ABSTRACT

AN ANALYSIS OF THE MECHANISM OF
L-RIBULOSE-5-PHOSPHATE 4-EPIMERASE FROM
AEROBACTER AEROGENES

By Jean D. Deupree

L-Ribulose-5-P 4-epimerase (EC. 5.1.3.) catalyzes the interconversion of L-ribulose-5-P and D-xylulose-5-P. The mechanism of epimerization was analyzed to determine whether NAD^+ was required as a participant in an oxidation-reduction mechanism similar to that of UDP-glucose 4-epimerase or whether the mechanism was different. This was prompted by the observation that the L-ribulose-5-P 4-epimerase is comparable to UDP-glucose 4-epimerase in that: (a) there is no T_2O or $H_2{}^{18}O$ exchange with the medium; (b) T is not lost from C-4 carbon of the substrate during epimerization; and (c) the enzyme does not require added NAD^+ for activity nor is it inhibited by NADase. However, differences in the mechanisms of the epimerases are implied from the observations that: (a) the kinetic isotope effects differ; (b) L-ribulose-5-P and D-xylulose-5-P are unique among the substrates for the 4-epimerases in that a carbonyl group is present two carbons removed from the site of epimerization; and (c) a sensitive catalytic assay did not detect NAD^+ bound to the L-ribulose-5-P 4-epimerase.

Jean D. Deupree

Homogeneous L-ribulose-5-P 4-epimerase was obtained from Aerobacter aerogenes grown on L-arabinose using the following purification steps: DEAE-cellulose chromatography, ammonium sulfate fractionation, calcium phosphate gel elution, Sephadex G-200 column chromatography, and DEAE-Sephadex column chromatography. Fine needle-shaped crystals of the 4-epimerase were obtained from an ammonium sulfate solution. The homogeneity of the 4-epimerase was based on the following observations: (a) the same specific activity was obtained before and after crystallization and recrystallization; (b) a single band was obtained on polyacrylamide gels electrophoresed at 3 different pH values; and (c) a constant molecular weight was obtained across the cell in high-speed equilibrium ultracentrifugation. A specific activity of 70 ± 10% at 28°C and pH 8.0 was obtained for the homogeneous 4-epimerase when Mn^{++} was the only divalent cation present.

A molecular weight of $1.14 \times 10^5 \pm 1.4 \times 10^3$ was obtained for the homogeneous enzyme by high-speed sedimentation equilibrium experiments.

NAD^+ was not tightly bound to the 4-epimerase as concluded from the following observations: (a) NAD^+ was not detected by a microbiological assay either before or after hydrolysis in acid or base; (b) ^{14}C-NAD^+ was not incorporated into the 4-epimerase isolated from a nicotinic acid-requiring mutant of A. aerogenes which was grown on

^{14}C-nicotinic acid and which produced ^{14}C-NAD$^+$; (c) the fluorescence and absorption spectra of the 4-epimerase were not characteristic of bound NAD$^+$ or NADH, and (d) an absorption spectrum with a maximum around 340 mμ was not obtained on incubation of the 4-epimerase with L-ribulose-5-P. In addition, the homogeneous 4-epimerase did not require NAD$^+$ for activity nor was the activity altered by the presence of NAD$^+$, NADH, NADP$^+$, NADPH in the assay mixture.

Divalent metal ions activated the 4-epimerase to varying extents with the order of activation being $Mn^{++} > Co^{++} > Ni^{++} > Ca^{++} > Zn^{++} > Mg^{++}$. Incubation of the enzyme with EDTA resulted in a loss of about 90% of the enzyme activity, and the activity was not recovered on passage of the enzyme through a Sephadex G-25 column. However, addition on the divalent metal ions reactivated the enzyme.

Indirect evidence is not consistent with lipoate, cystine, B_{12}-coenzyme, or an oxidized tryptophan derivative catalyzing 4-epimerization by oxidation-reduction. This is based on the facts that: (a) the absorption spectra of the 4-epimerase were not characteristic of B_{12}-coenzyme; and (b) the enzyme was stable to treatment with borohydride, sodium sulfite, or arsenite in the presence of a thio compound, and in the presence of NADH.

In additional tests of possible mechanisms, it was concluded that the substrate does not appear to form

a Schiff base with the 4-epimerase since the enzyme was stable to borohydride reduction in the presence and absence of substrate. Further, a carbanion intermediate could not be detected by incubating the enzyme-substrate complex with tetranitromethane.

Thus, it was ascertained that the mechanism of L-ribulose-5-P 4-epimerase is different than that of UDP-glucose 4-epimerase in that the mechanism of epimerization cannot use NAD^+ as an electron acceptor, and the indirect evidence is not consistent with an oxidation-reduction mechanism. A mechanism involving either dealdolization-aldolization or hydration-dehydration is proposed.

AN ANALYSIS OF THE MECHANISM OF L-RIBULOSE-5-PHOSPHATE 4-EPIMERASE FROM *AEROBACTER AEROGENES*

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1970

This thesis is dedicated to my uncle,

Dr. John F. Deupree

ACKNOWLEDGMENTS

The author extends her sincere appreciation to Professor W. A. Wood for his guidance during the course of this work. The author is further indebted to Dr. Roy H. Hammerstedt for his encouragement, continual interest, stimulating discussions and assistance offered. The author is also indebted to Dr. George N. Holcomb for initiating her into the world of research and for continually encouraging her to obtain a Ph.D. degree.

The financial assistance of the National Science Foundation and the Department of Biochemistry at Michigan State University is greatly appreciated.

VITA

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LIST OF ABBREVIATIONS

BAL	2,3-Dimercapto-1-propanol
DEAE	Diethylaminoethyl
Ru	Ribulose

INTRODUCTION

L-Ribulose-5-phosphate 4-epimerase (EC. 5.1.3.) catalyzes the interconversion of L-ribulose-5-phosphate and D-xylulose-5-phosphate. The enzyme is induced in Escherchia coli (1), Lactobacillus plantarum (2) and Bacillus subtilis (3) by growth on L-arabinose and is induced in Aerobacter aerogenes by growth on L-arabinose (4), L-xylose (5), and L-arabitol (6).

Seven different 4-epimerases have been identified thus far. The substrates for all of these, except that for L-ribulose-5-P 4-epimerase, are nucleotide-diphosphate aldoses which are held in the pyranose form by a glycoside linkage at C-1. In contrast, the open chain substrates, L-ribulose-5-P and D-xylulose-5-P, contain a free carbonyl two carbons removed from C-4 where epimerization occurs.

It is currently assumed that the mechanism of all 4-epimerases involves an NAD^+-catalyzed oxidation-reduction at C-4 without loss of the migrating hydride ion to the surrounding medium. Evidence for an oxidation-reduction type of epimerization is based solely on studies of UDP-glucose 4-epimerase. Lack of exchange of isotopic hydrogen and oxygen from labeled water (7, 8, 9) and an inverse isotope effect (10) eliminate an epimerization mechanism involving an Sn_2 inversion, carbon-carbon bond cleavage,

or dehydration. Evidence in support of an oxidation-reduction mechanism include: (a) all UDP-glucose 4-epimerases that have been studied either require added NAD^+ for activity or contain tightly bound NAD^+; (b) an absorption spectrum with a 345 mμ maximum was obtained upon incubation of the 4-epimerase from E. coli with substrate (11); (c) enzyme-bound NAD^+ was reduced with tritiated sodium borohydride, and the T was transferred from the enzyme-bound NADT to TDP-4-keto-6-deoxy glucose (12) to form a mixture of tritiated UDP-glucose and UDP-galactose. Further, the retention of T on the C-4 position of added UDP-hexose-4-T (13, 14) indicated that the hydride ion which is removed is not free to diffuse from the active site. It is still not known how many steps are involved in the epimerization of UDP-glucose, or whether hydrogen is transferred only to NAD^+ or also to some other component of the enzyme.

Of the other nucleotide-linked 4-epimerases, only UDP-N-acetylglucosamine 4-epimerase (15) has been shown to require NAD^+ for activity. There is evidence that some of the UDP-glucose 4-epimerases can catalyze the epimerization of nucleotide-linked aldoses in addition to UDP-glucose and UDP-galactose (16, 17, 18, 19, 20).

L-Ribulose-5-P 4-epimerase is similar to UDP-glucose 4-epimerase in that: (a) T or ^{18}O were not exchanged when the epimerization was run in T_2O or $H_2{}^{18}O$ (21); (b) T was

not lost from C-4 of D-xylulose-5-P-4T during epimerization;[1] (c) the enzyme was not inactivated by treatment with charcoal or NADase, nor did it require NAD^+ for activity (4, 2, 21). Although bacterial UDP-glucose 4-epimerases were not inactivated by charcoal or NADase, tightly bound NAD^+ was detected in all cases. However, bound NAD^+ was not detected on L-ribulose-5-P 4-epimerase from Lactobacillus plantarum using a sensitive catalytic assay (2). The fact that L-ribulose-5-P 4-epimerase was shown to have a different kinetic isotope effect[1] than UDP-glucose 4-epimerase, indicates that there are differences in the mechanism of these two epimerases; however, the differences may be only in the rate determining step.

Thus, there is a definite need to study the mechanism of 4-epimerases, other than that of UDP-glucose 4-epimerase, in order to substantially determine whether an NAD^+-catalyzed oxidation-reduction mechanism is always involved. L-ribulose-5-P 4-epimerase was chosen for this study since: (a) substantial evidence for the presence or absence of an NAD^+ requirement for epimerization has not been obtained; (b) epimerization is unknown and may be completely different than that of UDP-glucose 4-epimerase; (c) the substrates are not nucleotide-linked, and thus, it is clear that a nucleotide moiety does not participate in the epimerization in

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[1]Fossitt, D., Wood, W. A., Salo, W. L. and Kirkwood, S., unpublished results.

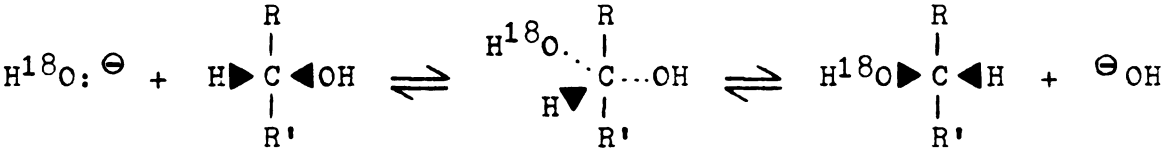
any manner; and (d) the enzyme is readily available and has been partially purified from A. aerogenes.

This thesis reports a purification procedure for obtaining crystalline and homogeneous L-ribulose-5-P 4-epimerase from A. aerogenes. A series of isotopic, optical and kinetic experiments show that NAD^+ is not bound to the isolated enzyme and is not involved in the epimerization of L-ribulose-5-P and D-xylulose-5-P. A series of kinetic experiments indicate that the epimerization is activated by specific metal ions. Further understanding of the mechanism of epimerization was obtained by determining the effect of inhibitors on enzyme activity. A discussion of the possible mechanism of epimerization is presented.

LITERATURE REVIEW

Chemical Mechanisms of Carbohydrate Epimerizations

Epimerizations of carbohydrates may occur at any carbon by an Sn_2 reaction commonly known as the Walden inversion. The general reaction is as follows:



In Sn_2 epimerizations the opposite epimer is always obtained. Reactions of this type are readily detectable, since, if the reaction is run in $H_2{}^{18}O$, the product will contain one atom of ^{18}O per molecule of product, as shown above. Likewise, if the initial substrate contains ^{18}O, the product will not contain ^{18}O.

Groups on carbon atoms adjacent to a carbonyl group are subject to Lobry de Bruyn-Alberda van Ekenstein transformations (22). The reaction, as shown in Figure 1, is thought to be a general acid, base-catalyzed enolization. To date, these transformations have only been studied to the extent of showing a significant epimerization of an aldose at C-2, considerably more isomerization to the corresponding 2-keto sugar, and slight epimerization at C-3.

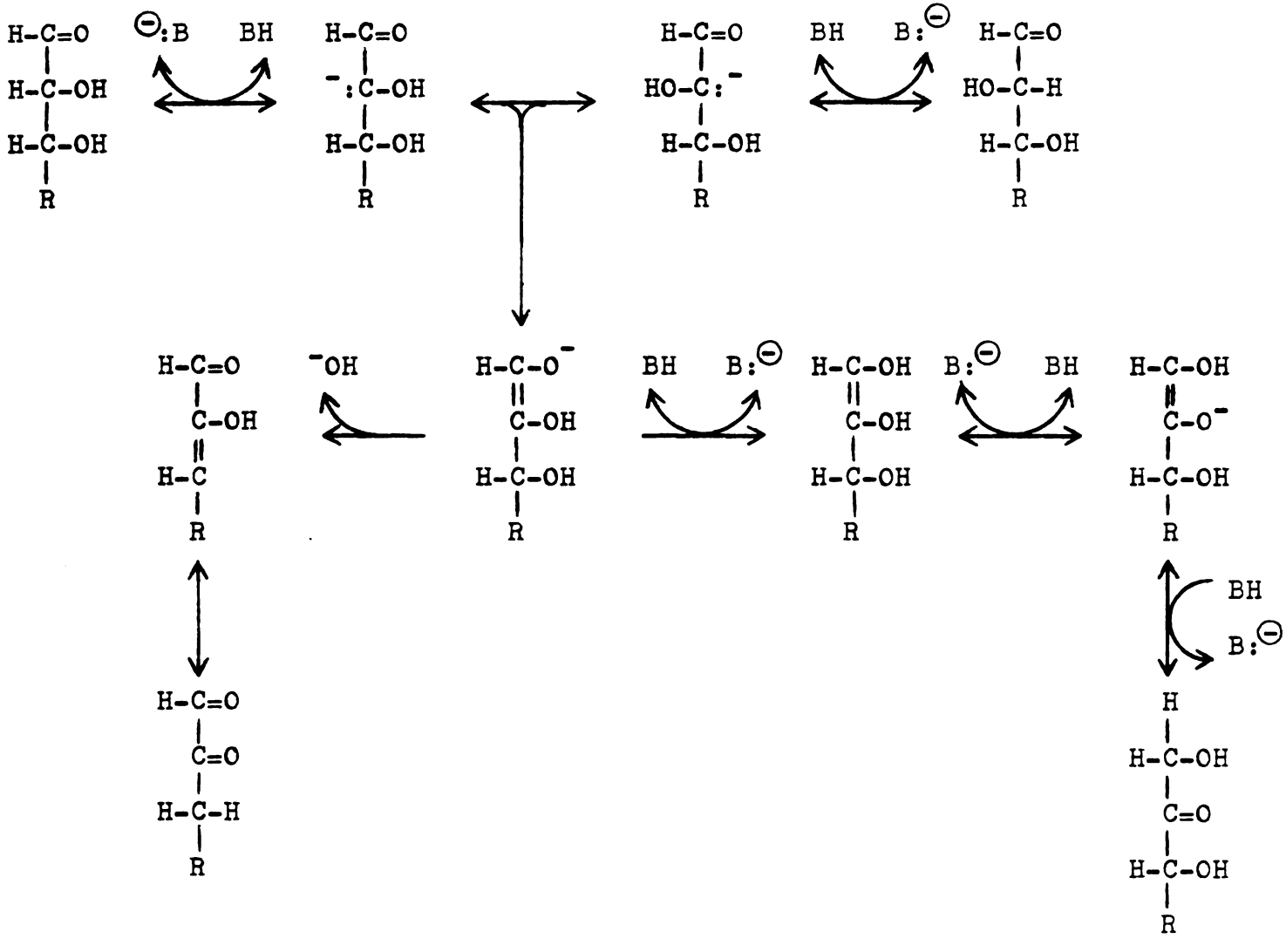
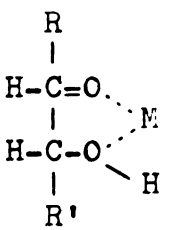


Figure 1. Mechanism of Lobry de Bruyn-Alberda van Ekenstein Transformations (22)

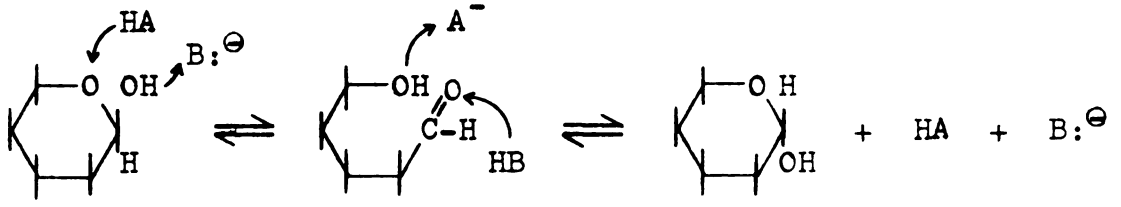
A mixture of C-2 and C-3 epimers and the isomerization product is thus obtained.

Rearrangement of the carbon chain has not been detectable during the transformations. Metal ions facilitate the removal of the α-proton from either an aldose or a ketose, probably by forming a complex such as:



Two of the side reactions of the Lobry de Bruyn-Alberda van Ekenstein transformations are dehydration and aldolization and/or dealdolization. Since dehydration is an irreversible reaction, it will not facilitate the formation of additional epimers. Either acid or base aldolizations could yield a mixture of four possible epimers. Assuming equal rates of formation of all epimers, the trans, or more thermodynamically stable epimers, would predominate. Aldolization of trioses to form hexoses have been reported in the literature. The conversion of D-fructose to D-sorbose is thought to occur by dealdolization followed by aldolization. Although aldolizations are acid or base-catalyzed, they occur during transformations carried out in the presence of a high concentration of free hydroxyl groups and rarely during acid transformations.

The mechanism of epimerization at C-1, or mutarotation, was first proposed by Lowry (23) as an acid, base-catalyzed reaction which involved the simultaneous transfer of a proton from the acid catalyst to the sugar in the same step that a proton was transferred from the sugar to the base catalyst yielding the sugar aldehyde or hydrate directly.



There is no indication of carbon-bound oxygen exchanging with the water during the course of the reaction. Others interpret the data as two consecutive bimolecular reactions, where a proton is added in one step and the second proton is removed in a separate step.

A mixture of epimers of carbohydrates could also be obtained either by oxidation of the one of the hydroxyls to give the carbonyl followed by reduction or by dehydration followed by rehydration.

Enzyme Catalyzed Epimerizations

One of the functions of an enzyme is to act as a site-directed catalyst by facilitating the perpetuation of a chemical reaction at a particular site on the substrate and by limiting the amount of the side reactions at other sites on the substrate. Thus the enzyme dictates the

epimers which will be formed and limits the amount of side reactions. Therefore, it is not unreasonable to expect most carbohydrate epimerizations to occur by the same mechanism as chemical epimerizations. In fact, many enzyme catalyzed epimerizations are thought to occur by the same mechanism as chemical epimerization although very little supportive data has been obtained. From our current knowledge, carbohydrate epimerases can be divided into 3-classes based on reaction mechanisms: (a) aldose-1-epimerases or mutarotases, (b) epimerizations adjacent to a carbonyl involving acid-base enolizations (Lobry de Bruyn-Alberda van Ekenstein transformations), and (c) oxidation-reduction mechanisms.

Aldose 1-Epimerases or Mutarotases

Epimerization by aldose 1-epimerases is thought to occur by the same acid, base-catalyzed mechanism discussed for chemical mutarotations. Studies by Bentely and Bhati (24) on the mutarotase from Penicillium notatum indicated that the mechanism did not involve a single displacement, dehydration, or a dehydrogenation reaction of any carbon-bound hydrogen or of a hydrated derivative of glucose aldehyde. These conclusions were based on the fact that (a) D and ^{18}O exchange with the solvent was negligible, and the extent of exchange was the same for the enzyme catalyzed mutarotations as for the spontaneous mutarotations, and (b) substitution of C-1 hydrogen by D had no effect on

the rate of spontaneous or enzyme catalyzed mutarotation.

Epimerizations Adjacent to a Carbonyl

Whereas the chemical model predicts exchange of carbon bound hydrogen with protons of the medium, this is not an obligatory process in the enzyme catalyzed processes. For instance in the mechanistically related phosphoglucoisomerase, Rose (25) found that the rate of incorporation of T into the hexose phosphate was a function of temperature. At lower temperatures T was not exchanged, and as the temperature increased the ratio of T exchange with solvent to T transfer between adjacent carbons also increased.

By conjecture 2-epimerases and 3-epimerases are thought to occur by keto-enolization mechanisms. Only in the case of the 3-epimerase has supportive evidence been obtained.

2-Epimerases: The only 2-epimerases which have been identified are N-acetylglucosamine-6-P 2-epimerase and N-acetylglucosamine 2-epimerase. Ghosh and Roseman (26) have reported a 200 to 300 fold purification of N-acetylglucosamine-6-P 2-epimerase from Aerobacter cloacae. N-glycolyl-D-glucosamine-6-P and N-glycolyl-D-mannosamine-6-P also served as substrates; however, N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, D-glucosamine-1-P, D-mannosamine-1-P, N-acetyl-D-galactosamine-6-P and D-mannose-6-P would not. The activity was not dependent on the addition of any cofactor.

Although N-acetylglucosamine-6-P 2-epimerase has not been found in animals, the corresponding N-acetylglucosamine 2-epimerase was found (27). Epimerization of N-glycolylglucosamine and N-glycolylmannosamine also occured with the latter enzyme. N-acetylglucosamine-1-P, UDP-N-acetylglucosamine, N-acetylmannosamine-6-P, N-acetylglucosamine-6-P, glucosamine, mannosamine, glucosamine-6-P, mannosamine-6-P, N-acetylgalactosamine, glucose, and mannose were not active as substrates. The purified enzyme had an absolute requirement for ATP, although there was no detectable conversion of ATP to ADP, or AMP. However, Datta and Ghosh (28) obtained evidence that ATP was acting as an allosteric effector with cooperative homotrophic interactions.

The mechanism of 2-epimerization has not been studied, although it is thought to occur by the same mechanism as the chemical epimerization of N-acetylglucosamine and N-acetylmannosamine at pH 11.0 (29, 30).

3-Epimerases: The existence of a 3-epimerase was first demonstrated by the identification and partial purification of D-xylulose-5-P 3-epimerase from rabbit muscle by Srere et. al. (31). Since then, the enzyme has been identified from numerous sources. The enzyme from Lactobacillus pentosus does not require a metal ion nor is it inhibited by 10^{-2}M EDTA (32). Only D-xylulose-5-P and D-ribulose-5-P were found to serve as substrates, although epimerization of tagatose-6-P and xylulose-di-P was tested

(33). The enzyme from A. aerogenes did not require NAD^+, nor was it inhibited by NADase. McDonough and Wood (21) reported that one atom of tritium was incorporated into an atom of pentose-5-P during the equilibration of D-ribulose-5-P and D-xylulose-5-P with D-ribulose-5-P 3-epimerase. These results are consistent with a keto-enolization mechanism; however, the possibility of an entirely different mechanism cannot be ruled out. Anderson and Wood (5) observed the 3-epimerization of L-xylulose-5-P and L-ribulose-5-P in extracts of A. aerogenes. Although the mechanism was not studied, by analogy a keto-enolization reaction is probably involved.

Epimerization by Oxidation-Reduction

Substantial evidence has been obtained for an oxidation-reduction mechanism of UDP-glucose 4-epimerase, and by inferance all other 4-epimerizations have been considered to occur by the same mechanism. It should be pointed out, however, that all but one of the known 4-epimerases catalyzes the epimerization of nucleotide-linked aldoses, in which the aldose is fixed in the pyranose ring. In contrast, L-ribulose-5-P 4-epimerase catalyzes the epimerization of the open chain substrates, L-ribulose-5-P and D-xylulose-5-P, at a carbon β to a carbonyl. Based on our knowledge of chemical epimerizations, it is conceivable that the mechanism of L-ribulose-5-P 4-epimerase involves keto-enolization of the carbonyl and thus may be quite different than nucleotide-diphosphate aldose 4-epimerases.

UDP-Glucose 4-Epimerase; Studies on the Mechanism:

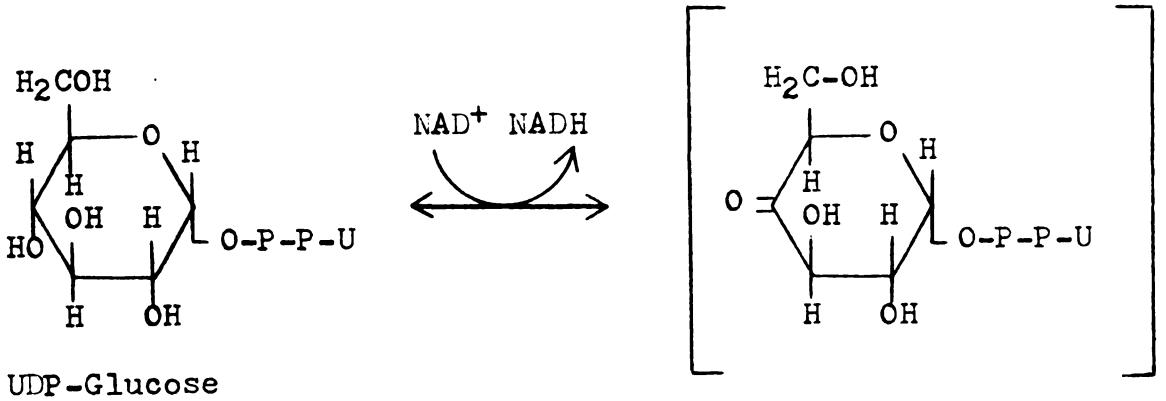
A number of mechanisms were originally proposed to explain the epimerization of glucose and galactose. These include: (a) multiple cleavage of the hexose with rearrangement of the carbon atoms to form the epimer, (b) cyclization of the carbon chain to form an inositol ring with cleavage of the ring at a different carbon to form the epimer; (c) attack at C-4 by a hydroxyl group in an Sn_2 (Waldenase) reaction with simultaneous elimination of a hydroxyl group at C-4; (d) dehydration of the substrate with formation of a double bond between C-3 and C-4 or C-4 and C-5 with rehydration to yield the epimer; (e) splitting of the carbon chain between C-3 and C-4 with reformation of the chain to give a mixture of epimers; and (f) oxidation at C-4 with formation of a carbonyl group followed by reduction at C-4 to form the epimer.

In 1950, Leloir (34, 35) showed that glucose and galactose were epimerized as nucleotide-linked sugars and not as the free hexoses. If epimerization occurs via inositol ring formation, the UDP moiety would have to be translocated to a different carbon atom. Epimerization by means of multiple bond cleavage of the hexose or by means of carbon atom rearrangement via inositol ring formation has been rather conclusively ruled out by a number of isotope studies which showed that: (a) identical labeling of liver glycogen was obtained from rats fed D_2O and a 60%

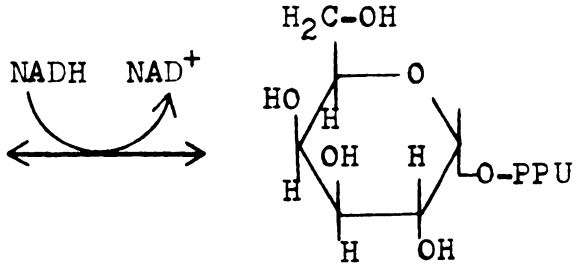
glucose or galactose diet (36); and (b) fasted rats fed glucose-1-^{14}C produced glycogen with ^{14}C exclusively in the C-1 position of glucose (37).

When UDP-glucose 4-epimerase and substrate were incubated in the presence of T_2O or $H_2{}^{18}O$, neither T nor ^{18}O were incorporated into either UDP-glucose or UDP-galactose (7, 8, 9). If epimerization involves an Sn_2 inversion, ^{18}O should have been incorporated into the sugars. If the mechanism involves dehydration followed by rehydration, some incorporation of T or ^{18}O might be expected. However, Rose (38) and Jencks (39) both concur that migration of protons or hydroxyl group from substrate to enzyme can proceed at a rate faster than exchange of the proton or hydroxyl group with the medium. Thus, it is conceivable that the same hydrogen or hydroxyl group which is removed is added back, one of the two being added to the opposite side of the substrate.

The first clue as to the mechanism of action of UDP-glucose 4-epimerase was discovered by Maxwell (40, 41) when she demonstrated that the purified UDP-glucose 4-epimerase from calf liver required the addition of NAD^+ for activity. The following mechanism was thus proposed:



UDP-Glucose



UDP-Galactose

where NAD^+ removes a hydride from C-4 of the hexose moiety leaving a 4-keto hexose. A hydride ion from NADH in turn reduces the oxidized intermediate.

Further experiments indicate that neither $NADP^+$, the α-isomer of NAD^+, acetyl-pyridine or pyridine-3-aldehyde analogue of NAD^+, nor deamino-NAD^+ could be substituted for NAD^+. NADase-treated NAD^+ was not active, indicating that NAD^+, and not a contaminant of NAD^+, was the activator. The proposed oxidized intermediate could not be trapped by running the reaction in the presence of thiosemicarbazide, hydroxylamine or hydrazine. Thus the intermediate must be tightly bound to the enzyme and inaccessible to the surrounding medium, or a carbonyl intermediate does not exist. The

epimerization of UDP-glucose or UDP-galactose was not inhibited by NADH oxidase, acetaldehyde or alcohol dehydrogenase. T was not incorporated into the substrate from exogeneous NAD^+-4-T or NADH-4-T which had been added to the reaction mixture. The rate of the reaction in 96% D_2O was identical to that in H_2O. These results indicate that if NAD^+ accepts a hydride ion from the substrate, there is a return of the hydride ion to the oxidized intermediate, and the NADH formed during the course of the reaction is not free to exchange with the medium.

Since that time, other UDP-glucose 4-epimerases have been found to require the addition of NAD^+ to the assay mixture for activity. These include UDP-glucose 4-epimerases found in rat tissue (42), human fibroblast lysates (43), tumor cells (44), hemolysates of infants and adults (45, 46), and wheat germ (20). The epimerizations in all cases were inhibited by NADH, and the rate of reaction was dependent on the ratio of NAD^+/NADH.

In contrast, bacterial UDP-glucose 4-epimerases do not require added NAD^+, nor are they inhibited by $NADP^+$ or NADH (7, 9, 48). Tightly bound NAD^+ was detected in the epimerase from both yeast (49, 50, 51, 52) and E. coli (8) by means of fluorescence in the presence of methyl-ethyl ketone and by enzymatic and fluorometric analysis of the NAD^+ released by acetone or perchloric acid denaturation of the enzyme. The yeast and E. coli 4-epimerases contained

1 mole of NAD^+ per mole of protein. The enzyme from yeast (9) and Lactobacillus bulgaris (7) were not inhibited by washing with charcoal or incubating with NADase.

Some of the characteristics of the yeast 4-epimerase have been reported in a series of papers by Maxwell et. al. (9) and Kalckar and associates (50, 51, 52, 53). A fluorescence emission spectrum with a maximum at 450 mμ was obtained by exciting the enzyme at 350 mμ. NADH had similar excitation and emission spectras. NAD^+ bound in the para position to a sulfhydryl group on an enzyme can exhibit characteristic NADH fluorescence as reported for glyceraldehyde-3-P dehydrogenase (54). The enzyme also contained bound NADH at a ratio of NAD^+/NADH between 3 and 10, and the more NADH bound to the enzyme the higher the level of fluorescence. Thus fluorescence could be due to NAD^+, NADH or both.

