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DIPHOSPHATE-GALACTOSE-4-EPIMERASE.

The Ohio State University, Ph.D., 1972
Biochemistry

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STUDIES ON THE MECHANISM OF ACTION OF URIDINE
diphosphate-galactose-4-epimerase

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Timple Go Wee, A.A., M.D.

The Ohio State University
1972

Approved by

[Signature]
Adviser
Department of Chemistry
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ACKNOWLEDGMENTS

I wish to express my gratitude to my adviser, Professor Perry A. Frey for suggesting the research problem, his continuous guidance throughout the investigation and in the preparation of this thesis.

I want to thank Carol Workman, Kyung-ja Oh and Kevin Quiggle for their technical assistance in the purification of UDP-galactose-4-epimerase.
To my mother
**VITA**

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Major Field: Biochemistry
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INTRODUCTION

In 1949, Caputto et al. isolated an enzyme system from \textit{Saccharomyces fragilis} which catalyzed the conversion of galactose-1-phosphate (Gal-1-P) to glucose-1-phosphate (G-1-P), thus causing an apparent inversion at the C-4 position (1). The enzyme system was named galactowaldenase. The transformation required a coenzyme which they subsequently characterized and named uridine diphosphoglucose (UDPG) (2-4). A similar activity was also isolated from lactating mammary gland by Caputto and Trucco and from \textit{Saccharomyces marxianis} by Garner and Grannis (5,6). The latter was called phosphogalactoisomerase.

Since that time the mechanism of conversion has been the subject of numerous investigations (7-23). Several mechanisms have been entertained, namely: (a) cyclization of a hexose to L-inositol, which on ring reopening could yield either glucose or galactose (7), (b) formation of a 1,4-cyclic monophosphate intermediate with simultaneous inversion at C-4 followed by deesterification involving the latter and phosphate (8), (c) cleavage between C-3 and C-4 of the hexose followed by recondensation (9,10), (d) nucleophilic attack by OH\(^-\) of the medium to bring about inversion of configuration (9), (e) removal of water between carbon atoms 3 and 4 or between carbon atoms 4 and 5 followed by rehydration, (f) dehydrogenation
between carbon atoms 3 and 4 or 4 and 5, to give an enediol intermediate, followed by rehydrogenation, and (g) oxidation of the secondary alcohol group at carbon 4 to a keto group, followed by reduction.

Fischer proposed the cyclization of a hexose to L-inositol (7). As shown in Figure 1, L-inositol could yield either glucose (ring opened at B) or galactose (ring opened at A). Stetten and Klein found that when glycogen was formed from deuterated glucose or galactose in rat liver, a portion of its carbon-bound hydrogen originated from the stable hydrogen of the sugar fed, the remaining coming from the body water (24). In addition, the transformation of galactose into glycogen in rats using [1-14C]-galactose resulted in the recovery of [1-14C]-glucose upon hydrolysis of liver glycogen (25). These findings excluded not only the inositol mechanism but also the cleavage and recondensation hypothesis. Furthermore, the latter mechanism entails the formation of triose, however, during the reaction no trace of free triose was detected even in the presence of cyanide, which would displace the equilibrium in favor of triose (16). Investigations utilizing [2-14C]-galactose resulted in the production of liver glycogen in which the bulk of the tracer remained at C-2; if inositol were the intermediate the carbon labeled would have been C-5 (19,20). Moser et al. administered [6-14C]-glucose to mice and found the recovered cerebroside galactose to contain labeling at C-6, further supporting nonrandomization during the interconversion.
Figure 1. Cyclization of glucose to L-inositol.
Figure 1
When UDPG alone was incubated with the yeast enzyme, 25% was transformed into uridine diphosphogalactose (UDPGal) (17). Leloir suggested a working hypothesis for hexose phosphate transformation to occur in two steps (10,16):

\[
\text{Gal-1-P} + \text{UDPG} \rightleftharpoons \text{G-1-P} + \text{UDPGal} \quad (1)
\]
\[
\text{UDPGal} \rightleftharpoons \text{UDPG} \quad (2)
\]

Kalckar et al. demonstrated that reaction (1) is an independent reaction by isolating the specific enzyme called Gal-1-P uridylyl transferase (26). They further proposed that since the term galactowaldenase was used for the overall interconversion of hexose phosphates and since the two enzymes involved were separated, that the protein catalyzing reaction (2) be called uridine diphosphogalactose-4-epimerase (12).

Anderson, Lendel and Diedrich studied the same enzyme obtained from *Lactobacillus bulgaricus* Cere A. They employed the bacterial system described by Hansen and Craine, in which the formation of G-1-P was detected as G-6-P in the presence of phosphoglucomutase when Gal-1-P was incubated with the enzyme preparation (27). They showed that the inversion did not involve exchange of $^{18}\text{O}$ between the substrate and the medium (13). Simultaneously, Kowalsky and Koshland confirmed the finding that neither $^{18}\text{O}$ nor tritium was taken up from $^{3}\text{H}_2^{18}\text{O}$ during the reaction. Prolonged dialysis of *bulgaricus* enzyme did not affect its activity but treatment with *neurospora* NADase did destroy the enzyme action, which suggested
that the enzyme contains a tightly bound NAD⁺ (14). Maxwell isolated epimerase from calf liver which required NAD⁺ to exert enzymatic activity. Its Km for NAD⁺ is 2 x 10⁻⁷ M (15). With the discovery of uridine diphosphoglucose dehydrogenase by Strominger et al., assay of epimerase became much easier (28,29). When UDPGal was incubated with the enzyme preparation in the presence of D₂O, the rate of conversion to UDPG was not different from that occurring in water (12). These evidences appeared to eliminate the simple substitution mechanism and to some extent the dehydrogenation-rehydrogenation as well. The most plausible mechanism is oxidation-reduction involving NAD⁺ with the formation of a keto-intermediate. Failure to transfer tritium labeled at the C-4 position of NAD⁺ to the hexose indicated that the hydrogen originally removed from the hexose in the first step is stereospecifically reintroduced into the isomeric hexose in the second step (13). The C-4 position is known to be the active site in reactions involving NAD⁺ (11,30). Likewise, the lack of tritium incorporation into the substrate was also found when NADH, labeled with tritium at both C-4 positions, was used. This would indicate that the reduced NAD⁺ formed as a result of oxidation of the hexose moiety of UDPG, never dissociates from the enzyme, but exists momentarily as enzyme-NADH-substrate complex. This was further supported by the finding that during the reaction, there was no detectable NADH accumulation, and coupling the reaction to other enzymatic systems which oxidize NADH had no
effect. Attempts to trap the intermediates with carbonyl reagents were unsuccessful (18, 21). Since the existence of NADH was not demonstrated then, one other possibility was that the pyridine nucleotide was never involved in the isomerization.

The in vitro experiments of Bevill et al. have shown that when \([4-^3H]-\text{glucose-1-phosphate}\) was added to a reaction mixture containing uridine triphosphate, uridine diphosphoglucose pyrophosphorylase, inorganic pyrophosphatase and \textit{S. fragilis} enzyme, the galactose isolated contained labeling at C-4 (22). Therefore, either the C-4' hydrogen is stereospecifically removed and reintroduced or it never departs from the molecule. The in vivo experiment of Kohn et al. showed that glucose obtained from liver glycogen of rats had a tritium to \(^{14}\text{C}\) ratio similar to that of the substrate, which was \([U-^{14}\text{C}]-\text{galactose}\) with tritium at position 4 (23). These experiments showed that there is no loss of tritium during epimerization. However, they do not in any manner exclude or include the possibility of involvement of NAD\(^+\) in the process.

Weismajer and Jordan were the first to use \textit{Escherichia coli} as a source of enzyme (32). They prepared UDPGal from \textit{E. coli} extract strain C7M. Imae et al. isolated epimerase from \textit{E. coli} K-12 strain (33). Of the three different sources of protein, namely liver, yeast and \textit{E. coli}, the \textit{E. coli} and yeast enzymes possess a tightly bound NAD\(^+\) which does not dissociate even on extensive purification. Using \textit{E. coli} strain K-12 gal\(^+\) (λdg), Wilson and
Hogness carried the purification to greater purity. Their purest enzyme isolated has a molecular weight of 79,000 with a specific activity of 13,000 units per mg of protein. The pH optimum is between 8 and 8.5 and, at equilibrium, the ratio of UDPG to UDPGal is 3.5, which is close to the finding of Leloir (34). Km's for UDPG and UDPGal are $1.0 \times 10^{-3} M$ and $1.6 \times 10^{-4} M$ respectively (33,34). The turnover number was estimated to be $5.0 \times 10^{2}$ per second on transforming UDPGal to UDPG. The protein has an amino acid sequence of 360 residues; each is represented twice within the native enzyme. The fact that guanidine hydrochloride reduces the molecular weight to one half and the presence of a pair of aspartic acid in the amino terminal per mole of protein are consistent with the simple conclusion that the sequence of 360 residues is contained within one single polypeptide. Therefore, the enzyme is a dimer held together by noncovalent bonds which contains one mole NAD$^+$ per mole of dimer. This further suggests that there is a single site for NAD$^+$ binding if the association of the two subunits creates an axis of two fold symmetry (35).

Studies on the structure of *S. fragilis* enzyme by several investigators indicated that the active protein has a molecular weight of 125,000 and each molecule contains 16 sulfhydryl groups per NAD$^+$ bound to it (42-45). The yeast enzyme exhibits a natural blue fluorescence when obtained from organisms induced with D-galactose (36,37). This was attributed to the presence of tightly
bound NADH molecules (38). The fluorescence spectrum is similar to 
that of NADH, with an excitation maximum at 350 µm and emission 
maximum at 450 µm (46).

The *S. fragilis* enzyme is stimulated by different cations 
such as Na\(^+\), Mg\(^{2+}\), spermine and spermidine (42). This was shown 
to be the result of a decrease in the Km of the enzyme for UDPGal, 
which may be attributed to the effects of cations on the enzyme 
conformation and aggregation. Depending on the concentrations of 
the cation, the enzyme could assume three different states of 
aggregation, 3.6S, 6.5S and 10.9S. They corresponded to inactive 
enzyme titrated with parachloromercuribenzoate (PCMB), partially 
active enzyme observed in solutions of low cation concentration, 
and fully active enzyme observed in the presence of relatively high 
concentrations of activating cations. These three stages of 
aggregation are caused by successive binary associations of subunits, 
resulting in molecular weights of 60,000, 125,000 and 250,000 
respectively (45).

Fluorescence of *S. fragilis* enzyme appeared to depend upon an 
ordered protein structure. Para-chloromercuribenzoate abolished 
the fluorescence and rendered the protein inactive. Upon addition 
of NAD\(^+\) and mercaptoethanol, the activity was restored but not the 
fluorescence property (36, 40). The dissociation appeared to be 
associated with loss of NAD\(^+\) which in turn was required for 
reassociation. This was further supported by the observation of
decreased fluorescence upon treatment of reduced epimerase with
guanidine hydrochloride; the latter caused subunit dissociation and
not necessarily dissociation of pyridine nucleotides. Sodium
borohydride caused an increase in the fluorescence of the yeast
enzyme which disappeared on removal of the reductant. This
fluorescence was five times enhanced relative to that due to an
equivalent concentration of NADH alone. Its intensity gradually
diminished in the absence of substrate. If this enzyme was allowed
to stand, the loss of fluorescence was accompanied by complete
restoration of activity. Substrate itself did not increase the
fluorescence of the enzyme, but small amounts of substrate
stabilized the reduced epimerase. The enhanced fluorescence
greater than NADH alone is probably caused by the active site
environment which probably determines the conformation of pyridine
nucleotide when bound at the active site. It is known that the
conformation of pyridine nucleotide affects the fluorescence property,
since α-NADH is not fluorescent but β-NADH is. Denaturation of
fluorescent protein released not only both pyridine nucleotides,
but also substrates. Reoxidation of some preparations of reduced
epimerase could be achieved by lactic acid dehydrogenase and
pyruvate (43).

Bhaduri et al. induced fluorescence in the yeast protein by
using substrate analogs (46). They incubated the epimerase with
5'-uridine mononucleotide (UMP) and one of the following simple
sugars, D-glucose, D-galactose, D-fucose, D-xylose, or L-arabinose,
in concentrations of $10^{-3} \text{M}$ to $10^{-2} \text{M}$. The most reactive sugar was L-arabinose. The fluorescence produced by D-glucose in the presence of UMP was slow to appear and stable (147-49). This suggested that most likely the sugar was oxidized in the process, because of the formation of reduced epimerase, but there was no indication that the sugar was epimerized. Under this condition, the binding of UMP to reduced epimerase, although not covalent, persisted after passage through five Sephadex G-50 columns. However, the bound UMP could be removed readily, together with the bound NADH, by titration of the available sulfhydryl groups with PCMB. The resulting apoenzyme would not bind UMP. Hence the binding of substrate or substrate analog to the enzyme is dependent both on the presence of and the oxidation state of the pyridine nucleotide. Using [2-$^{14}$C]-UMP, approximately one mole of UMP per mole of reduced dimer was released by PCMB together with the NADH (51,52). The reduced epimerase could be fully activated by long storage in dilute solutions at 4°C, presumably due to autooxidation. This may be attributed to a greater ease of dissociation of bound UMP or sugar derivatives in dilute solution. In more concentrated solutions, there may be aggregation of reduced epimerase molecules. Subunit interactions in aggregates may stabilize with respect to changes needed for reactivation.

Bertland and Kalckar incubated *S. fragilis* enzyme with UMP and glucose for 4 hours. The reduced protein revealed a rearrangement
in structure, as evidenced by changes in the ORD and CD spectra. This suggested that the fluorescent enzyme contains more β-structure and less α-helix than the native protein. The reactivation of reduced enzyme suggested reversibility of this alteration in structure (49). This phenomenon of reductive inactivation was also demonstrated in epimerase obtained from E. coli.

Unlike the yeast enzyme in E. coli, epimerase becomes fluorescent in the presence of a specific substrate such as UDPGal. The rate of increase in fluorescence is considerably faster than that observed with UMP and simple sugars. All the sugars which are active in the reduction depend on the presence of UMP with the exception of 2-deoxy and 3-deoxy sugars. These sugars bring about a limited reduction of 1.4 to 1.7-fold increase in fluorescence, however, on addition of UMP the reduction proceeds much further (50). The mechanism in this case is difficult to understand.

As early as 1952, Paladini and Leloir purified UDPG prepared enzymatically and found UDP-xylose (UDPX) to be a contaminant (57). Neufeld et al. found that extracts from mung bean seedlings (Phaseolus aureus) could catalyze the interconversion not only of UDPG and UDPGal but also of UDPX and UDP-L-arabinose (UDPA). This was interpreted then as a side reaction due to impure preparations of enzyme (58). The substrate specificity of yeast epimerase was further studied by Salo et al. who found that uridine diphosphate-D-fucose, UDPA, and UDPX were epimerized, while UDP-D-mannose, UDP-N-acetyl-D-galactosamine, UDP-D-allose, UDP-3-O-methyl-D-glucose were
not. Nucleotide sugars of purines and other pyrimidines were not epimerized. The epimerizations of UDP-pentoses could be due either to broad specificity of this enzyme or that the enzyme preparations used contained a contaminating protein capable of catalyzing the epimerization of UDP-pentoses \((59,60)\). These findings therefore suggested that ribose and uracil are essential to activity.

