyruvate persists.

Identification of Amino Acid Involved in Dinitrophenylation

The nature of the groups involved in dinitrophenylation was examined further. Dinitrophenylated enzyme containing 4 moles of DNP per mole of aldolase was prepared at pH 8.0 as already described. The protein was precipitated with ammonium sulfate and the pellet suspended and washed three times with 5 per cent TCA. The precipitate was suspended in constant boiling HCl and hydrolyzed in a sealed tube for 18 hours at 110°C. The HCl was removed by repeated lyophilization and the residue dissolved in water. The aqueous layer was extracted with ether and the ether layer was found to be devoid of DNP derivatives as measured by absorbancy at 360 mμ.

Chromatrography of the aqueous layer on paper, as described in Table 5, showed a single DNP derivative with R_f values identical to those of authentic ε-DNP lysine. In none of the solvent systems tested did the derivative behave as authentic α,ε-diDNP lysine, or mono DNP-S-cysteine.

TABLE 5.--Identification of dinitrophenyl amino acids obtained from KDPG-aldolase.

Test	ε-DNP lysine	S-DNP cysteine	α,ε-diDNP lysine	DNP-Derivative from Aldolase
	R_f	R_f	R_f	R_f
Paper Chromatography Butanol:acetic acid: water (60:15:25)	0.63	0.54	0.91	0.63
1.5 M K^+ phosphate pH 6.0	0.70	--	0.15	0.70
Butanol:water (Saturated butanol)	0.60	0.47	0.67	0.60

Inactivation of KDPG-Aldolase by 1.5-Difluorodinitrobenzene

Since KDPG-aldolase is inactivated by the apparent uptake of 4 moles of FDNB per mole of enzyme, it was considered possible that the difluoroderivative would also lead to inactivation and perhaps result in the formation of N^{ε}, $N^{\varepsilon'}$ -dinitrophenylene-bis-lysine. This compound may result by bridging two of the lysine residues involved

in the phosphate binding site if they were properly situated. The reaction with diFDNB was performed as described for FDNB (Legend, Table 3). The results in Table 6 show that diFDNB reacted with the aldolase in a manner analogous to reaction with FDNB. In a large scale reaction performed under the same conditions, the enzyme was hydrolyzed, after precipitation with ammonium sulfate, in constant boiling HCl for 18 hr at 110°C. Chromatography of the hydrolyzate in butanol:acetic acid:water (60:15:25 v/v) showed that the compound obtained on complete acid hydrolysis behaved in a manner similar to authentic N^{ε}, N^{ε}'-dinitrophenylene-bis-lysine and different than ε-DNP lysine as shown in Table 7.

The identification of N^{ε}, N^{ε}'dinitrophenylene-bis-lysine after treatment of KDPG-aldolase with 1,5-diflourodinitrobenzene suggests that this reagent reacts with, and inactivates, the aldolase in a manner analogous to monofluorodinitrobenzene. The results do not suggest conclusively that inactivation is the direct result of the bridged compound. For example, the primary reaction leading to inactivation may be due to a mono reaction only. A more comprehensive review of the possibilities is described in the Discussion.

TABLE 6.--The reaction of 1,5-difluorodinitrobenzene with KDPG-aldolase. The reaction conditions were identical to those described in Table 3 except that approximately 2,000 units of aldolase were used in each experiment.

Treatment	Reaction Behavior with KDPG-Aldolase
Treatment at pH 9.2	Completely inactivated after 20 min.
Treatment at pH 8.0	69% inactivation after 30 min.
Treatment at pH 8.0 in the presence of 20 μmoles KDPG	0% inactivation after 60 min.
Treatment at pH 8.0 in the presence of 20 μmoles PO_4	5% inactivation after 50 min.
Chromatography of an acid hydrolyzate in butanol: acetic acid: water	The component from KDPG-aldolase behaved as N^{ϵ}, $N^{\epsilon'}$ - dinitrophenylene-bis-L-lysine.[a]

[a]The authentic sample was a gift from Dr. Peter Marfey.

TABLE 7.--Chromatographic characteristics of product obtained after treatment of KDPG-aldolase with 1,5-difluorodinitrobenzene.

Compound	Chromatography in Butanol:Acetic Acid:Water
	R_f
ε-DNP lysine	0.71
N^{ε}, N^{ε}'-dinitrophenylene-bis-lysine	0.33
Product from aldolase hydrolysis	0.35

Mechanistic Studies of the KDPG-Aldolase Catalyzed Reaction

Reductive Binding of α-Keto Compounds in the Presence of Sodium Borohydride

Since KDPG-aldolase is completely inactivated by incubation with pyruvate in the presence of borohydride (33), this test could be used to measure azomethine formation by other compounds. Accordingly, α-carbonyl compounds, usually at 3×10^{-2} M concentrations, were incubated with the aldolase and $NaBH_4$, as previously described, to measure the specificity of this phase of the aldolase reaction. As shown in Table 8, a number of α-keto acids and monohydroxyacetone were able to inactivate in the presence of $NaBH_4$. Of the compounds tested, only pyruvate and α-keto-butyrate caused complete inactivation of aldolase, whereas α-keto-isovalerate, monohydroxyacetone, α-ketoglutarate,

TABLE 8.--Inactivation of KDPG-aldolase in the presence of sodium borohydride and α-carbonyl compounds. Each reaction mixture contained substrate in the concentration indicated and 3,000 to 4,000 units of aldolase in a total volume of 1.0 ml. $NaBH_4$ (0.01 ml of a 1.0 M solution) was added alternately with 0.005 ml of 2 M acetic acid at three min intervals. Enzyme activity was determined before and after sodium borohydride reduction as previously described (33).

Addition	Concentration	Cleavage[a]	Inactivation
	$Mx10^{-2}$	Per Cent	Per Cent
None			0
Pyruvic acid	0.4	---	100
Hydroxpyruvic acid	3.0	---	0
Acetone	3.0	---	0
Dihydroxyacetone	3.0	---	0
Monohydroxyacetone	3.0	---	25.7
α-Ketobutyrate	1.0	---	100
	3.0	0	100
α-Ketoisovalerate	3.0	0	43.4
α-Ketoglutarate	3.0	0	62.0
2-Ketoglutonate	3.0	0	0
5-Keto-4-deoxyglucarate	3.0	0	66.3
2-Keto-4-hydroxyglutarate	3.0	0.1	68.0
2-Keto-3-deoxygluconate	2.0	<0.1	69.0

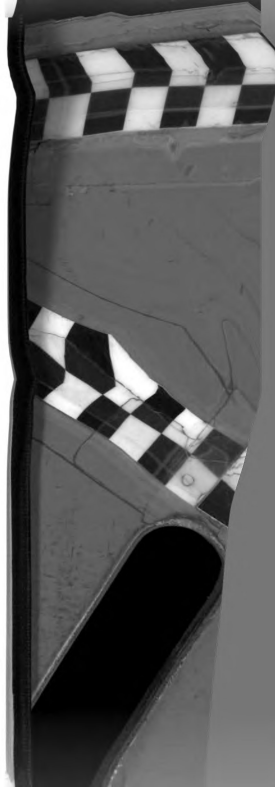
[a]Expressed as % of the rate for KDP-gluconate. A dash indicates that the test was not run.