Titration of half of the free sulfhydryl groups with p-hydroxymercuribenzoate resulted in complete loss in enzyme activity. Yet the fluorescence was not completely lost until all of the sulfhydryl groups had been titrated. Bound NAD^+ could not be detected in the p-hydroxymercuribenzoate treated enzyme. The activity, but not the fluorescence, was restored after precipitating the enzyme with ammonium sulfate and resuspending the enzyme in the presence of cysteine and NAD^+. It should be noted that the liver enzyme (40) was also inhibited by p-hydroxymercuribenzoate,

and the activity was restored by incubating with cysteine; yet neither cysteine nor p-hydroxymercuribenzoate had any effect on the activity of the E. coli enzyme.

Treatment of the enzyme with borohydride in the presence of substrate at the same concentration as the enzyme resulted in a 50-80% enhancement of fluorescence with a concomitant loss in enzyme activity. A similar enhancement of fluorescence was obtained when the enzyme was incubated for 6 to 10 hours with 5' nucleotides in the presence of free sugars. Nucleotides which elicited fluorescence were 5' UMP, CDP, TDP, UTP. Less than a 2-fold increase in fluorescence was obtained with UTP, UDPglucose, UDPgalactose, 5' CMP and 3' UMP. Of the sugars, D-fucose, D-galactose, and D-xylose produced the same increase in fluorescence which was greater than that obtained with L-arabinose, D-glucose, or D-ribose. Sucrose and L-fucose had a negligible effect on the fluorescence. Uridylic acid and galactose were rapidly incorporated into the protein. The bound galactose was not liberated even after digestion of the epimerase with pronase and trypsin, although it was released on denaturation with alcohol or heat. The bound galactose was converted to a unidentified product even in the absence of 5' UMP.

The 4-epimerases which had been either reduced with borohydride or incubated with 5' nucleotides in the presence of sugars were comparable in that: (a) the magnitude and

quantum yields of fluorescence were identical; (b) the enzymes had only 5% of their original activity; (c) less than 10% of the original NAD^+ was present; (d) only 30% of the increase in 340 mμ absorption could be accounted for as NAD-4-H, although the assay used may not have detected all of the NADH.

Not enough experiments have been carried out at this time to accurately interpret all of these results. The fluorescence enhancement may not be related to any mechanistic property of the enzyme, and the free nucleotides and sugars may be reacting at a site on the enzyme remote from the active site. However, it is possible that the 5' UMP and free sugars were responding in the same manner as true substrate, in which case, the sugar would have been oxidized at C-4 by transfer of a hydride to NAD^+. In the normal reaction the oxidized intermediate would be immediately reduced by NADH, so that at any one time the steady state level of NADH would have been too low to be detectable. However, in the presence of pseudosubstrate, the oxidized intermediate may not have been reduced due to the inability of the protein or substrate to make a necessary conformational change, or the oxidized intermediate may have been converted to a second non-reactive form. Thus, detectable levels of NADH would have been produced, and this would have been observed as an increase in fluorescence. Since the reduction of the enzyme with borohydride was carried

out in the presence of substrate, the borohydride may have reduced either NAD^+, the oxidized substrate or both, but in either case NADH would have formed which would have produced the increase in fluorescence.

Indirect evidence for a reduction of NAD^+ during the course of the epimerization was obtained by Wilson and Hogness (11). They found that incubation of the E. coli 4-epimerase with substrate produced an absorption spectrum with maximum at 345 mμ. If NAD^+ were reduced during the course of the reaction, one would expect an absorption maximum near 340 mμ; due to electron perturbation caused by the protein, the exact wavelength will vary from protein to protein. They calculated that 19% of the enzyme bound NAD^+ was in the reduced form in the presence of substrate. Their data are consistent with the following sequence of reactions:

$$NAD_{ox}\text{-}E + S_1 \rightleftharpoons NAD_{ox}\text{--}ES_1 \rightleftharpoons NAD_{red}\text{-}ES_{ox} \rightleftharpoons$$

$$NAD_{ox}\text{--}ES_2 \rightleftharpoons NAD_{ox}\text{-}E + S_2$$

where E is the enzyme and S_1 and S_2 are UDP-glucose and UDP-galactose.

Bevill et. al. (13) and Kohn et. al. (14) independently presented evidence for retention of T at C-4 during epimerization by the yeast enzyme. Thus, the T is either stereospecifically removed from the hexose and reintroduced, or the C-H bond is never broken.

To further elucidate the mechanism, Bevill and coworkers (10) studied the kinetic isotope effect observed when tritium was substituted for the hydrogen at C-4 of the hexose moiety. A mixture of either UDP-glucose-1-^{14}C and UDP-glucose-4-T or UDP-galactose-1-^{14}C and UDP-galactose-4-T was incubated with the yeast 4-epimerase for given periods of time. The rate of appearance of ^{14}C in the product was taken to be a measure of the rate of reaction of hexose with hydrogen at C-4, since any ^{14}C isotope effect should have been negligible. The ratio of $T/^{14}C$ at any given point in the reaction to $T/^{14}C$ at equilibrium was plotted versus % attainment of equilibrium. The plots were extrapolated to 0% attainment of equilibrium to obtain an estimate of k_T/k_H. An inverse isotope effect of either 1.5 or 3.0 was obtained depending on the direction of the reaction.

If T were transferred in the rate determining step, a positive isotope effect would have been expected. However, a small or even inverse primary isotope effect might have been obtained if hydrogen were bound more tightly in the transition state than in the starting state. Due to the α-hydrogen isotope effect of reactions involving a trigonal carbon, such as cleavage of C-O or C-C bonds at C_4, the unlabeled compound should react 12-25% faster than the T-substituted compound. A Sn_2 like mechanism could conceivably explain the inverse isotope effect; however,

^{18}O exchange with the medium would have been necessary for an Sn_2 reaction. An inverse isotope of 3 would be consistent with transfer of hydrogen to oxygen or carbon, but transfer of the hydride ion to sulfur atom is ruled out. Bevill proposed the following mechanism to explain their data:

$$E + SH \overset{k_1}{\rightleftharpoons} E\text{-}SH \overset{k_2}{\rightleftharpoons} E\text{-}H\text{----}S \overset{k_3}{\rightleftharpoons} E\text{-}H\text{----}S' \overset{k_4}{\rightleftharpoons}$$

$$E\text{----}HS' \overset{k_5}{\rightleftharpoons} E + S'H$$

where S and S' are UDP-galactose and UDP-glucose. K_3 is the rate determining step and might be a reorganization of the enzyme-substrate complex which allows the hydrogen to return to the opposite configuration. The secondary isotope effect would be due to a shift in the equilibrium of k_1 and k_5 or k_2 and k_3. If the reaction were occuring at a site on the enzyme which excludes water, the observed isotope effect would be consistent with the transfer of two-hydrogen ions and two-electrons from the substrate directly to NAD^+ with the C-4 hydrogen ion being transferred to the nitrogen of NAD^+.

A preliminary report by Nelsestuen and Kirkwood (12) has recently appeared presenting direct evidence for the formation of NADH during the course of the reaction. Incubation of the E. coli 4-epimerase with $NaBT_4$ resulted in loss in enzyme activity towards UDP-glucose and a concommitant reduction of NAD^+ to NADT. The NAD^+ was reduced in

the β side of the para-position as determined by denaturation of the protein and assaying NADT released with specific α and β-dehydrogenases. The activity was regained when the reduced enzyme was incubated with TDP-4-keto-6-deoxyglucose. Simultaneously, the NADH was oxidized and TDP-6-deoxyglucose-4-T and TDP-6-deoxygalactose-4-T were produced.

At the present time it is not known whether the uracil moiety of the UDP-hexose plays a significant role in the catalytic events, whether it is necessary for specificity of binding of the substrate to the enzyme, or if it is only present to maintain the substrate in the preferred conformation or in the pyranose form. If the latter were the only function of the uridine moiety, the enzyme should also epimerize other glycosides of glucose and galactose, such as glucose-1-P, but this has not been studied. However, a number of reaction mechanisms involving the uridine moiety have been proposed.

UDP-glucose can exist without steric hindrance in a folded conformation with the uracil moiety, particularly the acylamido (-CO-NH-CO-) group, held in close proximity to the 4-position of the hexose. Because of this, de Robichon-Szulmajster (55) proposed that one of the carbonyl groups of the uridine moiety displaces a proton from the C-4 hydroxyl group at the same time the NAD^+ removes a hydride from C-4. Due to migration of the proton

on the nitrogen, either carbonyl group of the uracil may become protonated. Thus a proton is freely accessible to either side of the hexose, and can be returned to the oxygen anion at C-4 at the same time the hydride ion is returned from the NADH. It is impossible to test this mechanism isotopically since oxygen-bound protons are freely exchangeable with the medium.

From a study of a number of enzymes using UDP-hexose substrates Budowsky et. al. (56) and Druzhinina et. al. (57, 58) proposed that the only analogues of UDP-glucose that can enter into a UDP-glucose-requiring-enzymatic reaction are those which contain an unchanged -C(X)-NH-C(X)- group in the uracil ring where X can be oxygen or sulfur. They proposed that the function of the acylamido group was to hold the UDP-glucose in a folded conformation by hydrogen bonding between: (a) the C-2 carbonyl group of uracil and the hydroxyl group at C-2 of ribose; (b) the nitrogen at C-3 of the uracil and the hydroxyl group at C-3 of glucose; and (c) the C-4 carbonyl group of uracil and the hydrogen at C-2 of glucose. Thus the more rigid uracil nucleus serves as a template for the conformationally labile monosaccharide moiety so that the hydroxyl groups at C-2 and C-3 always occupy the equatorial positions, which are the positions most available to hydrogen bonding. A number of analogues of UDP-glucose were investigated in support of the above theory. Analogues of the uracil ring

which were found to be epimerized by UDP-glucose 4-epimerases from calf liver were 5,6-dihydro-UDP-glucose, 2'deoxy-UDP-glucose, 6-aza-UDP-glucose, 4-thio-UDP-glucose and 2-thio-UDP-glucose all of which have an acylamido group, were found not to be epimerized by the 4-epimerase. However, Druzhinia et. al. (57) has recently discovered that ADP-glucose was epimerized by UDP-glucose 4-epimerase from both calf liver and mung bean. UDP-Glucose epimerization was inhibited by ADP-glucose indicating that UDP-glucose 4-epimerase was responsible for ADP-glucose epimerization. Since the adenine nucleus does not have an acylamido grouping, it would indicate that if the acylamido group were responsible for maintaining the secondary structure of the nucleotide-diphosphate hexose, this is not a necessary prerequisite for 4-epimerase activity of the calf liver and mung bean enzyme. However, Salo et. al. (16) reported that ADP-glucose was not epimerized by the E. coli enzyme. Druzhinina's results would also rule out de Robichon-Szulmajsters (55) proposed theory of a direct involvement of the uracil moiety in the epimerization.

Druzhinina et. al. (17) and Salo et. al. (16) have independently reported on the epimerization of a number of other nucleotide sugars by UDP-glucose 4-epimerase. Using a series of UDP-deoxyglucose analogues Druzhinina et. al. (17) found that UDP-4-deoxyglucose was the most effective competitive inhibitor of UDP-glucose 4-epimerase from calf

liver and mung bean. UDP-3-deoxyglucose was not epimerized itself, suggesting that the C-3 hydroxyl group was necessary for the actual epimerization reaction. Nevertheless, the C-3 hydroxyl group appeared to facilitate substrate binding, since the k_i for UDP-3-deoxyglucose was twice that of the 4-deoxyglucose derivative. UDP-6-deoxyglucose was not epimerized by the enzyme from calf liver, but was epimerized by the enzyme of plant origin, and was an inhibitor of UDP-glucose for both epimerases. Further, the specific activity of UDP-6-deoxyglucose 4-epimerase did not change on purification, which indicates that UDP-glucose 4-epimerase was responsible for the epimerization. UDP-glucuronic acid was also found to be inhibitory to the epimerization.

Using yeast UDP-glucose 4-epimerase Salo et. al. (16) was able to obtain epimerization of UDP-xylose, UDP-arabinose, β-L-arabinose-1-P, xylose, UDP-glucuronic acid, UDP-4-o-methyl-D-glucose, UDP-D-allose, UDP-D-mannose, UDP-N-acetylglucosamine, UDP-β-D-glucose, dUDP-D-glucose, ADP-glucose, CDP-glucose, GDP-glucose, IDP-glucose, or dTDP-glucose. The ratio of UDP-glucose epimerization to UDP-xylose, UDP-arabinose, and UDP-fucose epimerization increased on purification, and UDP-xylose, UDP-arabinose, and UDP-fucose did not inhibit epimerization of UDP-galactose. Thus there appeared to be 4-epimerases other than UDP-glucose 4-epimerase in the less pure preparations.

In summary, the lack of incorporation of T or ^{18}O

from the medium, and the negative isotope effect tend to rule out all proposed mechanisms of glucose and galactose epimerization except that involving oxidation-reduction. UDP-glucose 4-epimerase appears to require NAD^+ for activity, since all bacterial enzymes examined were shown to contain tightly bound NAD^+, and NAD^+ had to be added for activity of the epimerases of higher plants and animals. Activity of the epimerases requiring added NAD^+ was dependent on the NAD^+/NADH ratio in the medium; this may be a means of regulating the enzyme activity in the cell. Production of an absorption spectrum with a 345 mμ maximum when the enzyme was incubated with substrate provided substantial indirect evidence for NADH formation during the course of the reaction. The data of Nelsestuen et. al. (12) provides rather conclusive direct evidence for transfer of a hydride ion to NAD^+ and for the formation of a 4-keto derivative during the course of the epimerization. However, the hydride may be transferred to another group on the enzyme surface before or after reduction of NAD^+. There is no substantial evidence that the uracil moiety is involved in any way in the enzyme reaction.

UDP-Glucose 4-Epimerase; Other Characteristics: Darrow and Creveling (59) reported that the yeast 4-epimerase was activated by cations and organic amines. Maximal stimulation was obtained by preincubation with 50 mM NaCl, KCl, NH_4Cl, Na_2SO_4, $(NH_4)_2SO_4$ and with 5 mM $MgCl_2$, $MnCl_2$, and

$CaCl_2$. Monovalent organic amines at 100 mM concentration produced the same maximal activity elicited by cations. However, 10 mM spermine and spermidine produced twice the maximal rate obtained with metals. It should be noted, however, that the organic amines were neutralized with NaOH, and, therefore, it is not known what part of the activation was due to Na^+ and what part was due to the organic amine. Different levels of activity produced by equivalent levels of Cl^- indicated that the activation was due to either the cation or the amine and not to the Cl^-. In the absence of any metal ions or amines, the enzyme did not elicit true Michaelis-Menton kinetics; rather, there appeared to be product inhibition. Product inhibition has also been reported for the same enzyme from rats (42). In the presence of spermine the V_{max} was increased, but the K_m remained the same as that obtained with the liver enzyme, and the results gave linear Lineweaver-Burke plots. Thus the metal ion or amine activation appears to be non-specific and may reflect a change in conformation of the protein to a more favorable conformation or binding of the product by a metal ion and, thus, eliminating product inhibition.

Sucrose gradient experiments by Darrow and Rodstrom (60) indicated various degrees of aggregation of the yeast 4-epimerase under different conditions. Enzyme treated with p-hydroxymercuricbenzoate had the smallest molecular weight, possibly that of monomer. Native enzyme and enzyme

treated with spermine at 25°C or p-hydroxymercuricbenzoate treated enzyme which had been reactivated with cysteine had twice the molecular weight (dimer). Enzyme treated with spermine at 5°C aggregated to form a tetramer. Darrow and Rodstrom (61) also report that a molecular weight of 125,000 was obtained for the yeast enzyme as determined by low speed equilibrium centrifugation. One mole of NAD^+ was found per mole of enzyme; thus, one molecule of NAD^+ is bound per dimer.

Similarly Wilson and Hogness (11) reported a molecular weight of 7.9 (± 0.8) x 10^4 for the E. coli 4-epimerase as obtained from a series of high speed sedimentation equilibrium and sedimentation velocity experiments.

UDP-Glucose 4-epimerase from newborn and adult rats (42) appear to have the same stability characteristics; however, the enzyme from newborn rats had a V_{max} 5 times greater than that of the adult-rat enzyme. UDP-Glucose was a competitive inhibitor of UDP-galactose. Inhibition was also obtained with UDP-mannose, UMP, and TDP-galactose; however AMP, ADP, ATP, ADP-glucose, GDP-glucose and GDP-mannose were not inhibitory.

Other Nucleoside-Diphosphate 4-Epimerases: Other nucleoside diphosphates which are epimerized include: UDP-glucuronic acid (19, 62), UDP-xylose (16, 17, 18, 19, 20), UDP-N-acetylglucosamine (15, 63, 64), d-TDP-glucose (20, 65), and UDP-fucose (16, 17). In most cases it was

not reported whether the epimerization was catalyzed by UDP-glucose 4-epimerase or by separate epimerases.

Salo et. al. (16) reported that epimerizations of UDP-L-arabinose, UDP-L-fucose and UDP-xylose were not due to UDP-glucose 4-epimerase, since there was no competitive inhibition of UDP-glucose 4-epimerase by the other substrates, and UDP-xylose activity could be partially removed by purification. However, Druzhinina's results indicate that UDP-fucose and UDP-glucose are epimerized by the same enzyme from mung beans (17). Likewise, Ankel and Maitra (18) found that UDP-glucose and UDP-xylose are metabolized by the same 4-epimerases from E. coli, since the same ratio of UDP-glucose to UDP-xylose epimerase activity was found in three mutants of E. coli containing different levels of UDP-glucose epimerase. In addition UDP-xylose and UDP-glucose were competitive inhibitors of the 4-epimerase preparation, as determined by assaying the enzyme in the presence of varying quantities of both substrates. Feingold et. al. (19) have reported a separation of UDP-glucuronic acid 4-epimerase from UDP-L-arabinose 4-epimerase in mung bean, but they did not determine whether UDP-glucose was epimerized. Feingold et. al. (20) also reported a separation of UDP-glucose 4-epimerase activity from UDP-L-arabinose 4-epimerase and dTDP-glucose 4-epimerase activities present in wheat germ extracts. However, TDP-glucose and UDP-L-arabinose may be epimerized by the

same enzymes. Thus, there is a precedence for separate epimerases for all of these substrates; yet, in some organisms UDP-glucose 4-epimerase appears to be less specific and capable of epimerizing more than one substrate.

Of all of these other 4-epimerases only UDP-N-acetylglucosamine 4-epimerase from rabbit skin (15) has been shown to require NAD^+. It was not determined whether UDP-glucose 4-epimerase was present or required NAD^+. Thionicotinamide and acetylpyridine analogues of NAD^+ were found to be less active than NAD^+; however, the NAD^+ could not be replaced by $NADP^+$. The epimerization was inhibited by UDP, uridine, uracil, UDP-glucose and UDP-glucuronic acid.

L-Ribulose-5-Phosphate 4-Epimerase

In 1957 Wolin, Simpson and Wood (66) reported the conversion of L-ribulose-5-P and D-ribulose-5-P by crude extracts of Aerobacter aerogenes. Since then, the enzyme has also been identified in Lactobacillus plantarum (2) and Bacillus subtilis (3) and identified (1) and purified (67) from Escherichia coli.

L-Ribulose-5-P 4-Epimerase; Studies on the Mechanism: L-Ribulose-5-P 4-epimerase, like the bacterial UDP-glucose 4-epimerase, does not require NAD^+, nor is it stimulated by NAD^+ or $NADP^+$ (4). The enzyme was stable to charcoal treatment (4), and it was not inactivated by NADase (21, 2). Burma and Horecker (2) did not detect any bound NAD^+ or NADH

using a sensitive catalytic assay; however, the 260 mμ absorption was higher than expected of only protein, thus, indicating the presence of bound nucleotide. McDonough and Wood (21) did not find evidence for isotopic exchange from the medium into substrate when the reaction was run in $H_2{}^{18}O$ or T_2O.

Experiments to determine the kinetic isotope effects for L-ribulose-5-P 4-epimerase, comparable to those used for UDP-glucose 4-epimerase, were performed by the joint efforts involving W. A. Wood's and S. Kirkwood's laboratories.[1] D-xylulose-5-P-1-^{14}C and D-xylulose-5-P-4-T were obtained by the enzymatic conversion of D-xylose-1-^{14}C and D-xylose-4-T in the presence of D-xylose-isomerase and D-xylulokinase and ATP. D-xylulose-5-P-1-^{14}C and D-xylulose-5-P-4-T were combined in known ratios and incubated for given periods of time in the presence of L-ribulose-5-P 4-epimerase from A. aerogenes. The rate of the reaction of the two labeled species was determined from the rate of appearance of ^{14}C in L-ribulose-5-P. An estimate of the initial isotope effect (k_T/k_H) was obtained by determining isotope effect on the rate at various times prior to the obtainment of equilibrium and extrapolating to zero percent attainment of equilibrium. An isotope effect of $k_T/k_H = 1.00$ was obtained. Their data also demonstrated that T was maintained at C-4 during the epimerization. The absence of an isotope effect indicates that T cannot be transferred

during the rate determining step. However, in an enzymatic reaction the binding of the substrate to the enzyme or the conformational change of either the substrate or the enzyme may be the rate determining steps. Thus, carbon-hydrogen bond cleavage or an oxidation-reduction reaction in L-ribulose-5-P epimerization cannot be ruled out by the lack of an isotope effect. However, the mechanism of L-ribulose-5-P 4-epimerase must differ from that of UDP-glucose 4-epimerase in some respect; either a difference in the rate determining step or in the reaction mechanism.

In summary, at the present time, there is no substantial evidence that the mechanism of L-ribulose-5-P 4-epimerase is the same as that for UDP-glucose 4-epimerase. The facts that T_2O and $H_2{}^{18}O$ are not exchanged and that T is not lost from C-4 during epimerization are consistent with oxidation-reduction of substrate as in the case of UDP-glucose 4-epimerase. These results would rule out an Sn_2 inversion reaction. The L-ribulose-4-P 4-epimerase could contain tightly bound NAD^+ which is not removed by charcoal or NADase treatment, as found for bacterial UDP-glucose 4-epimerases; however, there is no substantial evidence for tightly bound NAD^+ on L-ribulose-5-P 4-epimerase. A $k_T/k_H = 1.00$ indicates that there is some departure from the mechanism of UDP-glucose 4-epimerase, although this may be minor. If a hydride ion were transferred during the rate-determining step, an isotope effect

greater than 1.0 would be expected. A chemical epimerization by the Lobry de Bruyn-Alberda van Ekenstein transformation at C-4 due to the functional carbonyl at C-2 should be very limited if it exists at all. In the presence of an enzyme, the chemical epimerization could be directed exclusively to the C-4 carbon, and the rate of reaction could be greatly accelerated. This mechanism is not feasible with UDP-glucose 4-epimerases due to the lack of a carbonyl group on the hexose moiety.

L-Ribulose-5-P 4-Epimerase; Other Characteristics: Wolin, Simpson and Wood (4) have reported a 223 fold purification of the enzyme from A. aerogenes. Only D-xylulose-5-P and L-ribulose-5-P were found to serve as substrates for the 4-epimerase; the other substrates tested were D-tagatose-6-P, D-xylose-1-P, D-xylose-5-P and D-ribulose-5-P.

Additional cofactors or metal ions were not required for activity by the enzyme from A. aerogenes (4), L. plantarum (2), or E. coli (67). The K_m of these enzymes for L-ribulose-5-P was 1×10^{-4}M, 1.1×10^{-3}M, and 9.5×10^{-5}M respectively. The pH optimum for the enzyme from A. aerogenes was between pH 8.5 and 9.5 with about 40% of maximal activity at pH 7.0 and no activity between pH 5 to 6. In contrast, a broad pH optimum between pH 7.0 and 10.0 was found for the L. plantarum (2) and the E. coli (67) enzymes. Spontaneous conversion of L-ribulose-5-P to D-xylulose-5-P does not occur even at pH 10.0. The equilibrium ratio of D-xylulose-5-P

and L-ribulose-5-P varied from 1.2 to 1.9 for the enzyme from L. plantarum (2) and A. aerogenes (67); these values are approximately comparable to those found for UDP-glucose/UDP-galactose.

L-Ribulose-5-P 4-epimerase from E. coli (67) was purified to homogeneity as determined by acrylamide gel electrophoresis and ultracentrifugation. The enzyme was crystallized; however, activity could not be recovered from the crystals. A molecular weight of 103,000 was obtained for the enzyme. Only one molecular weight species was apparent from acrylamide gel and molecular weight determinations.

UDP-Glucuronic Acid 5-Epimerase

The only 5-epimerase which has been reported to date is UDP-glucuronic acid 5-epimerase. Jacobson and Davidson (15) obtained evidence for the 5-epimerization of UDP-glucuronic acid to UDP-L-iduronic acid by extracts of rabbit skin. The epimerization required added NAD^+; $NADP^+$, thionicotinamide-ADP, and acetylpyridine-ADP were less active than NAD^+; and NADH was inactive. When the reaction was run in T_2O, T was not incorporated into the substrate, nor was there any spectral evidence for production of an α-β unsaturated carboxylic acid during the course of the reaction. Incubation of the enzyme with UDP-D-glucuronic acid and $UD^{32}P$ did not result in any exchange of $UD^{32}P$ into UDP-D-glucuronic acid. The tissue extracts did not have

any UDP-glucose 4-epimerase activity; however, UDP-N-acetylglucosamine 4-epimerase appeared in the same purification fractions as the UDP-glucuronic acid-5-epimerase activity. The UDP-N-acetylglucosamine 4-epimerase was inhibited by UDP-glucuronic acid and the UDP-glucuronic acid 5-epimerase was inhibited by UDP-N-acetylgalactosamine and UDP-N-acetylglucosamine. The 5-epimerase was also inhibited by UDP, uridine, and uracil. Thus the 5-epimerase appears to have some of the mechanistic characteristics of UDP-glucose 4-epimerase. This is difficult to explain. Since the sugar moiety exists in the pyranose ring, the formation of a carbonyl group by NAD^+ facillitated oxidation cannot occur. If the mechanism involves oxidation at C-4 followed by keto-enolization between C-4 and C-5, the α-β unsaturated carboxylic acid should have been detected spectrophotometrically if the steady state levels were high enough to be detected. There is precedence for this type of reaction, since 6-deoxy-sugar formation appears to involve oxidation at C-4 followed by enolization between C-4 and C-5 and dehydration between C-5 and C-6 (68, 69).

Control of L-Ribulose-5-Phosphate 4-Epimerase Formation

Pathway of L-Arabinose Metabolism in Bacteria

L-Ribulose-5-P 4-epimerase is induced in A. aerogenes by growth on L-arabinose (4), L-arabitol (6) or L-xylose (5), but not by growth on D-glucose, D-xylose, or D-arabinose (4).

Crude extracts of A. aerogenes grown on L-arabinose contain L-arabinose isomerase, L-ribulokinase and L-ribulose-5-P 4-epimerase, and the pathway of L-arabinose metabolism was thus deduced (71) to be:

$$\text{L-Arabinose} \xrightarrow{\text{Isomerase}} \text{L-Ribulose} \xrightarrow{\text{Kinase}} \text{L-Ribulose-5-P}$$

$$\xrightarrow{\text{4-Epimerase}} \text{D-xylulose-5-P} \longrightarrow \text{Pentose cycle}$$

L-Ribulose-5-P 4-epimerase is also induced in E. coli (1), L. plantarum (2), and B. subtilis (3) by growth on L-arabinose. The pathway of L-arabinose metabolism in all of these bacteria is essentially identical to that in A. aerogenes.

L-Arabinose Operon

Englesberg and his associates (1, 72, 73, 74) have extensively mapped the L-arabinose operon in E. coli. The L-arabinose operon codes for a regulatory protein, an initiator site, L-ribulokinase, L-arabinose isomerase and L-ribulose-5-P 4-epimerase in that order. The L-arabinose permease is located at a different site on the DNA and also contains an initiator site. The regulatory protein, or repressor, presumably is an allosteric protein containing a site for attachment of the regulatory protein to the initiator regions of both the L-arabinose operon, and the L-arabinose permease gene, and also a site for L-arabinose.

Binding of L-arabinose to the repressor is thought to produce an allosteric transition in the repressor to form the activator. The activator initiates the transcription of the genes coding for all of the proteins on the L-arabinose operon as well as for the L-arabinose permease. A mutation in the initiator gene which results in the production of either more or less of the L-ribulokinase also results in a coordinate increase or decrease in the level of L-arabinose isomerase and L-ribulose-5-P 4-epimerase indicating that synthesis of these three proteins is coordinately controlled.

Although the L-arabinose operon has not been mapped in A. aerogenes, Mortlock and associates have shown that L-arabinose isomerase, L-ribulokinase and L-ribulose-5-P 4-epimerase are coordinately controlled as in E. coli.[2]

Precedence for a Second L-Ribulose-5-P 4-Epimerase

L-Arabitol, L-xylose, and L-xylulose are not naturally-occuring pentoses, however, A. aerogenes is able to metabolize them. The pathway for metabolism of these pentoses was determined by W. A. Wood and associates (6, 76) to be as follows:

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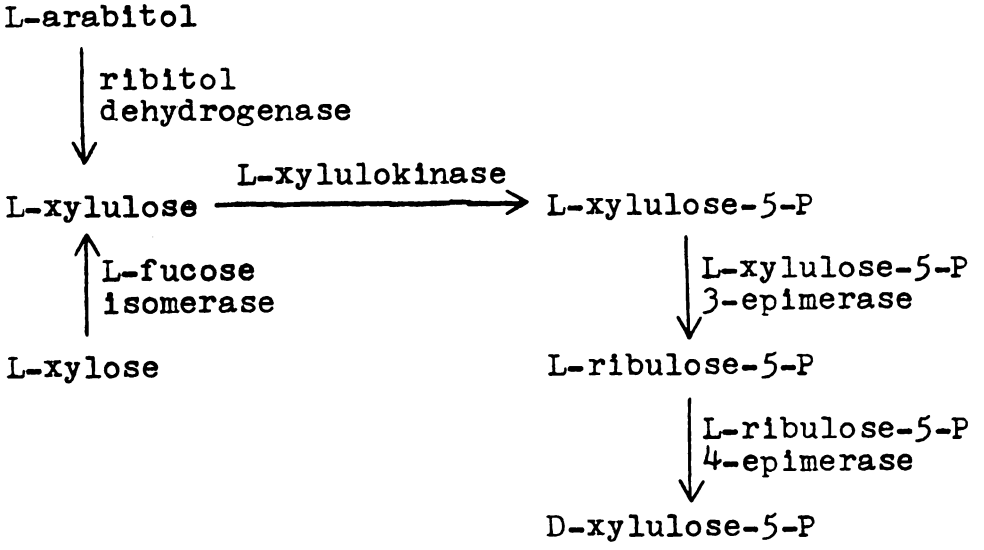
[2]LeBlanc, D., Mortlock, R. P., Deupree, J., Wood, W. A., unpublished results.



The questions which remain are: (a) what is the genetic location of the genes coding for all of these enzymes, and (b) is the L-ribulose-5-P 4-epimerase coded for by the gene in the L-arabinose operon, or is there a second gene. In order to answer these questions it is necessary to discuss the metabolism of pentoses and pentitols, by A. aerogenes in detail.

The arabitol dehydrogenase activity present after growth on L-arabitol is the result of a very low rate of oxidation by ribitol dehydrogenase. Further growth of the organism on L-arabitol results in the selection of mutants which are derepressed and constituitive for ribitol dehydrogenase (77). Likewise, L-fucose isomerase appears to be the enzyme involved in isomerizing L-xylose to L-xylulose, and growth of the organism on L-xylose resulted in the selection of mutants producing constituitive levels of L-fucose isomerase (78). Thus the L-arabitol dehydrogenase

and L-xylose isomerase activities appear to be due to proteins which are coded for by a gene for ribitol dehydrogenase and L-fucose isomerase, respectively. The pathway of ribitol and L-xylose metabolism is presented in Figure 2.