Bardowsky et al. speculated that the presence of an acylamide grouping of \(-\text{C}_2(\text{X})-\text{N}_3\text{H}-\text{C}_4(\text{X})-\)\(^1\), where \(\text{X}\) could be oxygen or sulfur, appeared necessary for enzymatic activity \((61)\). de Robichon-Szulmajster proposed that the pyrimidine ring of the substrate molecule actually participates in the reaction and that oxidation-reduction involves two hydrogen acceptors, uracil and NAD\(^+\). The situation was allowable by the molecular configuration of the substrate and the binding of uracil to enzyme \((62)\). Ankel and Maitra showed that partially purified UDP-L-arabinose-4-epimerase from \textit{E. coli} contained UDP-galactose epimerase activity. The authors used \textit{E. coli} mutants having either normal, decreased or non-detectable levels of UDP-galactose epimerase. In all the cases studied, UDP-L-arabinose-4-epimerase levels paralleled those of UDP-galactose epimerase almost exactly, indicating that UDP-galactose epimerase can actually use UDPX as substrate. Furthermore, kinetic experiments have shown that UDPX is a competitive inhibitor of UDP-galactose epimerase and UDPG is likewise a competitive inhibitor of UDP-arabinose

\(^1\)The number next to the atom refers to its position in the ring.
epimerase. The $K_I$'s of UDPG and UDPX were found to be $0.7 \times 10^{-3}$M and $1.8 \times 10^{-3}$M respectively. The $K_m$ of UDP-galactose epimerase for UDPX is $1.2 \times 10^{-3}$M (63).

These findings indicate that epimerization of UDPX to UDPA in *E. coli* is catalyzed by UDP-galactose epimerase. Mutations that affect UDP-galactose epimerase have the same effects on UDP-arabinose epimerase and hence position 6 of the pyranose ring is not important in catalysis.

Position 1 of the sugar moiety seems unlikely on chemical grounds to be directly participating in epimerization. Sayema and Kalckar failed to observe the appearance of tritium in the $NAD^+$ when UDP-[1-$^3$H]-galactose was incubated with epimerase (54). This finding, however, does not exclude the involvement of C-1. Other carbon atoms possibly involved in the epimerization are C-2, C-3, C-4 and C-5. The lack of epimerization of UDP-mannose, UDP-2-deoxyglucose, and UDP-N-acetylglucosamine indicates that C-2 may be necessary for catalysis (59). Furthermore, 2-keto sugar has been found to oxidize reduced epimerase (56). However, the direct participation of C-2 would require two consecutive tautomerationizations to achieve the isomerization, causing it to be mechanistically unlikely (Fig. 2). Carbons 3 and 5 are the next most probable sites. Oxidation at either position could facilitate epimerization at C-4 through the formation of enediol intermediate. The lack of hydrogen exchange between the substrate and the medium does not
Figure 2. Epimerization of UDP-glucose to UDP-galactose involving carbon 2 of pyranose ring.
entirely rule out this possibility, since the group accepting the hydrogen is not necessarily accessible to the medium. This possibility is consistent with the observed inability of the enzyme to catalyze epimerization of UDP-D-allose and UDP-3-O-methyl glucose. Furthermore, there was also no epimerization of UDP-β-D-glucose or UDP-D-glucuronic acid (59). Other possible roles of C-1, C-2, C-6 and perhaps C-3 are for binding of substrate to the enzyme such that only certain configurational orientation is presented to the active site. The presence of a bulky polar group at C-6, as in UDP-glucuronic acid, may result in distortion or poor fitting of the substrate.

Experimental evidences have shown that the oxidation-reduction is made possible because of the built-in NAD⁺ molecule (12-14). It has been shown that other epimerases such as UDP-glucuronic acid-4-epimerase also requires NAD⁺ for catalysis (64). It is premature to conclude that all epimerases effect their catalyses by mechanisms involving NAD⁺, but it is likely that UDP-galactose epimerase does. Wilson and Hogness' finding that 19% of the NAD⁺ bound to the E. coli enzyme becomes reduced at equilibrium in the presence of saturating concentrations of substrates was the first direct evidence for the participation of NAD⁺ in the initial reduction (34). Despite the fact that the E. coli enzyme is a dimer, there is no evidence for the involvement of another NAD⁺ or other coenzymes during the catalysis, however, the participation of a group on the enzyme has
not been ruled out and is a reasonable possibility. The situation may be similar to that of alcohol dehydrogenase, where tryptophan has been postulated to be directly involved in hydrogen transfer (65).

The direct transfer of hydrogen from C-4 to enzyme-bound NAD\(^+\) is the most popular hypothetical pathway (76,78). In other reactions involving enzyme bound NAD\(^+\), such as thymidine diphosphate-D-glucose oxidoreductase and UDP-D-glucuronate carboxy-lyase, oxidations at C-4 of the sugar moieties are involved (66,67). This would naturally suggest the UDP-galactose-4-epimerase also functions in the same manner. Thymidine diphosphate-D-glucose oxidoreductase catalyzes hydrogen transfer specifically to the \(\beta\) side of NAD\(^+\)(66). It appears likely that epimerase would behave similarly, however, it has never been possible to predict the stereochemistry of such reactions. Tracer studies using isotopically labeled substrates may be able to differentiate between reaction pathways involving oxidation at C-3 or C-4 of substrate sugar moieties.

Hydrogen transfer during epimerization may be intramolecular or intermolecular. Intermolecular transfer can occur by a variety of hypothetical pathways, but in particular it could entail the participation of another hydrogen acceptor on the enzyme other than NAD\(^+\). The resulting intermediate enzyme complex can assume one orientation where minimal conformational change is required. A hypothetical group \((Y)\) could function as intermediate hydride acceptor in one of the following ways:
(1) Intramolecular hydrogen transfer

\[
\begin{align*}
E^\cdot Y^\cdot NAD + SH & \rightarrow E^\cdot Y^\cdot NAD \rightarrow E^\cdot X^\cdot NAD \rightarrow E^\cdot Y^\cdot NAD \\
& \rightarrow E^\cdot PH^\cdot NAD \rightarrow E^\cdot NAD + PH
\end{align*}
\]

(2) Intermolecular hydrogen transfer

\[
\begin{align*}
E^\cdot YH' + SH & \rightarrow E^\cdot YH' \rightarrow E^\cdot X^\cdot NADH \rightarrow E^\cdot YH' \rightarrow E^\cdot PH' \rightarrow E^\cdot NADH + PH'
\end{align*}
\]

where \(SH\) = substrate, \(PH\) = product and \(X\) = intermediate. Pathway (1) would not result in isotope labeling of the enzyme but pathway (2) would, if substrate is labeled with tritium at C-4. The findings of isotopic labeling of enzyme or coenzyme during the conversion using UDP-[\(^4\)-\(^3\)H]-glucose would favor pathway (2). If a second hydride acceptor is not involved, catalysis of hydrogen transfer can still be achieved inter- or intramolecularly by inducing compensatory conformational changes of the intermediate or enzyme·intermediate complex after the initial reduction. Hydrogen could be transferred from one side of the pyranose ring to \(NAD^+\) and returned to the other side of the intermediate resulting in the formation of product. This requires at least 2 different conformations of intermediate or enzyme·intermediate complex.

Of course many questions about and possibilities for hydrogen transfer pathways can be entertained. Since the bacterial and yeast
epimerases are dimers, it is particularly interesting for one to consider the possibility of two binding sites, one for the substrate and one for the product. So far no evidence for such has been found.

The purpose of this investigation is to attempt to establish unambiguously the pathway of hydrogen transfer during the epimerization and to study the role of pyridine nucleotide and nature of the substrate-derived intermediates.
**MATERIALS AND METHODS**

**Reagents**

The following reagents were obtained from the indicated commercial suppliers:

- UDP-D-glucose, UDP-D-galactose, UDP-D-xylose, 5'UMP, EDTA tetrasodium dihydrate, N,N-bis(2-hydroxyethyl)glycine (bicine), A grade and 1,4-bis[2-(5-phenyloxazolyl)] benzene scintillation grade from Calbiochem; pyruvic acid, glutamic acid, potassium α-keto-glutarate, β-NAD⁺, glutathione (reduced form) and Trizma base reagent grade (Tris) from Sigma Chemical Corp.; UTP and NADH from Boehringer Mannheim; Biogel P-2 and analytical grade anion and cation exchange resins from BioRad Laboratories; tritiated water from New England Nuclear; UDP-D-[U-1⁴C]-galactose, D-[4-³H]-glucose, D-[3-³H]-glucose, D-[U-1⁴C]-glucose and [³H]-NaBH₄ from Amersham-Searle; semicarbazide hydrochloride, NaBH₄, L(+) arabinose, and D(+) xylose from Matheson, Coleman and Bell; cyclohexanone, cyclohexanol and D-glucose anhydrous analytical reagent from Mallinckrdot; Glucostat from Worthington Biochemical Corp.; α-bromoacetophenone from Aldrich Chemical Company; Sephadex G-25, medium, 100-200 mesh from Pharmacia Fine Chemicals, Inc.; 3,5-dinitrobenzoyl chloride and lactic acid from Baker Chemical Company; D-galactose, C.P. from Pfanstiehl Laboratories, Inc.; Naphthalene (certified) from Fischer Scientific.
Co.; 2,5-diphenyloxazole scintillation grade (PPO) from Research Products International Corp.

**Enzymes**

Lactic acid dehydrogenase, UDP-glucose dehydrogenase, UDP-glucose pyrophosphorylase, inorganic pyrophosphatase were purchased from Sigma Chemical Company. Glutamic acid dehydrogenase, hexokinase and phosphoglucomutase were purchased from Calbiochem. Acetoacetate decarboxylase was a gift from Prof. F. H. Westheimer, Department of Chemistry, Harvard University.

UDP-galactose-4-epimerase was purified in our laboratory from *E. coli* following the procedure of Wilson and Hogness (34). Protein determination during the early purification was done by using the Warburg and Christian method (70), however, after the hydroxylapatite chromatography (Step VI), protein concentrations were calculated according to the method of Wilson and Hogness from the optical density at 280 μm with 1 cm light path, on the assumption that 1 mg of protein per ml has an optical density of 1.05. For cases where exactly equivalent concentrations of native and reduced epimerase were desired, Lowry's method was employed (69). The final specific activity of the various preparations is usually about 10,000 units per mg. However, in some experiments enzyme preparations of specific activity as low as 6,000 units per mg protein were also used.
Bacteria

An E. coli strain K12 mutant which was operator constitutive was grown in the medium as described by Wilson and Hogness, except that D-fucose was omitted (34). Growth was carried out in 10-liter containers at 37°C with maximum aeration. The bacteria were harvested after the exponential growth phase using a Sorvall refrigerated centrifuge equipped with a continuous flow head. The cell pellets were frozen at -4°C and could be removed and thawed anytime.

Assay of UDP-galactose-4-epimerase

The enzyme was assayed according to the method of Wilson and Hogness (34). The reactions are as follow:

\[
\text{UDP-galactose-4-epimerase} \quad \text{UDP-galactose} \quad \text{UDP-glucose}
\]

\[
\text{H}_2\text{O} + \text{UDP} + 2\text{NAD}^+ \quad \text{UDP-glucose} \quad \text{UDP-glucuronate}^- + 2\text{NADH} + 3\text{H}^+
\]

dehydrogenase

The reaction mixtures contained, in a total volume of 0.50 ml, 0.13 umole of UDP-galactose, 0.63 umole of NAD\(^+\), 440 units of UDP-glucose dehydrogenase, 50 umoles of sodium bicinate buffer at pH 8.5 and less than 0.1 unit of UDP-galactose-4-epimerase. The reaction was allowed to proceed at 27°C and the increase in absorption at 340 m\(\mu\) was followed using a Norelco Unicam SP 800 spectrophotometer equipped with an external recorder (Sargent-Welch) and scale expansion accessory. The change in optical density per minute is converted to micromoles of UDP-glucose formed per minute by using the \(\epsilon_{340}\)
of $6.2 \times 10^3 \text{M}^{-1} \text{cm}^{-1}$ for NADH (71), and taking into consideration that 2 moles of NADH are formed per mole of UDP-glucose produced. One unit of enzyme activity is defined as that amount of enzyme which catalyzes the formation of one umole of UDP-glucose per hour under the conditions described. In this assay, the rate of NADH formation is not increased by increasing the UDP-glucose dehydrogenase concentration.

Chromatography

Gel filtrations were performed using Sephadex G-25 and Biogel P-2 columns. All paper chromatograms were developed descending using either Whatmann 3MM or Schleicher and Schuell paper in one of the following solvents: I, ethanol-1M ammonium acetate (pH 7.5) (7:3) (88); II, ethyl acetate-pyridine-water (12:5:4) (87); III, aqueous phenol, 88% phenol (82); IV, isobutyric acid-1 M NH$_3$ (5:3).

Commercial UDPG and UDP-[U-$^{14}$C]-galactose were purified by paper chromatography using solvent I at room temperature. Radioactive glucose was purified by paper chromatography using solvent II at 4$^\circ$. Glucose concentration was determined by Glucostat, and UDP-glucose and UDP-galactose concentrations were determined by their absorbance at 260 m$_\nu$ using $\epsilon_{260}$ of $9.72 \times 10^3 \text{M}^{-1} \text{cm}^{-1}$ and $9.6 \times 10^3 \text{M}^{-1} \text{cm}^{-1}$ respectively. The following solutions were used for reactions or elution of chromatography columns: Buffer A, $0.001\text{M K}_2\text{HPO}_4$ in $0.001\text{M EDTA}$; Buffer B, $0.003\text{M}$ potassium phosphate
in 0.001M EDTA pH 8.7; Buffer C, 0.01M K₂HPO₄ in 0.001M EDTA.

**Preparation of NaBH₄ solution**

This was done by cooling in an ice bath simultaneously a measured solution of 0.001M K₂HPO₄ and a weighed amount of NaBH₄ in two separate test tubes. The two were mixed in the cold immediately before use.

**Determination of radioactivity**

Paper chromatograms containing ^1⁴C or tritium were scanned with a Packard radiochromatogram scanner model 385. All liquid scintillation counting was done with a Packard Tricarb liquid scintillation spectrometer, Model 3310. Tritium or ^1⁴C-containing compounds were dissolved in vials containing 1 ml of water and 15 ml of scintillation fluid consisting of 1,4-dioxane which contained 7 gm of diphenyloxazole (PPO), 0.3 gm of 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP) and 100 gm of naphthalene per liter of dioxane.