5-keto-4-deoxyglucarate, 2-keto-4-hydroxyglutarate, and 2-keto-3-deoxygluconate inactivated to the extent of 25 to 70 per cent. Hydroxypyruvate, acetone, dihydroxyacetone, and 2-ketogluconate were without effect. In all cases, there was no loss of activity with $NaBH_4$ or with the carbonyl compound alone. Of the compounds tested, only 2-keto-4-hydroxyglutarate and KD-gluconate were cleaved (Table 8), as determined by the production of pyruvate. Activity with 2-keto-4-hydroxyglutarate was approximately 0.1 per cent compared to KDP-gluconate. Activity with KD-gluconate was somewhat lower than 0.1 per cent, but upon exceedingly long incubation the formation of pyruvate was measurable.

The inactivation of KDPG-aldolase in the presence of $NaBH_4$ and α-ketobutyrate suggested that this compound was stably bound to the active site lysine ε-amino group normally occupied by pyruvate or KDP-gluconate. If this were true, aldolase inactivated with α-ketobutyrate and $NaBH_4$ should be incapable of binding pyruvate-1-^{14}C. The results of Table 9 show that after treatment with α-ketobutyrate and $NaBH_4$, only 2 per cent of the usual amount of pyruvate was bound. Thus, it would follow that if α-ketobutyrate and pyruvate were competing for the same site, the presence of α-ketobutyrate should depress the amount of ^{14}C-pyruvate which would be reductively bound by $NaBH_4$ when both were present. In a preliminary experiment no such depression was noted when a ten-fold excess of α-ketobutyrate was used. However, as

TABLE 9.--The competition of α-ketobutyrate and pyruvate in azomethine formation. Each 1.0ml reaction contained: KDPG-aldolase; 200 μmoles of phosphate buffer, pH6.0; 20 μmoles of α-ketobutyrate or 1 μmole of pyruvate-1-^{14}C (specific activity, 5 μcuries per μmole), as indicated. The reductions were performed as described previously. In each case the protein was precipitated three times with 600 mg per ml of ammonium sulfate and redissolved in water. The final precipitate was dissolved in 1.0 ml of water and an aliquot counted.

Addition	Enzyme Units		Inactivation	Pyruvate-1-^{14}C Incorporation	
	Before	After	%	Dpm per mg	Moles per mole aldolase
Pyruvate-1-^{14}C	5,700	100	98.3	2.33×10^5	1.86
α-Ketobutyrate followed by pyruvate-1-^{14}C	5,700	150	97.5	0.05×10^5	0.04



seen in Table 10, when the α-ketobutyrate level was 100-fold greater than that of pyruvate, the stable incorporation of pyruvate-1-^{14}C was depressed 54 per cent.

In order to compare the relative abilities of pyruvate and α-ketobutyrate towards inactivation of KDPG-aldolase in the presence of $NaBH_4$, an experiment was designed whereby inactivation was determined as a function of substrate concentration. The results in Fig. 3 show that complete inactivation with α-ketobutyrate was obtained with a concentration equal to or less than 10 μmoles per ml, while complete inactivation with pyruvate was obtained at a concentration of approximately 3.0 μmoles per ml. In this experiment the single addition of borohydride was presumed to reduce the preformed azomethine completely as well as the excess substrate so that further azomethine formation was eliminated. The results of this experiment are important for the interpretation of the decreased rate of proton exchange into α-ketobutyrate which is described in the following section.

Proton Exchange Reaction of α-Ketobutyrate

The inactivation of KDPG-aldolase in the presence of α-ketobutyrate and $NaBH_4$ indicated that the enzyme was capable of forming an azomethine with α-ketobutyrate. It became imperative, therefore, to determine unequivocally whether or not the aldolase catalyzed a proton exchange between α-ketobutyrate and the medium. In a previous test

TABLE 10.--Competition of α-ketobutyrate and pyruvate-1-^{14}C for azomethine formation. Each 1.0 ml reaction mixture contained: KDPG-aldolase; 200 μmoles of phosphate buffer, pH 6.0; 20 μmoles of α-ketobutyrate where indicated; 0.2 μmoles of pyruvate-1-^{14}C (specific activity, 5 μcuries per μmole). The reductions and protein precipitations were as previously described.

Addition	Enzyme Units		Inactivation	Pyruvate-^{14}C	Incorporation
	Before	After	%	DPM per mg	Moles per mole of aldolase
Pyruvate-^{14}C	5,500	0	100	2.0×10^5	1.62
Pyruvate -^{14}C and α-ketobutyrate	5,500	0	100	0.92×10^5	0.74

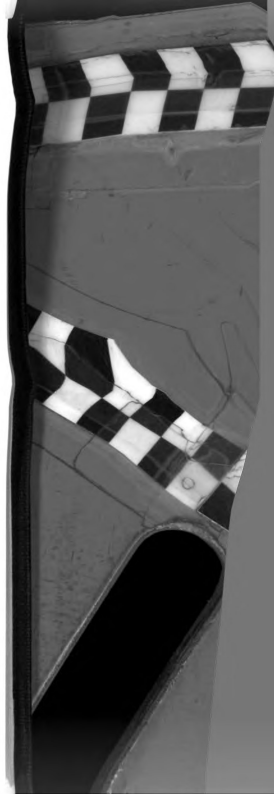
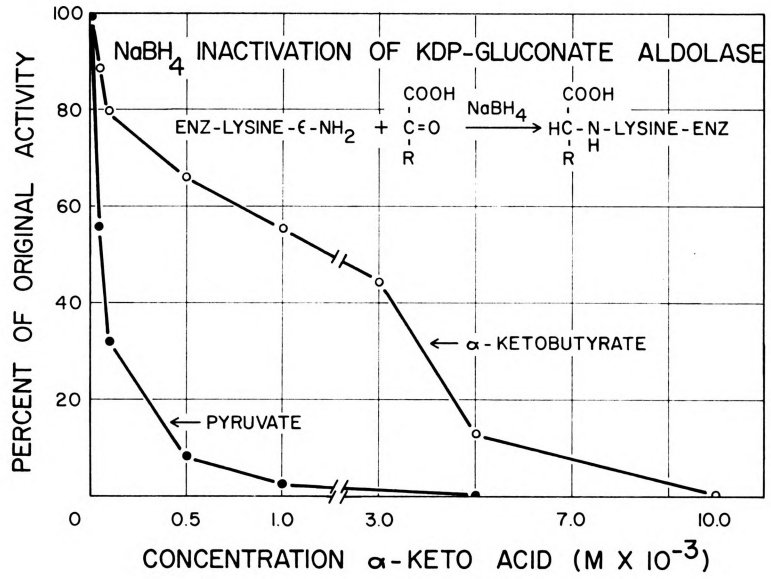


FIGURE 3.--Sodium borohydride inactivation of KDPG-aldolase in the presence of pyruvate or α-ketobutyrate. Each reaction mixture contained: about 1,000 units of aldolase, 100 μmoles of pH 6.0 phosphate buffer, substrate as indicated, and water to 1.0 ml. The reaction was pre-incubated for 5 min and 10 μmoles of $NaBH_4$ added. After 5 min 10 μmoles of acetic acid were added and the activity which remained was assayed as previously described.