If L-ribulose-5-P 4-epimerase was translated from the genes of the L-arabinose operon following growth on these substrates, all of the L-arabinose operon proteins should have been produced since the L-arabinose operon is thought to be coordinately controlled. However, extracts of L-arabitol and L-xylose grown cells do not contain L-arabinose isomerase or L-ribulokinase activities (79). In addition, the enzymes of the L-xylulose pathway must not be coded for by the genes in the L-arabinose operon nor are they produced constituitively, since L-xylulokinase and L-ribulose-5-P 3-epimerase activities were not present in extracts of cultures grown on L-arabinose (79). Thus, it seems reasonable to hypothesize that a L-xylulose operon exists and that it codes for L-xylulokinase, L-xylulose-5-P 3-epimerase and a second L-ribulose-5-P 4-epimerase. This implies that L-xylulose is found in nature and is metabolized by A. aerogenes.

Recently Mortlock[2] obtained a mutant of A. aerogenes which was constituitive for the L-arabinose operon and had a defective gene for L-ribulose-5-P 4-epimerase, in that 4-epimerase activity was not present in extracts of cultures grown on L-arabitol plus casamino acids. However, the crude

extracts were able to epimerize L-ribulose-5-P following growth on L-arabitol plus casamino acids. This is highly indicative of the fact that two L-ribulose-5-P 4-epimerases exist, and that each is under separate control and is coded for by separate genes.

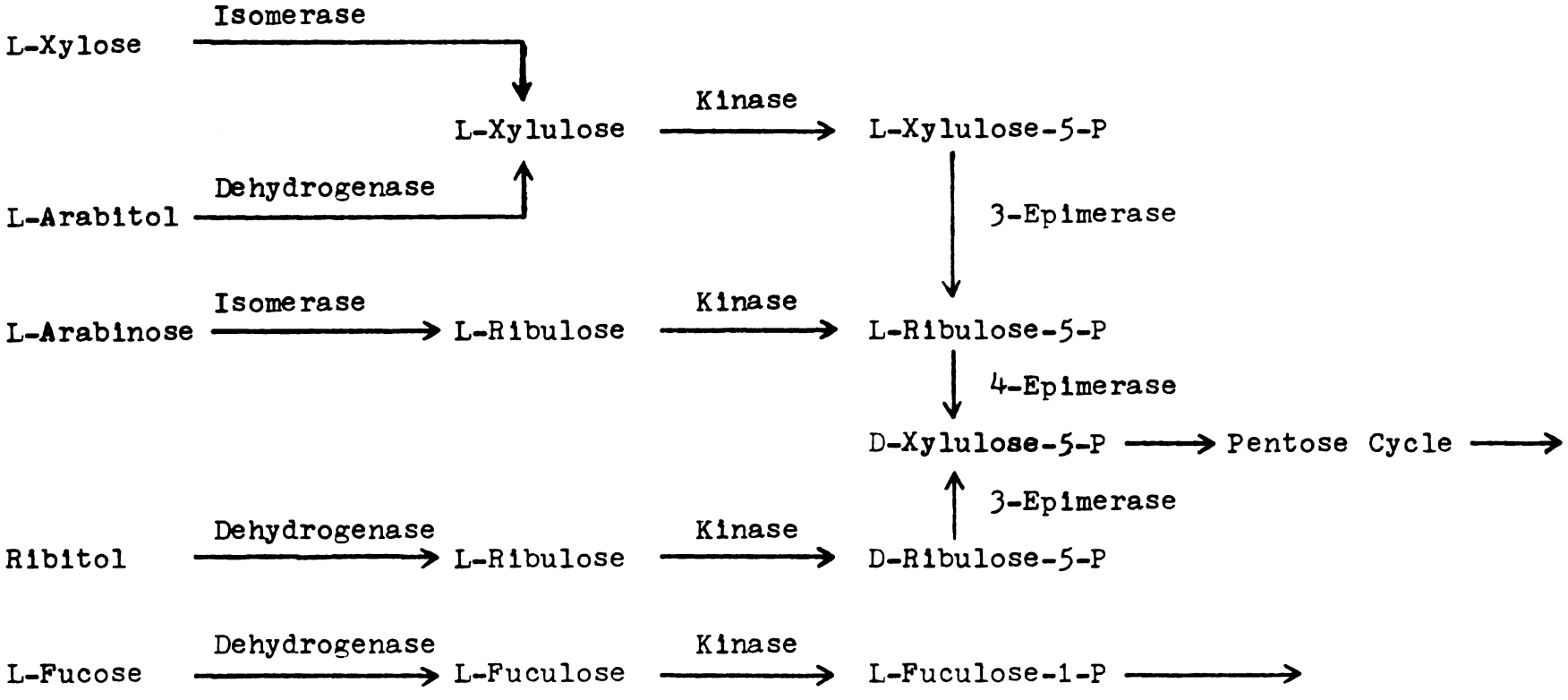


Figure 2. Pathways of Pentose, Pentitols and L-Fucose Dissimilation In <u>Aerobacter aerogenes</u>

MATERIALS AND METHODS

Bacteriological

Aerobacter aerogenes

L-Ribulose-5-P 4-epimerase was obtained from either wild type or mutant strains of A. aerogenes, PRL-R3. The cells were grown with aeration at 32° or 37°C on a minimal salts medium (76) composed of 0.15% KH_2PO_4, 1.42% Na_2HPO_4, 0.3% $(NH_4)_2SO_4$, 0.02% $MgSO_4$, 5 x 10^{-4}% $FeSO_4$, and 0.5% L-arabinose. An initial innoculum was grown in 10 ml quantities. After 24 hours the culture was transferred to one liter of medium in a 3-liter fernbach flask. Four liters of a 12-hour growth were used to innoculate 100 liters of media in a 130-liter fermenter (New Brunswick Co., Inc.). The culture was grown at 37°C at a stirring speed of 200 rpm and an aeration rate of 2.5 ft^3/min. until the absorbance at 660 mμ was 1.8 using a 1 cm light path. The cells were harvested prior to reaching stationary phase in a continuous centrifuge (Sharples Corporation), and the cells were stored frozen. The medium was not cooled during the harvesting since the bacteria produce a polysaccharide upon cooling.

A series of mutants derived from a uracil auxotroph of A. aerogenes PRL-R3 was obtained from Dr. R. P. Mortlock

of the University of Massachusetts, Amherst, Mass. The mutants were grown in the same manner as wild type with the addition of 0.005% uracil to all media. The u^-i^- mutant is constituitive for the L-arabinose operon and produces 3 times more L-ribulose-5-P 4-epimerase than the wild type.[2] Therefore it was used as a source of 4-epimerase for most of the experiments. The n^-u^- mutant requires nicotinic acid as well as uracil for growth, and it was grown on minimal salts medium supplemented with L-arabinose, uracil and 10^{-5}% nicotinic acid.

L-Ribulokinase was obtained from a u^-e^- mutant which did not produce an active 4-epimerase, but did synthesize active L-arabinose isomerase and L-ribulokinase. Similarly, a mutant of *E. coli*, $D-\frac{139}{139}$, obtained from Englesberg, was also used as a source of L-ribulokinase. The *E. coli* mutant is diploid for the L-arabinose operon, but it does not produce a functional 4-epimerase. Both mutants were grown according to the procedure of Anderson (80).

Leuconostoc mesenteroides

Leuconostoc mesenteroides (an uncharacterized strain) was grown, harvested, and the crude extract was prepared as described by Sapico and Anderson (81).

Lactobacillus arabinosus

Lactobacillus arabinosus 17-5 (ATCC 8014) was obtained from the American Type Culture Collection. The

organism was grown on Difco Micro Innoculum Broth and maintained on the same type of medium supplemented with 2% agar.

Chemicals

General Chemicals

Nicotinic acid (carboxyl-^{14}C) with a specific activity of 59.1 mC/mmole was obtained from Nuclear-Chicago. Spectrographically standardized $CoSO_4$, $MnSO_4$, $FeSO_4$, $MgSO_4$, $NiSO_4$, and $ZnSO_4$ were obtained from Johnson, Matthey and Co., Limited. L-Arabinose was purchased from Nutritional Biochemical Co. Calbiochem's triosephosphate isomerase-α-glycerolphosphate dehydrogenase mixture was used. Whatman DE-32 was obtained from Reeve Angel. Calcium phosphate gel was prepared according to the procedure of Keilin and Hartree (82).

L-Ribulose-5-P

L-Ribulose-5-P was prepared according to the procedure of Anderson (80) by a series of steps involving chemical isomerization of L-arabinose to L-ribulose, phosphorylation of L-ribulose in the presence of L-ribulokinase and purification of the L-ribulose-5-P obtained.

The L-ribulose was prepared using Anderson's modification (80) of the original procedure of Reichstein by refluxing 25 gm of L-arabinose in pyridine. A 10% yield of L-ribulose was obtained as determined by the cysteine-carbazole

method (83) run at 37°C for 20 min. Because of the specificity of the L-ribulokinase, the L-ribulose was not purified.

L-Ribulokinase was isolated from either an E. coli mutant or an A. aerogenes mutant which lacked an active L-ribulose-5-P 4-epimerase. The crude extract was partially purified to remove the competing ATPase. L-Ribulose-5-P which was prepared using the E. coli L-ribulokinase appeared to be partially contaminated with D-xylulose-5-P. This was probably due to the presence of some L-ribulose-5-P 4-epimerase activity in the L-ribulokinase preparation; however, the level of activity was too low to be detected in an enzyme assay. When this preparation of L-ribulose-5-P was used, it was necessary to pre-incubate the substrate in the reaction mixture prior to adding the 4-epimerase due to the initial consumption of D-xylulose-5-P by phosphoketolase.

L-Ribulose-5-P formed from the L-ribulokinase reaction was isolated as the Ba salt at pH 6.7 without any attempt to remove contaminating nucleotides with charcoal. When charcoal treatment was used, a removal of 50% of the L-ribulose-5-P resulted. Therefore the L-ribulose-5-P was purified using a Dowex-1-formate column according to the method of Simpson and Wood (84). Approximately 50% of the starting L-ribulose was obtained as the dried Ba salt of L-ribulose-5-P.

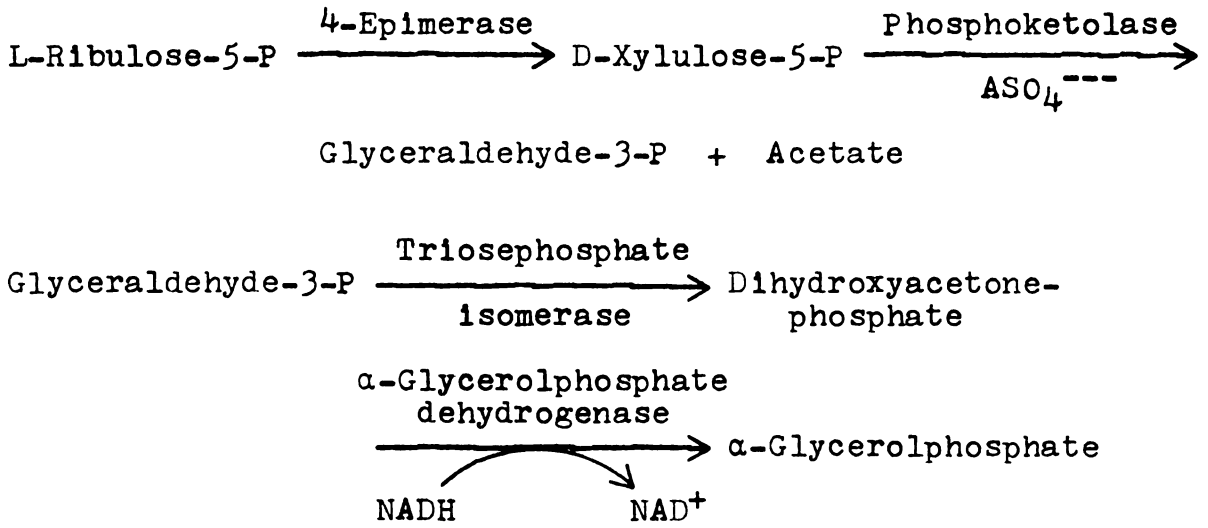
The L-ribulose-5-P was shown to be about 50% pure as determined enzymatically using an end point assay, wherein a limiting amount of L-ribulose-5-P was added to the 4-epimerase assay containing an excess of 4-epimerase. The amount of L-ribulose-5-P present was calculated from the total drop in 340 mμ absorbance as reported below for the 2-step assay. Quantitative assays for the amount of substrate produced are approximately 40% low due to the fact that the end point is only approached and never reached. Therefore the L-ribulose-5-P content of the barium precipitate obtained was determined chemically. A barium free solution of sugar was dephosphorylated with acid phosphatase in acetate buffer at pH 5.0. The supernatant was assayed for inorganic phosphate by the Fiske Subbarow method (85); for ribulose by the cysteine-carbazole method (83), and the orcinol method (86); and for L-ribulose-5-P concentration using the 4-epimerase assay. The substrate was found to be at least 70% pure by the chemical assay. D-Ribulose or D-xylulose were not detected in the supernatant using a D-ribuloreductase and D-xyluloreductase assay which should have detected a 6% or greater contamination of the D-ketopentose. The dephosphorylated sugar solution was deionized and chromatogramed using N-butanol:pyridine:water (6:4:3) and water saturated phenol solvents. Location of the sugars was detected by spraying with a mixture of orcinol, and trichloroacetic

acid in water saturated phenol (87) and with a mixture of dimethylphenaline and trichloracetic acid in water (88). Only one sugar was detected on chromatograms of the prepared sugar, and it migrated in both solvents with the same R_f as ribulose.

Enzymatic

L-Ribulose-5-P 4-Epimerase Assay

Continuous Assay: L-Ribulose-5-P 4-epimerase was routinely assayed at 28°C in a Gilford spectrophotometer using a minor modification of the continuous coupled assay previously reported by Anderson and Wood (76). The coupled assay is based on the following series of reactions:



The 0.2 ml reaction mixture contained 0.5 μmoles of L-ribulose-5-P, 0.1 μmole of thiamine pyrophosphate chloride, 1.0 μmole Na_2HAsO_4, 0.1 μmole NADH, 0.5 μmole of $MgCl_2$, 10 μmoles of imidazole buffer (pH 7.2) and an excess of α-glycerolphosphate

dehydrogenase, triosephosphate isomerase and D-xylulose-5-P phosphoketolase. The reaction was started by adding 4-epimerase. A molar extinction coefficient of 6.22×10^3 was used to convert the decrease in 340 mμ absorbance to μmoles of NAD^+ formed. This in turn is equivalent to μmoles of D-xylulose-5-P formed. Enzyme units are expressed as μmoles of D-xylulose-5-P formed/min. The continuous assay must be run at pH 7, since phosphoketolase is not active above this pH. However, it should be noted that the 4-epimerase has a pH optimum at 9.0 with 40% of the maximal activity at pH 7.0.

2-Step Assay: L-Ribulose-5-P 4-epimerase activity was also determined using a 2-step assay (4), which allowed the L-ribulose-5-P and 4-epimerase to be incubated together in the absence of other enzymes or cofactors. The 0.1 ml reaction mixture, containing 0.5 μmoles of L-ribulose-5-P and 5 μmoles of glycylglycine buffer (pH 8.0) was preincubated for 10 min. at 28°C. The reaction was started by adding 4-epimerase. Aliquots were removed at a given time and added to 2 μl of concentrated acetic acid to stop the 4-epimerase activity. The enzyme solution was heated in a boiling water bath for 1 min. and then neutralized with NH_4OH. The D-xylulose-5-P content, was determined by adding an aliquot of the reaction mixture to the remainder of the coupled 4-epimerase assay at 37°C and pH 7.2. The D-xylulose-5-P assay was always started by the addition of phosphoketolase. The decrease in absorbance due to dilution

was determined by running the assay without the addition of 4-epimerase. The D-xylulose-5-P content was determined from the decrease in 340 mμ absorbance minus the decrease in absorbance of the assay in the absence of enzyme. The decrease in absorbance was converted to μmoles as in the continuous assay. The rate of epimerization followed zero order kinetics up to 6 min. as determined by plotting μmoles of D-xylulose-5-P formed versus time.

The 2-step assay was used to determine the effect of metal ions on enzyme activity since all metals can be removed from the first step of the reaction mixture. To reduce the metal content of the assay to a negligible value, all glassware was soaked overnight in 3N HCl and rinsed with double quartz distilled water. The solutions were not allowed to come in contact with soft glass or metal. The reagents were stored in acid-washed polyethylene bottles. Tris-Hepes buffer was used, since Sigma's Tris has a negligible metal content, and Hepes buffer is a poor metal chelator (89). The pH of the L-ribulose-5-P was adjusted with Tris, and the buffer and the L-ribulose-5-P were passed through a chelex column to remove any contaminating metals. The Tris-Hepes buffer (pH 8.0) was used in the first step of the assay in place of glycylglycine buffer. No attempt was made to remove metals from the reagents used in assaying the amount of D-xylulose-5-P formed or in the continuous assay, since Mg^{++} is required by the phosphoketolase.

Glyceraldehyde-3-P Dehydrogenase Coupled Assay: An attempt was also made to assay L-ribulose-5-P 4-epimerase using a coupled assay containing phosphoketolase, and glyceraldehyde-3-phosphate dehydrogenase in the presence of AsO_4^{---}. A slower rate was always obtained using this system. Any contamination with triosephosphate isomerase resulted in a decrease in the rate of reduction of the NAD^+, presumably because of the turnover number of triosephosphate isomerase is 100 times faster than that of glyceraldehyde-3-P dehydrogenase. Thus the glyceraldehyde-3-P coupled assay was not used.

Phosphoketolase

D-Xylulose-5-P phosphoketolase was purified from Leuconostoc mesenteroides using a modification of Racker's (90) procedure as follows. The pH of the crude extract was adjusted to 4.55 with 1 M acetic acid at 0°C. The precipitate was removed by centrifugation, and the pH of the supernatant was readjusted to 6.0 with phosphate buffer. The enzyme was applied to a DEAE-cellulose column which had been equilibrated with 0.05 M phosphate buffer, pH 6.0 and 1 mM thioglycerol until the 280 mμ absorption of the eluate was less than 0.04. The phosphoketolase was eluted using a linear gradient between 0.1 M phosphate and 0.6 M phosphate buffer at pH 6.0 containing 1 mM thioglycerol. The enzyme was precipitated with 3 M ammonium sulfate. The precipitate was back extracted with 2.5 M,

2.0 M, and 1.5 M ammonium sulfate in 0.1 M phosphate buffer, pH 6.0 and 0.01 M mercaptoethanol. Most of the activity was recovered in the 2.0 M fraction and was stored frozen and used as such.

Determinations

Protein Determinations

The protein concentration was determined using the 280/260 ratio as described by Warburg and Christian (91). In preparations containing a very low 280/260 ratio the Lowry (92) procedure for protein determination was used as indicated. The specific activity is defined as units/mg of protein.

Polyacrylamide Gel Electrophoresis

Enzyme fractions were analysed by electrophoresis in 7.5% acrylamide gels according to the method of Davis (93) using square cross-section, optical quartz tubes (5 x 5 x 100 mm). The lower gel was electrophoresed in Tris-glycine buffer, pH 8.3, to remove the ultraviolet absorbing material prior to applying the enzyme. The enzyme was layered on the lower gel in 5% sucrose and electrophoresed in the same buffer at room temperature. The gels were scanned at 280 mμ in a Gilford spectrophotometer using a linear transport attachment, then removed from the tubes, stained with amido black, and scanned at 560 mμ.

In some experiments, the gels were sliced into 1.7 mm

sections using a lateral gel slicer (Canalco, Incorporated). The enzyme was eluted from the gel by incubation in 0.2 ml of 0.05 M phosphate buffer, pH 7.2, for 4 hours at 4°C. Aliquots were assayed for enzyme activity. The radioactivity was measured by washing the gel suspension into scintillation vials with water and counting in xylene-dioxane-cellosolve scintillation fluid (94) in a scintillation spectrometer.

Molecular Weight Determinations

The method of Yphantis (95) was used for high speed sedimentation equilibrium studies. The enzyme was sedimented in 12 mm double-sectored cells with sapphire windows in a Spinco Model E analytical ultracentrifuge which was equipped with a regulated temperature control unit and Rayleigh interference optics. The fringe displacement data were analyzed using the computer program of Small and Resnick (96) which had been modified for use on a Control Data 3600 computer. Density of the solvent was determined with a pycnometer.

Microbiological Assay for NAD^+

For quantitative determinations of nicotinic acid and NAD^+, the micorbiological assay of Snell (97) as described in the Difco Manual (98) was used. The extent of growth of L. arabinose was determined by measuring the turbidity at 660 mμ after incubating for 16 to 24 hours at

37°C. A standard curve was prepared from the extent of growth obtained with either nicotinic acid or NAD^+ run under similar conditions. The enzyme to be assayed was dialyzed overnight against water, and hydrolyzed for 30 min. in a boiling water in the presence of either 0.1 N NaOH or 0.1 N HCl. The samples were sterilized either by passage of 4-epimerase or hydrolysate through a millipore filter or by performing the hydrolysis under sterile conditions. As controls, NAD^+, glyceraldehyde-3-phosphate dehydrogenase, NADH, and bovine serum albumin were subjected to similar treatment.

Identification of ^{14}C-NAD^+

The ^{14}C NAD^+ from the u^-n^- mutant which was grown on ^{14}C nicotinic acid was partially purified and identified according to the procedure of Hagino and coworkers (99). The cells were suspended immediately after harvesting in a sufficient quantity of 70% perchloric acid to give a final concentration of 5% acid. The precipitate was removed by centrifugation and washed twice with 5% perchloric acid. The supernatant fluid and the washings were combined and adjusted to pH 7 with 5 M KOH. The potassium perchlorate was removed by centrifugation and the supernatant fluid was subjected to chromatography on a Dowex 1-X8 formate (200 to 400 mesh) column (1.5 x 20 cm). The ^{14}C compounds were eluted from the column with 75 mls of H_2O and 75 mls each of 0.05, 0.1, 0.25, 1.0, 2.0 and 4.0 M formic acid.

The fractions from each radioactive peak were combined and lyophilized. The residue was taken up in water and lyophilized two more times to remove the formic acid. The final residue from each radioactive peak was dissolved in water and mixed with cold NAD^+ and chromatographed in ascending 1 M ammonium acetate:ethanol (3:7), and 1-butanol:acetone:water (66:1.7:33), and in descending pyridine:water (2:1). In a like manner, the radioactive fractions were mixed with cold nicotinamide and chromatographed on thin layer plates using a chloroform:methanol:acetic acid (30:50:20), dioxane:acetic acid (100:1) and chloroform:methanol (90:10) solvents. The radioactive material was detected using a Packard Radiochromatogram scanner; the radioactivity of the paper chromatograms were also detected by audioradioaugraphy. The location of the cold NAD^+ and nicotinamide spots was determined by fluorescence produced by exposing to ultraviolet absorption.

RESULTS[3]

Rate of Growth And L-Arabinose Utilization By *Aerobacter Aerogenes*

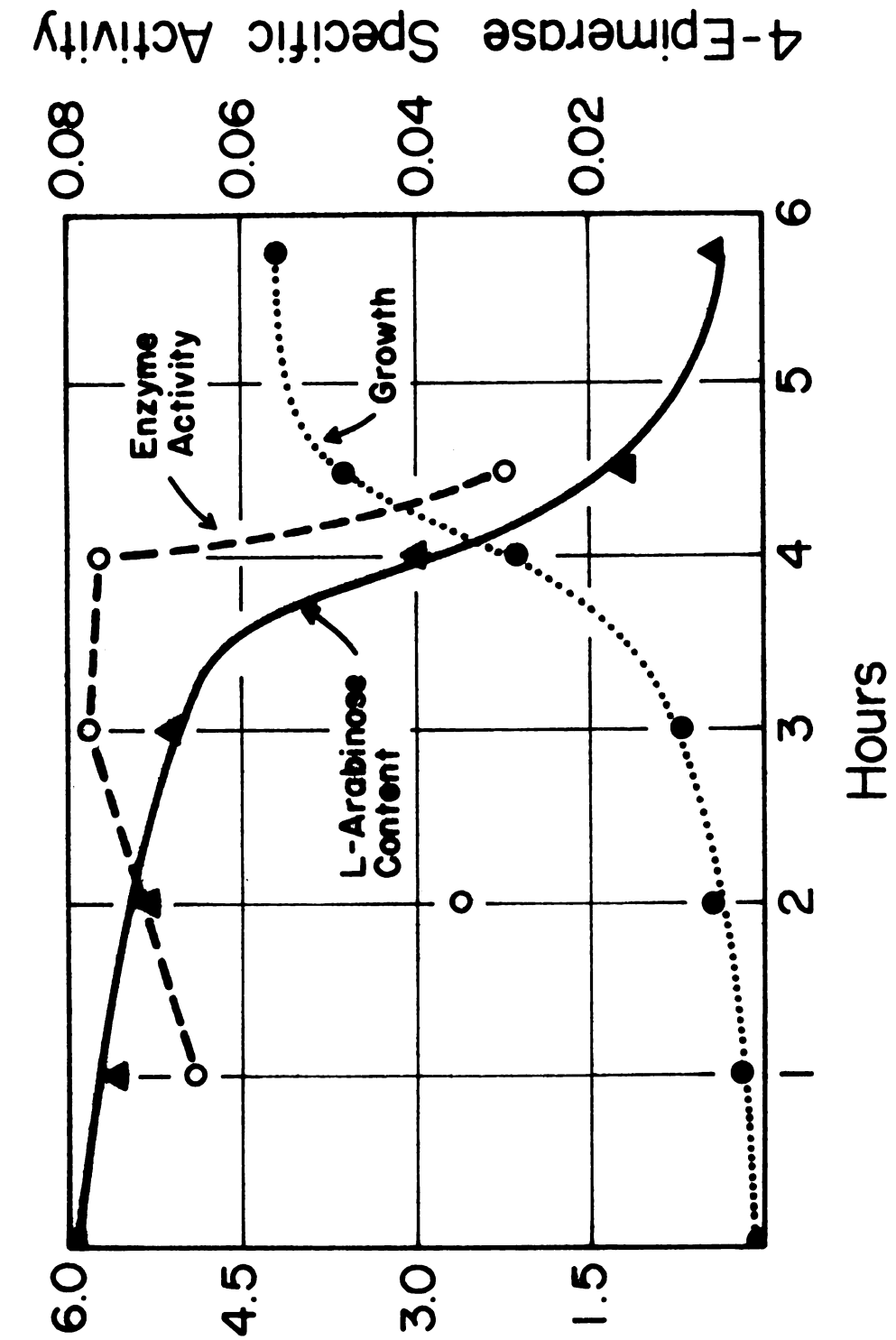
A study was made of the growth of *A. aerogenes* PRL-R3 as a function of L-arabinose utilization and 4-epimerase specific activity in order to determine the best time for harvesting the cells so as to obtain the maximum yield of 4-epimerase. A 100-liter culture of *A. aerogenes* was grown in the fermenter as described in Methods. Samples were withdrawn every hour, and the extent of growth was determined by measuring the turbidity at 600 mμ. The cells were removed by centrifugation and frozen, and the supernatant was assayed for L-arabinose content using the orcinol method (86). The frozen cells were subsequently thawed, diluted with two volumes of water, sonnicated, and assayed for 4-epimerase activity and for protein concentration.

As can be seen from Figure 3, the rate of L-arabinose utilization and the rate of drop in 4-epimerase specific activity followed the same curve. The bacteria reached the stationary phase as the L-arabinose content of the medium

[3]The purification, criteria of purity, molecular weight, all studies on the NAD^+ content, and the NAD^+ requirement of L-ribulose-5-P 4-epimerase are reported by J. D. Deupree and W. A. Wood in a paper to be published by Journal of Biological Chemistry.

Figure 3. The Rate of Growth of A. aerogenes PRL-R3 Versus L-Arabinose Utilization and 4-Epimerase Specific Activity

A. aerogenes PRL-R3 was grown in 100 liter quantities as described in Methods. Aliquots were withdrawn at the times indicated. The absorption at 660 mμ using a 1 cm light path was taken as a measure of growth. The cells were removed by centrifugation and assayed for 4-epimerase activity and protein concentration. The supernatant was assayed for L-arabinose content using the orcinol test (86).


Absorbance, 660 mμ
L-Arabinose Remaining (gm/liter)
6.0
4.5
3.0
1.5
0.08
0.06
0.04
0.02
4-Epimerase Specific Activity
1
2
3
4
5
6
Hours
Enzyme Activity
Growth
L-Arabinose Content

dropped below 0.1%. The 4-epimerase specific activity appeared to decrease during the last hour of the log phase. It should be noted, however, that 4-epimerase assays using crude extracts are always subject to large errors. This appears to be due to the large amount of NADH oxidase which is present in the extracts. As a control, the crude extracts were assayed in the absence of L-ribulose-5-P. The 4-epimerase activity was taken to be the difference between the rate of NAD^+ formation in the presence of L-ribulose-5-P versus the rate of NAD^+ formation in the absence of L-ribulose-5-P. In crude extracts 80% of the measurable NAD^+ formation was the result of NADH oxidase activity and 20% was due to 4-epimerase activity. Therefore, the level of 4-epimerase which can be added to the assay is at the lower level of activity which can accurately be measured. Thus, the validity of the 4-epimerase activity obtained for the crude extract is not known. The above experiment was only run once; however, the 4-epimerase activity values used were an average of more than one assay. In theory the 4-epimerase specific activity should have remained at the same level throughout most of the log phase, and the level should have decreased as the level of L-arabinose in the medium reached a minimum. The drop in the level of specific activity at 2-hours is unexplainable, and it was assumed that the level of 4-epimerase activity was actually higher, but was not measurable due

to the NADH oxidase activity present. Nevertheless, it can be reasonably assumed that to obtain the maximum amount of 4-epimerase the bacteria must be harvested such that the last of the cells are being recovered at the onset of the stationary phase. This assumption is further supported by the results of the following experiments.

The rate of growth of A. aerogenes PRL-R3 on 1-liter of minimal salts medium plus 0.5% L-arabinose was compared to that obtained with minimal salts, 1% casamino acid and 0.2% L-arabinose. The bacterial growth reached the same final level of absorbance in both cases. However, supplementing the medium with casamino acids shortened the growing time by 2-hours. The rate of L-arabinose utilization followed the rate of growth in both cases indicating that the L-arabinose was probably being used as a source of energy and the casamino acids as a source of amino acids.

The u^-i^- mutant was also grown on both mineral salts plus 0.5% L-arabinose and on minimal salts, 1% casamino acids and 0.2% L-arabinose. The results were the same as those obtained with the wild type, although a higher level of 4-epimerase was obtained by growth on 0.5% L-arabinose without casamino acids.

Purification of L-Ribulose-5-P 4-Epimerase

In order to study the reaction mechanism, it was first necessary to purify the enzyme to homogeneity.

Although a purification scheme had been worked out, it was not reproducible, and a new scheme had to be devised from which reasonable yields of 4-epimerase could be obtained in a reasonable period of time. A u^-i^- mutant was obtained after the purification had been worked out. The 4-epimerase specific activity of the crude extract from the mutant as well as the final yield of enzyme was three times higher than that obtained with wild type.[2] The 4-epimerases behaved identically during purification. Table 1 gives the results of a typical purification using the following purification steps.