**Preparation of UDP-glucose**

UDP-glucose was prepared enzymatically using a modification of the method of Wright and Robbins (73). The following reactions are involved:

\[
\text{Hexokinase} \\
\text{Glucose + UTP} \xrightleftharpoons{\text{Hexokinase}} \text{G-6-P + UDP} \\
\text{G-6-P} \xrightarrow{\text{G6Pase}} \text{6-1-P} \\
\text{UDPG} \xrightarrow{\text{UDPG pyrophosphorylase}} \text{UDP + PPI}
\]
The reaction was carried out by mixing glucose, UTP, Mg\(^{++}\), cysteine, glucose 1,6-diphosphate, hexokinase, phosphoglucomutase, inorganic pyrophosphatase, UDP-glucose pyrophosphorylase in Tris-HCl buffer at pH 7.8 in a total volume of about 2 ml. The reaction mixture was incubated for at least 3 hours in a 27\(^{\circ}\) bath. UDP-glucose formation was followed by taking aliquots at different intervals and assaying with UDP-glucose dehydrogenase and NAD\(^{+}\). Usually the amount of UDP-glucose formed reached a constant value after 3 hours. Figure 3 shows the time course for formation of UDP-glucose, in which 4 \(\mu\)moles of glucose were present as the starting material. The solution was heated to destroy the proteins, filtered, and concentrated to a small volume before passing through a Biogel P-2 column. Figure 4 shows UDP-glucose in the first and second peaks well separated from the nucleosides.\(^2\) The radioactivity was due to the fact that 6.00 \(\mu\)moles of D-[U-\(^{14}\)C]-glucose (specific activity 3.09 \(\times\) \(10^6\) cpm per \(\mu\)mole) were used to make UDP-[U-\(^{14}\)C]-glucose. The fractions in the first two peaks were pooled, concentrated and applied to S and S paper. Descending chromatograms were developed with solvent I at room temperature for 18 hours. The areas corresponding to UDP-glucose were identified by radiochromatogram scanner and UV light. They were

\(^2\)Repeated chromatography using Biogel P-2 column showed two UDPG peaks. Purification of both peaks separately revealed that both contained UDPG having identical specific radioactivity.
Figure 3. The rate of formation of UDP-glucose from glucose + UTP.

The procedure and reaction mixture is described in the text and also in Figure 4.
Figure 4. Separation of UDP-\([U-^{14}C]\)-glucose from other nucleosides.

The reaction mixture contained 6.05 \(\mu\)moles of purified D-\([U-^{14}C]\)-glucose (specific activity \(3.09 \times 10^6\) cpm per \(\mu\)mole), 12 \(\mu\)moles of UTP, 25 \(\mu\)moles of MgCl\(_2\), 12.5 \(\mu\)moles of cysteine, 0.02 \(\mu\)mole of glucose 1,6-diphosphate, 23 units of hexokinase, 15 units of phosphoglucomutase, 10 units of inorganic pyrophosphatase, 15 units of UDP-glucose pyrophosphorylase and 17.6 \(\mu\)moles of Tris-HCl buffer at pH 7.8, in a total volume of 1.80 ml. The solution was placed in a 27\(^\circ\) bath. The amount of UDP-glucose formed was measured by taking aliquots and assaying UDP-glucose using dehydrogenase and NAD\(^+\). At the end of 5\(\frac{1}{2}\) hours, the yield was 5.8 \(\mu\)moles. The solution was placed into a 100\(^\circ\) bath to denature the proteins; it was then filtered and concentrated on a rotary evaporator to approximately 1 ml volume. It was then placed on a Biogel P-2 column, size 1.5 x 42 cm, and eluted with buffer A. One ml fractions were collected at 5 min. intervals.

Symbols: \(\square--\square\), A\(_{280}\); 0---0, radioactivity.
Figure 4

Fraction Number

Radioactivity (cpm ml x 10^-5)

Absorbance

0.0

0.2

0.8

1.2

1.6

2.0

2.2

2.4

2.6

2.8
cut out, eluted from the paper, and rechromatographed using the same solvent. Final yield of UDP-[U-14C] glucose was 5.8 μmoles with a specific activity of 2.6 x 10^6 cpm per μmole.

**Formation of L-[2-3H]-glutamic acid from [3H]-NADH (83)**

To a 5.5 ml pooled solution of [3H]-NADH was added 82.5 μmoles of α-ketoglutarate, 1125 μmoles of NH₄Cl, 324 units of glutamic acid dehydrogenase and 375 μmoles of potassium phosphate buffer pH 7 in a total volume of 7.50 ml. Carrier NADH (64 μmoles) was added last after stirring the solution for 5 minutes. After 18 minutes, the reaction was complete as indicated by disappearance of A₆₄₀. The solution was heated at 100° for 1 minute and, while it was still hot, 100 mg of glutamic acid was added and stirred to dissolve. The mixture was filtered and concentrated to a smaller volume near saturation. Spontaneous crystallization occurred in the refrigerator overnight. The crystals were filtered, washed with ice-cold water, ethanol and ether.

**Formation of phenacyl lactate (84)**

The reaction mixture consisted of 120 μmoles of sodium pyruvate, 100 μmoles of NADH, 5.7 ml of [3H]-NADH solution, 50 μmoles of Tris-HCl buffer at pH 8 and 100 units of lactic dehydrogenase in a total volume of 5.95 ml at 27°. The reaction was followed by A₆₄₀ until nearly complete disappearance of NADH. After heating at 100° for 1 minute, 400 mg of activated charcoal were added and the solution stirred for 1 hour. Following centrifugation the supernatant
fluid was filtered through 5 layers of Whatmann No. 1 paper. Upon adding carrier lactic acid (244 mg), the solution was titrated to pH 5.1, combined with 5 ml of alcohol and 930 umoles of phenacyl bromide, and refluxed for 1 hour. The solution was concentrated to a smaller volume and placed in the refrigerator overnight. The precipitate was collected on a filter paper and recrystallized from benzene.

**Assay of UDPG and UDPGal**

A solution containing preferably less than 0.5 umole of UDP-hexose was added to a mixture of 0.62 umole of NAD⁺, 440 units of UDP-glucose dehydrogenase and 50 umoles of sodium bicinate at pH 8.5 in a volume of 0.5 ml inside a microcuvette. The amount of UDPG present was measured by the increase in $A_{340}$ until a constant level was obtained. Further increase in $A_{340}$ upon addition of 0.5 units of UDP-galactose epimerase was interpreted as that due to UDP-galactose. Appropriate blanks were employed.

**Formation of cyclohexyl 3,5-dinitrobenzoate (84)**

A pooled solution of 4.3 ml, supposedly containing [$^3$H]-cyclohexanol, was shaken in a Vortex-Genie with 1.0 ml of cyclohexanol (940 umoles) for 1 minute. After standing to allow time for separation, the top layer was decanted and added to 1900 umoles of 3,5-dinitrobenzyol chloride. The mixture was heated in a 100° bath for 5 minutes until a yellow liquid developed. Ten ml of cold water were added and the solution was cooled in an ice bath until product
solidified. The precipitate was collected on filter paper, washed with 10 to 15 ml of 2% sodium carbonate solution, and recrystallized from 5 to 10 ml of 60% ethanol. Crystals obtained melted at 110°.

Detection of tritiated water by tritium-hydrogen exchange (81)

A pooled fraction of supposedly radioactive water containing 1.04 x 10^5 cpm, was added to a mixture containing 673 umoles of acetone, 5 mg of acetoacetate decarboxylase (97 units) and 250 umoles of potassium phosphate buffer pH 5.9 in a total volume of 1.39 ml. The solution was incubated at 27° for 60 hours. Semicarbazide hydrochloride (894 umoles) was added with 0.15 gm of sodium acetate to make a total volume of 10.0 ml. The mixture was vigorously shaken and placed in a 100° bath and allowed to cool in an ice bath. The side of the vessel was scratched to cause spontaneous crystallization. Carrier semicarbazone of acetone (101 mg) was added and the crystals were removed by filtration and recrystallized from 50% ethanol. The M.P. was 182° compared to literature value of 187° (84). The reactions involving acetoacetate decarboxylase are shown in Figure 5. The exchange of acetone hydrogen with the medium involves equations (3) and (4).
Figure 5. Tritium-hydrogen exchange between acetone and tritiated water in the presence of acetoacetate decarboxylase.

The formation of $(\text{C}^3\text{H}_3)_2\text{CNNHCONH}_2$

The procedure and reaction mixture is described in the text.
\[ \text{CH}_3 - C - \text{CH}_2 - \text{CO}_2^- + \text{E}^+\text{NH}_2 \rightleftharpoons \text{CH}_3 - C - \text{CH}_2 - \text{CO}_2^- \]  
\[ \text{+ N-H} \]  
\[ \text{E} \]  

(1)

\[ \text{CH}_3 - C - \text{CH}_2 - \text{CO}_2^- \rightarrow \text{CH}_3 - C = \text{CH}_2 + \text{CO}_2 \]  
\[ \text{+ N-H} \]  
\[ \text{E} \]  

(2)

\[ \text{CH}_3 - C = \text{CH}_2 + ^3\text{H}_2\text{O} \rightleftharpoons \text{C}^3\text{H}_3 - C - C^3\text{H}_3 \]  
\[ \text{+ N-H} \]  
\[ \text{E} \]  

(3)

\[ \text{C}^3\text{H}_3 - C = C^3\text{H}_2 + \text{H}_2\text{O} \rightleftharpoons \text{C}^3\text{H}_3 - C - C^3\text{H}_3 + \text{E}^+\text{NH}_2 + \text{H}^+ \]  
\[ \text{+ N-H} \]  
\[ \text{E} \]  

(4)

\[ \text{C}^3\text{H}_3 - C = C^3\text{H}_3 + \text{H}_2\text{NNH}-\text{NH}_2 \rightarrow \text{C}^3\text{H}_3 - C = \text{NNH} - C - \text{NH}_2 + \text{H}_2\text{O} \]  
\[ \text{C}^3\text{H}_3 \]  

(5)

Figure 5
PART I
REDUCTIVE INACTIVATION OF URIDINE DIPHOSPHATE-GALACTOSE-4-EPIMERASE
INTRODUCTION

It has been repeatedly postulated by many authors that oxidation of carbon-4 in the hexose moiety is the first step in catalysis of the interconversion of UDPG and UDPGal, in which hydrogen is transferred from C-4 of the substrate sugar moiety to enzyme-bound NAD⁺ leading to the intermediate formation of NADH and UDP-4-ketoglucose (36,38-43, 46-51). Kalckar and coworkers demonstrated that NaBH₄ and a variety of sugars including glucose can reduce epimerase with production of fluorescence, undoubtedly due to formation of NADH. Reduction by sugars requires UMP and reduction by NaBH₄ is stimulated by UMP. This reduced epimerase is not only catalytically inactive but assumes a different conformation compared to that of native protein (49).

Wilson and Hogness observed, in the case of the E. coli epimerase, that the presence of substrates caused immediate partial reduction of enzyme-bound NAD⁺. They found that approximately 19% of the enzyme-bound NAD⁺ is in the reduced form in the presence of saturating substrates at equilibrium (34) and that the ultraviolet spectrum of enzyme-bound NADH exhibited a maximum at 345 μ and not the usual 340 μ. Kalckar et al. subsequently also showed that E. coli epimerase becomes fluorescent upon exposure to substrates (50).
In the present experiments, we were interested in further studying the nature of the reduced epimerase formed under different conditions and possibly the manner by which the reduction is brought about by the different reagents. Inasmuch as reduced epimerase evidently is a transient catalytic intermediate, it appeared that uridine nucleotide dependent reductive inactivation reactions might be partial reactions, or at least models of partial reactions. Knowledge of the mechanisms of such reactions could be valuable for understanding the overall mechanism of catalysis.

A. Results

I. Reduction by NaBH₄

a. Substrate and UMP-dependent reactions

Sodium borohydride has two possible roles in the reduction of epimerase. Since NaBH₄ is known to reduce NAD⁺, it is, therefore reasonable to expect that it could also reduce enzyme-bound NAD⁺, if the latter is accessible. This would lead to the formation of inactive epimerase. However, NaBH₄ can also reduce ketones. If the intermediate of the epimerization is UDP-4-ketoglucose, it may be trapped chemically by NaBH₄. The result would be the formation of UDPG or UDPGal, leaving the enzyme in the reduced form.

The rates of reduction by NaBH₄ may be different in the presence and absence of substrate. The accessibility of NAD⁺ may be facilitated by addition of substrate with enhancement in the rate of reduction by NaBH₄. Absolute substrate dependence could be
demonstrated by using modest concentrations of UDPG and small but not large concentrations of NaBH₄. Kalckar also found UMP-dependent NaBH₄ reduction under a different condition (50). This reaction was also re-examined under conditions comparable to substrate-dependent reactions. The two reactions appeared to be similar as described in the following.

Plots of activity against time are shown in Figures 6 and 7. Destruction of activity by NaBH₄ was 98% complete in the presence of UMP 2 minutes after incubation. In the presence of UDPG the inactivation was greater than 50% complete within 2 minutes. In the absence of UDPG or UMP, NaBH₄ caused only slight inactivation of epimerase, and UDPG or UMP alone hardly reduced the activity after 15 minutes of incubation.

(b) **Nature of reduced epimerase**

(i) **Formation and isolation of reduced epimerase**

Inactive epimerase produced in substrate-dependent reduction by NaBH₄ reducing a UDP-ketosugar intermediate or by substrate-stimulated direct NaBH₄ reduction of enzyme-bound NAD⁺. These two possibilities can be distinguished by the use of [³H]-NaBH₄ as reducing agent.

A reaction mixture containing epimerase, UDPG and [³H]-NaBH₄ was allowed to react at 27°C. After 10 minutes, an aliquot was removed for assay. When the specific activity fell to less than 5% of the original, the reaction mixture was subjected to gel
The reaction mixture consisted of 0.47 mg of epimerase (5500 units), 0.13 umole of NaBH₄, and 100 umoles of sodium bicarbonate buffer pH 8.5 in a total volume of 0.8 ml at 27°C. A second reaction mixture contained all of the above plus 0.3 umoles of UDPG. A control experiment was set up using the same amounts of epimerase and UDPG but with NaBH₄ omitted. From each of the 3 reaction mixtures, aliquots were taken at 0 and at other time intervals for assay of enzyme activity. The final concentration of NaBH₄ was 1.6 x 10⁻⁴ M and that of UDPG was 3.8 x 10⁻⁴ M.

Symbols: O—O, NaBH₄ alone; •—•, NaBH₄ + UDPG; □—□, UDPG alone.
Figure 6.
Figure 7. UMP-dependent reduction of UDP-galactose-4-epimerase by NaBH₄.

The reaction mixture consisted of 0.56 mg of epimerase (3500 units), 2.0 umoles of UMP, and 100 umoles of sodium bicininate buffer at pH 8.5 in a total volume of 0.8 ml. The reaction was allowed to proceed at 27⁰ and aliquots were taken at 0 and at other time intervals for assay of enzyme activity. The same experiment was repeated using the same reaction mixture plus 0.13 umole of NaBH₄. A control experiment was set up using the same amounts of epimerase and NaBH₄ with UMP omitted.

Symbols: 0—0, NaBH₄ alone; □—□, UMP alone; ●—●, UMP + NaBH₄.
filtration to separate protein from other small molecular weight compounds. To achieve good separation, the column was run in the cold at a slow speed.