NaBH4 INACTIVATION OF KDP-GLUCONATE ALDOLASE
ENZ-LYSINE-ε-NH2 + COOH C=O R NaBH4 COOH HC-N-LYSINE-ENZ R H
PERCENT OF ORIGINAL ACTIVITY
100
80
60
40
20
0
0.5
1.0
3.0
7.0
10.0
← α-KETOBUTYRATE
← PYRUVATE
CONCENTRATION α-KETO ACID (M X 10-3)

(1) using 140 units of aldolase and a 3 hr incubation, approximately 0.07 µatoms of hydrogen exchanged with α-ketobutyrate. However, if the incubation is performed with more enzyme and for a longer time a maximum of 1 µatom of hydrogen from the medium is incorporated per µmole of α-ketobutyrate (Fig. 4). Based upon the initial rate of proton exchange into pyruvate and making the assumption that the amount of incorporation in 0.5 hr measures the initial rate of exchange into pyruvate, then the rate of proton exchange into α-ketobutyrate (32 µatoms/hr vs. 0.086 µatoms/hr) is at least 37-times slower than the rate of exchange into pyruvate under identical reaction conditions. However, if the total enzyme units used (400) reflect the actual exchange capacity (9.6 µmoles per min) then the rate of exchange with α-ketobutyrate is at least 5,000-fold slower than the rate of exchange with pyruvate. It is reasonable to assume that each reaction was performed with a saturating level of substrate (see argument developed in Discussion). Under similar conditions neither hydroxypyruvate nor α-ketoglutarate exchanged protons to an appreciable degree. After 25 hr the incorporations were 5 per cent of one µatom. Although not established by appropriate controls it is suspected that these incorporations result from spontaneous enolization of these ketoacids.

FIGURE 4.--Exchange reactions catalyzed by KDPG-aldolase. The reaction mixtures contained: 400 units of aldolase, 10 μmoles of substrate, 50 μmoles of pH 8.0 imidazole buffer, 10 mcuries of T_2O (specific activity, 1 curie per g), in a total volume of 0.5 ml of water. The specific activity of the water was 4.0×10^5 dpm per μatom of hydrogen. At the end of each reaction, the mixture was transferred to a Dowex-1-Cl column (0.75 x 4 cm), washed well with water and eluted with 0.1 N HCl batch-wise. The α-keto acid was located and determined quantitatively by the semicarbazide reagent (1). The incorporation of tritium into the substrate was calculated as follows using the exchange into pyruvate in 0.5 hr as an example.

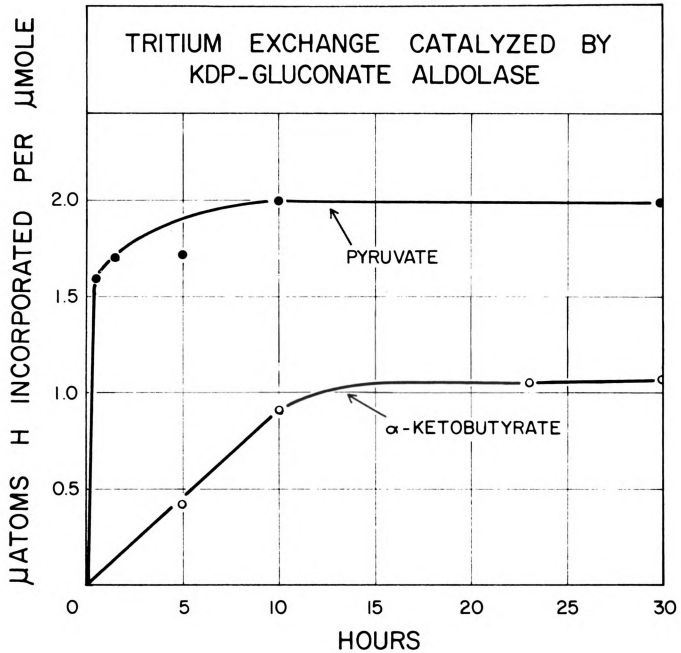
μMoles of pyruvate = 0.18

Cpm per 0.18 μmoles = 1.73×10^4

$$\text{Dpm per 0.18 } \mu\text{moles} = \frac{\text{cpm}}{\text{effeciency}} = \frac{1.73 \times 10^4}{0.15} = 1.15 \times 10^5$$

$$\text{Dpm per } \mu\text{mole} = \frac{1.15 \times 10^5}{0.18} = 6.4 \times 10^5$$

$$\text{Incorporation} = \frac{6.4 \times 10^5}{4.0 \times 10^5} = 1.6 \text{ μatoms per μmole}$$


TRITIUM EXCHANGE CATALYZED BY
KDP-GLUCONATE ALDOLASE
μATOMS H INCORPORATED PER μMOLE
2.0
1.5
1.0
0.5
0
5
10
15
20
25
30
HOURS
PYRUVATE
α-KETOBUTYRATE