Purification Steps

Crude Extract: One volume of A. aerogenes cells (u^-i^- mutant) was suspended in two volumes of cold distilled water (380 gm wet weight + 760 ml H_2O) and ruptured by treatment of 100-ml portions in a chilled 200-watt Raytheon sonicator for 20 min. Alternatively, the entire cell suspension was forced twice through a pre-chilled Manton-Gaulin laboratory homogenizer at 7000 psi. The suspension from either process was centrifuged at 20,000 x g for 20 min. and the pellet discarded. The supernatant solution was adjusted to pH 7.2 with ammonium hydroxide. The enzyme preparation was maintained at 4°C throughout the entire purification.

Chromatography on DEAE-Cellulose: The crude extract (59,500 mg/1100 ml) was stirred for 20 min. with approximately

Table 1. Typical Purification of L-Ribulose-5-P 4-Epimerase From A. aerogenes[a]

Step	Total Volume	Total Activity	Specific Activity	Recovery	Fold Purified
	ml	units	units/mg protein		
Crude extract	1100	1700[b]	0.029		
DEAE-Cellulose	820	1700	0.41	100	14
Ammonium sulfate back extraction	200	1200	0.40	70	14
Calcium phosphate gel	5.4	1200	3.0	70	102
Sephadex G-200	23	330	6.5	19	226
DEAE-Sephadex	8.5	320	13.0	19	445
First crystals[c]	5.9	104	12.0	6	445
Second crystals[d]			14.5		

[a]From 380 gm (wet weight) of cells.

[b]Although the assay indicated that the crude extract contained 600 units, it was assumed for the purpose of calculations that 1700 units were present.

[c]Performed on a small portion of the total fraction. The values shown for first crystals are those calculated for the total fraction.

[d]Performed on a portion of first crystals.

320 gm (dry weight) of DEAE-cellulose (Whatman DE-32), which had previously been washed with acid and base and equilibrated with 0.075 M phosphate buffer, pH 7.2. The DEAE-cellulose, with enzyme bound to it, was poured into a column (8.5 x 35 cm) and washed with 0.075 M phosphate buffer, pH 7.2, until the absorbance of the eluate at 280 mμ was less than 1.0. The protein was eluted from the column with a linear gradient prepared from 1500 ml each of 0.075 M and 0.50 M potassium phosphate buffer, pH 7.2. The 4-epimerase was eluted with approximately 0.35 M phosphate.

Ammonium Sulfate Back Extraction: The fractions from the DEAE-cellulose column containing 4-epimerase were pooled and ammonium sulfate and EDTA were added to 2.8 M and 10^{-3}M respectively. After stirring for one hour, the solution was centrifuged at 26,000 x g for 15 min. The resulting pellet was extracted with 2.0 M, 1.6 M, 1.1 M, and 0.5 M ammonium sulfate in 0.1 M phosphate buffer, pH 7.2, and 10^{-3}M EDTA by stirring with 40 ml of each ammonium sulfate solution for 30 min. followed by centrifugation at 26,000 x g for 15 min. About 70% of the 4-epimerase activity was extracted with the 1.1 M ammonium sulfate.

Calcium Phosphate Gel Fractionation: The ammonium sulfate fractions containing 4-epimerase were dialyzed for six hours against two 1-liter changes of distilled water. After diluting the protein with distilled water to 10 mg/ml,

calcium phosphate gel was added slowly at a ratio of 1 mg (dry weight) of gel per mg of protein and stirred for 20 min. The gel was pelleted by centrifugation and discarded. An additional 2 mg of gel per mg of protein were added to the supernatant solution; this adsorbed at least 90% of the activity. The 4-epimerase was eluted by washing the gel several times with 0.004 M phosphate buffer, pH 7.2.

Alternatively, the enzyme solution was dialyzed against 0.004 M buffer, and 3 mg of calcium phosphate gel per mg of protein were added. After removing the gel by centrifugation, the supernatant solution contained at least 90% of the 4-epimerase, and the same increase in specific activity was achieved.

The eluates from either procedure containing the 4-epimerase were combined, adjusted to 0.1 M phosphate concentration, pH 7.2, and solid ammonium sulfate added to 2.8 M. After stirring for 1 hour, the precipitate was removed by centrifugation and dissolved in a minimal volume of 0.05 M phosphate buffer, pH 7.2.

Chromatography on Sephadex G-200: The concentrated enzyme (400 mg) from the calcium phosphate gel step was dialyzed for 6 hours against three 1-liter changes of 0.05 M phosphate buffer, pH 7.2. The enzyme was applied to a column of Sephadex G-200 (2.5 x 100 cm) which had been equilibrated with 0.05 M phosphate buffer, pH 7.2. The column was developed with the same buffer, The

4-epimerase emerged after approximately 230 ml, and fractions containing most of the activity were pooled, precipitated with ammonium sulfate, and dialyzed as before against 0.05 M phosphate buffer, pH 8.0.

Chromatography on DEAE-Sephadex G-50: The enzyme (51 mg) was applied to a column of DEAE-Sephadex G-50 (50 to 150 mesh, 0.9 x 10 cm) which had been equilibrated with 0.05 M phosphate buffer, pH 8.0, and 0.05 M KCl. The enzyme was eluted with a linear KCl gradient in 0.05 M phosphate, pH 8.0, consisting of 40 ml each of 0.05 M and 0.40 M KCl. The distribution of the protein and the 4-epimerase activity in the eluate is shown in Figure 4.

Crystallization: To the combined fractions 106 to 124 from the DEAE-Sephadex column (Figure 4), solid ammonium sulfate was added to a concentration of 2.5 M. After stirring for one hour, the precipitate was collected by centrifugation at 26,000 x g for 15 min. The precipitate obtained was back-extracted with 2 ml each of 2.0 M, 1.8 M, 1.6 M, 1.4 M, and 1.2 M ammonium sulfate, pH 9.0, in 0.1 M glycine buffer, as before. After 24 hours at 0°C crystals appeared in the 1.6 M ammonium sulfate fraction. The fine colorless needle-shaped crystals as shown in Figure 5 contained no amorphous material. The crystals were washed with 1.8 M ammonium sulfate and dissolved in 0.05 M glycine buffer, pH 9.0, and assayed. The enzyme was recrystallized by back-extracting with ammonium sulfate

Figure 4. Elution of L-Ribulose-5-P 4-Epimerase from DEAE-Sephadex

The L-ribulose-5-P 4-epimerase was eluted from a DEAE-Sephadex column (0.9 x 10 cm) with a linear gradient consisting of 40 ml each of 0.05 M and 0.40 M KCl in 0.05 M phosphate buffer, pH 8.0. The 0.45-ml fractions were collected at 20-min intervals. The specific activity curve was obtained from the ratio of 4-epimerase activity to 280 mμ absorbance, both numbers being taken from the graph.

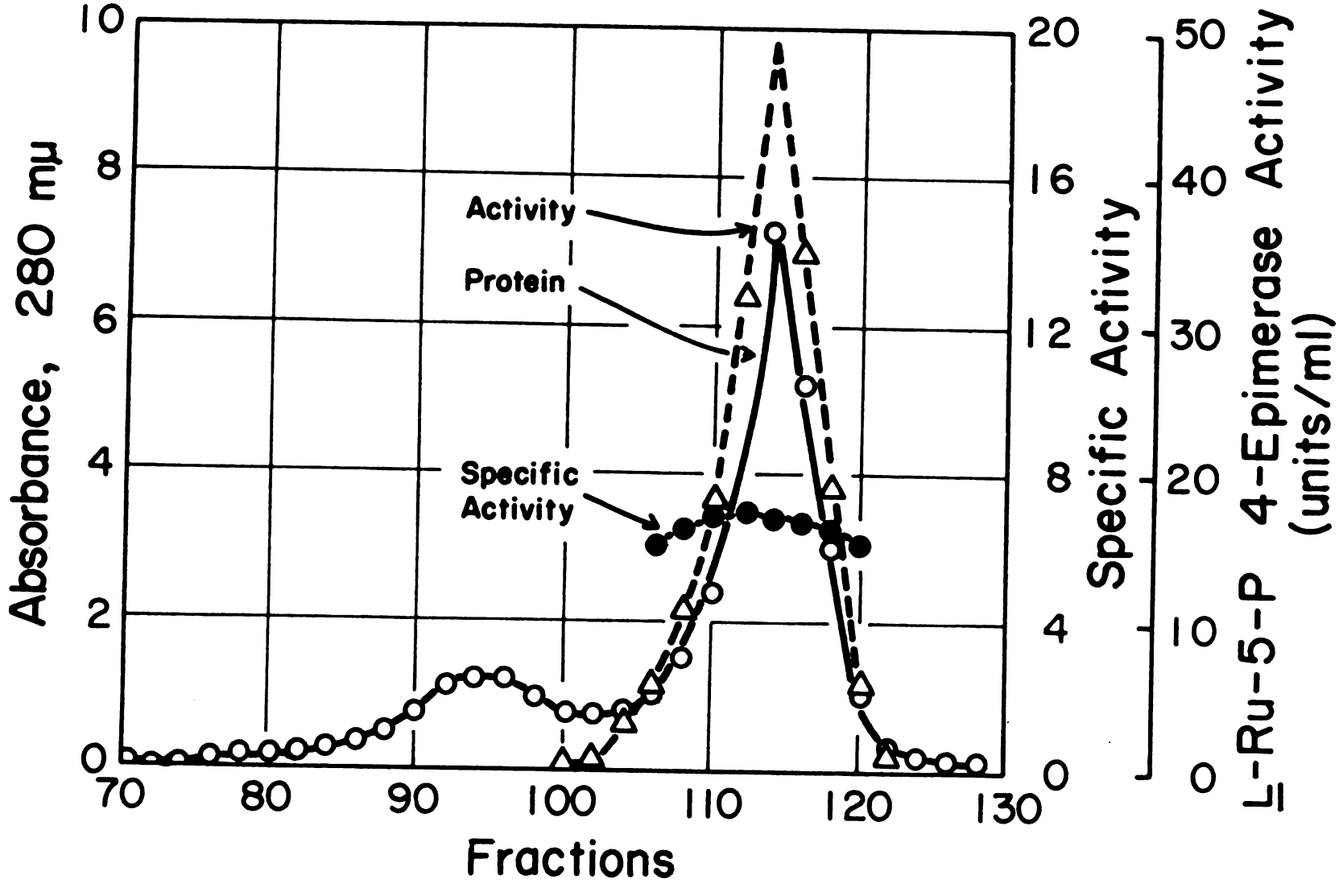

Absorbance, 280 mμ
Specific Activity
L-Ru-5-P 4-Epimerase Activity (units/ml)
Activity
Protein
Specific Activity
Fractions
0
2
4
6
8
10
0
4
8
12
16
20
0
10
20
30
40
50
70
80
90
100
110
120
130

Figure 5. Crystals of L-Ribulose-5-P 4-Epimerase in 1.6 M Ammonium Sulfate and 0.1 M Glycine Buffer, pH 9.0, at a Magnification of 4300x

as before. The needle-shaped crystals again appeared in the 1.6 M fraction.

Other Purification Steps Evaluated: In the course of developing a purification procedure for L-ribulose-5-P 4-epimerase, a number of purification steps were tried but were found to be of little value. The initial purification procedure required heating the enzyme to 60°C for 10 min. as the first step in the purification. It was subsequintly found that heating a partially purified enzyme solution resulted in a loss in specific activity. Thus the heat step causes an unnecessary loss in protein and does not accomplish any purification that is not accomplished by the DEAE-cellulose step. However, heating the enzyme to 60°C does denature the NADH oxidase present in the crude extracts. A reproducible assay of the crude extracts was thought to be obtained after the heat step had been carried out, although the level of activity was probably lower due to heat denaturation.

The acidity of the crude extracts was lowered to pH 5.0 with the loss of less than 15% of the activity, but essentially no increase in specific activity was obtained. When the pH was lowered to 4.5, more than 80% of the activity was lost, and the activity could not be recovered on readjusting the pH of either the supernatant or the resuspended precipitate to 7.0.

When the pH of the enzyme solution was raised to

10.0, less than 10% of the activity was lost, but an increase in specific activity was not obtained, and very little protein precipitated out of solution.

The addition of protamine sulfate to the crude extract following the heat step resulted in very little change in the 280/260 ratio. Since there is very little 260 absorbing material remaining after the DEAE-cellulose step, a protamine sulfate step was thought to be of little value.

Carboxymethyl cellulose bound less than 50% of a partially purified enzyme preparation which had been dialyzed against water. The carboxymethyl cellulose had been pre-equilibrated with 0.005 M phosphate buffer at pH 6.0, and it was added to the protein at a ratio of 1 gm (dry weight) of resin to 0.1 gm of protein.

A Sephadex G-200 column was found to give a better separation of protein than a G-100 column.

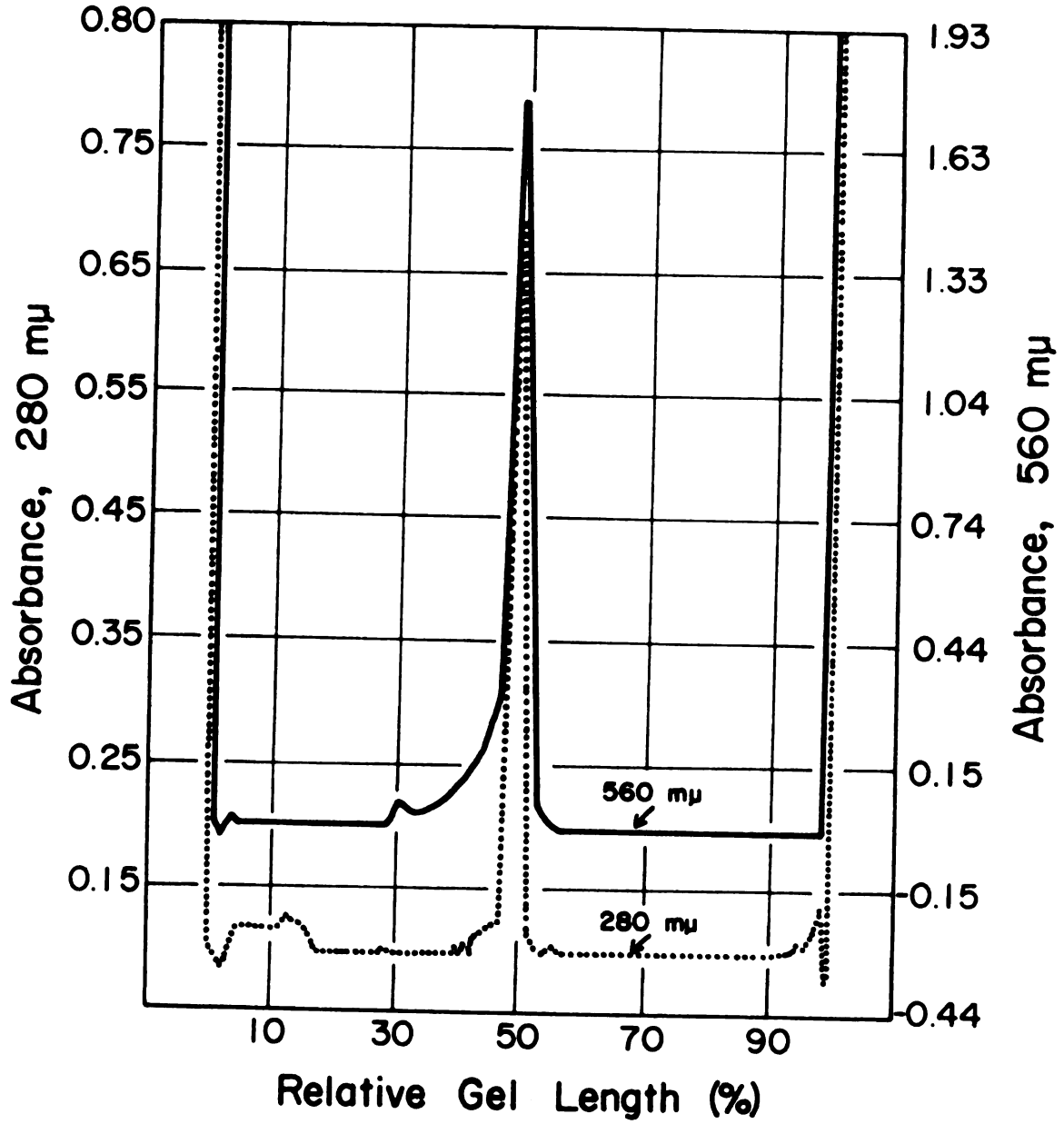
Criteria of Purity of the 4-Epimerase

The specific activity of the enzyme before and after crystallization and recrystallization was constant within experimental error and the same as that obtained from fractions 106 and 124 of the DEAE-Sephadex column (Figure 4).

The recrystallized enzyme was electrophoresed on polyacrylamide gel according to the procedure in Materials and Methods. As shown in Figure 6 only one protein band was detected by scanning at 280 mμ or by developing with

Figure 6. Tracings of Polyacrylamide Gels Containing Crystalline L-Ribulose-5-P 4-Epimerase

The 7.5% acrylamide gels in square quartz cuvettes (5 x 5 x 10 mm) were pre-electrophoresed in Tris-glycine buffer, pH 8.3, prior to applying the protein. Crystalline L-ribulose-5-P 4-epimerase (85 ug) in 5% sucrose was layered on the gel and electrophoresed at room temperature. The gels were scanned at 280 mμ, developed with amido black, and scanned again at 560 mμ in a Gilford spectrophotometer with a linear transport attachment. The tracings were redrawn and normalized to fit on the same axes.


0.80
0.75
0.65
0.55
0.45
0.35
0.25
0.15
Absorbance, 280 mμ
1.93
1.63
1.33
1.04
0.74
0.44
0.15
-0.15
-0.44
Absorbance, 560 mμ
560 mμ
280 mμ
10
30
50
70
90
Relative Gel Length (%)

amido black. A similar unstained gel was sliced and assayed for activity as described in Materials and Methods. L-Ribulose-5-P 4-epimerase was found only in the slices corresponding to the 280 mμ absorbing band. Similar results were obtained with the enzyme after purification through the DEAE-Sephadex step. When enzyme (specific activity, 13.0) was electrophoresed in the same manner at pH 7.0 in Tris-diethylbarbituric acid buffer (100), only one band was detected. A similar run at pH 10.0 in 0.215 M potassium glycinate buffer showed a minor 280 mμ absorbing peak accounting for less than 3% of the protein. High speed equilibrium ultracentrifugation of the purified enzyme resulted in the distribution of only one molecular weight species across the cell, indicating homogeneous protein as discussed below. A specific activity of 13.0 ± 10% at pH 7.2 and 28°C was obtained with the pure enzyme. As will be discussed later, this activity can be increased up to 5.4 fold by incubating with the appropriate cations.

Other Characteristics of Homogeneous L-Ribulose-5-P 4-Epimerase

Molecular Weight of the 4-Epimerase

The high speed equilibrium analysis of Yphantis (95) was used to determine the molecular weight of the L-ribulose-5-P 4-epimerase. A 50 μg sample of the enzyme (electrophoretically pure) was subjected to high speed ultracentrifugation as described in Methods. From the

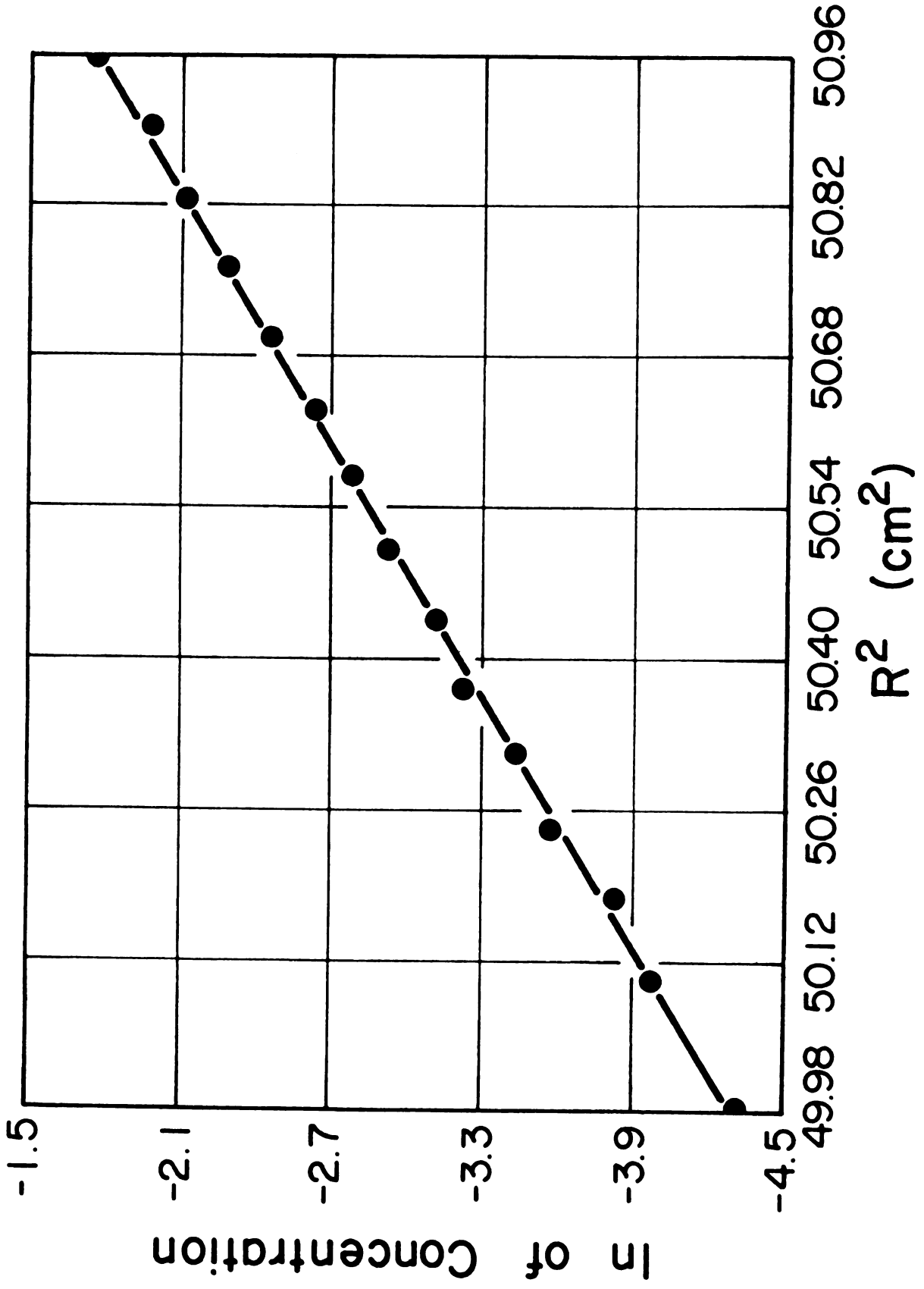
slope of the plot of ln of concentration versus the square of the distance from the center of the cell (Figure 7), and assuming a partial specific volume of 0.75, the weight average molecular weight over the entire cell was calculated to be $1.14 \times 10^5 \pm 1.4 \times 10^3$. The lack of additional molecular weight species on both ultracentrifugation and polyacrylamide gel electrophoresis indicates that the enzyme exists as a single molecular weight species under the conditions used.

NAD^+ Content of L-Ribulose-5-P 4-Epimerase

In studying the mechanism of L-ribulose-5-P 4-epimerase, it was first necessary to determine if L-ribulose-5-P 4-epimerase was similar to UDP-glucose 4-epimerase. The mechanism of UDP-glucose 4-epimerase has been studied extensively and shown to utilize an oxidation-reduction mechanism involving NAD^+ (11, 12). Thus if the mechanism of L-ribulose-5-P 4-epimerase is similar, one would expect to find NAD^+ either tightly bound to the enzyme or its addition to the assay mixture required for enzyme activity. To answer this question a series of experiments were performed to detect bound NAD^+ on the purified enzyme. These included a microbiological assay for nicotinic acid or NAD^+, determination of the ^{14}C content of purified 4-epimerase isolated from a nicotinic acid-requiring mutant grown on ^{14}C-nicotinic acid, and the measurement of the fluorescence

Figure 7. Molecular Weight Determination by Sedimentation Equilibrium

L-Ribulose-5-P 4-epimerase (50 μg; specific activity, 13.0) dialyzed against 0.05 M phosphate buffer, pH 7.2, and 0.2 M KCl overnight was used. The points were obtained as described in the text from a computer analysis of a 24-hour run at 20°C with a rotational velocity of 2.0396×10^4 RPM. The buffer density was 1.0161 and a partial specific volume of 0.75 was assumed.


ln of Concentration
-1.5
-2.1
-2.7
-3.3
-3.9
-4.5
49.98
50.12
50.26
50.40
50.54
50.68
50.82
50.96
R2 (cm2)

and absorption spectra. To determine if the reaction required added NAD^+, homogeneous enzyme was assayed in the presence and absence of added NAD^+.

Microbiological Assay for Nicotinic Acid and NAD^+

Lactobacillus arabinosus requires nicotinic acid for growth, although it can use NAD^+ or NADH as a source of nicotinic acid. Therefore, if L-ribulose-5-P 4-epimerase contains NAD^+, *L. arabinosus* should be able to use the enzyme or its hydrolysate as a source of NAD^+.

To test this possibility homogeneous L-ribulose-5-P 4-epimerase (specific activity, 13.0) was subjected to a microbiological assay for nicotinic acid using the conditions described under Materials and Methods (Table 2). With or without hydrolysis of the L-ribulose-5-P 4-epimerase the bacterial growth was always only slightly above background (Table 2). The enzyme contained less than 0.1 mole of NAD^+ per mole of 4-epimerase based upon a molecular weight of 1.4×10^5. One mole of NAD^+ per mole of 4-epimerase should have been readily detectable, since between 1.7×10^{-3} and 3.5×10^{-3} μmoles of enzyme were used, and the lower detection limit of the assay is 4.0×10^{-4} μmoles of NAD^+. To insure that the growth of *L. arabinosus* was not blocked by the presence of treated epimerase, NAD^+ was added to some of the assays as an internal standard prior to the hydrolysis. Under these conditions, the bacterial growth was only slightly higher than that obtained with

Table 2. NAD^+ Content of L-Ribulose-5-P 4-Epimerase Determined by a Microbiological Assay

L-Ribulose-5-P 4-epimerase (3.5×10^{-3} μmoles; specific activity, 13.0) was dialyzed against distilled water and was boiled for 30 min in either 0.1 N HCl or 0.1 N NaOH in the presence and absence of 7.5×10^{-4} μmoles of NAD^+. After neutralization, aliquots were assayed microbiologically for nicotinic acid as described in Materials and Methods. Standard curves were prepared using graded levels of NAD^+ treated under the same conditions. Glyceraldehyde 3-phosphate dehydrogenase and bovine serum albumin were also treated and assayed in a similar manner.

Protein	Treatment	Amount of Protein Assayed	NAD^+ Content
		μmoles	μmoles/μmole of protein
L-Ribulose-5-P 4-epimerase	None	2.9×10^{-3}[a]	3.5×10^{-2}
L-Ribulose-5-P 4-epimerase	0.1 N HCl	3.5×10^{-3}	4.3×10^{-2}
L-Ribulose-5-P 4-epimerase	0.1 N NaOH	3.5×10^{-3}	4.3×10^{-2}
L-Ribulose-5-P 4-epimerase + NAD (7.5 μmoles)	0.1 N HCl	3.5×10^{-3}	4.3×10^{-2}[b]
L-Ribulose-5-P 4-epimerase + NAD (7.5 μmoles)	0.1 N NaOH	3.5×10^{-3}	1.3×10^{-1}[b]
Glyceraldehyde 3-phosphate dehydrogenase	None	3.4×10^{-4}[c]	3.3

Glyceraldehyde 3-phosphate dehydrogenase	0.1 N NaOH	3.4×10^{-4}	3.5
Bovine serum albumin	None	1.5×10^{-2}[d]	0.0
Bovine serum albumin	0.1 N NaOH	1.5×10^{-2}	0.0

[a]Based on a molecular weight of 1.14×10^5.

[b]NAD^+ content in excess of that added as internal standard.

[c]Based on a molecular weight of 1.40×10^5 (101).

[d]Based on a molecular weight of 6.65×10^4 (101).

NAD^+ alone. Similar assays revealed the presence of 3.3 moles of NAD^+ per mole of glyceraldehyde-3-phosphate dehydrogenase with or without hydrolysis with acid or base. NAD^+ was not detected in the bovine serum albumin.

NAD^+ Content of the 4-Epimerase After Growth on ^{14}C-Nicotinic Acid

If L-ribulose-5-P 4-epimerase contained NAD^+, it would be predicted that the growth of a nicotinic acid-requiring mutant on ^{14}C-nicotinic acid would yield radioactive 4-epimerase.

Preliminary to this experiment it was necessary to (a) determine the minimum amount of nicotinic acid required by the organism for growth and (b) ascertain that the nicotinic acid mutant was not producing an altered 4-epimerase.

A minimum of 10^{-5}% nicotinic acid was required for growth of the n^-u^- mutant as determined by growing the mutant on graded levels of nicotinic acid. Cultures were also grown in 1-liter quantities in the presence of 10^{-5}% nicotinic acid. After reaching stationary phase a sample of the culture was removed, diluted, and plated on minimal salts-L-arabinose agar with and without nicotinic acid. The organism was still dependent on nicotinic acid for growth and, thus, had not reverted to the wild type. The culture was centrifuged under aseptic conditions, and the cells were used as a source of 4-epimerase. The supernate

was assayed for nicotinic acid content using the microbiological assay and found to contain only 10% of the added nicotinic acid. Thus, 10^{-5}% nicotinic acid is sufficient to prevent the selection of revertants and also allows most of the nicotinic acid to be consumed during growth.

The 4-epimerase was purified 43-fold from the n^-u^- cells obtained and was shown by polyacrylamide gel electrophoresis to be similar or identical in electrophoretic behavior to that of the u^-i^- mutant.