A typical chromatogram, as shown in Figure 8, reveals fairly good separation of epimerase from UDPG and other by-products of NaBH₄. A large amount of radioactivity is associated with reduced epimerase while only very small amounts of radioactivity are found in fractions containing UDPG. The specific radioactivity of epimerase was 1.21 x 10⁷ cpm per umole, compared to 1.08 x 10⁵¹ cpm per umole found in UDPG. The data lead to the conclusion that the overwhelmingly major part of the reaction proceeds by direct [³H]-NaBH₄ reduction of enzyme-bound NAD⁺.

(ii) Spectrum of reduced epimerase

To determine if NAD⁺ was reduced during inactivation with NaBH₄, the ultraviolet spectrum of a sample of the reduced epimerase isolated from the gel column was measured versus the same concentration of native epimerase. Figure 9 shows that the reduced epimerase has an absorption maximum at 345 μμ, which is similar to that found by Wilson and Hogness for epimerase in the presence of substrates at equilibrium (34).

(iii) Release of [³H]-NADH from the reduced epimerase

In order to explore the possibility that tritium in the

---

¹After correcting for free UDPG in excess of enzyme, which would dilute any radioactive UDPG produced if an enzyme-bound keto-intermediate had been trapped.
Figure 8. Biogel P-2 chromatography of epimerase reduced by [\(^3\)H]-NaBH\(_4\) in the presence of UDPG.

The reaction mixture contained 5.58 mg of epimerase (specific activity 8,300 units per mg), 2.4 umoles of UDPG, 0.26 umole of [\(^3\)H]-NaBH\(_4\) (140 uci per umole), in a total volume of 0.74 ml. The solution was incubated at 27\(^\circ\). At the end of 10 minutes, a sample of 0.01 ml was removed for assay. The specific activity had decreased to 138 units per mg. The solution was placed onto a 1.1 x 27 cm column of Biogel P-2. It was eluted with buffer A at 4\(^\circ\), and 1.1 ml fractions were collected at 5 minute intervals.

Symbols: O——O, \(A_{278}\) (protein); •——•, \(A_{260}\) (UDP-hexose); Δ——Δ, radioactivity.
Figure 9. The difference spectrum between reduced and native epimerase.

In a semimicro quartz cuvet was placed 1.07 mg of reduced epimerase in a total volume of 1.0 ml of buffer C. The sample was scanned between 300 and 370 m\(\mu\) versus an exactly identical amount of oxidized epimerase in the reference cell after careful matching of the 2 cuvettes.
Figure 9.
reduced enzyme was located in the pyridine nucleotide, NADH was
dissociated from the protein by heating in a solution containing
carrier unlabeled NADH. Separation of NADH and protein was
achieved by gel column chromatography. Carrier NADH was present to
provide a pool for the freed NADH and also to facilitate identifi-
cation of NADH released. The results are shown in Figure 10. There
was complete separation of protein and NADH. The enzyme fractions
retained only 3% of the original radioactivity, while NADH contained
the remainder of the total activity. There was no additional peak
of radioactivity which would represent significant amounts of
UDP-hexose released during the heat process.\(^2\) From the amount of
carrier NADH added and assuming that the amount of NADH in the enzyme
was negligible, the expected specific activity of NADH was calculated
to be \(1.51 \times 10^6\) cpm per umole. A value of \(1.52 \times 10^6\) cpm per umole
was found in the peak fraction.

(iv) Determination of the stereochemistry of tritium

labeling in the \([^3\text{H}]-\text{NADH}\) molecule

Lactic dehydrogenase is known to catalyze hydride transfer
from the A side of NADH to pyruvate (84). Lactic acid was prepared
from pyruvate and lactic dehydrogenase using the \([^3\text{H}]-\text{NADH}\) solution
obtained from the dissociation experiment (Fig. 10). The procedure was
described under "Materials and Methods." The lactic acid formed was

\(^2\)UDP-hexose would be located between protein and NADH peaks when
eluted from Sephadex G-25 column. See Part III.
Figure 10. Release of $[^3H]$-NADH from reduced epimerase.

Carrier NADH (0.5 umole) was combined with 1.0 mg of tritiated reduced epimerase (preparation described in the text), in a total volume of 1.0 ml in buffer A. The solution was heated at $100^\circ$ for 5 minutes, chilled, passed into a 1 x 37 cm column of Sephadex G-25 which was previously equilibrated with buffer B, and eluted with the same buffer. Fractions of 1.35 ml were collected at 5 minute intervals.

Symbols: $0--0$, $A_{278}$ (protein); $\bullet--\bullet$, $A_{340}$ (NADH); $\Delta--\Delta$, radioactivity.
Figure 10.
isolated as phenacyl lactate. The twice recrystallized crystals melted at 94.96° in agreement with the literature value of 96° (85). Only 1.42% of the 3.6 x 10^5 cpm was found in phenacyl lactate.

The B side labeling was determined by using glutamic acid dehydrogenase, known to transfer hydrogen from the B side of NADH to α-ketoglutarate (83). Radioactive glutamic acid was prepared from radioactive NADH solution obtained from the dissociation experiment (Fig. 10) by the procedure of Levy and Vennesland (84). The procedure was described under "Materials and Methods." The twice recrystallized glutamic acid had a melting point of 195-196° which was similar to that of carrier glutamic acid (195-198°). It contained 90.1% of the 3.8 x 10^5 cpm used in the reaction.

(c) Reducibility of epimerase briefly exposed to substrate

To determine if substrate induces an irreversible or slowly reversible conformational change of the enzyme which survives substrate dissociation, a solution containing epimerase and UDP-[U-14C]-galactose was allowed to react for 5 minutes in buffer C. Upon separation by gel filtration, the amount of radioactivity in the epimerase would indicate the amount of substrate bound to it. To determine whether enzyme recovered from the column exhibited enhanced reactivity with NaBH₄, samples were treated with 1.6 x 10^{-4}M NaBH₄ in the presence or absence of UDPG and assayed under the conditions of Figure 6. Two other control experiments were done using untreated native epimerase. Aliquots were taken at 0, 3, and 11
minute intervals and assayed for activity.

Figure 11 shows the separation of epimerase and UDP-[U-14C]-hexose. Repeated similar experiments revealed that the residual radioactivity associated with the epimerase ranged between 3.6 and 17.8% of the specific radioactivity of the substrate used. The enzyme with the largest labeling was used to test for reducibility with NaBH₄. The various reaction mixtures are tabulated in Table 1-A, while the different degrees of inactivation are shown in Table 1-B. In the presence of UDPG, both treated and untreated epimerase were reduced to the same extent. In the absence of added UDPG neither the treated nor untreated enzyme exhibited enhanced reactivity with NaBH₄. It is concluded that no irreversible or slowly reversible conformation changes could be detected by this method.

(d) Determination of Km for UDPG and UMP during NaBH₄ reduction

Rates at which 1.6 x 10⁻⁴M NaBH₄ destroys the activity of UDP-galactose-4-epimerase were measured at various UDPG or UMP concentrations. These measurements were made in order to obtain information about what concentrations of these compounds would produce half-maximal rates of UDPG or UMP-dependent reductive inactivation by NaBH₄. These values could be compared with Km values for substrates and the Kᵢ for UMP in the catalytic reaction. Early data established saturation kinetics with respect to these compounds, wherein the inactivation rates were independent of nucleotide concentrations in the 10⁻² to 10⁻⁵M range. The rates were smaller
Figure 11. Resolution of UDP-[U-14C]-galactose from its mixture with epimerase.

Epimerase (3.3 mg) was combined with 0.6 umole of UDP-[U-14C]-galactose (specific activity 6.93 x 10^5 cpm per umole) in 1.0 ml of buffer C. The solution was incubated at 27° for 5 minutes, applied to a 1.5 x 42 cm Sephadex G-25 column and eluted with buffer B. Fractions of 1.1 ml were collected at 5 minute intervals. Symbols: O--O, A_{278} (protein); Δ--Δ, A_{280} (UDP-hexose); □--□ radioactivity.
Figure 11.
Table 1-A
THE COMPOSITIONS OF THE REACTION MIXTURES USED FOR DETERMINATION
OF REDUCIBILITY OF EPIMERASE BY NaBH₄

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Reaction Mixture&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Epimerase treated</td>
<td>-</td>
</tr>
<tr>
<td>untreated</td>
<td>+</td>
</tr>
<tr>
<td>UDPG</td>
<td>+</td>
</tr>
<tr>
<td>NaBH₄</td>
<td>+</td>
</tr>
<tr>
<td>Buffer C</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> + = presence of the indicated compound  
- = absence of the indicated compound

The procedure is described in the text.
TABLE 1-B  

$\text{NaBH}_4$ INACTIVATION OF EPIMERASE, EPIMERASE PLUS UDPG,  
UDP-[U-$^{14}$C]-GALACTOSE-TREATED EPIMERASE AND UDP-[U-$^{14}$C]-GALACTOSE- 
TREATED EPIMERASE PLUS UDPG  

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Time (min)</th>
<th>Specific activity (units/mg)</th>
<th>Percent inactivation</th>
<th>Specific activity (units/mg)</th>
<th>Percent inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt; $\frac{1}{2}$ hour</td>
<td></td>
<td>$\frac{1}{2}$ hours</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Specific activity</td>
<td>Percent inactivation</td>
<td>Specific activity</td>
<td>Percent inactivation</td>
</tr>
<tr>
<td>I</td>
<td>0</td>
<td>11,400</td>
<td>--</td>
<td>10,280</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3,060</td>
<td>73.2</td>
<td>3,550</td>
<td>65.4</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>868</td>
<td>92.4</td>
<td>1,150</td>
<td>89.6</td>
</tr>
<tr>
<td>II</td>
<td>0</td>
<td>9,710</td>
<td>--</td>
<td>10,890</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9,150</td>
<td>5.6</td>
<td>10,650</td>
<td>2.2</td>
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<td>11</td>
<td>8,950</td>
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<td>10,200</td>
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<tr>
<td>III</td>
<td>0</td>
<td>10,750</td>
<td>--</td>
<td>11,600</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1,165</td>
<td>89.2</td>
<td>2,270</td>
<td>80.4</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>403</td>
<td>96.2</td>
<td>1,020</td>
<td>91.2</td>
</tr>
<tr>
<td>IV</td>
<td>0</td>
<td>10,900</td>
<td>--</td>
<td>8,390</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9,530</td>
<td>12.5</td>
<td>7,930</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>8,410</td>
<td>22.8</td>
<td>7,260</td>
<td>13.4</td>
</tr>
</tbody>
</table>

Each reaction mixture contained 0.02 umole (6,800 units) of epimerase,  
0.13 umole of $\text{NaBH}_4$, 80 umoles of sodium bicinate and enough of 10^{-2}M  
phosphate buffer in 10^{-3}M EDTA at pH 8.6 to make up a total volume of  
0.8 ml. Reaction mixtures I and III also contained 2 umoles of UDPG.  
Untreated epimerase was used in reaction mixtures I and II while UDP- 
[U-$^{14}$C]-galactose treated epimerase was used in reaction mixtures III  
and IV. Samples were taken for assays at 0, 3 and 11 minutes after  
reaction.
at $10^{-5} - 10^{-7}$ M.

Varying concentrations of UMP and UDPG ranging from $10^{-7}$ to $10^{-5}$ M were added to epimerase, followed by reduction with NaBH$_4$. Assays were taken at 0 and 2 minutes. Table 2A shows the rate of reduction using 3 different concentrations of epimerase. Table 2-B was the same experiment using 3 higher concentrations of UDPG and assays were taken at longer intervals. Both experiments show a dependency of hydride reduction on UDPG or UMP concentration. A Lineweaver-Burk plot of the results in Table 2 is shown in Figure 12. The apparent $K_m$ of UDPG is $3.0 \times 10^{-6}$ M and that of UMP is $6.8 \times 10^{-7}$ M. The apparent $K_m$ for UDPG in this reaction is considerably smaller than the $K_m$ of native epimerase for UDPG in the epimerization reaction, which is $1.0 \times 10^{-3}$ M.

II. UMP-dependent reduction of UDP-galactose-4-epimerase by glucose

Davis and Glaser found a kinetic isotope effect of 3 when 3-deuteroglucose was used in the UMP-dependent reductive inactivation of UDP-galactose-4-epimerase. They proposed a model for epimerization involving UDP-3-ketoglucose. However, they were unable to demonstrate incorporation of tritium into NADH when either $[3-^3H]$-glucose or $[4-^3H]$-glucose was used in the reaction. They further explained that the radioactivity must have been incorporated into the protein (73). We re-examined this possibility by reacting glucose labeled with tritium at C-4 or C-3, with epimerase in the presence of UMP.
Table 2-A

THE DEPENDENCE OF RATE OF NaBH₄ REDUCTION ON CONCENTRATION OF UDPG OR UMP

<table>
<thead>
<tr>
<th>Trial No</th>
<th>[Substrate]</th>
<th>[Epimerase]</th>
<th>( \nu )</th>
<th>Degree of inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>2.03 x 10⁻⁵</td>
<td>4.26 x 10⁻⁶</td>
<td>5.95</td>
<td>48.5</td>
</tr>
<tr>
<td></td>
<td>4.05 x 10⁻⁶</td>
<td>'</td>
<td>3.99</td>
<td>32.5</td>
</tr>
<tr>
<td></td>
<td>2.03 x 10⁻⁶</td>
<td>'</td>
<td>3.45</td>
<td>29.2</td>
</tr>
<tr>
<td>II</td>
<td>1.01 x 10⁻⁵</td>
<td>6.3 x 10⁻⁶</td>
<td>2.28</td>
<td>45.2</td>
</tr>
<tr>
<td></td>
<td>4.05 x 10⁻⁶</td>
<td>'</td>
<td>1.67</td>
<td>37.0</td>
</tr>
<tr>
<td></td>
<td>2.03 x 10⁻⁶</td>
<td>'</td>
<td>1.36</td>
<td>30.1</td>
</tr>
<tr>
<td>III</td>
<td>1.08 x 10⁻⁵</td>
<td>1.75 x 10⁻⁵</td>
<td>11.75</td>
<td>46.7</td>
</tr>
<tr>
<td></td>
<td>4.35 x 10⁻⁶</td>
<td>'</td>
<td>8.60</td>
<td>31.4</td>
</tr>
<tr>
<td></td>
<td>2.16 x 10⁻⁶</td>
<td>'</td>
<td>6.10</td>
<td>20.4</td>
</tr>
<tr>
<td>IV</td>
<td>3.6 x 10⁻⁶</td>
<td>1.41 x 10⁻⁵</td>
<td>8.35</td>
<td>33.4</td>
</tr>
<tr>
<td></td>
<td>1.8 x 10⁻⁶</td>
<td>'</td>
<td>7.26</td>
<td>29.6</td>
</tr>
<tr>
<td></td>
<td>7.25 x 10⁻⁷</td>
<td>'</td>
<td>6.10</td>
<td>24.0</td>
</tr>
</tbody>
</table>

a [Substrate] = UDPG
b [Substrate] = UMP
c NaBH₄ concentration in all trials was 1.6 x 10⁻⁴ M
d \( \nu \) = initial velocity measured 2 minutes after addition of NaBH₄
e Inactivation 2 minutes after addition of NaBH₄

The calculated amounts of epimerase were combined with the measured amounts of UDPG or UMP in a total volume of 0.8 ml of sodium bicinate buffer (100 umoles) pH 8.6 at 27°C. The reactions were initiated by adding 0.13 umole of NaBH₄. Aliquots of 0.01 ml were removed at 0 and 2 minutes for assay of activity.
Table 2-B

THE DEPENDENCE OF RATE OF NaBH₄ REDUCTION ON CONCENTRATION OF UDPG

<table>
<thead>
<tr>
<th>Time (Min)</th>
<th>[UDP] (M)</th>
<th>Percent Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.75 x 10⁻³</td>
<td>1.86 x 10⁻³</td>
</tr>
<tr>
<td>2</td>
<td>53.2</td>
<td>55.5</td>
</tr>
<tr>
<td>4</td>
<td>72.6</td>
<td>78.5</td>
</tr>
<tr>
<td>10</td>
<td>80.0</td>
<td>84.5</td>
</tr>
<tr>
<td>20</td>
<td>82.3</td>
<td>88.7</td>
</tr>
<tr>
<td>30</td>
<td>90.1</td>
<td>91.5</td>
</tr>
</tbody>
</table>

The reaction mixtures contained 0.23 mg of epimerase (2710 units), the indicated amounts of UDPG, and 100 umoles of sodium bicine buffer pH 8.5 in a total volume of 0.8 ml. The reactions were initiated by 0.13 umole of NaBH₄. The solutions were incubated at 27°C and aliquots of 0.01 ml were removed at 0 and other intervals for assay of activity.
Figure 12. Lineweaver-Burk Plot of the initial rate of epimerase inactivation by NaBH₄ in the presence of UDPG.