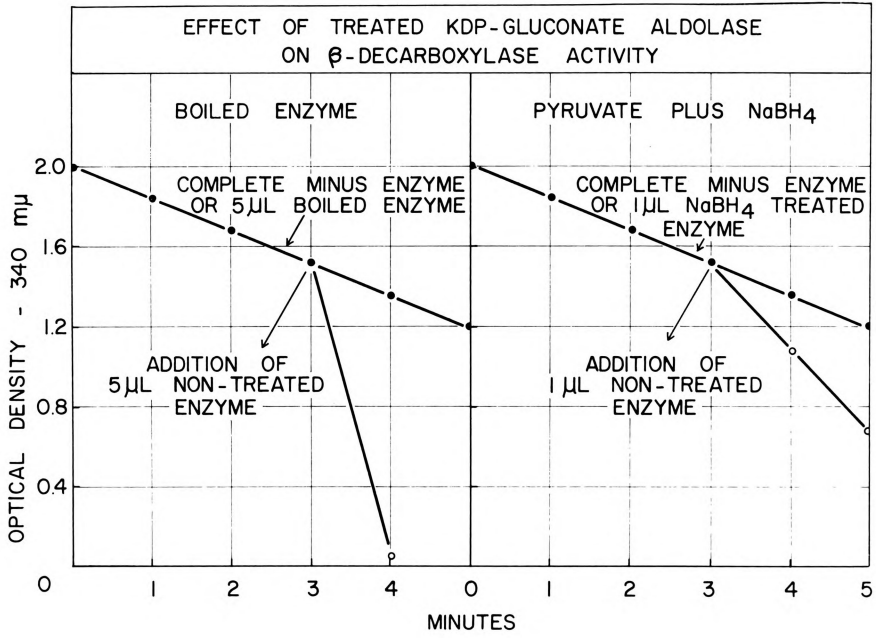
β-Decarboxylation of Oxalacetic Acid

Hamilton and Westheimer have previously reported that acetoacetic acid decarboxylase catalyzes the decarboxylation of acetoacetate through the formation of a Schiff base between the carbonyl portion of the substrate and a lysine residue in the enzyme (56). The enzyme was also inactivated in the presence of substrate and $NaBH_4$ (57). Since oxalacetate resembles pyruvate, the substrate for KDPG-aldolase, and also resembles other α-keto acids which were shown to form azomethines with the enzyme, it was anticipated that KDPG-aldolase might catalyze the β-decarboxylation of oxalacetate. Under conditions of the assay described in Fig. 5, the aldolase was observed to catalyze the formation of pyruvate from oxalcetate at approximately 0.5% the rate of cleavage of KDPG. Although the spontaneous rate of β-decarboxylation was high, the results in Fig. 5 show that the enzyme previously rendered inactive by formation of its N^6-propionyl derivative by use of pyruvate and $NaBH_4$ (33) or inactivated by heating no longer exhibited oxalacetate decarboxylase activity.

Characteristics of the Peptide Containing the Lysine Residue Responsible for Azomethine Formation

Following the reaction of KDPG-aldolase with pyruvate-1-^{14}C and $NaBH_4$ as described previously, the precipitated, derivatized enzyme was subjected to pepsin hydrolysis as described under methods. Upon termination of hydrolysis, the

FIGURE 5.--KDPG-aldolase-catalyzed β-decarboxylation of oxalacetate. In the first experiment aldolase (about 8,000 units) was boiled in 1.0 ml of 0.1M imidazole buffer, pH 8.0. A control reaction was identical except that it was not boiled. After cooling, 5 µl of the boiled preparation were added to the reaction cuvette containing an assay mixture similar to that described for KDPG-aldolase except that 0.5 µmoles of oxalacetate was substituted for KDPG. In the second experiment aldolase (about 8,000 units) was reduced with borohydride in the presence of pyruvate as previously described. In a control, the enzyme was reduced in the absence of pyruvate. One µl samples were added to the reaction cuvettes as described previously.


EFFECT OF TREATED KDP-GLUCONATE ALDOLASE
ON β-DECARBOXYLASE ACTIVITY
BOILED ENZYME
PYRUVATE PLUS NaBH4
COMPLETE MINUS ENZYME
OR 5 µL BOILED ENZYME
ADDITION OF
5 µL NON-TREATED
ENZYME
COMPLETE MINUS ENZYME
OR 1 µL NaBH4 TREATED
ENZYME
ADDITION OF
1 µL NON-TREATED
ENZYME
OPTICAL DENSITY - 340 mµ
2.0
1.6
1.2
0.8
0.4
0
1
2
3
4
0
1
2
3
4
5
MINUTES

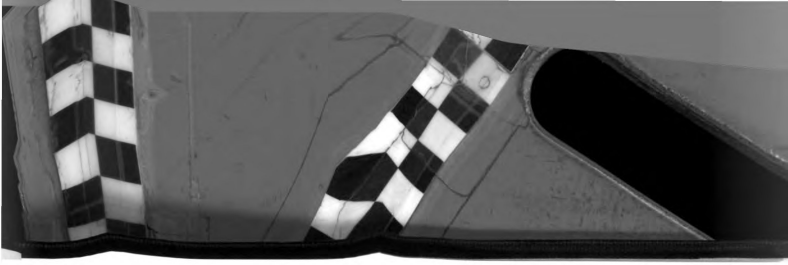
peptide mixture was placed on a Sephadex G-25 column previously equilabrated with 0.05M NH_4OH. The column was eluted with the same concentration of NH_4OH at a flow rate of 40 ml per hr. Seven ml fractions were collected and assayed for ^{14}-C content and ninhydrin reactivity (48). The elution pattern of a typical chromatogram is shown in Fig. 6. In this particular chromatogram, 2.2×10^5 cpm were placed on the column and 2.4×10^5 cpm were recovered. Of the two peaks containing radioactivity there was approximately ten times more ^{14}-C in peak one than peak two. The fractions of peak one were pooled and concentrated in vacuo and then chromatographed in the solvent systems described in Table 11. The migratory characteristics of the labeled peptide in both solvent systems indicate that it moved more rapidly than the majority of the ninhydrin reactive

TABLE 11.--Chromatographic characteristics of the ^{14}C-labeled peptide obtained from KDPG-aldolase. The aldolase, 2.5 mg (specific activity, 10,000) was reduced with $NaBH_4$ in the presence of pyruvate-1-^{14}C. The protein was precipitated and washed three times with 5 per cent trichloroacetic acid, resuspended in 0.01 N HCl and hydrolyzed with 0.1 mg of pepsin for one hour at 37°C. The digest was eluted from Sephadex G-25 as described above.

Solvent System	R_f Value
Butanol:acetic acid:water (60:15:25)	0.81
Phenol:water (100:20)	0.86

FIGURE 6.--Sephadex G-25 chromatography of pepsin digested KDPG-aldolase.

OPTICAL DENSITY 570mμ (——)
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60
70
FRACTION NUMBER
500
400
300
200
100
CPM PER 0.1 ML (----)
①
②



material migrated with an R_f value of 0.60 or less. At least 90 per cent of the radioactivity recovered on the chromatogram migrated with an R_f value as reported in Table 11. Minor radioactive components were detected in the region of high ninhydrin reactivity and no attempt was made to isolate or purify these fractions.

Since the separation of the labeled peptide from the non-labeled material was so discrete, this procedure was used for the large scale preparation of peptide. Peptide prepared in this manner was electrophoresed in pyridine:acetate buffer at pH 6.5. Under these conditions the peptide was homogeneous. Approximately 0.05 μmoles of the purified peptide were completely hydrolyzed with 6N HCl and the amino acid composition determined with a Beckman-Spinco amino acid analyzer. The results of the determination are shown in Table 12. A most interesting finding is that 65 per cent of the amino acids present are of the "non-polar" type. This group included; leucine, isoleucine, valine, alanine, and proline.

Amino Acid Composition of KDPG-Aldolase

Amino acid determinations were performed as described under Methods. Table 13 shows the amino acid composition of KDPG-aldolase based upon a molecular weight of 87,000 (1).