To obtain ^{14}C-labeled NAD^+, the u^-n^- mutant was grown on two liters of minimal medium plus L-arabinose supplemented with 10^{-5}% nicotinic acid (carboxyl-^{14}C) (specific activity, 59.1 mC/mmole), as described in Materials and Methods. The 4-epimerase was purified as before, and the activity and ^{14}C content measured for each step (Table 3). The ratio of ^{14}C to 4-epimerase was initially very high, but decreased to approximately 1.0 following chromatography on DEAE-cellulose, ammonium sulfate fraction, and treatment with calcium phosphate gel. Although there was considerable overlap, the ^{14}C and 4-epimerase eluted from the DEAE-Sephadex columns (Figure 8) at different salt concentrations. In fractions 61 through 67, the molar ratio of ^{14}C to 4-epimerase was less than 0.15. Fraction 61 was then electrophoresed on polyacrylamide gel. After scanning at 280 mμ and 260 mμ, the gel was sliced and the enzyme eluted. Figure 9 indicates that the 4-epimerase

Table 3. Purification and ^{14}C Content of L-Ribulose-5-P 4-Epimerase After Growth of the u^-n^- Auxotroph on Nicotinic Acid (Carboxyl-^{14}C)

Purification Step	Total Activity	Specific Activity	Total Radioactivity	^{14}C Nicotinic Acid[a] / 4-Epimerase[b]
	units	units/mg protein	dpm	mole/mole
Crude extract	20.2	0.021	9.35×10^7	52.8
DEAE-Cellulose	35.0	0.35	3.16×10^6	1.03
Ammonium sulfate back extraction	25.0	0.47	1.57×10^6	0.71
Calcium phosphate gel	19.8	1.05	1.24×10^6	0.71
DEAE-Sephadex I	6.91	3.00	6.08×10^4	0.10
DEAE-Sephadex II	2.7	9.2	2.06×10^3	0.01

[a] $\text{mmoles } ^{14}C\text{-nicotinic acid} = \text{total dpm} \times \frac{1 \text{ mC}}{2.2 \times 10^9 \text{ dpm}} \times \frac{1 \text{ mmole}}{59.1 \text{ mC}}$

[b] $\text{mmoles 4-epimerase} = \text{total units} \times \frac{1 \text{ mg}}{13 \text{ units}} \times \frac{1 \text{ mmole}}{1.14 \times 10^5 \text{ mg}}$

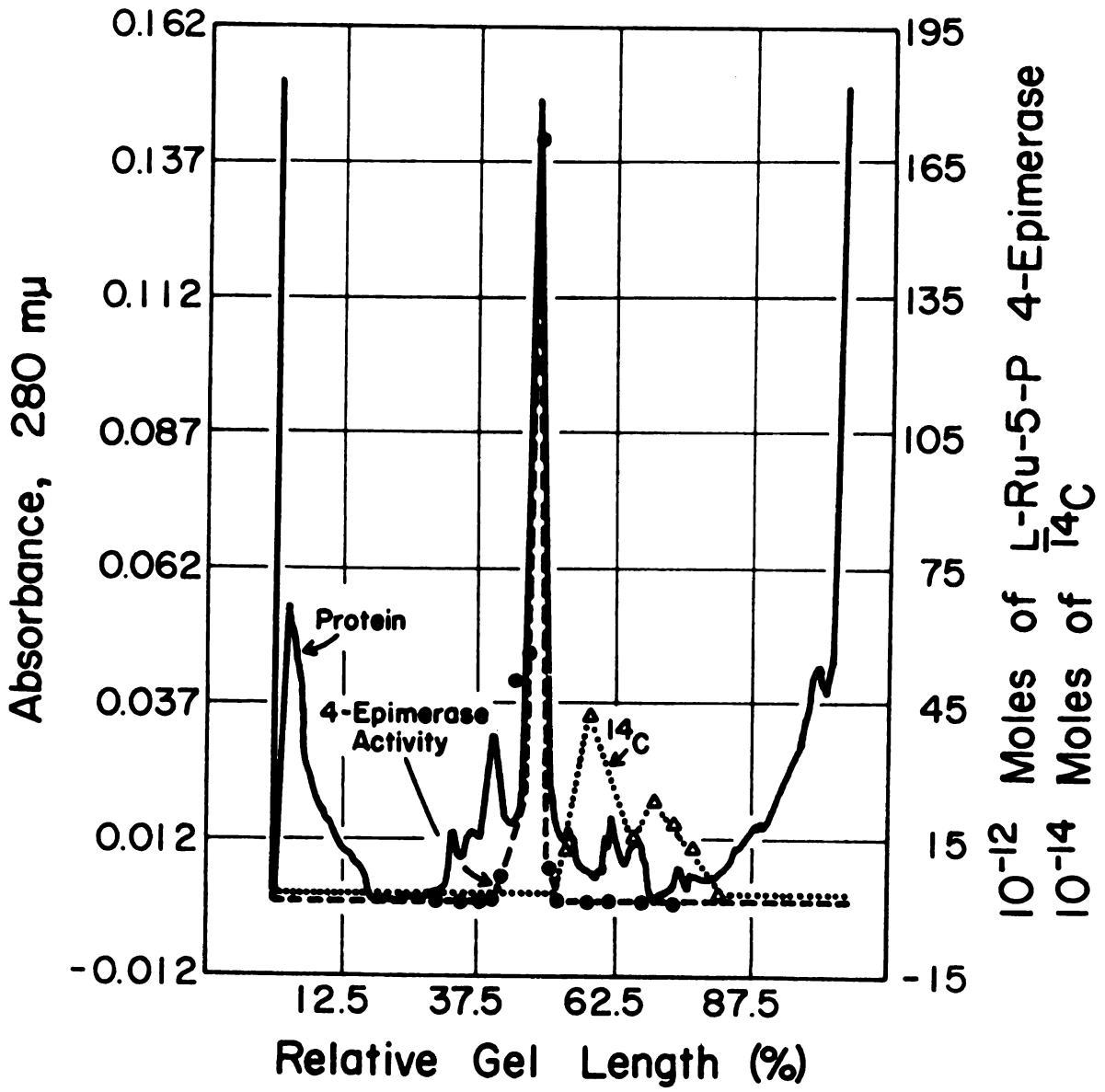
Figure 8. Separation of L-Ribulose-5-P 4-Epimerase and ^{14}C on a Column of DEAE-Sephadex

The protein purified through the calcium phosphate gel step (Table 3) was applied to a DEAE-Sephadex column and eluted as in Figure 1.

Absorbance, 280 mμ
2.5
2.0
1.5
1.0
0.5
0
40
45
50
55
60
65
70
Fractions
15
12
9
6
3
0
(cpm/ml) x 10⁻⁴
10
8
6
4
2
0
L-Ru-5-P 4-Epimerase Activity
(units/ml)
Enzyme Activity
¹⁴C
Protein

Figure 9. Separation of ^{14}C and L-Ribulose-5-P 4-Epimerase by Electrophoresis on Polyacrylamide Gel

The experimental procedure was the same as for Figure 6. After scanning at 280 mμ, the center of the gel was sliced into 2-mm sections and the enzyme was eluted from each slice by incubation in 0.2 ml of 0.05 M phosphate buffer, pH 7.2, for 4 hours at 4°C. Aliquots were assayed for 4-epimerase activity, and the remainder used for radioactivity determinations.


0.162
0.137
0.112
0.087
0.062
0.037
0.012
-0.012
Absorbance, 280 mμ
195
165
135
105
75
45
15
-15
10-12 Moles of L-Ru-5-P 4-Epimerase
10-14 Moles of 14C
Protein
4-Epimerase Activity
14C
12.5
37.5
62.5
87.5
Relative Gel Length (%)

activity and remaining ^{14}C were completely separated by the electrophoresis.

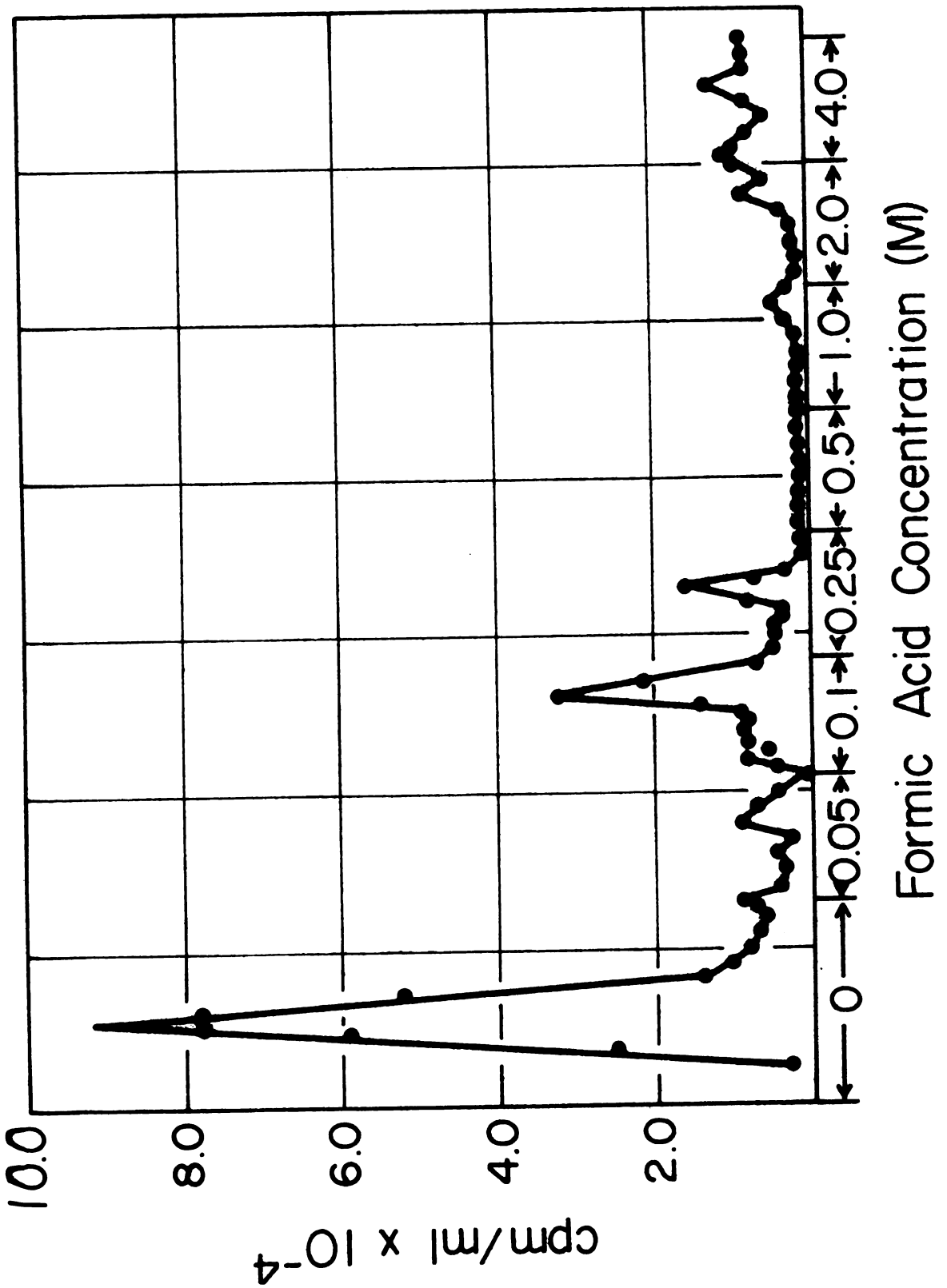
Fractions 61 through 67 from the DEAE-Sephadex column were combined, dialyzed against 0.05 M phosphate (pH 8.0) and 0.15 M KCl, and applied to a second DEAE-Sephadex G-50 column (9.9 x 10 cm) which had been pre-equilibrated with 0.05 M phosphate buffer, pH 8.0, and 0.15 M KCl. The enzyme was eluted with a 40 ml linear salt gradient between 0.15 M and 0.6 M KCl in 0.05 M phosphate, pH 8.0. The molar ratio of ^{14}C to 4-epimerase was 0.01 for the combined 4-epimerase fractions from the DEAE-Sephadex column.

Although the assumption that ^{14}C nicotinic acid would be incorporated into NAD^+ seemed reasonable, the above experiment was further validated by demonstrating that ^{14}C-nicotinic acid was converted to ^{14}C-NAD^+ under the growth conditions employed. To test for ^{14}C-NAD^+ in the cells, 10% of the cells grown on ^{14}C-nicotinic acid were suspended immediately after harvesting in 5% perchloric acid. The radioactive material was recovered and chromatographed on a Dowex-1-Formate column as described in Materials and Methods. The ^{14}C elution profile is shown in Figure 10.

The lyophilized residue from the major radioactive peak of the H_2O, 0.1, and 0.25 M formic acid eluates were mixed with either cold NAD^+ or cold nicotinamide and

Figure 10. Dowex-1-Formate Elution Profile of the Perchloric Acid Treated Cells of the n^-u^- Mutant Which Was Grown on ^{14}C Nicotinic Acid

The perchloric acid-treated cells of the n^-u^- mutant which was grown on ^{14}C nicotinic acid was applied to a (1.5 x 20 cm) Dowex 1-8X Formate (200 to 400 mesh) column as described in Methods. The ^{14}C nicotinamide derivatives were eluted with 75 ml of H_2O and 75 ml each of 0.05, 0.1, 0.25, 1.0, 2.0 and 4.0 M formic acid. Fractions of 5 ml were collected at 15 min intervals.


cpm/ml x 10-4
10.0
8.0
6.0
4.0
2.0
0
0.05
0.1
0.25
0.5
1.0
2.0
4.0
Formic Acid Concentration (M)

chromatographed as described in Materials and Methods. As shown in Table 4, the NAD^+ ultraviolet absorbing material migrated in all the solvents used with approximately the same R_f as one of the radioactive spots from the 0.25 M formic acid eluate. To further assure that the ultraviolet absorption and the radioactivity on the paper chromatogram coincided, the chromatograms were exposed to x-ray film. One of the radioactive spots in all three solvents of the chromatograms of the 0.25 M formic acid residue corresponded to the NAD^+ fluorescent spot in both shape and location. Since nicotinamide is a degradation product of NAD^+ the presence of ^{14}C nicotinamide in the 0.1 M formic acid fractions, as shown in Table 5, further confirmed the presence of ^{14}C NAD^+ in the u^-n^- cells grown on ^{14}C nicotinic acid.

Absorption Spectrum of the 4-Epimerase

An indication of cofactors tightly bound to an enzyme surface can, in many cases, be obtained from the light absorption characteristics of the protein. Enzymes containing NAD^+ usually have a 280/260 ratio around 1.0 due to the 260 absorption of the adenine moiety. A 280/260 ratio of 1.3 would be expected for L-ribulose-5-P 4-epimerase containing 1 mole of NAD^+/mole of protein, based on a molar extinction of 18.0×10^3 at 259 mμ and a 280/260 ratio of 0.234 for NAD^+, and assuming pure protein has a 280/260 ratio of 1.6. However a 280/260 ratio of 1.78 was

Table 4. Identification of ^{14}C NAD^+ by Paper Chromatography

The lyophilized residue of the major radioactive peaks of the H_2O, 0.1 M and 0.25 M formic acid eluates of the Dowex-1-formate column (Figure 10) were mixed with cold NAD^+ and chromatogramed in ascending 1 M ammonium acetate: ethanol (3:7) and 1-butanol:acetone:water (66:1.7:33), and in descending pyridine:water (2:1). The radioactive material was detected using a Packard Radiochromatogram Scanner, and the location of the NAD^+ was determined by the fluorescence produced on exposing to ultraviolet light.

Residue from the Dowex-1-Formate Column Eluate (Fig. 10)	Thin Layer Chromatography Solvent	R_f of Ultraviolet Absorbing Spot	R_f of ^{14}C Spot
H_2O	Ammonium acetate:Ethanol	0.29	0.58 0.81
0.1 M Formic Acid	Ammonium acetate:Ethanol	0.22	0.26 0.32
0.25 M Formic Acid	Ammonium acetate:Ethanol	0.23	0.27 0.82
Water	Butanol:Acetone:Water	0.36	0.38 0.64
0.1 M Formic Acid	Butanol:Acetone:Water	0.39	0.16 0.25

0.25 M Formic Acid	Butanol:Acetone:Water	0.40	0.26 0.44 0.83
0.1 M Formic Acid	Pyridine:Water	0.58	0.61 0.31 0.72 0.81
0.25 M Formic Acid	Pyridine:Water	0.59	0.63 0.73

Table 5. Identification of ^{14}C Nicotinamide on Thin Layer Chromatography

The lyophilized residue from the major radioactive peaks of the H_2O and 0.1 M formic acid eluates of the Dowex-1-formate column (Figure 10) were mixed with cold nicotinamide and chromatogramed on thin layer plates using chloroform: methanol:acetic acid (30:50:20), dioxane:acetic acid (100:1) and chloroform: methanol (90:10) solvents.

Residue from the Dowex-1-Formate Column Eluate (Fig. 10)	Thin Layer Chromatography Solvent	R_f of Ultraviolet Absorbing Spot	R_f of ^{14}C Spot
H_2O	Chloroform:methanol: acetic acid	0.40	0.26 0.46
0.1 M Formic Acid	Chloroform:methanol: acetic acid	0.76	0.76
H_2O	Dioxane:acetic acid	0.60	0.28 0.57
0.1 M Formic Acid	Dioxane:acetic acid	0.59	0.59
H_2O	Chloroform:methanol	0.29	0.20
0.1 M Formic Acid	Chloroform:methanol	0.47	0.47

obtained for 4-epimerase preparation (electrophoretically pure) which had been dialyzed overnight against 0.05 M phosphate buffer pH 8.0.

An epimerase solution at 5.5 mg/ml (4.8×10^{-5}M) was scanned between 700 mμ and 310 mμ at a full scale deflection of 0.1 A. The divergencies were less than 0.02 A of a scan of bovine serum albumin under identical conditions. With these conditions any cofactor with a molar extinction coefficient greater than 4.2×10^{2} would have been detectable.

Fluorescence Measurements on the 4-Epimerase

UDP-Glucose 4-epimerase isolated from yeast has a fluorescence excitation and emission spectra characteristic of NADH (50) even though other studies have shown that NAD^{+} was bound to the 4-epimerase. In preliminary experiments, a solution containing 2 ml of 0.05 M glycylglycine buffer and 10 mmoles of L-ribulose-5-P 4-epimerase was excited at 350 mμ. The fluorescence at 450 mμ was the same as background. Using 10 mmoles of NADH, a strong fluorescence emission at 465 mμ was obtained and a characteristic 15 mμ shift (50) in fluorescence to 450 mμ was observed when 8 nmoles of NADH were added to 2 nmoles of crystalline lactic dehydrogenase under the conditions used with the 4-epimerase.

Absorption Spectrum of the 4-Epimerase-Substrate Complex

If bound NAD^+ were reduced during the course of the enzyme reaction, it is possible that there would be a sufficient steady state level of NADH on the 4-epimerase to produce an absorption peak in the 340 mμ region when the enzyme was incubated with substrate. Such a peak was obtained by Wilson and Hogness (8) for the UDP-galactose 4-epimerase from E. coli. In similar experiments, the absorption spectrum in the 340 mμ region was determined for L-ribulose-5-P 4-epimerase using a Cary spectrophotometer equipped with double compartment microcuvettes. A solution of 9.15×10^{-6}M L-ribulose-5-P 4-epimerase, and 4.3×10^{-4}M L-ribulose-5-P in 0.05 M glycylglycine buffer, pH 8.5, was used in one compartment of the sample cuvette and buffer was added to the second. The same concentrations of enzyme and substrate were placed in separate compartments of the reference cuvette. There was no detectable increase in the 340 mμ absorption. Increases in absorption of less than 0.01 A were considered unreliable. Thus, at the epimerase level used, and assuming one NAD^+ per epimerase molecule, a reduction of more than 20% of the NAD^+ would have been required to be detectable.

The Effect of NAD^+ on 4-Epimerase Activity

The possibility exists that NAD^+ functions in the reaction mechanism, and that NAD^+ is furnished in the assay by the NADH preparation used or by contaminating proteins

added with the coupling enzyme. Accordingly, a two-step assay was performed as described in Materials and Methods wherein L-ribulose-5-P was incubated with the 4-epimerase, and aliquots were removed for enzymatic determination of the amount of D-xylulose-5-P produced. To avoid the possibility of any protein containing NAD^+ being added to the assay, crystalline 4-epimerase was used. The first four lines of Table 6 show that the formation of D-xylulose-5-P occurs in the absence of NAD^+ and that the rate is approximately equivalent to that observed in the coupled assay using 0.05 M imidazole buffer, pH 7.2, at 28°C for both assays. The addition of NAD^+, NADH, $NADP^+$, and NADPH, as well as a 1-hour pre-incubation with NAD^+, did not alter the rate. Similar results were obtained with 4-epimerase (specific activity, 3.0) which had been purified from ^{14}C-nicotinic acid-grown u^-n^- mutant and freed of ^{14}C by electrophoresis on polyacrylamide gel. In addition, the enzyme was not inhibited by a 30 min. incubation with NADH, as determined by assaying in the coupled assay.

Other Studies on the Mechanism of Action of L-Ribulose-5-P 4-Epimerase

General Discussion

The preceeding results indicate that 4-epimerization of L-ribulose-5-P and D-xylulose-5-P does not involve an oxidation-reduction mechanism which requires NAD^+; however, another electron acceptor could be utilized, or an entirely

Table 6. The Effect of Pyridine Nucleotides on Rate of L-Ribulose-5-P 4-Epimerization

The 0.2 ml reaction mixture contained 5 μmoles of glycylglycine buffer, pH 8.5; 10 μmoles of L-ribulose-5-P, and additions of pyridine nucleotides and crystallized 4-epimerase (specific activity, 13.0) as noted in the Table. After preincubation at 37°C, 4-epimerase was added to initiate the reaction and 50 μl aliquots were withdrawn at 1, 2, 4, and 6 minutes, added to acetic acid, heated in a boiling water bath for one minute, and neutralized with ammonium hydroxide prior to assaying for D-xylulose-5-P. The D-xylulose-5-P concentration was calculated from the decrease in absorbance at 340 mμ as described under Materials and Methods.

Additions	Concentration	4-Epimerase (units x 10^3)	
		Added[a]	Found[b]
--	--	3.8	6.6
--	--	7.7	8.3
--	--	9.6	9.9
--	--	19.2	19.0
NAD^+	10^{-3}	9.6	9.2
NAD^+	10^{-6}	9.6	9.7
NADH	10^{-3}	9.6	11.5
NADH	10^{-6}	9.6	10.3
$NADP^+$	10^{-3}	9.6	9.3
$NADP^+$	10^{-6}	9.6	9.3
NADPH	10^{-3}	9.6	9.0
NADPH	10^{-6}	9.6	10.2
NAD^+[c]	10^{-6}	9.6	6.6

[a]Based upon continuous assay at pH 7.2 and 28°C.

[b]Based upon two step assay at pH 8.5 and 37°C.

[c]The L-ribulose-5-P 4-epimerase was incubated with the NAD^+ and glycylglycine buffer for one hour. The reaction was started by adding L-ribulose-5-P to the incubation mixture.

different mechanism could be involved. There are 4-different means by which the enzymatic epimerization of L-ribulose-5-P and D-xylulose-5-P could be accomplished. These include:

1. An oxidation-reduction mechanism catalyzed by an electron carrier other than NAD^+.
2. Sn_2 (Waldenase) inversion involving the displacement of the C-4 hydroxyl group by a hydroxyl group of water.
3. A dealdolization-aldolization mechanism involving carbon-carbon bond cleavage between C-3 and C-4.
4. Base catalyzed dehydration between C-3 and C-4.

Enzymatic electron acceptors, which would facilitate oxidation at C-4 of the pentose, include the B_{12}-coenzyme, an oxidized-indolenine group of tryptophan, lipoate or cystine.

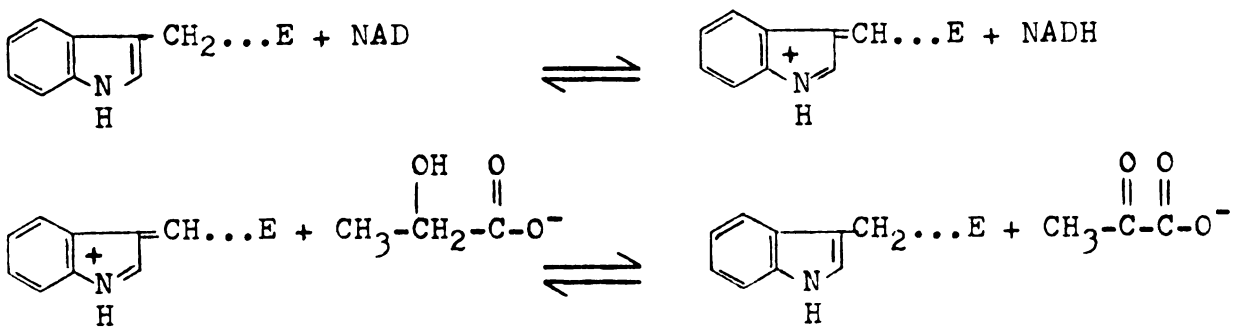
In most enzymes requiring the B_{12}-coenzyme the cobamide coenzyme appears to catalyze the exchange of hydrogen atoms and some group between adjacent carbon atoms of the substrates. This mechanism has been shown for glutamate mutase, methylmalonyl-CoA mutase, glycerol dehydrase, dioldehydrase and ethanolamine deaminase (102, 103). In these reactions the hydrogen migration occurs without exchange with the solvent. The cobamide coenzyme-dependent ribonucleotide diphosphate reductase also involves the displacement of a group by hydrogen, but it differs in that the

hydrogen arises from a second substrate, usually lipoate (104, 105, 106). Abeles and associates (107) showed that tritium was transferred to the coenzyme from dl-1,2-propanediol-^{13}H in the presence of dioldehydrase. The tritium was transferred from the coenzyme to the reaction intermediate when the tritiated coenzyme was incubated with dl-1,2-propanediol and apoenzyme. The coenzyme was tritiated exclusively at the 5' position of the adenyl moiety. This suggests that the mechanism involves the abstraction of the hydrogen from C-1 of dl-1,2-propanediol, and the transfer of it to the coenzyme, where it becomes equivalent with at least one, but probably not both, hydrogens at the C-5' position of the adenine of cobamide coenzyme. The cobamide coenzyme appears to function in a similar manner in the presence of the cobamide-dependent ribonucleotide reductase from Lactobacillus leichmannii (108), methylmalonyl-CoA isomerase, and glutamate isomerase (107). The 5' carbon is also covalently bound to cobalt, and it has been proposed that the carbon-cobalt bond is split during the reaction to provide a position for the hydrogen atom.

Thus it is conceivable that cobamide coenzyme could facilitate hydrogen atom translocation in the L-ribulose-5-P 4-epimerase by a similar mechanism. The reaction would differ, however, in that the hydrogen would displace a group to the opposite side of the carbon rather than to an

adjacent carbon.

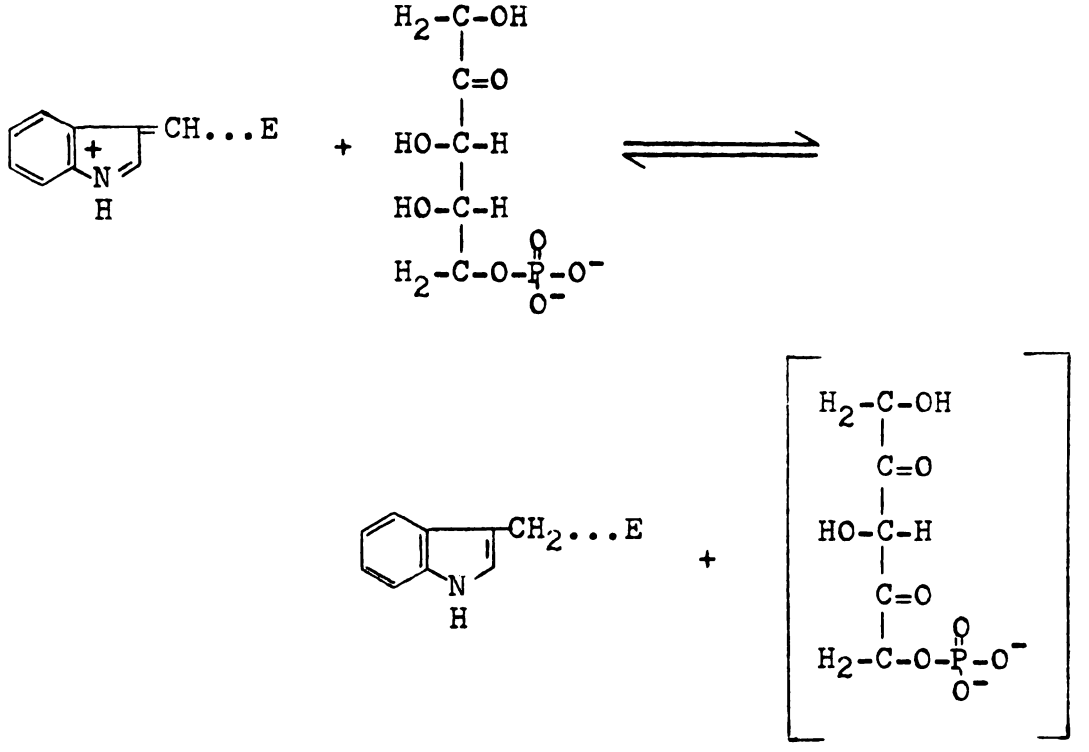
The possibility of a tryptophan involvement in the oxidation-reduction reaction is based on Schellenberg's proposal that tryptophan is an intermediate in the dehydrogenase reaction (109, 110, 111, 112). The proposed reaction sequence for the oxidation of lactate by lactic dehydrogenase involves the NAD^+-catalyzed oxidation of the indole group of tryptophan to indolenine followed by reduction of the indolenine by the substrate as follows:



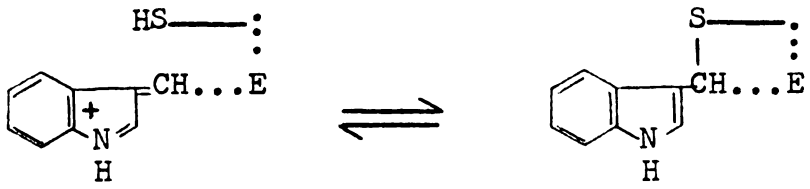
Evidence in support of this mechanism includes: (a) lactic dehydrogenase contained tritium in the methylene group of tryptophan after incubation with NAD^+ and lactate-2-T followed by denaturation with perchloric acid or urea, (b) renatured, tritiated enzyme retransferred the tritium to oxidized NAD^+ in the presence of the reduced substrate, and (c) renaturation of the tritium-labeled enzyme in the presence of NAD^+ resulted in the transfer of NAD^+ to NADT. The T was transferred from the NADT to acetaldehyde in the presence of alcohol dehydrogenase. The lactate did not

appear to be non-specifically bound to the enzyme, since enzyme incubated with lactate-1-^{14}C did not retain the label on denaturation. Thus, the evidence strongly suggests that the tryptophan group can be reduced by the hydride ion from the substrate; however, this may only be a side reaction and may not be an obligatory part of the dehydrogenation reaction.

If tryptophan were involved in the oxidation of L-ribulose-5-P and D-xylulose-5-P, the enzyme would have to maintain tryptophan in the oxidized form. The indolenine group would be reduced and reoxidized during the course of the reaction as follows:



The indolenine could be stabilized by a nearby cysteine residue



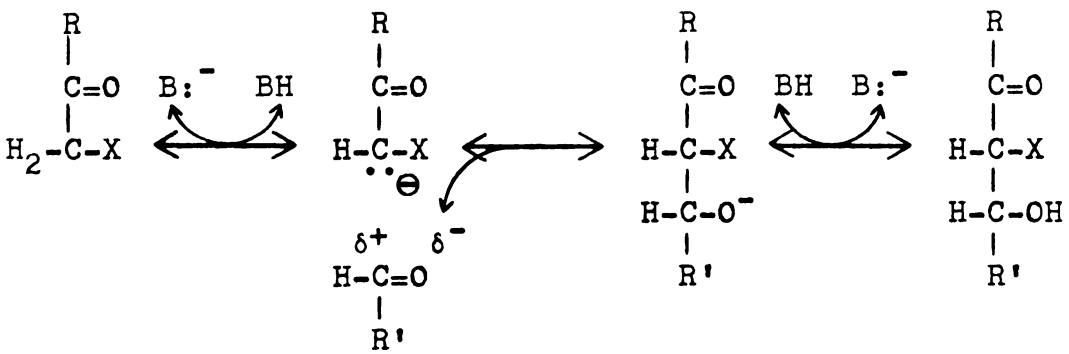
as proposed by Schellenberg (111).