The procedure is described in Table 2-A.
Figure 12.

$1 \iff$ unit$^{-1}$

$K_m = 3.0 \times 10^{-6}$ M (UDP)

$K_m = 6.8 \times 10^{-7}$ M (UMP)
and then isolating both the enzyme-NADH complex.

The reduction was carried out following the procedure of Bertland et al. except that both UMP and glucose concentrations were 10^{-3} M \text{ (47)}. Incubations were performed at pH 8.5, in 27^\circ \text{ bath} for 22 hours. When the specific activity of epimerase fell to 10\% of the original value, the solution was subjected to gel filtration in the cold. A control experiment was performed using [U-^{14}\text{C}] \text{-glucose. The amounts of radioactivity of protein isolated from the columns were determined.}

The elution profiles for the chromatography columns are shown in Figures 13-A, B, C and are summarized in Table 3. There was insignificant labeling of either NAD^{+} or epimerase. The data are inconsistent with the involvement of either C-3 or C-4 of the glucose molecule in the reaction. There was also considerable reactivation of epimerase upon gel filtration.

III. Reductive inactivation by UDP-glucose

During catalysis, the amount of keto-intermediate is probably small. However, preliminary experiments indicated that prolonged incubation with substrate caused activity losses. Prolonged incubation of epimerase with substrate may gradually transform larger amounts of free or active forms of enzyme into some unknown inactive reduced forms as a result of its reaction with the substrate molecules.

To determine whether such interactions might be important,
Figure 13. UMP-dependent reductive inactivation of UDP-galactose-4-epimerase by [4-3H]-glucose and [3-3H]-glucose.

The reaction mixtures consisted of 1.0 μmole of glucose, 3 mg of epimerase (21,500 units), 1.0 μmole of UMP, and 100 μmoles of sodium bicinate buffer at pH 8.5 in a total volume of 1.0 ml, incubated at 27°C. When the specific activity fell to less than 10% of the original value (22 hours) each reaction mixture was placed on a 1.1 x 40 cm Sephadex G-25 column and eluted with buffer B. Fractions of 1.0 ml were collected at 5 minute intervals. Specific activity of [3-3H]-glucose was 2.5 x 10^7 cpm per μmole, that of [4-3H]-glucose was 2.02 x 10^7 cpm per μmole, and that of [U-14C]-glucose was 8.16 x 10^7 cpm per μmole.

Symbols: O—O, A278 (protein); 0—0, A260 (UMP); △—△, radioactivity.
Figure 13-A. The separation of epimerase UMP and $[3^{-3}H]$-glucose.
Figure 13-B. The separation of epimerase, UMP and [\(4-{\text{H}}\)]-glucose.
Figure 13-C. The separation of epimerase, UMP and [U-14C]-glucose.
Table 3
RADIOACTIVITY OF PROTEIN ISOLATED FROM REACTION MIXTURES
CONTAINING RADIOACTIVE GLUCOSE; EPIMERASE AND UMP.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>% Inactivation before passage through column</th>
<th>% inactivation after passage through column</th>
<th>Specific radioactivity (cpm/umole)</th>
<th>Extent of labeling in the enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3-(^3^H)]-glucose</td>
<td>91.4</td>
<td>43.5</td>
<td>2.56 x 10^5</td>
<td>2.50 x 10^7</td>
</tr>
<tr>
<td>[4-(^3^H)]-glucose</td>
<td>96.0</td>
<td>64.4</td>
<td>1.06 x 10^8</td>
<td>2.02 x 10^7</td>
</tr>
<tr>
<td>[U-(^14^C)]-glucose</td>
<td>92.0</td>
<td>46.5</td>
<td>1.63 x 10^8</td>
<td>8.16 x 10^7</td>
</tr>
</tbody>
</table>

\[ a = \frac{\text{specific radioactivity of epimerase}}{\text{specific radioactivity of glucose}} \times 100 \]

The composition of the reaction mixtures and the procedure of these experiments were discussed in Figure 13.
UDPG was added to a 1 ml cuvette containing epimerase in sodium bicinate buffer. Total difference spectra of the enzyme were taken between 300 and 370 m\(\mu\) at different intervals by using native epimerase and UDPG, each placed separately in 2 different cuvettes, in the reference positions. Aliquots were also taken for assay of activity.

All spectra showed an absorption maximum at 345 m\(\mu\). Figure 14 shows an increase in \(A_{345}\) and a concomitant decrease in specific activity. There was a rapid phase in the first 1.5 to 2 hours followed by a very slow steady inactivation. At the end of 330 minutes, the residual activity was 39.3% of the original value while the amount of active, oxidized enzyme was 36.5% as calculated from the absorbance at 345 m\(\mu\) and \(\varepsilon\) of 6,200 M\(^{-1}\) cm\(^{-1}\). The two values corresponded fairly close to each other. Incubation of epimerase in buffer C under similar conditions in the absence of UDPG revealed insignificant loss of activity over the same period of time (75).
Figure 11. Reductive inactivation of UDP-galactose-4-epimerase by UDP-glucose.

The reaction mixture contained 2.9 mg of epimerase (10⁴ units per mg), 2.15 umoles of UDPG and 100 umoles of sodium bicinace buffer pH 8.5 in a 1 ml cuvette, was placed into sample position #1 of the spectrophotometer. Difference spectra were obtained between 300 and 370 μm at different time intervals versus two reference cells containing the same concentrations of the different compounds. Reference cell #1 contained epimerase in buffer C, while reference cell #2 contained UDPG and sodium bicinace. Sample cell #2 contained only buffer C. Aliquots of 0.01 ml were taken from sample cell #1 and diluted into 1.0 ml buffer C in an ice bath and catalytic assays were performed within 30 minutes after sampling.

Symbols: •----•, specific catalytic activity; 0—0, A₃₄₅.
Figure 1A. CATALYTIC ACTIVITY (units/mg protein x 0.03)

TIME (min.)

A 545

0.1

0.02
DISCUSSION

From the data presented, the reduction of *E. coli* epimerase by NaBH$_4$ is dependent on the presence of UDPG or UMP. The effective concentration of NaBH$_4$ used in the reaction was $10^{-4}$ M. A NaBH$_4$ concentration of 10 to 100 times larger is necessary to produce the same degree of inactivation in the absence of substrate. Furthermore, the E\cdot NADH produced by reduction of epimerase with NaBH$_4$ alone, is unstable and is subject to autooxidation.$^4$ These findings are similar to the observations of Kalckar and coworkers on the fluorescence property of *S. fragilis* epimerase. The authors produced fluorescence of epimerase by addition of NaBH$_4$ into enzyme solution in the presence and absence of UDPG (49). In the latter situation, the fluorescence, supposedly due to the existence of enzyme-bound NADH, was short-lived. This suggests that upon binding of UDPG to the enzyme, there is a conformational change on the protein. The resultant altered protein structure induced by UDPG facilitates the reduction of NAD$^+$, either by increasing its reactivity or exposing it to the reductant. The conformational change probably does not take place in E\cdot NADH because reductive inactivation of epimerase by NaBH$_4$ alone is transient and reversible (48). This new conformation of the enzyme

$^4$The topic will be dealt with in Part II of the text.
is maintained as long as the nucleotide portion of the substrate molecule or the whole substrate molecule remains enzyme-bound.

The data also showed that the degree of NaBH₄ reduction is related to UDPG concentration. The apparent Km of epimerase for UDPG during reduction was in the range of $10^{-8}$ to $10^{-7}$M and is $1/1000$ that of the Km for UDPG in the epimerization reaction. This can be interpreted as a greater affinity of the reduced protein for substrate. If one assumes that (a) in the presence of substrate, reduction with NaBH₄ occurs only after formation of E·S complex, (b) NaBH₄ does not bind to the protein but to E·S to effect reduction, and (c) substrate can associate with both oxidized and reduced epimerase, the following can be written:

$$
E + S \xrightleftharpoons{K_1} ES
$$
$$
EH + S \xrightleftharpoons{K_2} EHS
$$
$$
ES + NaBH₄ \xrightleftharpoons{K_1'} ES·NaBH₄ \xrightarrow{k} EHS
$$

$$
\nu = k [ES·NaBH₄]
$$

$$
[E_T] = [E] + [ES] + [EH] + [ES·NaBH₄] + [EHS]
$$

$$
= \frac{K_1K_1'[ES·NaBH₄]}{[S][NaBH₄]} + \frac{K_1'[ES·NaBH₄]}{[NaBH₄]} + \frac{K_2[EHS]}{[S]}
$$

$$
+ [ES·NaBH₄] + [EHS]
$$
\[
[E_T]-[EHS] \left(1 + \frac{K_2}{[S]}\right) = [ES \cdot NaBH_4] \left(1 + \frac{K'_1}{NaBH_4}\right) \frac{K_2}{[S][NaBH_4]}
\]

\[
v = \frac{k[[E_T]-[EHS]\left(1+\frac{K_2}{[S]}\right)]}{1+\frac{K'_1}{NaBH_4} \frac{K_1K'_1}{[S][NaBH_4]}}
\]

If initial rate is measured, \([EHS] = 0\)

\[
v = \frac{k[E_T]}{1 + \frac{K'_1}{NaBH_4} + \frac{K_1K'_1}{[S][NaBH_4]}}
\]

\[
\frac{1}{V} = \frac{1}{k[E_T]} + \frac{K'_1}{k[E_T][NaBH_4]} + \frac{K_1K'_1}{k[E_T][NaBH_4]}, \quad \frac{1}{[S]}
\]

In our experiment \([NaBH_4]\) and \([E_T]\) were constant, then \(v\) is dependent on \([S]\), \(K_1\) and \(K'_1\). The equation fits our findings although it is hard to see from our data the relationship of \(v\) to \(E_T\). Sodium borohydride is so reactive that it may not have to bind first to the protein to cause reduction. This possibility was not investigated, however, and the meaning of the experimentally measured values will depend upon whether prior adsorption of \(NaBH_4\) is involved.

There are two possible ways by which \(NaBH_4\) could cause substrate-dependent reduction of enzyme-bound \(NAD^+\), if epimerization is to occur following this hypothetical pathway:

\[
E \cdot NaD^+ + SH \Longleftrightarrow E \cdot NaD^+ \cdot SH \Longleftrightarrow E \cdot NaDH \cdot X \Longleftrightarrow E \cdot NaD^+ \cdot PH \Longleftrightarrow E \cdot NaD^+ + PH
\]
The X in complex III may be UDP-4-ketoglucose or UDP-3-ketoglucose which epimerizes by enolization at C-4. Reduction of enzyme by NaBH₄ if it is to be substrate dependent has to occur in either the Michaelis complexes (II and IV) or complex III. The hydride may be transferred to NAD⁺ or X, the keto-intermediate. When tritiated NaBH₄ is the reducing agent, the labeling is expected to be found in the NADH or substrates or both. In either case, NAD⁺ would be reduced on the enzyme with or without keto-intermediate or substrate bound to it. We found the overwhelming majority of the radioactivity associated with the protein and only insignificant amounts in the substrate fractions. In addition, upon dissociation of [³H]-NADH from the enzyme, nearly 97% of the radioactivity eluted from the gel filtration column, was found in the NADH fractions, indicating that labeling was mainly on the pyridine nucleotide and not on any other hydride acceptors of the protein. Therefore, the substrate-dependent reduction of epimerase by NaBH₄ occurs on the NAD⁺ bound to the protein. The reduction of X, the keto-intermediate, probably does not take place because of its inaccessibility or low reactivity to NaBH₄. If the enzyme-bound X is reduced by [³H]-NaBH₄ in the process, the amount of radioactive substrates will be small because of the low concentration of complex III. In the dissociation experiment, the elution profile failed to show a radioactive peak in the fractions supposed to correspond to elution of substrates from the column, indicating that if the reduced radioactive enzyme
contained bound substrate, the latter was not labeled (Fig. 10).

Nelsestuen and Kirkwood studied the stereochemistry of
$[^{3}\text{H}]-\text{NaBH}_4$ reduction of epimerase and reported that the $[^{3}\text{H}]-\text{NADH}$ isolated carried at least 76% of the radioactivity on the B side of the nicotinamide ring (68). Our experiments using the A and B specific dehydrogenases revealed that at least 90% of the tritium was transferred from $[^{3}\text{H}]-\text{NADH}$ to $\alpha$-ketoglutarate, a reaction known to be specific for hydrogen transfer from the B side of NADH, while only 1.4% of the tritium could be transferred to pyruvic acid, a reaction catalyzed by A-side specific lactic dehydrogenase. These results further support the contention that reduction was on the NAD$^+$ bound to the protein and not on free NAD$^+$, since the latter would produce non-specific labeling. Reduction of bound NAD$^+$ is possible only on the side of the molecule exposed to the reactant.