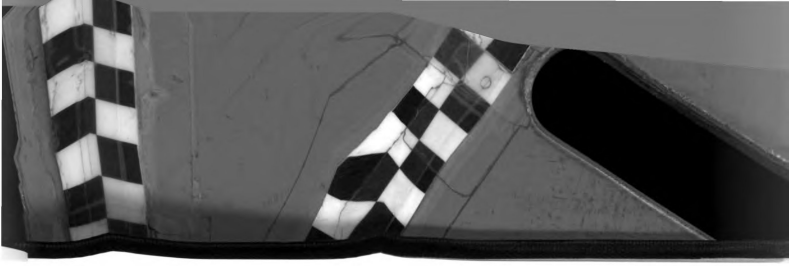


TABLE 12.--Amino acid composition of ^{14}C-labeled peptide obtained by pepsin hydrolysis of KDPG-aldolase. The peptide was prepared and purified as described previously and hydrolyzed in 6N HCl for 18 hr.

Amino Acid	Experimental Value[a]	Residue Number
Lysine	0.91	1
Histidine	0.27	1
Arginine	0.87	1
Aspartate	2.14	2
Threonine	1.00	1
Serine	1.05	1
Glutamate	3.09	3
Proline	1.45	1
Glycine	2.14	2
Alanine	5.68	6
Valine	4.00	4
Methionine	0	0
Isoleucine	2.13	2
Leucine	4.09	4
Tyrosine	0	0
Phenylalanine	0	0
Unidentified (166 min)	1.09	1

[a]Values expressed assuming threonine equals one residue.

TABLE 13.--Amino acid composition of KDPG-aldolase.

Amino Acid	Experimental Value[a]	Number of Residues[b]
Cysteic acid[c]	-	15
Aspartate	10.2	78
Threonine	6.7	52
Serine	5.9	48
Glutamate	10.2	80
Proline	6.7	52
Glycine	10.4	84
Alanine	13.4	108
Cystine	1.0	8
Valine	6.9	50
Methionine[c]	2.8	20
Isoleucine	7.1	56
Leucine	8.2	72
Tyrosine	1.0	8
Phenylalanine	2.8	24
Lysine	4.3	36
Trypotophan[d]	-	16
Histidine	0.6	6
Arginine	4.95	42
		Molecular weight = 88,348

[a]Average nmole value determined with one chromatogram of a 24 hr hydrolysis assuming tyrosine equal to one residue. These values were multiplied by 8 to determine the number of residues per molecular weight of 87,000 for the aldolase.

[b]Average of two determinations each for 24 and 48 hr hydrolysis.

[c]Average of two determinations for 24 hr hydrolysis only.

[d]Determined spectrophotometrically.

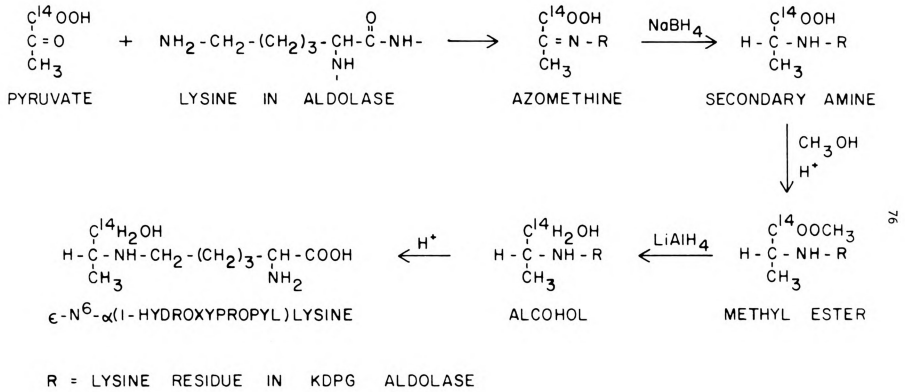
CHAPTER V

DISCUSSION

Active Sites of KDPG-Aldolase

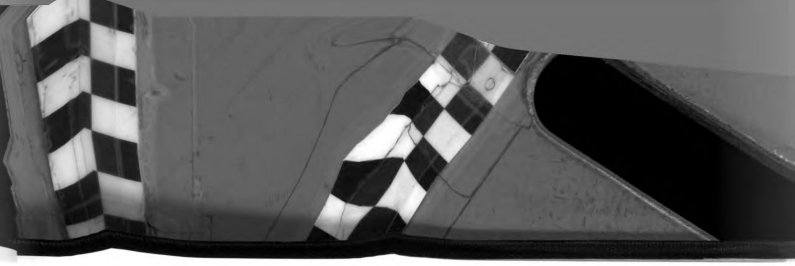
The experiments described for the inactivation of KDPG-aldolase in the presence of pyruvate and $NaBH_4$ and the inactivation in the presence of FDNB indicate that three lysine residues per active site may play important roles in catalytic activity. The isolation of a radioactive component with properties identical to those of N^6-α-(1-hydroxypropyl) lysine is considered to result from the following reaction sequence (Fig. 7). Pyruvate, a substrate for the aldolase, forms an azomethine bond with an ε-amino group of a lysine residue in the active site. Reduction of this linkage to the secondary amine results in the observed loss in activity. Radioactivity is covalently bound in amounts equivalent to two moles of pyruvate per mole of aldolase. The methyl ester of the presumed secondary amino propionic acid derivative was then formed by treatment with methanolic HCl and the ester reduced to the primary alcohol with $LiAlH_4$. Subsequent hydrolysis liberated about one-half of the radioactivity as a compound identical to N^6-α-(1-hydroxypropyl) lysine. The identification of this stable derivative is firm evidence that N^6-α-propionyllysine is the derivative formed when

FIGURE 7.--Chemical sequence for the identification of the amino acid involved in azomethine formation.


PYRUVATE
LYSINE IN ALDOLASE
AZOMETHINE
NaBH4
SECONDARY AMINE
CH3OH
H+
METHYL ESTER
LiAlH4
ALCOHOL
H+
ε-N6-α(1-HYDROXYPROPYL) LYSINE
R = LYSINE RESIDUE IN KDPG ALDOLASE

native aldolase covalently binds pyruvate or KDPG. These results are in accord with those obtained with muscle FDP-aldolase, transaldolase, and DR-aldolase (54, 36).