Since NAD^+ is known to catalyze the oxidation of UDP-glucose and UDP-galactose in the presence of UDP-glucose 4-epimerase, it can be reasoned that any constituent on the enzyme surface with an oxidation potential similar to that of NAD^+ should also accept electrons from the substrate. Lipoic acid ($E_o' = -0.29$) and cystine ($E_o' = -0.33$) have reduction potentials comparable to that of NAD^+ ($E_o' = -0.32$). Therefore, it is conceivable that cystine and lipoic acid could act as an electron acceptor and donor in the oxidation-reduction of L-ribulose-5-P and D-xylulose-5-P in the presence of L-ribulose-5-P 4-epimerase, assuming the reduction potentials for the pentulose phosphates are similar to that of UDP-glucose and UDP-galactose.

If the L-ribulose-5-P 4-epimerase mechanism consisted of an Sn_2 (Walden) inversion at C-4, the hydroxyl at C-4 would have to be displaced by a hydroxyl group from water. However, Wood and McDonough (21) have reported that equilibration of L-ribulose-5-P and D-xylulose-5-P in the presence of $H_2{}^{18}O$ and 4-epimerase resulted in incorporation

of less than 13% of the ^{18}O expected. This result indicates that a mechanism other than Sn_2 inversion must be involved.

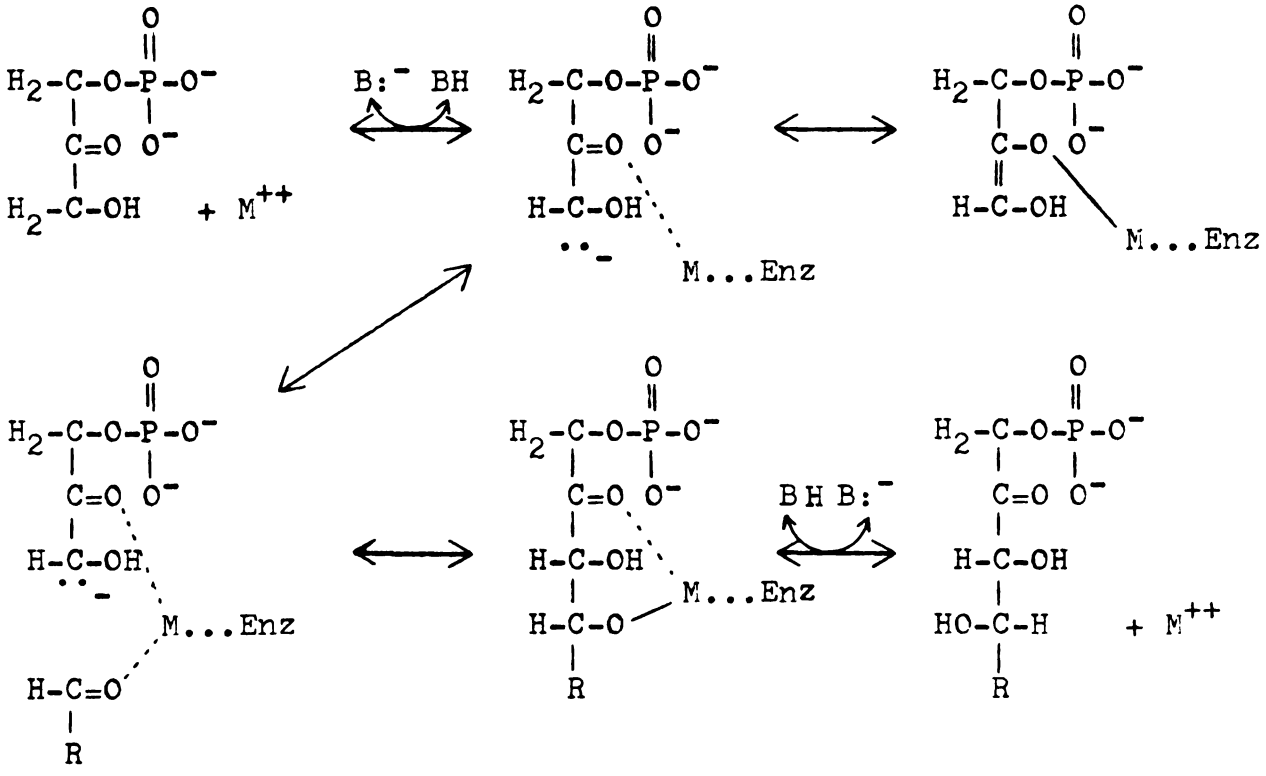
Chemical epimerization by an aldolization-dealdolization mechanism have been reported as discussed in the Literature Review. Enzymatically catalyzed aldolizations have been studied extensively as indicated in recent reviews by Morse and Horecker (113) and by Rutter (114, 115). Aldolases facilitate the base catalyzed aldolization between a ketone and an aldehyde as follows:



The enzyme catalyzes the exchange of a proton between the carbanion and water. The carbanion is stabilized by dispersing the negative charge into an electron sink at the active site of the aldolase either by means of a metal ion or by reasonance of a schiff base with the enamine form.

The metaloaldolases (Class II) are found in bacteria and blue green algae (114, 115). All the enzymes in this class require metal ions for activity, and in most cases the metal is tightly bound Zn^{++}, but loosely bound Fe^{++} or Co^{++} have been reported for fructose-1,6-diphosphate aldolase

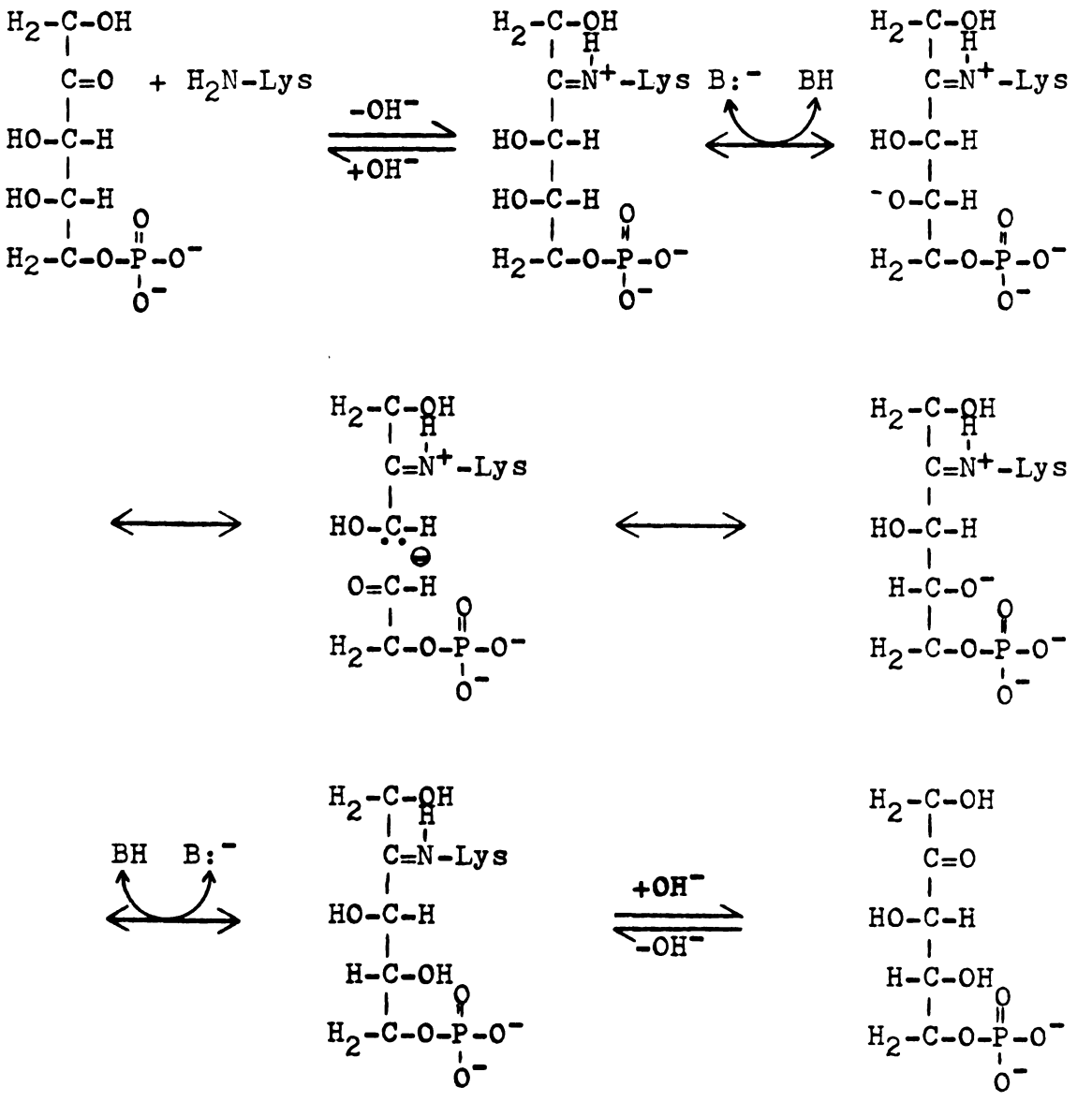
from Clostridium and Anacystis. The reaction is completely inhibited by metal chelators, and the inhibition is reversed by the addition of divalent cations. The activity is markedly stimulated by K^+ and NH_4^+ at a concentration of 10^{-1}M. The substrate does not appear to form a Schiff base with the enzyme since the reaction is not inhibited by sodium borohydride. Rutter (114) has proposed the following mechanism for fructose-diphosphate aldolase which utilizes the metal as an electron sink as follows:



In contrast Class I aldolases do not require metal ions for activity, nor are they inhibited by metal chelators (113). The carbonyl group forms a Schiff base with lysine as demonstrated by reducing the ketimine Schiff base with

$NaBT_4$ and identifying the tritium labeled intermediate. In addition, the carbonyl oxygen is exchanged for ^{18}O of $H_2{}^{18}O$. The proposed mechanism (113) for Class I fructose-1,6-diphosphate aldolase is presented in Figure 11.

Based on the mechanism proposed for fructose-diphosphate aldolase, the following mechanism can be proposed for L-ribulose-5-P 4-epimerase:



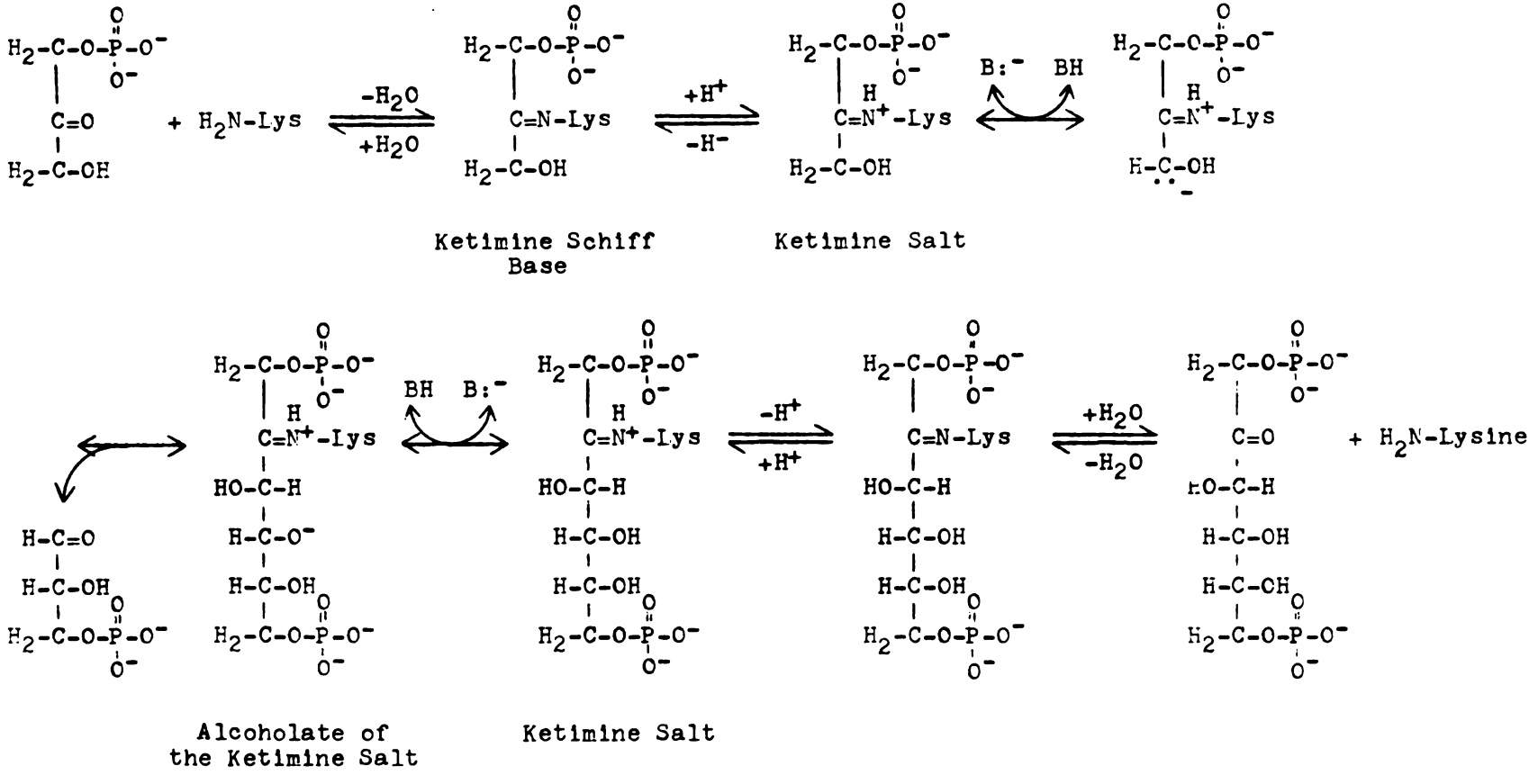
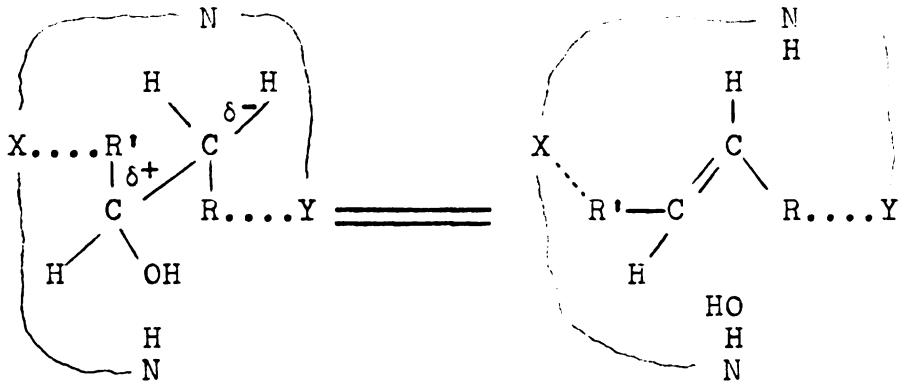


Figure 11. Proposed Mechanism for Class I Fructose-1,6-Diphosphate Aldolase (113)

The enzyme could also act as an electron sink by forming a Schiff base between the substrate and enzyme or by means of a metal ion. The 2- and 3-carbon intermediates would not have to be released from the enzyme surface, and thus there would not be necessary for any proton exchange at C-3 as found for transaldolase where dihydroxyacetone remains tightly bound to the enzyme surface and protons are not exchanged with the medium (113). The 4-epimerase would have to prevent the racemization of the hydroxyl at C-3 and permit the racemization of the hydroxyl group at C-4, with the trans or more thermodynamically stable isomer predominating. In contrast, aldolases catalyze either a trans- or a cis-, but not both, arrangement of the hydroxyl groups on carbon-carbon bond formation. This appears to be a property which is dictated by the fructose-diphosphate aldolase since the condensation of pyruvate and glyceraldehyde-3-P by 2-keto-3-deoxy-6-P gluconate aldolase always gives 2-keto-3-deoxy-6-P gluconate and not 2-keto-3-deoxy-6-P galactonate (116).

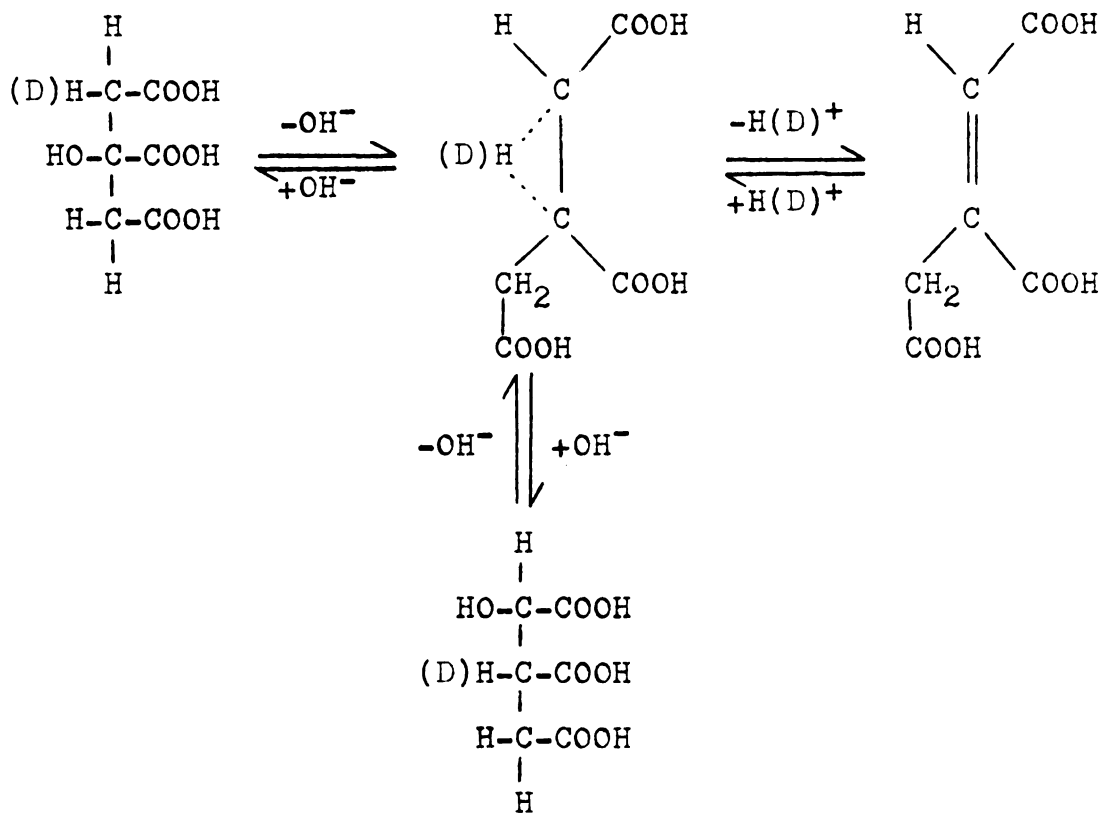
Dehydrations of hydration reactions between carbons αand β to a carbonyl are catalyzed by a number of different enzymes including fumarase, aconitase, enolase, and nucleotide diphosphate aldose reductase. There are at least 4-classes of hydrases and dehydrases (117) including: (a) enzymes requiring cofactors, (b) enzymes requiring both a metal ion and a reducing agent, (c) enzymes requiring a

divalent metal ion, and (d) enzymes requiring pyridoxal phosphate. Of the two basic mechanisms, only the one of which fumarase and enolase are characteristic is applicable to L-ribulose-5-P 4-epimerase. In both of these enzymes, two-imidazole groups, one charged and one uncharged, are believed to be present in the active site and are believed to act as proton donors and acceptors as follows:



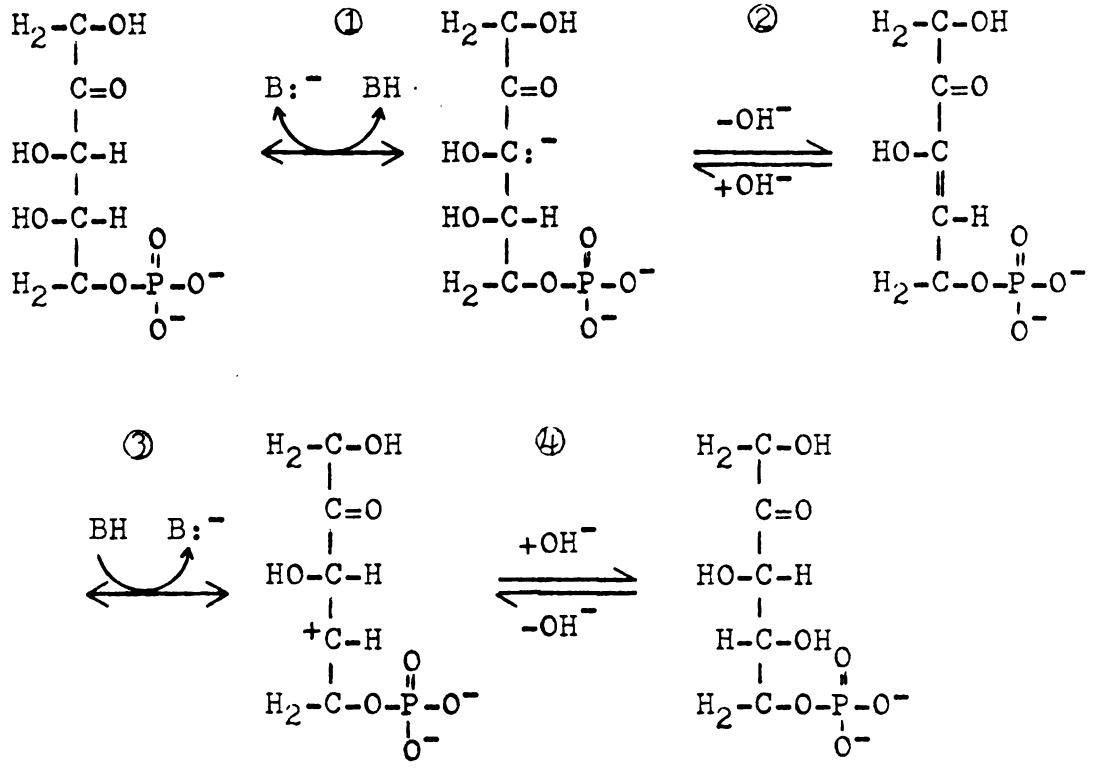
This mechanism requires a polarization in the substrate which could be induced by a dipole in the catalyst, so that the proton donor and acceptor could also be responsible for this polarization of the substrate. However, in the case of imidazole groups, where the proton acceptor is uncharged, this does not seem possible. Instead, the polarization is thought to be induced via the interaction of the substrate binding groups, x and y, which would include cofactors such as Mg^{++} in enolase. The free energy changes associated with hydration-dehydration reactions are, in general, small so that these processes have equilibrium contents close to unity. The overall reaction catalyzed by

aconitase is similar to that expected for L-ribulose-5-P 4-epimerase in that with aconitase a hydroxyl group is translocated to an adjacent carbon, whereas, with L-ribulose-5-P 4-epimerase the hydroxyl group would be translocated to the opposite side of carbon atom. The proposed aconitase mechanism is (118):

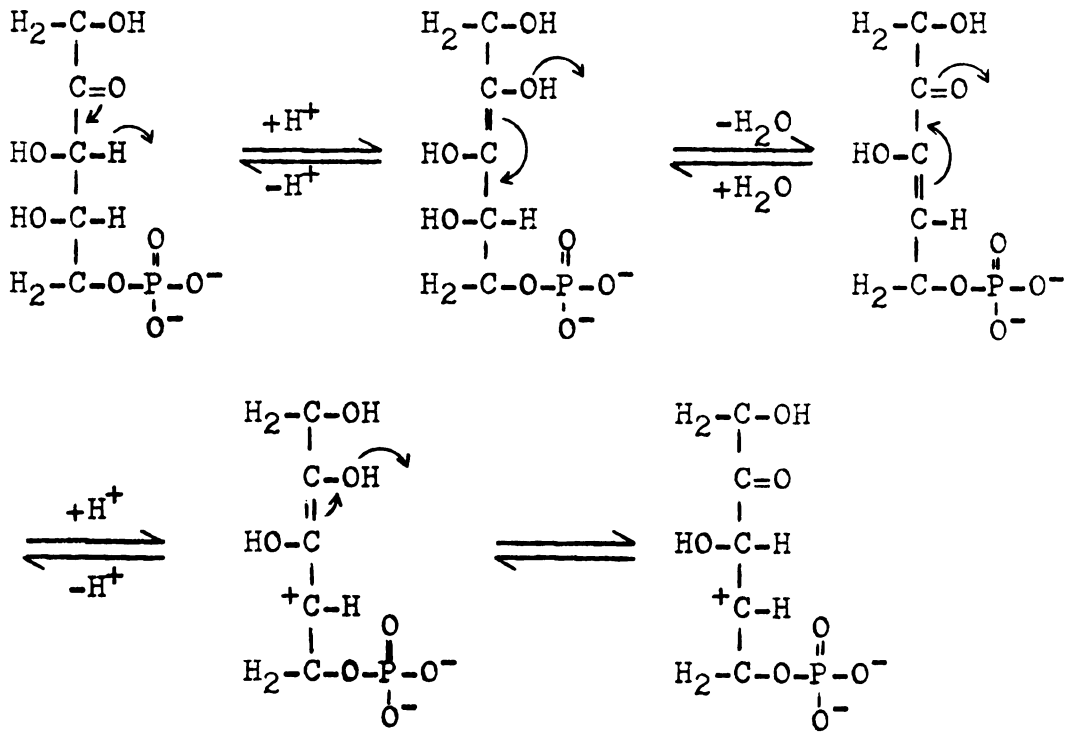


which proceeds by both a <u>trans</u> addition and a <u>trans</u> elimination of water. Aconitase requires both cysteine and Fe^{++}.

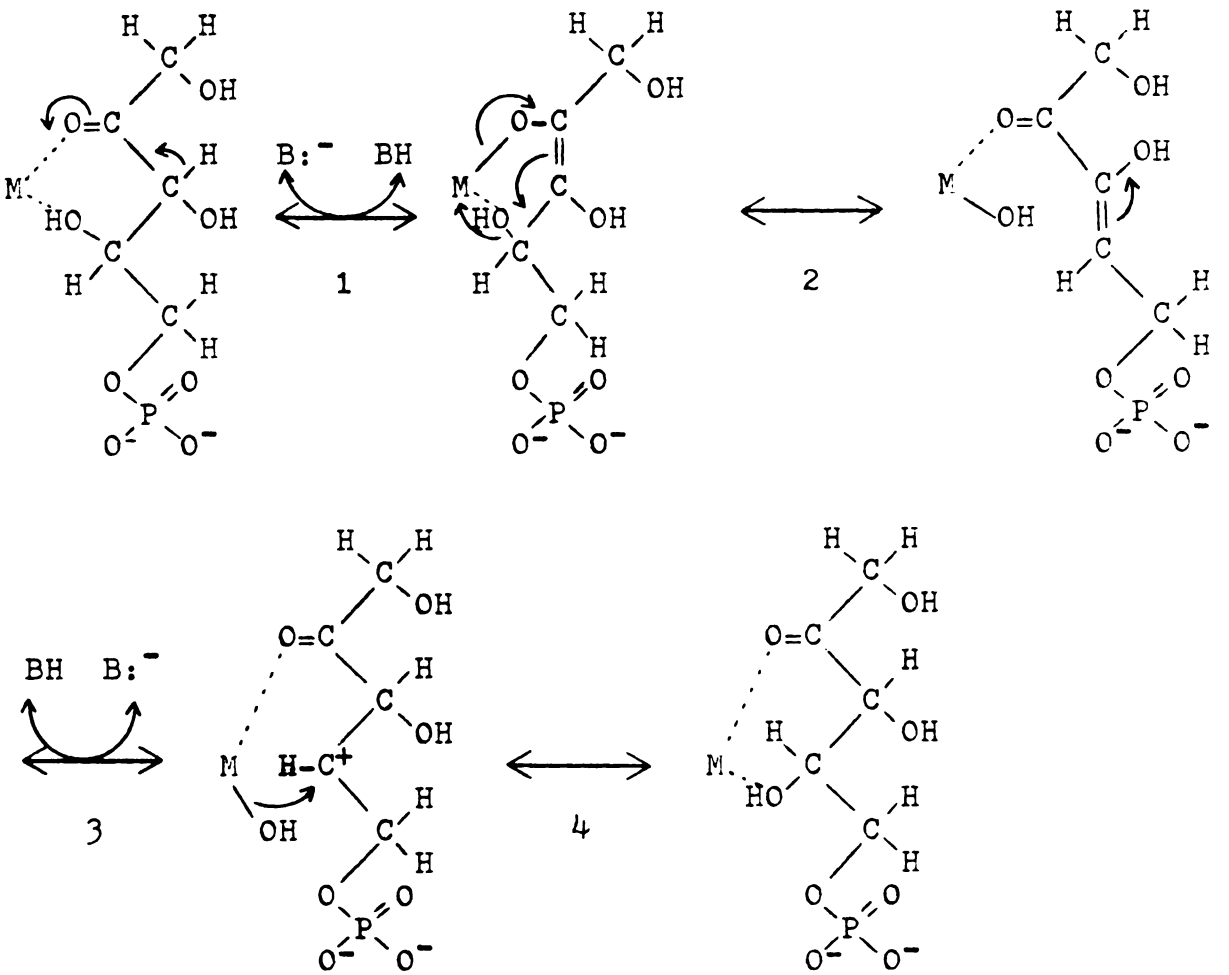
In comparison to known hydration and dehydration reactions, the following mechanism can be proposed for L-ribulose-5-P 4-epimerase:



Slight variations in the above mechanism could exist, such as steps 1 and 3 could be acid catalyzed and involve keto-enolization as follows:



A metal could act as an electron sink at the carbonyl group and as a Lewis acid to accept the hydroxyl group at C-4 as follows:



A base is required to accept the proton from the α-carbon since the α-proton does not appear to be excahngeable with the medium (21). The metal ion could facilitate the translocation of the hydroxyl to the opposite side of C-4, or the carbonium ion could rotate. In either case there should be an equal probability of the hydroxyl group returning to either side of C-4, and the more thermodynamically stable

epimer should predominate. The above reaction is not freely reversible since the equilibrium of step 2 is far to the right. However, starting with either L-ribulose-5-P or D-xylulose-5-P a mixture of L-ribulose-5-P and D-xylulose-5-P would be formed from the preceeding mechanism.

A better understanding of the mechanism of L-ribulose-5-P 4-epimerase could be obtained if it was established whether (a) metal was involved in the enzyme reaction, (b) the substrate formed a Schiff base with the enzyme, (c) a carbanion was formed during the course of the reaction and (d) an electron acceptor other than NAD^+ was present on the enzyme surface. The following experiments were undertaken to answer these questions.

The Role of Divalent Metal Ions

4-Epimerase Activity in the Presence of Metal Complexing Agents: L-Ribulose-5-P 4-epimerase did not require the addition of divalent metal ions for activity at any stage of the purification; however, the appropriate precautions were never taken to remove divalent metal ions from the enzyme of the assay mixture. A more definitive indication of whether an enzyme requires a metal ion for activity can be obtained by assaying the enzyme in the presence of metal chelators. Reactivation by addition of divalent metal ions would be indicative of a metal requirement by the enzyme. Therefore, the 4-epimerase activity was determined in the presence of a number of metal chelators.

The continuous coupled assay could not be used to determine the activity of the 4-epimerase in the presence of metal chelator, since Mg^{++} is required by the phosphoketolase, one of the coupling enzymes. Therefore, the enzyme was assayed in 2-steps. Step 1 consisted of incubating the 4-epimerase with L-ribulose-5-P in the presence of glycylglycine buffer. The reaction was stopped by the addition of acid, and the enzyme was irreversibly denatured by heating in a boiling water bath for one minute as described in Methods. Step 2 consisted of assaying for the amount of D-xylulose-5-P which was formed in step 1. This was accomplished by adding an aliquot of the reaction mixture from step 1 to the coupled assay containing phosphoketolase, triosephosphate isomerase, and α-glycerolphosphate dehydrogenase as described in Methods. The D-xylulose-5-P concentration was calculated from the amount of NADH oxidized during the course of the reaction.