Kalckar et al. pioneered in the formation of reduced epimerase by reacting enzyme with glucose and UMP (47). We wanted to show if there was specific labeling of NAD$^+$ by incubating protein with UMP and glucose labeled with tritium at C-3 or C-4. [$U$-$^{14}\text{C}]$glucose was employed in the control experiment to detect non-specific binding of glucose to epimerase. In all the reaction mixtures, epimerase was successfully reduced but none of the protein recovered had significant amounts of radioactivity, indicating that the hydrogen transferred from the sugar molecule to the NAD$^+$ does not involve positions C-4 or C-3 of the glucose molecule. Davis and Glaser found
a kinetic isotope effect of 3 using D-glucose-3d but they failed to observe a transfer of tritium to NAD$^+$ when [3-$^3$H]-glucose or [4-$^3$H]-6-deoxyglucose was incubated with the enzyme and UMP (72). They proposed a model for the catalysis of UDP-galactose-4-epimerase involving a 3-keto-intermediate. The lack of tritium incorporation into the NADH was attributed to the presence of a hydride acceptor on the protein. The present experiments contradict this hypothesis.

Ketley and Schellenberg while incubating epimerase with UMP and sugar, observed a direct transfer of tritium from [4-$^3$H]-galactose to the B side of the pyridine nucleotide but not in the case of [4-$^3$H]-glucose under similar conditions of incubation (65). Recently Sayema and Kalckar isolated the corresponding aldonic acid of the sugar used and claimed that the oxidation probably involved position 1 of the sugar (53). When [1-$^3$H]-galactose and UMP were used in the reductive inactivation of epimerase, there was isotopic dilution of the bound NADH. All of these observations seem to suggest that the UMP-dependent reduction of epimerase by different sugars involves pathways different from that of the normal catalysis. Both position 1 of D-glucose and D-xylose and either position 1 or 4 of D-galactose, D-fucose and L-arabinose are involved. It is possible to rationalize these observations speculatively. If one assumes anomeric specificity for $\alpha$-D sugars, then the fact that hydrogen transfers from both C-1 and C-4 of galactose suggests that only equatorial hydrogen is removed in the process. In $\alpha$-D-glucose and
α-D-xylose, if the molecule is rotated 180° along a horizontal axis, equatorial C-1 hydrogen will be in the transferring position and C-2, C-3 will assume the same relationship as C-3 and C-2 of α-D-galactose. For α-D-galactose, α-D-fucose and α-L-arabinose, the molecule assumes the same C-2 to C-4 relationship whether it is rotated or unrotated, therefore, the hydrogen transfer may occur at either C-4 or C-1. This accounts for the large isotopic dilution of NADH observed (53). Figure 15 shows the structural relationship of the different sugar molecules represented in the chair conformation. If oxidation occurs at C-4, the 4-ketosugar may undergo further cleavage into trioses or dehydration reaction involving C-5 and C-6. The combined size of sugar and UMP is smaller than the substrate, and this may allow free rotation of glucose at the active site such that it can bind with either C-1 or C-4 hydrogen close to the NAD⁺. Other positions such as C-2 and C-3 may function in the binding of sugar to the protein; this may partially explain the kinetic isotope effect observed when position 3 was deuterated. This more than one way of binding of glucose to the protein would be an example of a poor fitting of substrate to the active site and hence a slow rate of reduction.

The reactivation of reduced epimerase obtained from UMP-dependent reduction by glucose is possible if the aldonic acid can be reduced by the enzyme-bound NADH in situ to form the corresponding sugar and subsequent dissociation of UMP and sugar from the oxidized
Figure 15. The structural relationship of the simple sugars used in the UMP-dependent reduction of UDP-epimerase.

α-D-glucose

α-D-galactose

α-D-fucose

α-L-arabinose

α-D-xylose
enzyme complex. The same activation can be achieved by direct
dissociation of aldonic acid and UMP from the reduced enzyme complex.
The resultant E•NADH may undergo autooxidation with restoration of
catalytic activity. Table 3 shows a 50% reactivation of all the
reduced epimerases isolated upon passage through gel columns. This
suggests that in contrast to the S. fragilis enzyme, the UMP molecule
does not bind tightly to the reduced E. coli epimerase and
dissociation is fairly easy.

Prolonged incubation of UDPG and enzyme results in decrease of
catalytic activity and reduction of NAD$^+$ bound to epimerase. This
was verified by Nelsestuen and Kirkwood in independent experiments
(74). The amount of inactivation corresponds fairly well to the
amount of reduced epimerase formed. The process is biphasic,
characterized by an initial burst followed by a very slow steady
inactivation. This reduced epimerase cannot be E•NADH, the species
capable of self-reactivation, because it is stable. One possible
cause of inactivation is spontaneous dissociation of NADH from the
protein, although all the spectra taken during the process were
found to have an absorption peak at 345 μm and not 340 μm. This
remains a possibility because the peak absorption would depend on the
relative amounts of NADH and E•NADH in the reaction mixture. Other
possibilities include formation of a stable substrate-reduced
epimerase complex, or substrate-triggered dissociation of the protein
core into single subunits with or without the pyridine
nucleotide remaining bound. The former possibility is likely since one notices that the apparent Km for UDPG decreases on reduction of the enzyme. However, one has to account for the dissociation of the keto-intermediate from E'NADH·X before binding of substrate unless the substrate is bound to another site. The second possibility is somewhat improbable since most enzymes become more stable in the presence of their specific substrates and do not undergo dissociation into inactive subunits. A reduced enzyme formed under this condition is somewhat reactivated on passage through a gel column indicating that the process is reversible either by reassociation of subunits or other mechanisms. This problem will be further clarified in Part III.
PART II

REOXIDATION AND REACTIVATION OF REDUCED URIDINE DIPHOSPHATE-GALACTOSE-1'-EPIMERASE
INTRODUCTION

Following reduction of epimerase bound NAD\(^+\), a subsequent step in catalysis involves reoxidation of the enzyme-bound NADH with reduction of the intermediate. If the intermediate is UDP-4-keto-glucose, this would require that the intermediate assume a second conformation at the active site or that a more extensive conformational change involving the E·NADH part of the E·NADH·X complex occur such that hydride can be transferred to the epimeric side of the keto-intermediate. In the absence of conformational change of either X or E·NADH·X, catalysis might be achieved by mediation of hydride transfer by an adjacent group on the enzyme acting as a second hydride acceptor and donor. The absence of tritium exchange with the medium and the observation that hydrogen transfer in epimerization is intramolecular (12-14, 86) do not support this, but neither do they exclude it. Conformational change of the reduced S. fragilis epimerase relative to native enzyme has been reported by Bertland and Kalckar (49).

Creveling et al. observed the reversible fluorescence property of epimerase reduced by NaBH\(_4\) alone (43). This implies that the reduced epimerase formed under such conditions is unstable and is subject to autooxidation. The E·NADH probably has not undergone a considerable conformational shift because of the absence of
substrate; it may lose its hydride to another group on the enzyme and become oxidized spontaneously. If this is taking place, one might expect exchange of the hydrogen of this hypothetical group with the medium if it is a disulfide and is accessible. If it is not exposed, exchange may still be possible under partial denaturing conditions. This also assumes that the hypothetical disulfide group is adjacent to NADH only in the native conformation. Following this line of speculation, epimerase reduced in the presence of substrate should be reactivated by processes capable of removing or destroying UDPG or UMP.

Nelsestuen and Kirkwood found that acetone could bring about autooxidation when added to a solution of epimerase reduced by NaBH₄ alone (74). Acetone has potentially a dual function in this case; it can remove the excess hydride and it might also be reduced by NADH, thereby reactivating the enzyme. Of course, one has to be able to differentiate this kind of reactivation from the spontaneous oxidation that will occur irrespective of the addition of acetone. However, if a non-specific ketone, such as acetone can be reduced by NADH, then other ketones and aldehydes such as cyclohexanone should be able to act in the same manner.

A. Results

I. Spontaneous reoxidation

This phenomenon was observed when epimerase was reduced with NaBH₄ alone in the absence of UDPG or UMP. When 1.3 umoles of
NaBH₄ was added to 1.7 mg of epimerase in 1.0 ml of buffer C at
27° the absorbance at 345 mµ increased to 0.058 within 12 minutes,
indicating a 45% reduction. The A₃₄₅ then fell gradually to 0 after
25 minutes. The determination of catalytic activity taken at this
stage, showed that the specific activity was 11,000 units per mg of
protein, which was the original activity of the enzyme before re-
duction. A repeat of this experiment under similar conditions
showed that A₃₄₅ increased to 0.074 (65% reduction) within 10
minutes and then decreased to 0 after 17 minutes.

In contrast to this E-NADH complex prepared by reduction
of epimerase by NaBH₄ alone, the reduced epimerase obtained from
similar reaction mixture in the presence of UDPG is relatively
stable. A sample of the stable reduced epimerase, prepared according
to the procedure described in Figure 8, with a specific activity of
554 units per mg was passed through a Sephadex G-25 column. Upon
its recovery the specific activity was 1,120 units per mg, which is
only about one tenth of the original activity. Reactivation was
significant but relatively small.

Another sample of reduced epimerase was obtained by addition
of [³H]-NaBH₄ into a reaction mixture containing epimerase and UDPG
incubated for over 2 hours.¹ The protein was subjected to dialysis
against buffer C with change of dialyzing fluid every 12 hours. At
different intervals, the solution was removed from the bag and protein
concentration, enzymatic activity and radioactivity were determined.

¹Details of the preparation will be described in Part III.
The absorption peak shifted from 270 to 278 μm, while R values \( \left( A_{278} / A_{262} \right) \) increased from 1.15 to 1.36 after 48 hours. The specific radioactivity decreased from \( 4.08 \times 10^5 \) to \( 1.18 \times 10^5 \) cpm per mg while there was little change in specific activity of the enzyme (Table 4-A).

Another preparation of reduced epimerase was obtained by \( \text{NaBH}_4 \) reduction of a reaction mixture containing epimerase and \( \left[4^{-3}\text{H}\right]-\text{UDP-hexose} \) incubated for over 2 hours.\(^1\) Dialysis was performed as above. The results, as shown in Table 4-B, reveal an increase of R from 1.33 to 1.37, with an increase in specific activity from 4,200 to 7,320 units per mg while the specific radioactivity decreased from 5,060 to 1,530 cpm per mg of protein.

We have repeatedly observed that reduced epimerase freshly prepared would have a specific activity of about 100 units per mg. After separation by gel filtration, the specific activity increased to between 500 to 1,000 units per mg. If this enzyme preparation was allowed to age in the cold for several months, the specific activity would gradually increase to as high as 4,000 units per mg.

II. Effects of acetone and UDPG on reoxidation

Kirkwood and coworkers found that acetone, when added to a solution of epimerase reduced by \( \text{NaBH}_4 \) alone, would reactivate the protein. This effect was abolished by addition of TDP-glucose (68,74). We investigated this observation by adding acetone, in
Table 4-A

DIALYSIS OF REDUCED EPIMERASE<sup>a</sup>

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>R&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Specific radioactivity (cpm/mg)</th>
<th>Specific enzyme activity (units/mg)</th>
<th>Absorption peak (μ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.15</td>
<td>4.08 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>4,120</td>
<td>270</td>
</tr>
<tr>
<td>24</td>
<td>1.28</td>
<td>3.84 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>5,630</td>
<td>277</td>
</tr>
<tr>
<td>48</td>
<td>1.36</td>
<td>2.86 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>5,480</td>
<td>278</td>
</tr>
<tr>
<td>72</td>
<td>1.33</td>
<td>2.36 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>1.34</td>
<td>1.72 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>4,440</td>
<td></td>
</tr>
<tr>
<td>144</td>
<td>1.30</td>
<td>1.16 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>4,200</td>
<td></td>
</tr>
<tr>
<td>166</td>
<td>1.29</td>
<td>1.18 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>4,900</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>The enzyme preparation was kept in the cold for almost 1 month.

<sup>b</sup>R<sub>R</sub> = \( \frac{A_{72}}{A_{24}} \)

The procedure for preparing reduced tritiated enzyme and for this experiment is described in the text.
Table 4-B

DIALYSIS OF REDUCED EPIMERASE

<table>
<thead>
<tr>
<th>Time</th>
<th>R&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Specific radioactivity (cpm/mg)</th>
<th>Specific enzyme activity (unit/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>--</td>
<td>5,060&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4,200&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>18</td>
<td>1.34</td>
<td>5,060</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>1.33</td>
<td>2,520</td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>1.33</td>
<td>1,820</td>
<td></td>
</tr>
<tr>
<td>98</td>
<td>1.34</td>
<td>1,510</td>
<td>7,320</td>
</tr>
<tr>
<td>120</td>
<td>1.37</td>
<td>1,530</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> \[ R = \frac{A_{278}}{A_{282}} \]

<sup>b</sup> Value was taken before Biogel P-2 column chromatography.

<sup>c</sup> Value was taken after its exit from the Biogel P-2 column.

The procedure is described in the text.
concentration comparable to that used by Kirkwood, to a solution of epimerase and NaBH₄ prepared in a similar manner. The specific activity of the enzyme was followed.

Figure 16-A shows the results obtained from two identical solutions of epimerase and NaBH₄ with or without acetone. Both curves showed an initial decrease followed by a slow recovery of specific activity, but the solution containing acetone (lower curve) exhibited a greater and more prolonged activity loss and a lesser extent of recovery of activity. The reactivation in the lower curve was probably due to spontaneous reactivation which is an inevitable event. This was not the effect of acetone since greater reactivation was detected in the solution not containing acetone. When acetone was added early in the reaction, it caused a further fall in activity (Fig. 16-B, curve I). When acetone was added later, after the spontaneous oxidation had taken place, it caused a further diminution with a slight recovery of activity (Fig. 16-B, curve II). If UDPG was added to a solution containing pre-treated epimerase, repeatedly reduced with NaBH₄, the reduced epimerase became stabilized and would remain reduced (Fig. 16-C). The administration of acetone did not affect its activity. The rise in A₃₄₅ after the addition of acetone was probably an artifact. It may be due to bubbling on mixing the solution since there was no change in the specific activity. An increase in specific activity after the third addition of NaBH₄ might

---

2The procedure for the preparation of this form of enzyme has been described in Figure 11.
Figure 16-A. Effect of acetone on reactivation of reduced epimerase.

Epimerase (0.77 mg) was combined with 6.5 umoles of NaBH₄ in 1.0 ml of buffer C at 27°C. Acetone (675 umoles) was added 12 minutes after the addition of NaBH₄. An identical reaction mixture was set up without acetone. Aliquots were removed for assay of activity. The point of acetone addition is indicated by the upward vertical arrow in the lower curve.
Relative Specific Activity (units)

Figure 16-A
To a 1.0 ml cuvette was added 0.84 mg of epimerase (specific activity 9,060 units per mg), 6.5 umoles of NaBH₄, in buffer C at pH 8.7 and 27°C. Acetone (675 umoles) was added 6 minutes after the start of the reaction. An identical reaction mixture was set up with acetone added 56 minutes after mixing epimerase with NaBH₄. Aliquots were removed at different time intervals for assay of activity. The times of acetone addition are shown as downward vertical arrows in the plot.
Relative Specific Activity (units)

Figure 16-B.
Figure 16-C. Effects of UDPG and acetone on $A_{345}$ and activity of epimerase pre-treated with NaBH$_4$.