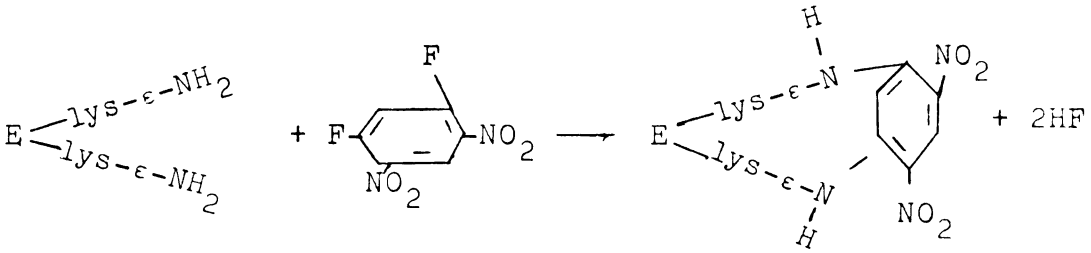
Treatment of KDPG-aldolase with FDNB, as described for ribonuclease by Hirs (58) and for transaldolase by Rowley et al. (39) caused complete inactivation and yielded exclusively ε-DNP lysine upon hydrolysis. No stimulation of activity was observed as for FDP-aldolase (40) and no evidence for S-DNP cysteine was obtained. The arylation reaction was prevented by inorganic phosphate, KDPG, and DL-glyceraldehyde-3-PO_4. Since no protection was afforded by pyruvate, another substrate for the aldolase reaction, it is concluded that these lysine residues are concerned with binding the phosphate group of KDPG. The same amount of dinitrophenylation occurred with aldolase previously rendered completely inactive by reductive binding with borohydride of two moles of pyruvate per mole of aldolase. That the lysine residues concerned with the dinitrophenylation are located relatively near the lysine involved in azomethine formation is indicated by the fact that after dinitrophenylation of four lysine residues per mole of aldolase, reductive binding of pyruvate-^{14}C with borohydride never exceeded 50 per cent of the value obtained with the native enzyme, presumably due to steric effects of the dinitrophenyl groups. It is considered unlikely that some arylation of the lysine residue involved in azomethine



formation had occurred because the stoichiometry and kinetics of arylation are the same with both the native and N^6-α-propionyl enzyme. The fact that dinitrophenylated enzyme is capable of binding pyruvate-^{14}C, although having lost the ability to catalyze the cleavage reaction indicates that, by this means, cleavage and binding of pyruvate may be artificially separated into distinct reactions. A similar separation was noted by Rutter, Richards, and Woodfin (14) with muscle aldolase. Upon treatment with carboxypeptidase, the cleavage reaction decreased 20-fold, whereas the exchange reaction between dihydroxyacetone phosphate and water protons decreased 500-fold. On this basis cleavage must be distinct from exchange.

The formation of N^{ϵ}, N^{ϵ}'-dinitrophenylene-bis-lysine upon treatment of the aldolase with 1,5-difluorodinitrobenzene shows that reactive lysine groups are sufficiently close together so that two arylation reactions are possible from a single reagent as follows. First, the diFDNB may react with one lysine residue involved in the phosphate binding site and thereafter with a nearby lysine residue not specifically involved in catalysis. The reverse seems unlikely because mono- FDNB reacts only with substrate protectable lysine residues. Second, the reagent may react with both lysine residues producing the anionic binding site by either of two mechanisms. For either of the two latter possiblities, it must be assumed that the lysine

residues are sufficiently together so as to successfully bind the relatively small phosphate moiety. A bridged N^{ε}, N^{ε}'-dinitrophenylene-bis-lysine would result if the lysine residues were in close proximity by virtue of their situation in the primary sequence, that is, separated by only a few amino acids within a linear portion of a peptide chain. Also, the bridged derivative would be formed if the two ε-amino groups of the lysine residues were ideally situated by virtue of their spatial orientation while actually residing on two chains or on unrelated portions of the same chain. If the latter were the case an unlimited number of amino acid residues might separate the important groups. The reaction postulated to occur is illustrated below without regard for the spatial relationships of the groups involved.



An interesting speculation arising from the latter two possibilities discussed above concerns the distance which actually separates the residues. The isolation of a pure peptide from KDPG-aldolase containing the bridged double derivative might answer whether the groups are closely related in primary sequence, or closely related due to enzyme conformation

To function as a phosphate binding site, the lysine residue involved must be protonated because at the optimum pH for the reaction the phosphate group of KDPG exists as a dianion. However, at the same pH, the ε-amino group of the lysine residue involved in azomethine formation must be unprotonated because it is believed that a non-protonated amino group is required for azomethine formation (59). It is possible, due to local inductive effects, that at the same pH, an ε-amino group involved in azomethine formation can exist in the non-protonated form, whereas two others concerned with binding can exist in protonated forms. Further, in a pH region near the pK of the lysine ε-amino group, interconversion of the protonated and non-protonated forms by mass action would be predicted when one of these is removed by reaction or binding.

Mechanistic Studies of the KDPG-Aldolase Catalyzed Reaction

Previous studies concerning the reaction mechanisms of FDP-aldolase, DR-aldolase, and KDPG-aldolase have demonstrated the importance of Schiff base formation. The reaction occurs between the carbonyl carbon of the substrate and an ε-amino group of an enzyme lysine residue. Whether Schiff base formation completely accounts for the detailed reaction mechanism is a problem of current research. Presumably as a result of Schiff base formation FDP-aldolase catalyzes a stereospecific proton exchange between dihydroxyacetone phosphate and the medium (2) while DR-aldolase and

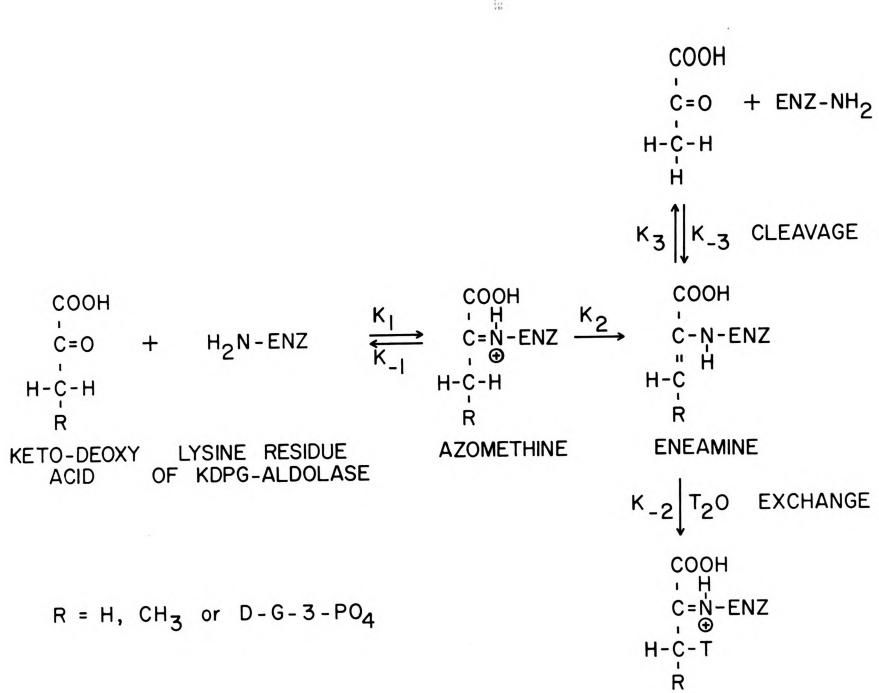
KDPG-aldolase (41, 1) catalyze non-specific exchanges of solvent protons with all three methyl hydrogens of acetaldehyde and pyruvate, respectively. The present investigation demonstrates that KDPG-aldolase also exchanges one hydrogen, presumably the methylene hydrogen, of α-ketobutyrate with the protons of water. Thus, a degree of assymetry, as compared to pyruvate, is introduced by the additional methyl group of α-ketobutyrate. During the present investigation, Rosen and co-workers (41) reported that DR-aldolase also exchanges, stereospecifically, a single methylene hydrogen of propionaldehyde with water.