To determine the effect of metal chelators on enzyme activity, 4-epimerase (50% pure), which had been dialyzed overnight against 0.05 M glycylglycine buffer, pH 8.0, was incubated at room temperature in the same buffer with 1 mM o-phenanthroline, 0.1 M dithioerythritol, 0.1 or 1 M mercaptoethanol, 2×10^{-3}M and 8×10^{-2}M 8-hydroxyquinoline sulfonate, 10^{-3}M BAL and 10^{-3} or 10^{-2}M EDTA. Aliquots were withdrawn at given time intervals and assayed for 4-epimerase activity. Precautions were not taken to remove the divalent

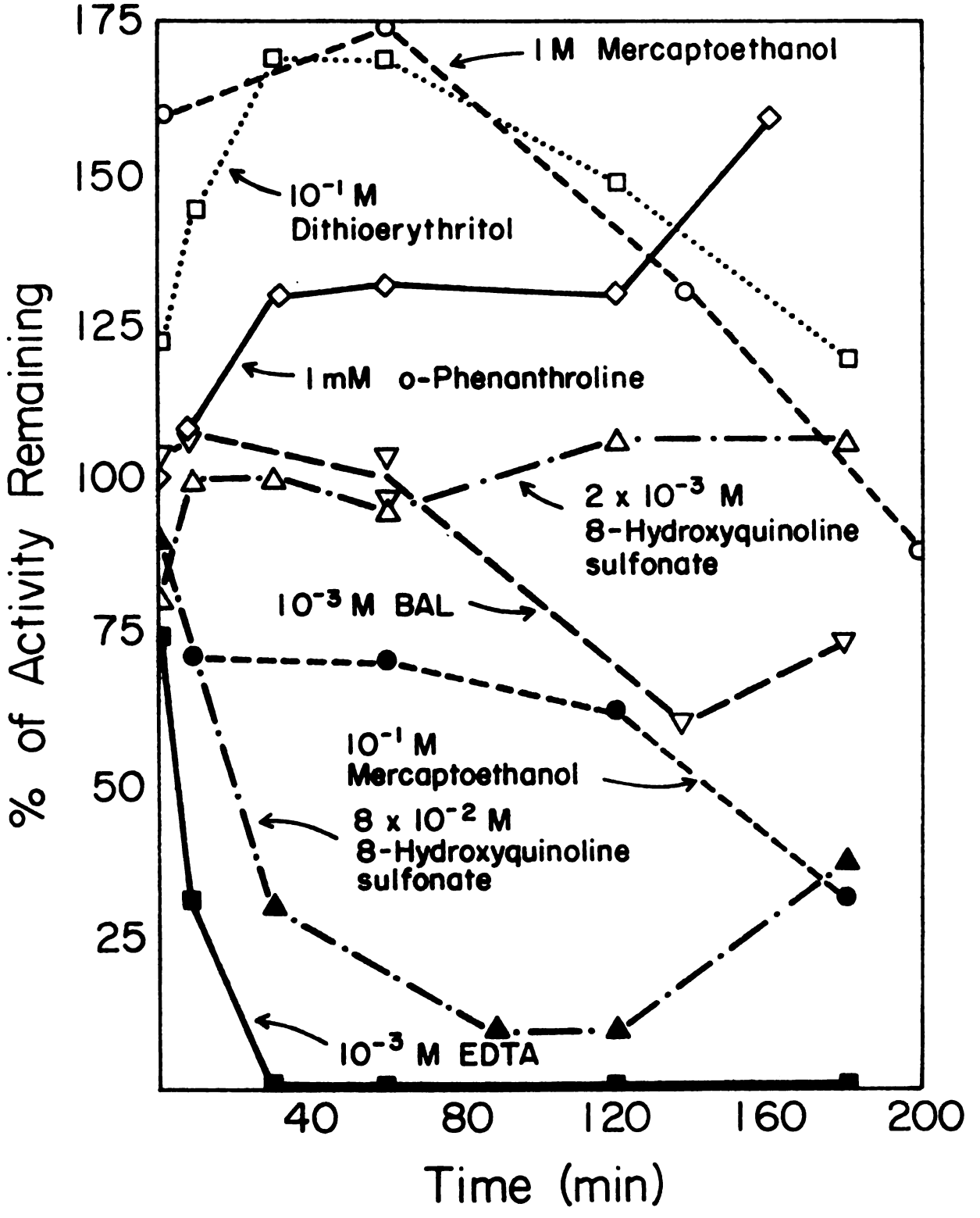
cations already present in the assay buffer or the glassware used in either the first or second step of the assay. The addition of metal chelator to the second step of the assay did not interfer with the assay for D-xylulose-5-P when added at a concentration comparable to that carried over from the first step of the assay. However, o-phenanthroline at levels higher than 1 mM did interfere with the D-xylulose-5-P assay.

A plot of the percent of activity remaining after given periods of time is illustrated in Figure 12. Without metal chelator the 4-epimerase lost 20% of its activity in the first 20 min. and remained stable thereafter. Only EDTA and 0.08 M 8-hydroxyquinoline sulfonate inactivated the enzyme within 20 min. The enzyme was initially activated by 1 M mercaptoethanol, dithioerythritol, and o-phenanthroline, and even after 3 hours the level of activity remaining was still higher than that of enzyme incubated in the absence of metal chelators. The extent of activation was always less than 2-fold. BAL and 2×10^{-3}M 8-hydroxyquinoline sulfonate had essentially no effect on enzyme activity.

The effects of metal chelators on the 4-epimerase activity may be due to their ability to remove divalent cations or else they may be due to a nonspecific reaction of the enzyme with the chelator. Based on the list of stability constants for metal chelator complexes given in

Figure 12. The Effect of Metal Chelators on Enzyme Activity

The 4-epimerase preparation approximately 50% pure, was dialyzed against 0.05 M glycylglycine buffer, pH 8.0 and incubated with metal chelator in 0.05 M glycylglycine, pH 8.0, at room temperature. An aliquot was withdrawn at the times indicated and assayed for 4-epimerase activity in the 2-step assay at 37°C and pH 8.0 as described in Methods. All values are expressed as percentage of the activity remaining compared to the activity prior to the addition of the chelator.


175
150
125
100
75
50
25
% of Activity Remaining
1 M Mercaptoethanol
10⁻¹ M
Dithioerythritol
1 mM o-Phenanthroline
2 x 10⁻³ M
8-Hydroxyquinoline
sulfonate
10⁻³ M BAL
10⁻¹ M
Mercaptoethanol
8 x 10⁻² M
8-Hydroxyquinoline
sulfonate
10⁻³ M EDTA
40
80
120
160
200
Time (min)

the Appendix, BAL, glycylglycine, and 8-hydroxyquinoline sulfonate, and o-phenanthroline bind Zn^{++}, and Ni^{++} better than Mg^{++} and Ca^{++}, and EDTA binds all metals tightly. Thus EDTA is probably complexing all the divalent cations present in the enzyme solution, whereas the other chelators should be complexing specific divalent cations and leaving other metal ions still free in solution. Hence, the results presented in Figure 12 could be explained if the 4-epimerase were able to bind all metal ions in solution but was activated to different extents by the different metal ions, with Ca^{++} being more active than Zn^{++}. Thus, the purified enzyme may consist of enzyme molecules with various activities which are dependent on the metal ion which is bound. Chelators, such as BAL, may remove the metals from the medium which have lower abilities to activate the 4-epimerase and, thus, allow the binding of divalent metal ions which produce higher activities.

Inactivation produced by metal chelators only after a lag of 3 or more hours was probably a non-specific inactivation. The metal chelators should have rapidly bound the cations in solution. The rate of complexing of metal ions bound to the enzyme surface would depend on the rate of release of the metal ion from enzyme surface. However, most metal-containing enzymes are inactivated in less than 3 hours.

Activity of the 4-Epimerase in the Presence of Specific Divalent Metal Ions: In order to determine if EDTA was inhibiting the enzyme by removal of the divalent cations or by some other means, it was necessary to show that the enzyme could be reactivated by metal ions after treatment with EDTA. Thus, if EDTA were inactivating the enzyme by some other means, the activity should be regained on removal of the EDTA. If EDTA were inactivating the enzyme by complexing a necessary metal ions, the enzyme activity should be regained only after addition of the metal ion.

To test this, it was necessary to remove all metal ions from the solutions and the glassware as described in Methods. A Sephadex G-25 column (0.6 x 11 cm) was extensively washed with 10^{-2}M EDTA to remove metal ions and was then extensively washed with metal free 0.05 Tris-Hepes buffer, pH 8.0. Following incubation with 10^{-2}M EDTA for 1 hour, there was no detectable epimerase activity in the 2-step assay, but a specific activity of 2.2 was obtained with the continuous assay containing Mg^{++} compared to a specific activity of 3 by both assays prior to the EDTA inhibition.

The enzyme inactivated by EDTA was passed through a Sephadex G-25 column which had previously been shown to clearly separate ^{32}P and blue dextran. In order to insure separation of EDTA and 4-epimerase only the fractions

containing most of the enzyme were collected. After passage through the Sephadex column the enzyme had no detectable activity in the 2-step assay but had a specific activity of 4.2 in the continuous assay where Mg^{++} was present. This experiment was repeated 5 times, and the activity in the 2-step assay was recovered after the Sephadex step only when metal ions were present.

In preliminary experiments, when the enzyme was incubated at room temperature with a series of metal chloride salts at 10^{-3}M concentration and assayed in the 2-step assay at 28°C, activities comparable to those presented in Table 7 were obtained. Of the divalent metals studied only Cu^{++} and Fe^{++} were found to interfere with the second step of the assay when added directly to an assay of a known amount of D-xylulose-5-P. In addition the other divalent cations did not cause the chemical epimerization of L-ribulose-5-P as determined by adding the cation to the first step of the assay in the absence of 4-epimerase.

The results of the 2-step assay indicated that more than 90% of the enzyme activity was lost on incubating the enzyme with EDTA. Although EDTA in solution should have been separated from the 4-epimerase on passage through a Sephadex G-25 column, tightly bound EDTA may not have been removed.

The enzyme activity lost on EDTA treatment was recovered on the addition of the divalent metal ions indicating that the enzyme requires metal ions for activity.

In addition, as much as a 17-fold activation over the activity after dialysis was obtained depending on the metal ion added. The order of activating effect of the metal ion was $Mn^{++} > Co^{++} > Ni^{++} > Ca^{++} > Zn^{++} > Mg^{++}$. The enzyme in the presence of 10^{-3}M Mg^{++} regained less than 40% of the original activity.

It should also be noted, that when the enzyme was incubated overnight at 4°C with 10^{-2}M EDTA, only about 50% of the protein expected was recovered from the Sephadex G-25 column, indicating that the enzyme had probably been denatured or adsorbed. Metal containing proteins are often denatured when completely depleted of metal ions, although the EDTA may inactivate the enzyme in other ways over the extended period of time.

In order to show more conclusively the order of activation of the divalent cations, the 4-epimerase after recovery from the Sephadex G-25 column was assayed in the presence of varying quantities of cations. The metal salts used were freshly prepared solutions of spectrographically analyzed metal sulfates containing less than 5 parts per million of most other metals. Since the same activity was obtained when the enzyme was preincubated with 10^{-3}M Co^{++} for 0, 10 or 30 min. the enzyme was not preincubated with metal ion prior to assaying. Rather metal ion and substrate were preincubated to allow temperature equilibration of the assay mixture, and the reaction was started by the addition of enzyme.

The results presented in Table 7 indicated that the highest 4-epimerase activity at the lowest divalent cation concentration was obtained with Mn^{++}. A 17-fold stimulation over starting activity or a 70-fold stimulation over activity of the enzyme in the absence of any metal ion was obtained with Mn^{++} at 10^{-5}M, whereas higher concentrations were required for the maximal activation possible by Ni^{++} and Mg^{++}

These results also substantiated the hypothesis that EDTA inhibited the enzyme by removing necessary cations and not by binding to the enzyme. If EDTA were still bound to the enzyme after its recovery from the Sephadex G-25 column, and if the inhibition of the enzyme were due to the presence of the EDTA, the initial activity should have been recovered with Zn^{++} and Ni^{++} at 10^{-4}M or at a lower concentration than would be obtained with Mn^{++}, since the stability constant for Zn^{++} and Ni^{++} EDTA complexes are 10^{4}-fold higher than that of Mn^{++}. However, enzyme activity was obtained with Mn^{++} at a 10-fold lower concentration than with Zn^{++}.

In additional experiments the enzyme after dialysis and after recovery from the Sephadex G-25 column was diluted in 10^{-4}M $CoSO_4$ and 10^{-3}M $MnSO_4$, $ZnSO_4$, $MgSO_4$ and $NiSO_4$ and assayed in the continuous assay. Neither the assay mixture nor the glassware had been treated to remove metal contaminants and a syringe with a metal needle was

Table 7. Divalent Metal Ion Activation of L-Ribulose-5-P 4-Epimerase Based on the 2-Step Assay

The 4-epimerase (85% pure) was dialyzed overnight against 0.05 M Tris-Hepes buffer, pH 8.0, incubated for one hour with 10^{-2}M EDTA and passed through a Sephadex G-25 column (0.6 x 11 cm) which had been washed free of cations with EDTA and equilibrated with 0.05 M Tris-Hepes buffer, pH 8.0. The 2-step assay mixture contained spectropure metals at the levels indicated in the Table. Precautions were taken to remove the contaminating metals from the glassware and the reagents as described in Methods.

		Metal Ion Concentration (M) Specific Activity			
Conditions	None	10^{-6}	10^{-5}	10^{-4}	10^{-3}
After dialysis	3.4				
After dialysis and EDTA	0.85				
$MnSO_4$		51	57	59	60
$CoSO_4$		32	37	50	60
$NiSO_4$		2.2	4.3	21	18
$CaCl_2$[a]			8.0		9.6
$ZnSO_4$		0.93	3.9	3.3	3.4
$MgSO_4$		0.38	0.29	0.29	1.3

[a] $CaCl_2$ was Malinckrodt analytical reagent grade.

used to measure the enzyme aliquots. The results of the continuous assay, as presented in Table 8, do not reflect the activation of the 4-epimerase by Mn^{++}, Zn^{++}, or Mg^{++} that was obtained with the 2-step assay (Table 7). The difference in activities obtained by the two assays is most likely due to the presence of contaminating metal ions in the continuous assay. However, results of the continuous assay do indicate that Co^{++} and Ni^{++} can activate the metal free 4-epimerase, although the activity as determined by the continuous assay was only 50% of that obtainable by the 2-step assay. The exceptionally low activity obtained with Mn^{++} is probably due to the fact that phosphoketolase is inhibited by high concentrations of Mn^{++} (119). Likewise any of the metals may be inhibiting the coupling enzyme in the assay, and thus the reaction may not be zero order. Hence, it is not possible to clearly interpret the results of the continuous assay, and the only conclusion which can be drawn is that an accurate measure of enzyme activity in the presence of specific divalent metal ions cannot be obtained with the continuous assay.

It should also be noted that enzyme which has not been freed of contaminating metal, cannot be activated more than 2-fold by any metal as determined by the 2-step assay. For this experiment the 4-epimerase (90% pure) was dialyzed for 2 hours against 0.05 M barbital buffer, pH 8.0,

Table 8. Divalent Metal Ion Activation of 4-Epimerase Based on the Continuous Assay

The enzyme was treated as described in Table 7. After dialysis and EDTA additions, and after elution from the Sephadex column, the enzyme was diluted in 10^{-4}M $CoSO_4$ and 10^{-3}M Mn^{++}, Zn^{++}, Mg^{++}, and $NiSO_4$ (spectropure) and assayed in the continuous assay as described in Methods.

Conditions	Specific Activity		Ratio of:
	After Dialysis	After EDTA and Elution from Sephadex G-25	Continuous Assay[a] / 2-Step Assay[b]
None	3.27	4.06	1.2
$MnSO_4$	2.23	5.9	0.098
$CoSO_4$	2.93	22.1	0.43
$NiSO_4$	3.52	8.1	0.45
$ZnSO_4$	1.86	2.22	0.65
$MgSO_4$	2.53	3.08	2.4

[a]The specific activity obtained after EDTA treatment and passage through a Sephadex G-25 column.

[b]The specific activity obtained from the 2-step assay (Table 7).

incubated for 1 hour with 10^{-2}M Co^{++}, Mn^{++}, Zn^{++}, and Mg^{++} chloride salts, and assayed in the 2-step assay. Contaminating metals were not removed from any of the glassware or reagents. The results presented in Table 9 indicate that only Mn^{++} can stimulate the enzyme in the presence of "endogeneous" metal ions. However, only a 2-fold stimulation was obtained indicating that Mn^{++} was not able to completely overcome inhibition by the other cations, since a 17-fold activation is obtained in the absence of any competing metal ions. Neither Co^{++} nor Mg^{++} markedly changed the activity, but it is not possible to determine from this experiment whether Co^{++} or Mg^{++} became bound to the enzyme. These results also substantiated the 2-fold stimulation observed with some of the metal chelators.

The observation, that Co^{++} appeared to activate the enzyme 5-fold as determined by the continuous assay and not at all in the 2-step assay, can be explained by the fact that a metal complexing buffer was used in the continuous assay and not in the 2-step assay. Imidazole binds heavy metals readily and prevents inactivation by these metals, whereas barbital is a very poor metal chelator and thus does not remove any of the competing metals ions (see Appendix).

Since the preceeding results strongly indicated that L-ribulose-5-P 4-epimerase was activated by metal ions, Mn^{++} being the most active, it was necessary to

Table 9. Divalent Metal Ion Activation of the 4-Epimerase Based on the 2-Step Assay

The L-ribulose-5-P 4-epimerase (specific activity 10.0) was dialyzed for 2 hours against 0.05 M barbital buffer pH 8.0 and incubated for 1 hour with 10^{-2}M Co^{++}, Mn^{++}, Zn^{++}, and $MgCl_2$ and assayed in the 2-step assay in the presence of glycylglycine buffer pH 8.0 and any metals already present in the buffer, substrate or glassware.

Conditions	% of Original Activity
$CoCl_2$	89
$MnCl_2$	205
$ZnCl_2$	13
$MgCl_2$	78

redetermine the specific activity of homogeneous 4-epimerase in the presence of Mn^{++}. Therefore 4-epimerase which was 80% pure as determined by acrylamide gel electrophoresis, was crystallized twice as previously described. The second crystals were washed with 1.8 M ammonium sulfate, collected by centrifugation and resuspended in 0.05 M Tris-Hepes buffer, pH 8.0. The 2nd crystals were at least 98% pure as determined by polyacrylamide gel electrophoresis. The enzyme was treated with 10^{-2}M EDTA, and the EDTA was removed by passing through a Sephadex G-25 column as before. The metal-free enzyme was incubated with 10^{-4}M $MnSO_4$ (spectropure) and assayed with the 2-step assay to which 10^{-5}M $MnSO_4$ had been added. A specific activity of 70 ± 10% was obtained for the pure L-ribulose-5-P 4-epimerase.

EDTA at a concentration of 10^{-3}M completely inhibited the enzyme in the presence of 10^{-4}M Mn^{++}, however, 10^{-3}M 8-hydroxyquinoline sulfonate did not inhibit the enzyme when this concentration of Mn^{++} was present. After the excess Mn^{++} had been removed by passage through a Sephadex G-25 column, incubation with 10^{-3}M 8-hydroxyquinoline sulfonate resulted in a 50% inhibition, whereas incubation at a concentration of 10^{-2}M resulted in complete loss of enzyme activity. These results indicated that the enzyme can be inactivated by chelators other than EDTA. The differences in levels of the two chelators required to

inhibit the enzyme can be explained by the fact that the binding constants of EDTA for Mn^{++} is 10^{-3} times higher than that of 8-hydroxyquinoline sulfonate.

In order to determine if Mg^{++} could inhibit the 4-epimerase in the presence of Mn^{++}, the enzyme was pre-incubated with either Mn^{++} or Mg^{++} and assayed immediately in the presence of the opposite metal. Only 10% of the activity was lost when the enzyme in the presence of 10^{-4}M Mn^{++} was diluted in 10^{-3}M Mg^{++} and assayed immediately in the presence of 10^{-3}M Mg^{++}, however 50% of the activity was lost when the enzyme was assayed one hour after diluting. Likewise, when the enzyme in the presence of 10^{-3}M Mg^{++} was diluted and assayed in 10^{-4}M Mn^{++}, the enzyme was only 50% as active as it was in the absence of Mn^{++}. Thus Mn^{++} is capable of inhibiting the enzyme in the presence of Mn^{++}.

Test for a Carbanion Intermediate

Tetranitromethane reacts with carbanions with the liberation of nitroform which absorbs at 350 mμ. Riordan (120, 121) has reported that tetranitromethane reacts with the enzyme-substrate complex of both yeast (Class II) and muscle (Class I) fructose diphosphate aldolases. The theory that tetranitromethane is reacting with the carbanion intermediate of the aldolase reaction is based on the following observations: (a) the rate of production of nitroform from tetranitromethane is markedly enhanced

by the presence of substrate, (b) the rate of nitroform production is directly proportional to the concentration of active enzyme, (c) the substrate concentration resulting in half-maximal rate of nitroform production (K_m') was virtually identical with the values of K_m of fructose 1,6-diphosphate and fructose-1-phosphate determined enzymatically, (d) phosphate competitively inhibits the tetranitromethane reaction, and (e) the substrate is consumed as a function of time. Thus it is reasonable to assume that if 4-epimerization of L-ribulose-5-P and D-xylulose-5-P is proceeding via a carbanion intermediate; it may be possible to detect the carbanion by conducting the epimerization in the presence of tetranitromethane.

To test this the 4-epimerase was dialyzed for 5 hours against 3-changes of 0.05 M glycylglycine buffer, pH 8.0. The 0.2 ml reaction mixture contained 0.05 M buffer, pH 8.0, 4.2×10^{-4}M tetranitromethane, L-ribulose-5-P, and 4-epimerase. The initial change in absorbance at 350 mμ was used as a measure of the rate of the tetranitromethane reaction.

As illustrated in Table 10, both the substrate and the 4-epimerase (80% pure) reacted with tetranitromethane when incubated separately with tetranitromethane in all three buffers. However, pure 4-epimerase did not react with tetranitromethane in Tris buffer.

Tetranitromethane did not appear to react to the

Table 10. Test for a Carbanion Intermediate Using Tetranitromethane

The 0.2 ml reaction mixture contained 0.05 M buffer, pH 8.0, 4.2×10^{-4}M tetranitromethane, substrate, and 4-epimerase as indicated. The initial change in absorbance at 350 mμ was determined for the reaction run at 28°C in a Gilford spectrophotometer.

Buffer	L-Ribulose-5-P	4-Epimerase	Initial Rate ($A_{350\ m\mu}$/min)			
			L-Ru-5-P + Buffer	4-Epimerase + Buffer	L-Ru-5-P + 4-Epimerase + Buffer	Difference
	M x 10^3	M x 10^{10}				
Imidazole	1.25	3.4[a]	0.025	0.017	0.138	-0.005
Imidazole	1.25	6.8[a]	0.035	0.056	0.086	0.005
Imidazole	2.5	10.2[a]	0.051	0.028	0.088	0.009
Glycylglycine	1.25	3.4[a]	0.056	0.072	0.164	0.036
Glycylglycine	1.25	6.8[a]	0.062	0.126	0.170	0.020
Tris	1.25	3.4[a]	0.023	0.030	0.055	0.002
Tris	1.25	20.0[b]	0.039	0.002	0.050	0.009
Tris	1.25	40.0[b]	0.038	0.002	0.052	0.012
Tris	1.25	80.0[b]	0.042	0.003	0.050	0.005

[a] 4-epimerase (8% pure)

[b] 4-epimerase (pure)

enzyme-substrate complex, since the rate of the tetranitromethane reaction with L-ribulose-5-P in the presence of 4-epimerase was not significantly greater than the combined rate of the tetranitromethane reaction with substrate alone and enzyme alone. In addition increasing the amount of enzyme did not increase the rate of the tetranitromethane reaction with enzyme-substrate complex. However, using the same conditions, it was possible to obtain a net initial increase in 350 mμ absorbance for muscle fructose-diphosphophate aldolase when tetranitromethane was incubated with the enzyme fructose-diphosphate complex. The rate of the tetranitromethane reaction was dependent on the aldolase concentration.

The reaction of the substrate with tetranitromethane may be due to the ready removal of the α-proton on the substrate at basic conditions. This would result in a carbanion formation which could be stabilized by keto-enolization of the carbonyl group. Also the substrate may form a Schiff base with the glycylglycine thus facilitating the removal of the α-proton.

The reaction of tetranitromethane with 4-epimerase (80% pure) was probably due to the reaction of tetranitromethane with the contaminating protein. Tetranitromethane is known to react with both tyrosine and sulfhydryl groups on proteins (122).

Under the conditions used a carbanion intermediate

of the L-ribulose-5-P 4-epimerase complex was not available to react with tetranitromethane in quantities which could be detected. Thus either a carbanion intermediate is not formed during the course of the epimerization, it is too short lived to react with tetranitromethane, or tetranitromethane is not freely accessible to the site of carbanion formation.

Effects of Borohydride on Enzyme Activity

If the enzymatic 4-epimerization of L-ribulose-5-P and D-xylulose-5-P proceeds by either a dealdolization-aldolization or dehydration-hydration mechanism, the reaction would be facilitated by acceptance of the electrons from the carbonyl group of the substrate by a group on the enzyme, as previously discussed. In Class I aldolases this is accomplished by formation of a Schiff base between the substrate and the lysine residue of the enzyme (112). The Schiff base is readily reducible in all Class I aldolases. Therefore, it can be reasoned that if a Schiff base is formed between either L-ribulose-5-P or D-xylulose-5-P and the enzyme, the enzyme-substrate complex should be reduced by sodium borohydride rendering the enzyme inactive. However, the enzyme should not be inactivated on incubation with borohydride in the absence of substrate.

If the mechanism of epimerization involves an oxidation-reduction mechanism a substituent on the enzyme

such as lipoate, cystine, B_{12}-coenzyme or oxidized tryptophan would have to act as an electron acceptor or donor as previously discussed. Lipoate (123), cystine, and the indolenine group of oxidized tryptophan (110) are reducible by borohydride. Therefore, if the mechanism involves oxidation-reduction, it may be possible to inhibit the enzyme by borohydride reduction of the electron acceptor; however, the substrate should protect against the borohydride reduction.

The borohydride reduction was performed using the procedure reported by Inghram and Wood (124). To 1.6 x 10^{-4} μmoles of L-ribulose-5-P 4-epimerase (85% pure) in 0.2 ml of 0.05 M phosphate buffer (pH 7.0) was added 2 μl of 1 M $NaBH_4$ followed three min. later by 2 μl of 1 M acetic acid. The $NaBH_4$ and acetate additions were repeated three times after which an aliquot of the enzyme was removed and assayed for enzyme activity. Three more additions of $NaBH_4$ and acetate were made and the enzyme was reassayed. Likewise, the reaction was run in the presence of 2.5 x 10^{-3}M L-ribulose-5-P and additional substrate was added after the first of the three additions of $NaBH_4$. The reaction was also run in the presence of enzyme L-ribulose-5-P and 10^{-4}M $CoCl_2$. As shown in Table 11, only when the enzyme was incubated with $NaBH_4$ was as much as 30% of the activity lost. Part of the loss can be attributed to loss in activity of the enzyme on standing in buffer during the

Table 11. The Effect of Borohydride on Enzyme Activity

The 0.2 ml reaction mixture contained 1.6×10^{-4} umoles of L-ribulose-5-P 4-epimerase (85% pure) and 10 umoles of phosphate buffer, pH 7.0. To this was added three additions of 2 ul each of $NaBH_4$ followed three minutes later by 2 ul each of 1 M acetic acid. An aliquot was removed and assayed for enzyme activity, after which the additions were made again. The same reactions were also run in the presence of 2.5×10^{-3}M L-ribulose-5-P and 10^{-4}M $CoCl_2$ as indicated. L-Ribulose-5-P was added a second time at the beginning of the second borohydride treatment.

Additions	Activity Remaining (%)	
	1st Borohydride Treatment	2nd Borohydride Treatment
None	90	70
+ L-Ribulose-5-P	96	80
+ L-Ribulose-5-P and $CoCl_2$	98	

first hour. The other reactions were run later and had a lower 4-epimerase activity prior to the addition of substrate.

Likewise the 4-epimerase at pH 6.5 in 0.05 M phosphate buffer was not inactivated by borohydride either in the presence or absence of substrate, however, NAD^+ was reduced under these conditions.

These results strongly indicate that a Schiff base is not being formed during the course of the reaction; however, one cannot rule out the possibility that a Schiff base may be forming at a site on the enzyme which is not accessible to borohydride. The results also support the theory that the enzyme does not contain bound NAD^+, since the enzyme should have been inactivated by borohydride in the absence of substrate. Both lipoate and cystine are readily oxidized by O_2, and thus if they were reduced during the course of the reaction, they may have been reoxidized prior to assaying; hence, inactive enzyme would not have been detected.

Test for an Electron Acceptor on the Enzyme Surface

The epimerization of L-ribulose-5-P and D-xylulose-5-P may proceed by an oxidation-reduction mechanism using enzyme bound lipoic acid or cystine as an electron acceptor, as previously discussed. If this were true, then the 4-epimerase would probably be inhibited by either arsenite or sulfite, since dihydrolipoate and cysteine

irreversible react with arsenite, and lipoate and cystine irreversibly react with sulfite.

To test this L-ribulose-5-P 4-epimerase (85% pure) was incubated with 10^{-1}M, 10^{-2}M, 10^{-3}M and 10^{-4}M sodium sulfite as described in Table 12 and assayed for enzyme activity in the continuous assay. In similar manner, the 4-epimerase was incubated with either 10^{-2}M mercaptoethanol or 10^{-3}M dithiotheritol to reduce the disulfide bond. The "reduced" enzyme was then incubated with sodium arsenite and assayed for enzyme activity in the continuous assay.

As illustrated in Table 12, L-ribulose-5-P 4-epimerase was not inactivated by sulfite or by arsenite in the presence of either mercaptoethanol or dithiotheritol. If either lipoic acid or cystine was present in the active site, the enzyme should have been inactivated by either of these treatments. However, the enzyme may have protected the lipoic acid or cystine from reacting with the arsenite or the sulfite.

Other Characteristics of the Enzyme

Anomalous Fast Activity of the Crude Extracts

Two levels of 4-epimerase activity, which differed by a factor of 3 to 5, were found in the crude extracts. The higher level of 4-epimerase activity was always lost on the first purification step, and only the lower level of 4-epimerase activity was recovered. Although this

Table 12. Test for Lipoic Acid and Cystine at the Active Site of L-Ribulose-5-P 4-Epimerase

The 0.4 umoles of L-ribulose-5-P 4-epimerase (85% pure) was incubated at room temperature with 10^{-1}M, 10^{-2}M, 10^{-3}M and 10^{-4}M Na_2SO_3 in 0.05 M Tris-Hepes buffer pH 8.0. The activity remaining after 30 min. was determined using the continuous assay as described in Methods. Alternatively the enzyme was incubated as above with 10^{-2}M mercaptoethanol or 10^{-3}M dithiotheritol. After 30 min. sodium arsenite was added to a concentration of 10^{-1}M or 10^{-4}M. Aliquots of the enzyme were removed after 5 min. and assayed in the continuous assay.