The reaction mixture contained 0.82 mg of pre-treated epimerase (specific activity of 7,900 units per mg, prepared as described in Figure 11), and 100 umoles of sodium bicinate in 1.0 ml of buffer C, pH 8.7 at 27°. NaBH$_4$ (0.65 umole) was added at 0, 8 and 12 minutes, UDPG (0.04 umoles) was added at 20 minutes. This was followed by 675 umoles of acetone at 55 minutes. $A_{345}$ was followed and aliquots were removed at different time intervals for assay of activity. The vertical arrows indicate the times of addition of the different reagents.

Symbols: $\circ$---$\circ$, $A_{345}$; $\Delta$---$\Delta$, specific activity.
Figure 16-C

Absorbance x 10^2

Specific Activity (unit·mg⁻¹ x 10⁻³)

NaBH₄
UDPG
Acetone
represent an early sign of spontaneous oxidation which was then suppressed by UDPG addition.

III. Reactivation by cyclohexanone

(a) Reactivation of reduced epimerase by [3H]-NaBH$_4$ during brief incubation with UDPG

(i) The reactivation

Cyclohexanone was examined for its ability to reactivate reduced epimerase because it is a cyclic ketone as is 4-ketoglucose. The $A_{450}$ of a solution containing tritiated reduced epimerase, sodium bicinate and cyclohexanone, was measured versus an appropriate reference, and the specific catalytic activity was followed by taking aliquots for assay.

The data are given in Figure 17-A, which shows a decrease in $A_{450}$ and a steady increase in specific activity. After 2 hours of incubation, the degree of reactivation was 25% as calculated from the increased activity and 57% reoxidation was calculated from the decrease in $A_{450}$. A logarithmic plot of $A_{450}$ was slightly curved, indicating that the process is not a simple first order reaction (Fig. 17-B).

Another identical experiment was performed, but in this case epimerase was separated from cyclohexanone on a column of Sephadex G-25. After 3 hours of incubation, reactivation was 48% while reoxidation was 60%. The elution profile (Figure 18-A) shows two radioactive peaks, the first of which corresponds to the enzyme while
Figure 17-A. Reactivation of reduced epimerase by incubation
with cyclohexanone.³

Difference absorption at 345 m\(\mu\) were taken using 4 cuvettes
each containing a volume of 1.15 ml. Sample cell #1 contained
1.66 mg of reduced epimerase (specific activity of 1,330 units
per mg), 100 umoles of sodium bicinate and 480 umoles of cyclo-
hexanone in a total volume of 1.15 ml. Sample cell #2 contained
buffer C. Reference cell #1 contained the same amount of epimerase
in buffer C and reference cell #2 had cyclohexanone, sodium
bicinate and buffer C. The final concentration of cyclohexanone
was 0.417 M. The specific activity was measured in aliquots
taken at different intervals.

Symbols: O—O, \(A_{345}\); Δ—Δ, specific activity.

³The reduced epimerase was prepared by NaBH₄ reduction of epimerase
briefly incubated with UDPG.
Figure 17-A.
Figure 17-B. Logarithmic plot of the data given in Figure 17-A.

The final concentrations of epimerase and cyclohexanone were 2.1 x 10^{-5} M and 0.417 M, respectively.
Figure 18.A. Sephadex G-25 gel filtration chromatography of tritiated reduced epimerase partially reactivated and reoxidized by incubation with cyclohexanone.

The reaction mixture contained 0.97 mg of reduced epimerase (specific catalytic activity 870 units per mg protein and specific radioactivity 7.07 x 10^7 cpm per umole), 480 umoles of cyclohexanone and 100 umoles of sodium bicinate buffer pH 8.5 in a total volume of 1.15 ml at 27°C. Absorbance at 345 μm and specific activity were measured at different time intervals. The solution was passed into a 1.1 x 37 cm Sephadex G-25 column and eluted with buffer B. Fractions of 2.0 ml were collected at 5 minute intervals.

Symbols: O——O, A_{278} (protein, 1st peak), A_{275} (cyclohexanone, 2nd peak); Δ——Δ, radioactivity.
Figure 18-A.
a second moves close to but was not identical with cyclohexanone. About 71% of the total radioactivity was associated with the second peak and only 18.4% remained associated with the enzyme fractions. The identity of the major radioactive peak is unknown. It may either be dissociated NADH, NAD+, cyclohexanol or even tritiated water.

If Biogel P-2 instead of Sephadex G-25 were used in the separation, the chromatogram had 3 radioactive components. While the first was due to protein, the other 2 had chromatographic mobilities different from cyclohexanone (Fig. 18-B).

(aa) Identification of tritiated water

Pooled fractions of peak 3 from Figure 18-B was subjected to tritium hydrogen exchange using acetoacetate decarboxylase (81). The procedure was described under "Materials and Methods". One reaction mixture was incubated for 3½ hours and another trial for 18 hours. The radioactivity transferred from the water to acetone was calculated to be 18% and 43% respectively for the two.

(ii) Dependence of epimerase reactivation on cyclohexanone concentration

Reactivation was performed in the usual manner using two different concentrations of cyclohexanone. The rate of activation is proportional to the cyclohexanone concentration (Fig. 19-A). A logarithmic plot (Fig. 19-B) of the percent increase in specific activity versus time shows that at lower concentration of cyclohexanone, the reactivation seems to follow a first order mechanism, while at higher concentration the rate deviates negatively.
Figure 18-B. Biogel P-2 gel filtration chromatography of tritiated reduced epimerase partially reactivated and reoxidized by incubation with cyclohexanone.

The procedure and symbols are as in Figure 18-A except that a Biogel P-2 column was used for the separation of the reaction mixture.
Figure 18-B.
Figure 19 A and B. Reactivation of reduced epimerase using varied concentrations of cyclohexanone.

The reactivation was performed in the usual manner as described in Figure 17-A. The concentration of epimerase was $7.75 \times 10^{-8}$ M and the concentration of cyclohexanone was 0.412 M for curve I and 0.019 M for curve II. The B figure is a logarithmic plot of the same data.
Figure 19-A

% increase in specific activity x 10^{-2}

TIME (min)

I

II

III

IV
Figure 19-B

% increase in specific activity vs. Time (min)

Line I: 
- Points: O
- Description: Curve indicating a significant increase in specific activity over time.

Line II: 
- Points: □
- Description: Linear increase in specific activity over time.

Time (min): 100, 200, 300

Specific activity: 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1000
(iii) **Effect of UMP on cyclohexanone reactivation of epimerase**

The previous results indicate that cyclohexanone reactivates the reduced epimerase and causes its reoxidation. The following experiments were performed to determine if UMP would have an affect on the reoxidation. UMP may bind to the enzyme making NADH more accessible to cyclohexanone. If the reduced enzyme is an E·NADH·substrate complex, and cyclohexanone reactivates it by causing the dissociation of substrate from the complex, UMP may have no, or an adverse effect on the reoxidation.

The reaction was carried out in a solution containing reduced epimerase, cyclohexanone with or without UMP. Specific activity was followed by taking aliquots for assay at different time intervals.

Table 5 shows a small degree of reactivation with cyclohexanone alone. However, in the presence of UMP, there was a decrease of the activity to an even lower level. There was a slight reactivation but the level was never above the original specific activity of the reduced epimerase used.

(b) **Reactivation of epimerase reduced by $[^{3}H]$-NaBH$_4$ after two hours of preliminary incubation with UDPG**

The reaction mixture contained epimerase, UDPG and sodium bicinate buffer pH 8.7, at 27°, after 2 hours of incubation when the specific activity was 5,000 units per mg protein and NaBH$_4$ was added.

*Delayed $[^{3}H]$-NaBH$_4$ trapping experiments will be discussed in Part III. The reduced enzyme was subsequently identified to be E·NADH·substrate complex.*
Table 5
EFFECT OF UMP ON CYCLOHEXANONE REACTIVATION
OF EPIMERASE

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Specific Activity (Unit/mg)</th>
<th>Without UMP</th>
<th>With UMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>300</td>
<td>296</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>350</td>
<td>226</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>490</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>400</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>500</td>
<td>193</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>520</td>
<td>178</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>540</td>
<td>184</td>
<td></td>
</tr>
<tr>
<td>210</td>
<td>870</td>
<td>131</td>
<td></td>
</tr>
</tbody>
</table>

The reactivation was carried in the usual manner in the presence and absence of UMP. Activity was followed by taking aliquots for assay at different time intervals. The final concentration of cyclohexanone was 0.048 M, that of UMP was $1.82 \times 10^{-3}$ M and that of epimerase was $1.38 \times 10^{-5}$ M.
The specific activity fell to 70 units per mg, the solution was separated by gel filtration. The separated reduced epimerase had a specific activity of 807 units per mg. Three hours after addition of cyclohexanone the specific activity was 2,560 units per mg. The solution was then passed through a Biogel P-2 column.

The results are shown in Figure 20, which shows that there are four radioactive peaks, the protein peak, followed by three others. None of these radioactive peaks corresponds to the position of cyclohexanone in the elution profile.

(i) **Identification of tritiated NAD⁺**

A portion of the pooled fractions from peak 3 of Figure 20 were subjected to paper chromatography. Figure 21 shows the radiochromatographic scan in which the radioactivity moves with the NAD⁺ marker.

(bb) **Formation of [³H]-NADH and CH₃CHNNHCONH₂**

The radioactive compound located in Figure 21 was eluted from the paper. It was then added to a solution containing ethanol, semicarbazide, glutathione (reduced form), carrier NAD⁺, and alcohol dehydrogenase in pyrophosphate buffer. The reaction was monitored by the appearance of NADH at 340 μm and the acetaldehyde formed was converted in situ into its semicarbazone to pull the reaction towards NADH formation. When the reaction was 95% complete, it was stopped by heating and the different compounds were separated by Biogel P-2 column.
Figure 20. Biogel P-2 molecular Sieve Chromatography of reduced tritiated epimerase partially reactivated in the presence of cyclohexanone.4

The reaction mixture contained 1.04 mg of reduced epimerase (specific activity of 1,330 units per mg, specific radioactivity of 2.16 x 10^7 cpm per umole), 480 umoles of cyclohexanone, and 100 umoles of sodium bicinate at pH 8.5 in a total volume of 1.0 ml at 27°C. After 3 hours the specific activity had increased to 2,560 units per mg, and the solution was applied to a Biogel P-2 column (1.5 x 42 cm) and eluted with buffer A. Fractions of 0.9 ml were collected at 5 minute intervals.

Symbols: 0—0, A_{278} (protein, 1st peak); A_{275} (cyclohexanone 2nd peak); □—□, radioactivity.
Figure 20

Absorbance

Radioactivity (cpm/mL x 10^-4)

Fraction Number
Figure 21. Paper Chromatogram of the Pooled Fractions of Peak 3 in Figure 20.

The pooled radioactive fractions from Peak 3 of Figure 20 were concentrated and spotted on washed S and S paper. The descending chromatogram was developed with solvent I at room temperature for 18 hours. The identification of the nucleotides was aided by UV lamp. The area containing pyridine nucleotide was cut into 1/2 inch wide strips for radiochromatographic scanning.
Figure 22 shows a fair separation of NADH and the semicarbazone of acetaldehyde. Essentially all of the radioactivity was associated with NADH, indicating that the original solution contained radioactive NAD$^+$.  

(ii) Absence of tritiated cyclohexanol

Another portion of the pooled fractions from peak 3 of Figure 20 was shaken with 1.0 ml of cyclohexanol, but only 10% of the radioactivity was transferred to the cyclohexanol layer. The radioactive cyclohexanol was isolated as an ester by reacting it with 3,5-dinitrobenzoyl chloride as described under "Methods and Materials" (84). The purified derivative melted at 110$^\circ$ (84). The amount of tritium found in the crystals was 1% of the total radioactivity in the reaction.

(iii) Identification of NADH

Another portion of the pooled fractions from peak 3 of Figure 20 was subjected to transfer experiment using glutamic acid dehydrogenase as described under "Methods and Materials." The amount of labeling found in glutamic acid was 4.4% of the total radioactivity used. Therefore, [4-$\beta$-$^3$H]-NADH could not have constituted more than 4.4% of the radioactivity in peak 3.

(iv) Identification of peaks 2 and 4

Peak 2 was suspected to be UDP-hexose and peak 4 to be tritiated water. However because of small amounts of radioactivity present, it was difficult to verify these possibilities.
Figure 22. Separation of $[^3]H$NADH and CH$_3$CHNNHCONH$_2$

To a 0.40 ml radioactive solution containing 7.76 x $10^4$ cpm in NAD$^+$ was added 3.9 mmoles of ethanol, 225 umoles of semicarbazide hydrochloride, 5.9 umoles of glutathione (reduced form), 4 units of alcohol dehydrogenase, 130 umoles of sodium pyrophosphate buffer in glycine, and 10 umoles of unlabeled NAD$^+$ in a total volume of 2.68 ml. The mixture was incubated at 27° at pH 7.8, and the course of the reaction was followed by appearance of NADH. At the end of 20 minutes, after 9.5 umoles of NADH had appeared, the solution was heated for 1 minute at 100°, filtered, and concentrated on a rotary evaporator to about 1 ml. It was passed into a Biogel P-2 column (1.5 x 42 cm) and eluted with buffer A. Fractions of 0.7 ml were collected at 5 minute intervals.

Symbols: ○——○, A$_{340}$ (NADH); △——△, A$_{225}$ (semicarbazone); O——O, radioactivity.
Figure 22

Absorbance

Radioactivity (cpm x 10^-3)

Fraction Number
(c) Reactivation of reduced epimerase prepared by $[^3H]$-NaBH$_4$ reduction of enzyme previously incubated with UDPG for 2 hours and precipitated with ammonium sulfate

The reduced epimerase prepared as in (b), was precipitated with 40% ammonium sulfate. It was dissolved in 1 ml of buffer C and dialyzed against 2 liters of the same buffer. The post-dialyzed volume was 1.25 ml with practically no loss of radioactivity and no change in specific activity (140 units per mg). The solution was passed into a Sephadex G-25 column size 1.1 x 37 cm and eluted with buffer B. The specific radioactivity of the reduced epimerase from the column was unchanged compared to the value before the precipitation. The protein fractions were stored frozen for 2½ months.

When the protein preparation was thawed for reactivation by cyclohexanone, the specific enzyme activity was 4,050 units per mg. It fell to 2,730 units after 105 minutes of incubation with cyclohexanone. At this point, the solution appeared cloudy. It was transferred to a Biogel P-2 column. The elution profile contains three radioactive peaks (Fig. 23).

(i) Identification of peak 2

Pooled fractions from peak 2 of Figure 23 were concentrated and spotted on S and S paper developed with solvent I. The radioactivity moved with UDPGal. Subsequent elution from paper and assay for UDP-hexose revealed the compounds to be UDPG and UDPGal, both were radioactive. Unfortunately, no attempts were made to identify NAD$^+$ in these fractions.
Figure 23. Reactivation of reduced epimerase by cyclohexanone.\textsuperscript{5}

The procedure was as described in Figure 20.

Symbols: 0—0, $A_{278}$ (protein, 1\textsuperscript{st} peak); $A_{262}$ (nucleotide, 2\textsuperscript{nd} peak); $A_{275}$ (cyclohexanone, 3\textsuperscript{rd} peak); $\triangle--\triangle$, radioactivity.