An important observation in the present investigation is the fact that α-ketobutyrate exchanges solvent protons at a rate at least 37-times slower than pyruvate (or 5,000-fold slower based upon the total exchange capacity of the aldolase). The point may be argued that perhaps this is a Km effect because the Km of pyruvate and α-ketobutyrate with KDPG-aldolase is not known. The results of the borohydride inactivation experiment (Fig. 3) in the presence of various concentrations of pyruvate or α-ketobutyrate suggest that the Km for α-ketobutyrate is not significantly different than that of the pyruvate since an essentially similar inactivation occurred with the same levels of each substrate. Complete inactivation with pyruvate was achieved at a concentration of 3 μmoles per ml and at 10 μmoles per ml with α-ketobutyrate. The manner in which the reduction was

performed is based upon the assumption that the enzyme must be fully charged with substrate prior to the addition of $NaBH_4$. As a result of these considerations it is assumed that the concentration of α-ketobutyrate used in the exchange reactions (20 μmoles/ml) was saturating and therefore its binding (reaction K_1, Fig. 8) was not a limiting factor. Therefore, the decreased rate of proton exchange into α-ketobutyrate must be due to a slower release of the methylene hydrogen, or a slower incorporation of a triton as shown in reactions K_2 and K_{-2} of Fig. 8. On a statistical basis one would expect that the rate of exchange into α-ketobutyrate would be three times slower than the rate with pyruvate since the three methyl hydrogens of pyruvate are essentially equal. It is obvious that this value does not account for the discrepancy found for the exchange rates of α-ketobutyrate and pyruvate. The above experiments suggest that the degree and rate of azomethine formation for pyruvate and α-ketobutyrate is similar but the rate of proton exchange is slower with α-ketobutyrate. Since the proton exchange reaction is slower than azomethine formation then it must be a process distinct from it.

This situation is analogous to that observed for carboxypeptidase treated FDP-aldolase (14) wherein the rate of dealdolization is decreased but the rate of proton exchange suffers a considerably greater attentuation. The explanation offered is that following carboxypeptidase

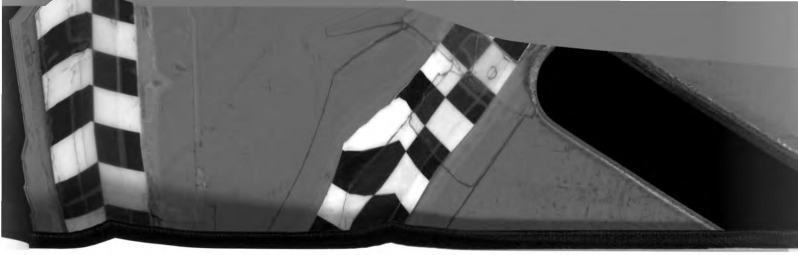
FIGURE 8.--Reaction sequence catalyzed by KDPG-aldolase leading to exchange or cleavage.


COOH
C=O
H-C-H
R
+ H2N-ENZ
K1
K-1
COOH
H
C=N-ENZ
H-C-H
R
K2
COOH
C-N-ENZ
H
H-C
R
COOH
C=O
H-C-H
H
+ ENZ-NH2
K3
K-3
CLEAVAGE
K-2
T2O
EXCHANGE
COOH
H
C=N-ENZ
H-C-T
R
KETO-DEOXY ACID
LYSINE RESIDUE OF KDPG-ALDOLASE
AZOMETHINE
ENEAMINE
R = H, CH3 or D-G-3-PO4

treatment the rate of proton neutralization becomes the rate determining step in the cleavage of FDP. Since the making, or breaking, of the C-H bond is rate determining then a pronounced isotope effect is revealed by the drastic rate decrease of the tritium exchange reaction. Therefore, the rate of proton neutralization of the enzyme-bound DHAP carbanion is slower than azomethine formation, as compared to the native enzyme, and hence exchange must be a reaction distinct from azomethine formation.

From the reported experiments with KDPG-aldolase, it has now been demonstrated that the rate of proton exchange may be decreased relative to the rate of azomethine formation by substituting α-ketobutyrate for pyruvate. Thus, azomethine formation can be visualized as a process distinct from the exchange reaction (Fig. 8); that is azomethine formation is independent of exchange.

The fact that KD-gluconate binds to the enzyme, as demonstrated by borohydride inactivation (Table 8), and that the cleavage of this compound is greatly decreased relative to KDPG, indicates the important role of the substrate phosphate group in cleavage as well as in binding (60). The importance of the substrate phosphate group has also been discussed in the previous section concerning the inactivation of the enzyme in the presence of FDNB. Although a direct determination of the Km of KDG is not possible an



approximate value may be arrived at. It has been shown by Hartman and Barker (60) that the binding due to the phosphate groups on the substrate, FDP, for muscle FDP-aldolase could account for the Km value of FDP. The Km values for FDP-aldolase and KDPG-aldolase for FDP and KDPG are essentially equal at 6×10^{-5} M and 10×10^{-5} M respectively (37,6). The Km value for dihydroxyacetone phosphate is 2×10^{-3} M and, while not determined rigorously, the value for pyruvate deduced from the effect of pyruvate concentration on borohydride inactivation is approximately 1×10^{-3} M. On this basis it is assumed that the Km for KDG would approximate the Km value of F-1-PO_4 for FDP-aldolase which is 1×10^{-2} M (37). While the production of pyruvate was measurable after prolonged incubation of KDG with the aldolase, there was no appreciable rate as determined spectrophotometrically, although the level of substrate was at least 10-fold greater than the suspected Km value. Since KDG is bound to the aldolase in the presence of borohydride, but exhibits no appreciable cleavage, it is concluded that the phosphate group of KDPG contributes more than increased binding capacity and that cleavage is not a direct result of azomethine formation. Further support for this latter view is the fact that FDNB reacts with the ε-amino groups of four lysine residues which function as a phosphate binding site. With arylation of these residues the enzyme lost the ability to cleave

KDPG while the ability to form an azomethine with pyruvate was readily apparent (Table 5).

In this connection the β-decarboxylation of oxalacetate is believed to be the direct result of Schiff base formation between the substrate and the enzyme with the subsequent formation of the eneamine species (Fig. 8). This would result in a decreased electron density about the C_3 - C_4 bond and result in β-decarboxylation presumably in the absence of additional forces supplied by the enzyme. These data also suggest that carbon dioxide is an excellent leaving group subsequent to azomethine formation.