Additions	Relative Activity
None	100
10^{-4}M Na_2SO_3	98
10^{-3}M Na_2SO_3	100
10^{-2}M Na_2SO_3	90
10^{-1}M Na_2SO_3	98
10^{-3}M $NaAsO_2$	90
10^{-1}M $NaAsO_2$	96
10^{-3}M Dithiotheritol	100
10^{-3}M Dithiotheritol + 10^{-4}M $NaAsO_2$	95
10^{-3}M Dithiotheritol + 10^{-3}M $NaAsO_2$	100
10^{-2}M Mercaptoethanol	100
10^{-2}M Mercaptoethanol + 10^{-4}M $NaAsO_2$	95
10^{-2}M Mercaptoethanol + 10^{-3}M $NaAsO_2$	95
10^{-2}M Mercaptoethanol + 10^{-2}M $NaAsO_2$	90
10^{-2}M Mercaptoethanol + 10^{-1}M $NaAsO_2$	90

phenomenon was not conclusively explained, a number of studies were conducted to help clarify this situation.

The anomalous activity of the crude extracts consisted of an initial fast rate of NADH oxidation which tapered off within 5 min. to a slower linear rate. The fast rates were not reproducible and did not follow zero order kinetics. However, an accurate determination of the 4-epimerase specific activity is difficult to obtain for the crude extracts due to NADH oxidase activity as discussed previously. To denature the NADH oxidase, the crude extracts were heated to 60°C at pH 7.0 or 8.0 in either Tris or phosphate buffer. Although there may have been some loss in 4-epimerase activity, the anomalous fast activity was still present in the heat treated extracts. If an aliquot of the extract was preincubated with the reaction mixture for 10 min. prior to the addition of L-ribulose-5-P, the initial fast rate was no longer observed. All of the components of the reaction mixture were necessary to obtain the fast activity.

Additional experiments indicated that the anomalous fast activity was lost if the enzyme solution was allowed to remain at 4°C for 3 to 4 days, but it was not lost during storage at -20°C. The fast activity was also lost on dialysis, ammonium sulfate fractionation or on DEAE cellulose chromatography. The total enzyme activity recovered from the DEAE-cellulose step was comparable to that calcu-

lated from the slow linear rate observed with the crude extracts, but this was three to five times lower than that calculated from the initial fast rate. There was no enhancement in activity when either boiled crude extract, filtrate from enzyme concentrated by ultrafiltration, or when concentrated dialysis buffer was added to an enzyme assay.

The high level of activity found in the extracts from the u^-i^- mutants does not appear to be related to the high level of activity found in extracts of the wild type, since the high level of enzyme activity found in extracts of the mutant was not lost on purification, and the level of pure enzyme obtained from the mutant was approximately three times higher than that obtained from wild type.

The anomalous fast activity found in some of the crude extracts may be comparable to the actual activity of the enzyme in the intact cell. On rupturing the cell the protein may irreversibly change conformations to a less active form. The rate at which the protein changes conformation may vary from one cell preparation to another depending on the harshness of the treatment used in rupturing the cell wall.

On the other hand, the anomalous fast activity may be related to the ability of divalent metal ions to activate the purified enzyme. One would expect most divalent metal ions to be present in the crude extracts. However,

the concentration of metal ions will depend on the extent of binding of the metal ions to all proteins present. Thus the activity of the 4-epimerase should be a reflection of the different divalent metal ions which are free in solution. In some of the crude extracts the contaminating protein may be able to chelate most of the free metal ions which do not activate the 4-epimerase to any extent, and the more effective cations may be free in solution and thus able to activate the 4-epimerase. However, on purification both the concentration of free metals in solution and the concentration of contaminating proteins changes, and the less activating metal ions will probably be present to compete with the more efficient cations for the active site of the 4-epimerase.

DISCUSSION

The L-ribulose-5-P 4-epimerase from A. aerogenes with a specific activity of 13 at pH 7.0 and 28°C appeared to be homogeneous as determined by a constant specific activity before and after crystallization and recrystallization, by a single band on polyacrylamide gel electrophoresis, and by high speed ultracentrifugation. The specific activity of 13 is not a true measure of the potential activity of the enzyme since this was determined by the continuous assay in the presence of Mg^{++} and other divalent cations. When Mn^{++} is the only divalent cation present, a specific activity of 70 is obtained with the homogeneous enzyme at 28°C and pH 8.0.

The L-ribulose-5-P 4-epimerase from A. aerogenase appears to be similar to the same enzyme purified from E. coli (67). Both enzymes exist as a single molecular weight species on polyacrylamide gel electrophoresis and on high speed ultracentrifugation. A molecular weight of 1.14×10^5 for the 4-epimerase from A. aerogenase is comparable to the molecular weight of 1.04×10^5 which was reported for the enzyme from E. coli (67). A specific activity of 19.0 at pH 7.5 and 37°C was reported for the 4-epimerase from E. coli based on an assay similar to the continuous assay used here. However, the E. coli 4-epimerase

has a pH optimum between 7 and 10 and, thus, was assayed at its optimum pH. The effect of divalent metal ion on the activity was not reported. In contrast, A. aerogenes has a pH optimum at 9.0 with 40% of the maximal activity at pH 7.0. However, the pH optimum may vary depending on the metal ion present. Consequently, it is not possible to compare the activities obtained by the two enzymes.

The L-ribulose-5-P 4-epimerases from A. aerogenes and E. coli were stable to extensive dialysis and chromatography on Sephadex G-200, indicating that neither enzyme contained loosely bound cofactors which were not present in the assay mixture.

The 4-epimerase from A. aerogenes appears to be activated by specific divalent metal ions, and the maximal activity obtainable is dependent on the metal ion present, with the order of activation being $Mn^{++} > Co^{++} > Ni^{++} > Ca^{++} > Zn^{++} > Mg^{++}$. Mn^{++} appeared to be the most effective of the metal ions since the highest enzyme activity was obtained with this metal, and maximal activity was obtained at the same or lower concentration than the other metal ions tested. Metals from virtually all groups and periods of the periodic table have been found to activate various enzymes; therefore, there may be other metal ions which were not tested which will give higher 4-epimerase activity than Mn^{++}.

Metals may participate in enzyme catalysis nonspeci-

fically by creating ionic or electrostatic environments which stabilize the protein, or by complexing with inhibitors. The metal ion may also be an essential participant in the reaction. Although a metal enzyme may have activity in the absence of metal ion, the presence of the metal ion must change the reaction mechanism. According to Malmstrom (125) a metal ion cannot be said to definitively participate in the catalytic reaction unless the influence of the metal ion concentration follows the appropriate kinetic parameters, and unless the thermodynamic measurements on interaction of the metal ion with components of the reaction system are consistent with the postulated reaction steps and with the kinetic information. However, in cases where the most obvious artifacts have been excluded and where the rate is greatly increased on the addition of metal ions, it is a reasonable working hypothesis that the metal ion participates in the catalytic reaction. Hence, since (a) L-ribulose-5-P 4-epimerase can be activated over 70 fold by the addition of Mn^{++} to the metal free enzyme, (b) different divalent metal ions gave varying degrees of activity, and (c) the enzyme was inactivated by EDTA and 8-hydroxyquinoline sulfonate, it is reasonable to hypothesize that the metal ion participates in the 4-epimerization reaction. Substantial evidence awaits more thorough kinetic analysis of the metal binding and metal activation properties.

It is not unreasonable to find that the 4-epimerase is activated by more than one divalent metal ion since most enzymes requiring metals for catalysis are activated by a series of metal ions. The most efficient metal ion is not always the metal ion which gives the highest activity since other metal ions may activate at lower concentrations (125). In general metals ions which activate enzymes usually have similar atomic radii (125). All the metal ions which were tested with the 4-epimerase have similar radii. There is no correlation between metal ion activation and coordination number of configuration of complexes either in general or for L-ribulose-5-P 4-epimerase.

The theory that EDTA inhibited the enzyme by complexing necessary metals is based on the following observations: (a) after removal of free EDTA by passage through a Sephadex column, the enzyme was still inactive and could be activated by the addition of metal ions; and (b) EDTA binds Zn^{++} and Ni^{++} tighter than Mn^{++} yet the enzyme is activated by Mn^{++} at a lower concentration than that required for Zn^{++} and Ni^{++} activation.

The fact that the other metal chelators did not inhibit the enzyme is not unreasonable, since most metal chelators are somewhat specific for certain divalent cations. Thus the chelators may remove some metal ions from solution, but metal ions which are not bound by the chelator are free to bind and activate the 4-epimerase.

The possible functions of a divalent catalysis in the metal ion of an enzyme reaction include (126):

(a) metal ions may form complexes with donor atoms of either the enzyme or the substrate and thereby enhance their tendancy toward reaction;

(b) metal ions may serve merely as a bridge through common coordination to bring the enzyme and the substrate into proximity;

(c) while serving function (b) metals may provide as well a chemical activation influence; and

(d) while coordinated to either the enzyme or the substrate, metal ions may appropriately orient groups undergoing reaction.

It should be possible to determine whether the divalent cations are complexing with the substrates or with the 4-epimerase by determining the extent of binding of the metal ion to the 4-epimerase and to L-ribulose-5-P and D-xylulose-5-P. Since Mn^{++} appears to activate the enzyme, it should be possible to determine the binding constants by nuclear magnetic resonance and electron spin resonance. An extensive kinetic analysis would also elucidate the role of the divalent cation in 4-epimerization. However, as previously discussed a metal ion would facilitate the enzymatic catalysis of an dealdolization-aldolization reaction by acting as an electron sink, and it would facilitate the proposed dehydration-hydration mechanism by acting as

both an electron sink, and as a Lewis acid in removing the alpha proton. In addition the metal could facilitate the translocation of the migrating hydroxyl group and could act as a common coordinate to bring the enzyme and the substrate into close proximity.

Metal enzymes can contain strongly bound metals and are referred to as metallo enzymes, or they can contain freely dissociable metals and are referred to as metal-enzyme complexes. In order to be classified as a metallo enzyme the metal-enzyme complex must have the following characteristics as outlined by Vallee (127): (a) the ratio of moles of protein to metal must be an integral number; (b) the ratio of metal to coenzyme, when the latter is part of the active complex must be an integral number; (c) the highly purified protein can be isolated with its full metal complement and full activity; and (d) the ratio of moles of metal to protein or coenzyme must be a small number, conforming with the law of multiple proportions. Vallee (127) also points out that the characteristics of metal-enzyme complexes include: (a) complete removal of the metal ion may not result in complete abolition of activity; (b) the association constant of the metal ion with the reactive group of the protein molecule will be low, and the metal ion will therefore be readily removed by dialysis; removal will be accompanied by a partial loss of activity and may be

restored by the addition of the metal ion; and (c) different metals may substitute for one another in bringing about activation of the enzyme activity. Since the purified 4-epimerase was not subjected to a metal analysis, it is not known how many of the criteria for metallo enzymes are met. However, it is known that more than one metal can activate the 4-epimerase and activation by Mn^{++} is partially inhibited by Mg^{++}. If the inhibition by Mg^{++} and other metal ions were competitive, it would indicate that Mn^{++} was readily displaced by the other metals, or, in other words, the Mn^{++} easily dissociates from the enzyme surface and is readily replaced by other metals. If the metal is not tightly bound to the enzyme surface, then 4-epimerase falls into the category of metal-enzyme complexes. This is consistent with the fact that Mg^{++} and Mn^{++} usually form weak complexes with proteins (125). A study of the binding properties of the different metal ions for L-ribulose-5-P 4-epimerase would also help to determine how the metal ion functions in the catalysis.

Of the other 4-epimerases which have been studied only UDP-glucose 4-epimerase (59) and UDP-N-acetylglucosamine (63) have been reported to be activated by metal ions. The UDP-glucose 4-epimerase activation (59) appeared to be nonspecific since the activity was independent of the metal ion added, and activation was also obtained with amines. The metal appeared to prevent product inhibition,

and thus the metal may bind to the product and change its properties resulting in a shift in the equilibrium of the reaction and a decrease in the amount of product readily accessible to the protein.

Glaser (63) reported that UDP-N-acetylglucosamine 4-epimerase was stimulated by Mg^{++} at a maximal concentration of 2×10^{-3}M and was inhibited by concentrations of Mg^{++} higher than 1×10^{-2}M. The pH optimum of the enzyme shifted from 8.5 to 9.5 when Mg^{++} was removed from the assay. However, the enzyme preparation had only been partially purified, and it was not reported whether the enzyme had activity in the absence of any metal ion or whether other metal ions had any effect on the enzyme activity. Insufficient data were reported to ascertain whether the metal ion was necessary for catalysis or whether the metal ion only had a non-specific effect on the enzyme. The presence of specific divalent metal ions may also shift the pH optimum of L-ribulose-5-P 4-epimerase.

The spectrum of the 4-epimerase indicated that the enzyme did not contain any tightly bound cofactors which absorb between 300 mμ and 700 mμ with a molar extinction coefficient greater than 4×10^2. Thus assuming that cobamide coenzyme, flavins, NADH or pyridoxal phosphate have approximately the same extinction coefficients when bound to an enzyme as when free in solution, these cofactors could not be tightly bound to the 4-epimerase. Likewise

a 280/260 mμ absorption ratio of 1.78 is indicative of the absence of a bound adenine moiety. A characteristic reduced NAD^+ or flavin fluorescence spectrum was not produced by the enzyme, and the enzyme was not inactivated by washing with charcoal or incubating with NADase (21).

The microbiological assay for NAD^+ and the lack of bound ^{14}C-NAD^+ on the purified 4-epimerase more definitively indicated that L-ribulose-5-phosphate 4-epimerase does not contain bound NAD^+. If NAD^+ were bound either covalently or ionically, the entire molecule or the nicotinamide moiety would have been released by hydrolysis in acid or base. L. arabinosus would be expected to grow on any of the released forms. Hence, the inability of L. arabinosus to use the 4-epimerase as a source of nicotinic acid constitutes strong evidence in support of the theory that NAD^+ is not present.

In all organisms which have been studied (128), the de novo synthesis of NAD^+ involves the reaction of quinolinic acid with 5-phosphoribosyl-1-pyrophosphate to form nicotinic acid mononucleotide which is then converted to NAD^+. Organisms whose de novo synthesis of NAD^+ is blocked must rely on the conversion of nicotinic acid to nicotinic acid mononucleotide for the synthesis of NAD^+. Thus, growing nicotinic acid auxotrophs in nicotinic acid (carboxyl-^{14}C) should have resulted in the radioactive labeling of all the nicotinic acid nucleotides. The fact that both

^{14}C-NAD^+ and ^{14}C nicotinamide could be identified chromatographically in the crude extracts indicates that ^{14}C-nicotinic acid was converted to ^{14}C-NAD^+ and ^{14}C-nicotinamide under the conditions of these experiments.

The ability to reduce the ^{14}C content of the 4-epimerase preparation from an auxotroph grown on ^{14}C-nicotinic acid to less than 0.01 mole of ^{14}C per mole of 4-epimerase constitutes further substantial evidence that neither NAD^+ nor any other nicotinic acid derivative is bound to the 4-epimerase. Further, the 4-epimerase had activity in the absence of added NAD^+, and the activity of the enzyme was not increased by NAD^+, NADH, $NADP^+$, and NADPH.

Thus, it appears that the mechanism of L-ribulose-5-P and D-xylulose-5-P 4-epimerization does not involve a NAD^+-catalyzed oxidation-reduction as has been rather substantially shown to occur with UDP-glucose 4-epimerase. However, not enough definitive data has been obtained to conclusively rule out an oxidation-reduction mechanism facilitated by an enzyme-bound electron acceptor other than NAD^+. The electron acceptor would have to be tightly bound to the enzyme surface since enzyme does not appear to require the addition of any cofactor other than metal ions. It does not seem reasonable that the metal is acting as an electron acceptor. The reduction potential for Mn^{+++}/Mn^{++} is 1.7 volts removed from NAD^+/NADH reduction potential. Since the overall epimerization reaction requires both an

oxidation and a reduction step, energy will be released on one side of the transition step and will be required on the other side of the transition step, and the net energy change will be zero. Thus, 39 kcal/mole would be released on reduction of Mn^{+++}, assuming the reduction potential of the 4-ketopentose phosphate to L-ribulose-5-P and D-xylulose-5-P was similar to the reduction potential of acetaldehyde to β-hydroxybutrylaldehyde and assuming the reduction potential of Mn^{+++}/Mn^{++} is the same when the metal is bound to the protein as when it is free in solution. This same amount of energy would be required to reduce the oxidized intermediate of the pentose phosphate.

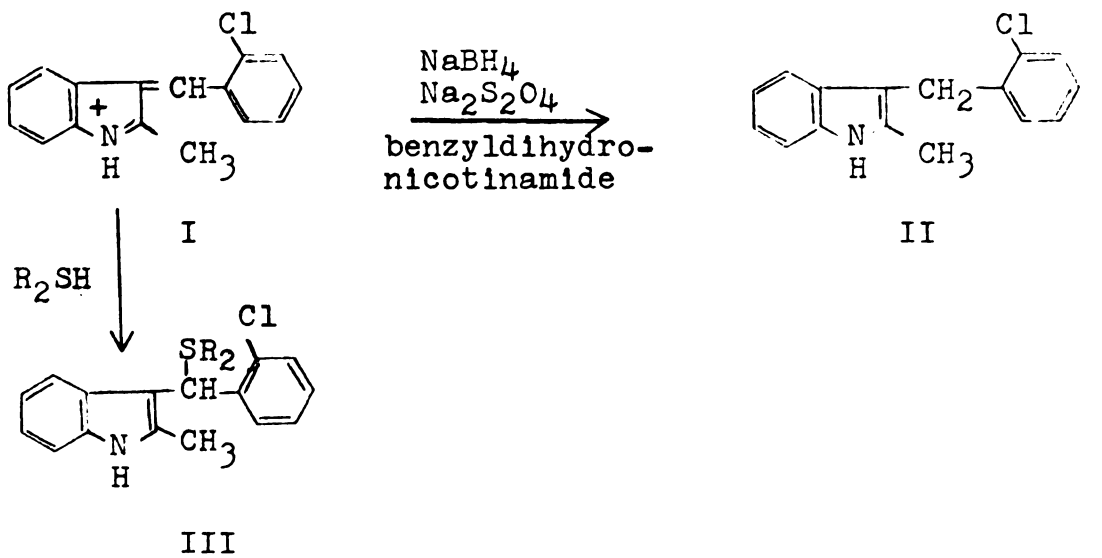
Other cofactors which could accept the hydride ion from the pentose-5-P include cobamide coenzyme, cystine, lipoate and the oxidized derivative of tryptophan as previously discussed. The cobamide coenzyme contains Co^{++} bond to the 5' carbon of adenine, which is thought to be the hydride accepting site of the coenzyme (105, 106). The Co-carbon bond is readily cleaved by light and acid hydrolysis. Thus, most enzymes containing bound cobamide-coenzyme are sensitive to light and are salmon colored. L-Ribulose-5-P 4-epimerase is colorless even in crystalline form and is not sensitive to light, nor does it have an absorption spectrum characteristic of free cobamide-coenzyme. However, mammaliam methylmalonyl-CoA isomerase contains tightly bound cobamide coenzyme and was not inactivated by treatment with

charcoal, light, cyanide or extrinsic factor. The enzyme could be resolved by acid treatment, and the apoenzyme was reactivated by dimethylbenzimidazole and benzimidazole coenzyme, but not adenylcobalamine coenzyme (129). Since cobamide coenzyme contains carbon bound Co^{++}, the Co^{++} is not freely exchangeable with other metal ions in solution. Although the 4-epimerase is activated by Co^{++}, Mn^{++} is the more efficient metal activator, and the metal ion is freely exchangeable with other metal ions in solution.

Cystine and lipoic acid have reduction potentials comparable to that of NAD^{+} and could, thus, participate in the epimerization reaction. Both disulfide compounds would be active in the oxidized form, and, since disulfides are readily oxidized by air, the oxidized form would predominate on the enzyme surface. Although lipoic acid absorbs at 330 mμ, its extinction coefficient was too low to be readily detected. Borohydride should have reduced the disulfide, but, the disulfide may have been reoxidized prior or during the assay. However 4 hours are usually required for air oxidation of cysteine to cystine in protein hydrolysates (130). In addition, enzyme activity was not lost on a one-hour incubation of the enzyme with 1 M mercaptoethanol assayed in the presence of 0.05 M mercaptoethanol, which are the conditions which are usually sufficient to maintain a disulfide in the reduced state. Although 50% of the activity was lost on incubating for an

additional hour, the activity was not recovered on passage through the Sephadex column indicating the inactivation was due to some phenomenon other than reduction of a disulfide at the active site. Further arsenite should have reacted with the reduced disulfide and inactivated the enzyme. Likewise, sulfite should have reacted with the disulfide forming the stable S-sulfonated derivative and inactivating the enzyme.

There are no substantial data to indicate the involvement of an indolenine intermediate of tryptophan as the electron acceptor in the 4-epimerization. Schellenberg (111) was able to reduce the indolenine salt (o-chlorophenyl (2-methyl-3H-indolylidine) methane hydrochloride) (I) to the indole (II) by sodium borohydride, dithionite, and 1-benzyl-1,4-dihydronicotinamide. The indolenine salt (I) readily added to mercaptobenzene, benzylmercaptan, and methyl thioglycolate to give the corresponding thioether indole (III).



Similarly, if the indolenine derivative of tryptophan were present in the 4-epimerase it would have been expected to be reduced by borohydride in the absence of substrate, whereas substrate would have protected against such an inhibition. The above results indicate that the indolenine salt could be stabilized by reaction with a sulfhydryl group on the enzyme surface, however, the indolenine salt must be generated first. This presumably could be accomplished by NAD^+ in solution. Likewise, NADH in solution may be able to reduce the indolenine derivative, however, a 30 minute incubation of the enzyme with NADH did not inactivate the 4-epimerase, and the enzyme was active in the presence of NADH and the absence of NAD^+ in the assay medium.

Consequently, the results are not consistent with the electron-acceptor being NAD^+, cobamide coenzyme, lipoate or cystine, or an oxidized indolenine derivative of tryptophan.

The presence of a carbonyl group β to the site of epimerization in L-ribulose-5-P and D-xylulose-5-P would greatly facilitate an epimerization mechanism involving carbon-carbon bond cleavage or a dehydration-hydration mechanism of epimerization, as previously discussed. If the mechanism involves carbon-carbon bond cleavage, the 4-epimerase appears to be more comparable to Class II aldolases than to Class I aldolases. This is based on the

observation that: (a) the enzyme-substrate complex is not reduced by sodium borohydride; (b) the enzyme is activated by metal ions; (c) the enzyme is inhibited with metal chelators; and (d) there is no ^{18}O exchange (21). Although tightly bound Zn^{++} is predominate in aldolases, most classes of enzymes are not consistent as to the type of metal required for enzyme activity. No one has looked for ^{18}O exchange with Class II aldolases, but there is no need for ^{18}O exchange based on the mechanism proposed by Rutter (114, 115). The dihydroxyacetone carbanion intermediate would not have to dissociate from the 4-epimerase surface, and, thus, there is no need for T exchange with the medium. This is substantiated by the fact that the dihydroxyacetone intermediate in transaldolase does not dissociate from the enzyme, nor is there T exchange with the medium (113). Although the carbanion intermediate was detected in fructose-diphosphate aldolase, this could be due to the fact that dihydroxyacetone-P can bind to the aldolase in the absence of glyceraldehyde-3-P. Thus, the bound dihydroxyacetone-P should exist in resonance with the carbanion intermediate and would be freely accessible to attack by tetranitro-methane. However, there is no reason for a glycoaldehyde intermediate to dissociate from L-ribulose-5-P 4-epimerase, and the carbanion intermediate in the epimerization reaction is probably short lived and inaccessible to attack by tetra-nitromethane.

The proposed dehydration-rehydration mechanism of epimerization is based on known chemical and enzymatic reactions as discussed previously. Although the mechanism involves a carbanion intermediate, it would be stabilized by tautomerization to the enol intermediate and would be very short lived and probably not accessible to attack by tetranitromethane. The removal of both a proton and a hydroxyl group from an enzyme usually leads to incorporation of a proton or hydroxyl from the medium. Thus, T or ^{18}O exchange would have been expected if epimerization were proceeding by this mechanism. However, both Jencks (39) and Rose (38) point out that protons transfer can occur at a rate faster than diffusion into the medium. Since the proton from C-3 of the pentulose-5-P returns to the same stereochemical position, it may not be freely accessible to exchange with the medium. Although the hydroxyl from C-4 is returning to the opposite side of the carbon, it could be translocated to a site on the enzyme located central to the C-4 position, and, thus, the hydroxyl would be freely accessible to both sides of the carbon and may not have a chance to exchange with the medium. As with other hydrases and dehydrases this mechanism would be facilitated by the presence of the metal ion as previously discussed. This mechanism would also be facilitated by Schiff base formation; however, Schiff base formation has not been indicated in other hydrases or dehydrases and is

not indicated here since there is no ^{18}O exchange or reduction of the L-ribulose-5-P enzyme complex by borohydride.

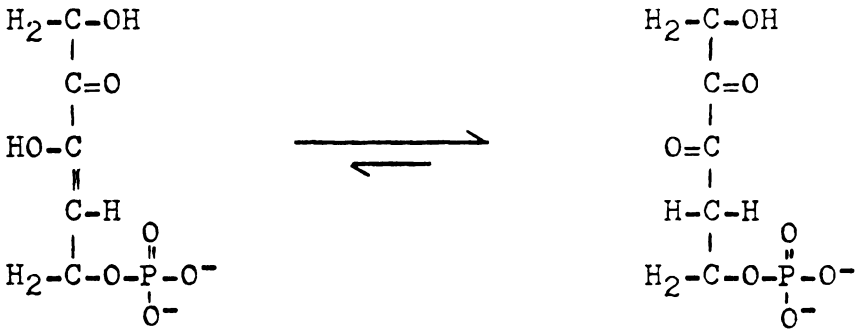
The most obvious way to definitively distinguish between an oxidation-reduction, dehydration-hydration or dealdolization-aldolization mechanism, would be to incubate the enzyme with the intermediate and show that both D-xylulose-5-P and L-ribulose-5-P were formed. The difficulty is in obtaining the intermediates.

The proposed intermediate for the oxidation-reduction reaction is L-2,4-diketopentulose-5-P, a relatively unstable compound. D-Ribulose-1-P (L-4-ketoribitol-5-P) is an intermediate in the D-arabinose metabolism of E. coli (131) and might serve as a pseudointermediate. It could probably be oxidized at C-2 by Gluconobacter oxydans to give the desired L-2,4-diketopentulose-5-P which would probably readily tautomerize, epimerize at C-3, and possible cleave between C-2 and C-3 or C-3 and C-4. However, incubation of the more stable L-4-ketoribitol-5-P with the enzyme which had been reduced with $NaBT_4$, might result in incorporation of T to give D-xylulose-5-P-4-T and L-ribulose-5-P-4-T. This experiment is dependent on the presence of an electron acceptor on the enzyme surface which can be reduced by borohydride and maintained in the reduced state by keeping the enzyme under anerobic conditions.

The intermediate of the proposed dealdolization-aldolization reaction include dihydroxyacetone carbanion

and glycoaldehyde phosphate. Glycoaldehyde phosphate is not readily available. A proton would have to be removed from the dihydroxyacetone which is not a normal step in the proposed epimerization reaction. However, incubation of the enzyme with the two intermediates may result in formation of D-xylulose-5-P and L-ribulose-5-P.

The intermediate for the proposed dehydration-hydration mechanism would be 2-keto-3,4-pentene-1,3-diol-5-P which would spontaneously rearrange to the more stable 2,3-diketo-1-pentitol-5-P.



Thus either 2-keto-3,4-pentene-5-P or 2-keto-3-methoxy-3,4-pentene-1ol-5-P would be needed to stabilize the double bond between C-3 and C-4. However, without the hydroxyl group at C-3 these compounds may not bind to the active site of the 4-epimerase. If they did, it could be reasoned that D or ^{18}O should be incorporated into the intermediate if the enzyme was equilibrated with D_2O or $H_2{}^{18}O$. Since this mechanism requires a site on the enzyme which binds both the hydroxyl group and proton from the substrate, in the absence of substrate the proton or hydroxyl group from

water should be bound to these sites and would thus available for transfer into the intermediate.

All of these methods would be dependent on the ability of the intermediate or pseudointermediate to enter the active site of the enzyme.

SUMMARY

A specific activity of 70 ± 10% was obtained for the homogeneous L-ribulose-5-P 4-epimerase from A. aerogenes at pH 8.0 and 28°C when Mn^{++} was the only divalent metal ion present. Colorless needles of the 4-epimerase crystallized from an ammonium sulfate solution. A molecular weight of $1.14 \times 10^5 \pm 1.4 \times 10^3$ was obtained for the homogeneous enzyme by high speed ultracentrifugation experiments. Tightly bound NAD^+ was not detected on the 4-epimerase, and NAD^+ was not required for activity nor was the activity enhanced by NAD^+, NADH, $NADP^+$, or NADPH. Divalent metal ions activated the 4-epimerase to varying extents with the order of activation being $Mn^{++} > Co^{++} > Ni^{++} > Ca^{++} > Zn^{++} > Mg$. EDTA inactivated the 4-epimerase and the activity was not recovered on the removal of the EDTA. Borohydride did not inhibit the 4-epimerase either in the presence or absence of substrate. Sulfhydryl reagents such as mercaptoethanol, dithiotheritiol, arsenite, and sulfite, did not inhibit the enzyme at a concentration below 10^{-2}M. The mechanism of 4-epimerization could be oxidation-reduction, dealdolization-aldolization, or dehydration-hydration; however, the indirect evidence is not consistent with an oxidation-reduction mechanism.

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APPENDIX

Appendix. Stability Constants of Metal Complexes (132)

Name	Type of Stability Constant	Log of Stability Constant							
		Mg	Ca	Mn	Fe	Co	Ni	Cu	Zn
Bal	K_1			5.2			45.6		13.5
	B_2			10.4			22.3		23.0
EDTA	K_1	8.7	10.6	14.0	14.2	16.0	18.6	18.8	16.4
	K_1 app (7.0)	5.4	7.3	10.7	10.9	12.7	15.3	15.5	13.1
	K_1 app (8.0)	6.4	8.3	11.7	11.9	13.7	16.3	16.5	14.1
o-Phenanthroline	K_1	1.5	0.5	3.9	5.9	7.3	8.6	6.3	6.4
	B_2			7.0		14.0	14.0	12.5	12.1
	B_3			10.0	21.0	20.0	20.0	18.0	17.0
8-Hydroxyquinoline 5-sulfonic acid	K_1	4.8	3.5	6.9	8.4	8.8	9.8	12.5	8.7
	B_2	8.4			15.1	15.9	18.5	23.1	16.2
Glycylglycine	K_1	1.1	1.2	2.2		2.9	4.5	6.0	3.8
	B_2					5.2	7.9	11.6	6.6
Imidazole	K_1		(0.1?)	1.6	3.3	2.4	2.9	4.2	2.0
	B_2			2.9	6.4	4.4	5.4	7.7	4.2
	B_3					6.0	7.3	10.1	6.6
	B_4					7.2	8.7	12.5	9.5

$K_1 = \frac{[ML]}{[M][L]}$ M - metal L - ligand

$K_2 = \frac{[ML_2]}{[ML][L]}$

$B_2 = K_1K_2 \qquad B_3 = K_1K_2K_3 \qquad B_4 = K_1K_2K_3K_4$