As demonstrated with KDG, cleavage is not the direct result of azomethine formation. It is proposed that the phosphate group of KDPG, in addition to promoting binding, may aid catalysis by imposing stress upon the C_3 - C_4 bond of KDPG by inducing an enzyme conformation change or by substrate stretching. The possibility that the Δ-carboxyl group of 2-keto-4-hydroxyglutarate binds in the same fashion as the phosphate group of KDPG may explain the reasonable rate of cleavage of this compound.

A separation of the various phases of the KDPG-aldolase catalyzed reaction is possible through the use of substrate analogs. The results obtained with α-ketobutyrate suggest that binding and azomethine formation are distinct from proton removal or neutralization.

The results with KDG suggest that cleavage is not a direct result of azomethine formation and that the C_6-phosphate group of KDPG contributes more to catalysis than increased binding efficiency. A reaction mechanism based upon these results is shown in Fig. 8. Whether any or all of these steps are enzyme catalyzed has not been determined rigorously. A recent report by Meloche (61) suggests that bromopyruvate reacts with a catalytically active base within the active site of the aldolase. The evidence suggests, therefore, that the proton exchange step may be enzyme catalyzed. The evidence that KDPG-aldolase proceeds via Schiff base formation is further clarified by the discovery of oxalacetate decarboxylase activity. Rutter and co-workers (37) predicted that Class I aldolases may structurally resemble pre-existing enzyme forms, such as a β-decarboxylase. This is the first demonstration of the fact that an aldolase does have β-decarboxylase activity.

It is evident from the variety of substrates which inactivate KDPG-aldolase in the presence of $NaBH_4$, that the specificity for azomethine formation is not high. However, the substitution of a polar function, such as a hydroxyl group on the C_3 position, prevents azomethine formation. Steric hindrance on the C_3 position of pyruvate must be ruled out as an inhibitor of azomethine formation since the addition of a methyl group rather than a hydroxyl results in a pronounced inactivation in the presence of borohydride.

Substrates which maintain non-polarity at the C_3 of pyruvate, such as α-ketobutyrate, α-ketoisovalerate, α-ketoglutarate, 2-keto-4-hydroxyglutarate, and 2-keto-3-deoxygluconate inactivated in the presence of $NaBH_4$. The above observations clearly indicate that a non-polar C_3 position is an essential requirement for the binding of a substrate. Perhaps of relevance in this connection is the isolation of the 30 amino acid peptide from the active site region of KDPG-aldolase which was shown to contain approximately 65 per cent non-polar amino acids. If the non-polar amino acids are concentrated in the azomethine region a pronounced exclusion of protons necessary to neutralize an enzyme-bound carbanion may well explain the decreased rate of proton exchange into α-ketobutyrate. For example, the addition of a non-polar methyl group could conceivably prohibit the necessary unmasking of the site, thereby preventing the rapid entrance of solvent protons needed for the neutralization of the carbanion.

The fact that other compounds, such as α-ketoglutarate, and 2-keto-4-hydroxyglutarate also inhibit the aldolase in the presence of borohydride indicates that hydroxyl groups and a ring configuration are probably not required for azomethine formation. In the case of FDP-aldolase, however, hydroxyl groups at carbons 3 and 4 may play an important role (60). In contrast, for KDPG-aldolase, the presence of a hydroxyl group at the C_3 position of the

substrate or substrate analog renders the compound inactive for azomethine formation. This is seen from the complete inability of 2-ketogluconate, hydroxypyruvate and dihydroxyacetone to form azomethines as measured by borohydride inactivation.

Amino Acid Composition of Native KDPG-Aldolase and N^6-α-Propionyllysine Peptide

The amino acid analysis of native KDPG-aldolase indicates a molecular weight of 88,348 as compared to 87,000 determined by pyruvate-^{14}C binding and 89,500 determined by equilibrium ultracentrifugation (1). The data also show low values for tyrosine, histidine, and methionine residues. The absence of free thiol groups, viz cysteine, may explain a preliminary observation that KDPG-aldolase is extremely resistant to thiol inactivating agents such as p-mercuribenzoate. The amino acid composition offers no obvious explanation for the extreme acid stability of KDPG-aldolase.

The amino acid composition of the N^6-α-propionyl lysine peptide reflects to some degree the overall amino acid composition of the native protein. The marked absence of tyrosine, methionine, and cystine residues is indicative of the low values for these residues found in the native molecule.

CHAPTER VI

SUMMARY

The formation of an azomethine involving an ε-amino group of lysine and pyruvate-1-^{14}C was established by chemical treatment of the aldolase and by hydrolysis to yield a radioactive component whose properties were identical to authentic N^6-α-(1-hydroxypropyl) lysine. Four moles of fluorodinitrobenzene reacted with the aldolase with accompanying inactivation. The reaction was inhibited by phosphate, 2-keto-3-deoxy-6-phosphogluconate, and DL-glyceraldehyde-3-phosphate, but not by pyruvate. Only ε-dinitrophenyl lysine was found upon complete acid hydrolysis. The same amount of dinitrophenylation occurred with aldolase already inactivated by reductive binding with borohydride of two moles of pyruvate per mole of enzyme. These experiments constitute evidence for a role of three lysine residues per active site in the action of KDPG-aldolase

The specificity for azomethine formation was studied by incubating the aldolase in the presence of α-carbonyl compounds and sodium borohydride. Both pyruvate and α-ketobutyrate produced complete inactivation whereas monohydroxyacetone, α-ketoisovalerate, α-ketoglutarate, 5-keto-4-deoxyglucarate, 2-keto-4-hydroxyglutarate, and 2-keto-3-deoxygluconate were partially

inhibitory. Hydroxypyruvate, dihydroxyacetone, and 2-ketogluconate were not inhibitory. It was concluded from these data that the only restriction against azomethine formation was the presence of a hydroxyl group at carbon three. The formation of a stabilized azomethine with α-ketobutyrate prevented subsequent azomethine formation with pyruvate and similarly α-ketobutyrate competed favorably with pyruvate in azomethine formation indicating that each substrate reacted with the same lysine ε-amino group.

The aldolase catalyzed the exchange of one proton from water into α-ketobutyrate. The initial rate of exchange was at least 37-times slower than with pyruvate. The aldolase also catalyzed the cleavage of 2-keto-4-hydroxyglutarate at 0.1% the rate of KDPG cleavage whereas KD-gluconate was cleaved at a definite, but much slower rate. Oxalacetate was decarboxylated at a rate 0.5% of the rate of KDPG cleavage. The data obtained with the exchange and cleavage reactions were interpreted as demonstrating a dissociation of azomethine formation, exchange, and cleavage phases of the KDPG-aldolase catalyzed reaction.

Methods are described for the isolation of a radioactive peptide containing the lysine-azomethine forming active site. The amino acid composition of this peptide together with the composition of the native protein is described.

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