\textsuperscript{5}Preparation of reduced epimerase as described in text.
(ii) Identification of peak 3

 Portions of fractions in peak 3 of Figure 23 was concentrated to dryness using a rotary evaporator. Ninety-five per cent of the total radioactivity was recovered in the trapping receptacle, which indirectly suggests that it was tritiated water.

 Tritium hydrogen exchange was carried out using acetoacetate decarboxylase as described under "Methods and Materials" (81). The radioactivity found in the acetone was 84% of the total radioactivity used.
DISCUSSION

Autooxidation is a phenomenon observed when epimerase is reduced by NaBH₄ in the absence of substrate. This particular form of enzyme is unstable and is unusually reactive. Hydride may be lost from the enzyme-bound NADH, resulting in the regeneration of the native protein. This particular reduced protein probably assumes a structure similar to the native protein. Under this peculiar spatial arrangement, a hypothetical group, which is otherwise inaccessible during ordinary catalysis, may be accessible to reduction by NADH. The reduced enzyme is difficult to isolate because of its transient existence. This makes the study of hydrogen exchange of the hypothetical group with the medium impossible. The way to stabilize this enzyme is to add substrate, which produces a stable enzyme and autooxidation is thus prevented.

Any procedure capable of removing the bound substrate from the reduced enzyme usually results in reactivation of protein. Passage through gel columns, aging and dialysis of a sample of stable reduced epimerase all resulted in gradual dissociation of substrate and increase in catalytic activity (Table 4 A and B). The gradual shift of the absorption peak from 270 mµ to 278 mµ of the dialyzed reduced enzyme, indicates a slow dissociation of a nucleotide, which normally absorbs at 262 mµ. The dissociation of this
nucleotide which is supposed to be UDP-hexose leads to a fall in
radioactivity content and an increase in enzyme activity due to
autooxidation of the E-NADH produced. The substrate in these
enzyme preparations was supposed to be radioactive. Another reason
for the drop in radioactivity was due to the simultaneous
autooxidation taking place with gradual relaxation of protein
structure.

Acetone does not itself cause autooxidation. Its only
observable effect is one of deactivation of enzymatic activity and
suppression of spontaneous reoxidation. The amount of acetone in
the reaction mixture was about 26 times that of the hydride, and it
is entirely possible that the high concentration has a denaturing
effect on the protein.

Epimerase reduced in the presence of substrate behaves
differently. It is stable. Cyclohexanone can reactivate this
enzyme leading to an increase in activity and decrease in A454.
However, this effect is not as complete as spontaneous reoxidation,
since a maximally reactivated enzyme regains only 50% of its
original activity. The reactivation seems to depend on the concen-
tration of cyclohexanone, but the effect is not a simple first order
process, and cyclohexanone may behave differently at high concentrations.

The mechanism by which cyclohexanone brings about reactivation
is not known. The possibility that cyclohexanone, because of its
structural relationship to ketosugar, may become reduced to
cyclohexanol is unsupported, since the cyclohexanol isolated after
reactivation of tritiated $E^2\text{NAD}^3\text{H}$ was never significantly radio-active. The chromatogram showing several peaks not corresponding to cyclohexanone would suggest formation of other radioactive compounds during the reactivation. From our results, they are not cyclohexanol and NADH, but are most likely UDP-hexose, $\text{NAD}^+$ and tritiated water in their order of appearance from the Bio gel P-2 column. A peak corresponding to radioactive UDP-hexose is discernible only when the reduced epimerase used was obtained from the delayed trapping experiments in which tritiated substrates and unlabeled $\text{NaBH}_4$ were used to produce the reduced complex. Cyclohexanone triggered the dissociation of substrate from the reduced enzyme complex and allowed the autooxidation to take place. This would revert the conformation of the protein back to its native form in which hydride transfer between NADH and an adjacent group on the protein is possible. This explanation is further strengthened by the observation that UMP prevents cyclohexanone reactivation. UMP may replace the substrate removed and stabilize the reduced enzyme against reoxidation and reactivation.

Comparing the extents of reactivation to reoxidation, the value for reoxidation was larger. This may indicate that the two values do not correspond to one single process and that reactivation does not necessarily parallel the formation of $\text{NAD}^+$. On the other hand, the discrepancy may be explained by the denaturation brought about by cyclohexanone. Simultaneous denaturation may result in slower and less extensive increase in activity as compared with the rate and extent of reoxidation. This interpretation is supported
by the data of Figure 17-A in which the activity increases to a maximum and then decreases, whereas the $A_{645}$ merely decreases to a constant low level. This role played by cyclohexanone is understandable from its concentration of 0.417 M used in most reactions. The initial partial unfolding of structure is probably accompanied by the release of bound stabilizing substrate and a shift of the position of the hypothetical disulfide group to a reactivating orientation for accepting hydride from NADH. The exchange of hydrogen between the acceptor and the medium and the release of NAD$^+$ are late effects. The transfer of hydride to such an acceptor probably does not occur in catalysis. This follows from the fact that tritiated water and tritiated NAD$^+$ are both isolated from cyclohexanone reactivated enzyme-NAD$^+$, which means that reoxidation of NADH under these conditions is not stereospecific. It has been reported that spontaneous reoxidation of *S. fragilis* epimerase-NAD$^+$ produces tritiated NAD$^+$ (53). Nelsestuen and Kirkwood, however, claim that spontaneous reoxidation of *E. coli* epimerase-NAD$^+$ does not produce tritiated NAD$^+$ or NADH (74). Under the conditions herein described both are produced.

The processes discussed can be schematically represented by the following equations:

$$E\cdot\text{NAD}^+ + H^- \longrightarrow E\cdot\text{NADH} \quad (1)$$

$$E\cdot\text{NAD}^+ + SH \rightleftharpoons E\cdot\text{NAD}^+\cdot\text{SH} \quad (2)$$
Reduction in the absence of substrate is represented by equation (1), while reduction in the presence of substrate is represented by equations (2), (3), and (5). Normal catalysis involves equations (2), (3) and (4). Cyclohexanone acts on equation (6) causing it to shift to the right while UMP reverses it. Equation (7) outlines the event in spontaneous reoxidation and the late effects of cyclohexanone.

Adair et al. and subsequently Nelsestuen and Kirkwood reoxidized reduced epimerase with TDP-4-keto-6-deoxyglucose and both claimed that the position of epimerization involves C-4 (74, 75, 78). Davis and Glaser used the same compound to reactivate reduced epimerase obtained from glucose and UMP reduction (72). Other observations of reactivation using 2-keto sugars makes this conclusion ambiguous (55).
PART III

PATHWAY OF HYDROGEN TRANSFER
Prolonged incubation of substrate with epimerase causes a progressive decrease in catalytic activity. As shown in Part I, the activity decrease is accompanied by an increase in $A_{545}$, indicating a simultaneous increase in the concentration of reduced enzyme. During normal catalysis, the only possible form of reduced enzyme is $E\cdot$NADH$\cdot$X, where X is a substrate-derived intermediate. However, this is not an inactive species because, upon transfer of hydrogen back to the intermediate, the enzyme is reactivated. As shown in Part I, the additional reduced enzyme is formed gradually only after prolonged incubation with substrate, therefore, it is an enzyme form which differs in some way from $E\cdot$NADH$\cdot$X. There are a number of ways to account for this behavior. It may be that the intermediate X or NADH itself or both can dissociate slowly from the $E\cdot$NADH$\cdot$X intermediate complex. In this case the dissociation phenomena would have to be reversible and reach an equilibrium, because the activity of the enzyme does not approach zero. If such dissociations proceeded to essential completion the activity would approach zero. Alternatively, it is possible that occasionally a mistake in hydrogen transfer occurs, such that hydrogen is transferred to enzyme-bound NAD$^+$ from some substrate carbon atom not normally
involved in the epimerization pathway. This would produce an abortive inactive complex \( E \cdot NADH \cdot X' \) in which \( X' \) differs from the \( X \) species in the intermediate complex. Again, this process would have to be reversible to account for the fact that the activity of the enzyme does not approach zero. Finally, it may be that prolonged interaction of the enzyme with the substrates induces a slow change in the enzyme structure, such that modified enzyme differs in catalytic properties from the native enzyme. In this case the intermediate complex could be designated \( E' \cdot NADH \cdot X \), in which the differences between \( E \) and \( E' \) cause the equilibrium concentration of \( E' \cdot NADH \cdot X \) to be larger than that of \( E \cdot NADH \cdot X \) and the turnover number for \( E' \) to be smaller than that for \( E \).

The results given herein resolve the question of the nature of this substrate induced change in the properties of UDP-galactose-4-epimerase. They show that substrate induces the release of the intermediate \( X \) from the \( E \cdot NADH \cdot X \) intermediate complex and that \( X \) is UDP-4-keto-sugar. These results strongly support the hypothesis that the hydrogen transfer pathway involves UDP-4-ketosugars as enzyme-bound intermediates. Furthermore, they constitute direct proof that, under certain special conditions, UDP-4-ketosugars are free intermediates.
A. Results

(1) UDP-xylose-induced changes in activity and $A_{445}$ of UDP-galactose-4-epimerase

The apparent reductive inactivation of epimerase by UDPG was repeated using another known substrate of the enzyme, UDP-D-xylose. The reaction mixture consisted of epimerase and UDP-D-xylose in sodium bicinate buffer in a $27^\circ$ bath. Specific activity and $A_{445}$ were both measured at various times over a 10 hour period.

Figure 24 shows the decrease in activity with a concomitant increase in $A_{445}$ over a period of 6 hours. After 10 hours of incubation, inactivation calculated from the activity loss was 84.2% and reduction calculated from increase in $A_{445}$ was 84.8%. The process is biphasic, as indicated by a relatively fast burst followed by a steady increase in $A_{445}$. The changes are similar to but more extensive than those observed with UDPG under comparable conditions (Part I).

(2) Effect of UMP on the activity of epimerase

The possibility that substrate decreases the enzyme activity by inducing a conformational change is tested in this experiment by reacting epimerase with UMP, a molecule having many of the structural features of substrate and the same electrostatic charge. Epimerase in bicine buffer was incubated with UMP and the reaction
Figure 2. Reductive inactivation of epimerase by UDP-D-Xylose

The final concentration of UDP-D-Xylose was $1.95 \times 10^{-3}$M and that of epimerase was $1.32 \times 10^{-5}$M. The procedures were similar to those described in Figure 13 of Part I.

Symbols: $0--0$, $A_{345}$; $\circ--\circ$, specific catalytic activity in units per mg protein.
Figure 24.

![Graph showing catalytic activity over time](image-url)
was followed as described previously. As shown in Figure 25, the inactivation was slight, temporary and possibly reversible.

(3) Effect of NAD$^+$ and mercaptoethanol on UDPG inactivation of epimerase

The inactivation of epimerase by substrates could conceivably result from dissociation of NADH from the E·NADH·X complex which leads to the formation of E·X and subsequently of E plus X. NADH has been found to inhibit epimerase activity at least in the case of the liver enzyme. This may be attributed to its possible exchange with bound NAD$^+$ (15). Adenosine diphosphate ribose is also a known inhibitor of this enzyme (77). The present experiment was designed to determine the effect of NAD$^+$ and 2-mercaptoethanol on the substrate induced inactivation.

Four reaction mixtures were prepared which were identical with respect to epimerase and UDPG concentrations. One test tube contained both NAD$^+$ and 2-mercaptoethanol, another two test tubes contained either NAD$^+$ or 2-mercaptoethanol, and the last test tube was a control which contained neither of the two compounds. The catalytic activities in the four solutions were measured as a function of time.

In Table 6 it can be seen that NAD$^+$ alone increases the degree of inactivation while mercaptoethanol slightly protects epimerase from inactivation. When both are present, mercaptoethanol appears to abolish the effect of added NAD$^+$. The effect of UDPG is not abolished by either reagent.
Figure 25. Effect of UMP on epimerase activity.

The procedure was similar to that of Figure 24. The final concentration of UMP was $2.4 \times 10^{-3}$ M, and that of epimerase was $3.5 \times 10^{-5}$ M.
Figure 25.

[Graph showing specific activity vs. time (Min).]
### TABLE 6

EFFECT OF NAD⁺, MERCAPTOETHANOL ON UDP-GLUCOSE INACTIVATION OF EPIMERASE.

| Time (min) | Specific Activity (units/mg) |  
|------------|-------------------------------|---
|            | I    | II    | III   | IV   |
| 0          | 10,550 | 10,550 | 10,550 | 10,550 |
| 6          | 9,050  | 5,880  | 9,600  | 10,150 |
| 10         | 6,800  | 4,130  | 7,250  | 7,800  |
| 18         | 6,470  | 3,700  | 7,420  | 6,950  |
| 30         | 6,510  | 2,680  | 7,340  | 6,270  |

% residual activity at 30

|            | 61.6 | 25.4 | 69.5 | 59.4 |

Reaction mixture I contained 0.55 mg of epimerase, 0.63 umoles of NAD⁺, 5 umoles of 2-mercaptoethanol, 0.94 umole of UDPG and 50 umoles of sodium bicinate pH 8.5 in a total volume of 0.5 ml. In reaction mixture II, 2-mercaptoethanol was omitted, and in reaction mixture III, NAD⁺ was omitted, and in reaction mixture IV, both NAD⁺ and mercaptoethanol were removed. The solutions were incubated at 27°C, aliquots of 0.01 ml were taken and diluted into 1.0 ml of ice-cold buffer C. Assays were done within ½ hour after sampling.
One explanation for substrate-induced inactivation of epimerase postulates spontaneous dissociation of the keto-intermediate from the E-NADH-X complex. The longer the incubation, the greater is the amount of intermediate freed. In this case reduction by $[^3H]$-NaBH$_4$ would result in the formation of radioactive UDP-hexose. This exhaustion of the intermediate would prevent it from recombining with E-NADH and thereby reactivating the enzyme. With the removal of the keto-intermediate, the enzyme would probably exist in an inactive reduced form which can be temporarily called an abortive complex. The abortive complex is probably not simply E-NADH, because it does not appear to undergo spontaneous reactivation.

Epimerase and UDPG were incubated for 2 hours. When the specific activity had decreased to 44% of the original, $[^3H]$-NaBH$_4$ was added to the reaction mixture. Another identical reaction mixture was reduced with $[^3H]$-NaBH$_4$ almost immediately after mixing UDPG and enzyme. A third control experiment was done by adding $[^3H]$-NaBH$_4$ to UDPG in buffer C. The components in the reaction mixtures were separated by gel filtration.

The results are represented in Figure 26, A, B and C. In Figure 26-B, a large amount of radioactivity is associated with the protein and only radioactivity is found in fractions associated with UDP-hexose. Figure 26-A shows less radioactivity in the protein fractions while a considerable amount of radioactivity appears
Figure 26. Biogel P-2 chromatography of reduced epimerase and UDP-hexose

A. A reaction mixture containing 4.55 mg of epimerase (specific activity of 8,720 units per mg) and 3.06 umoles of UDPG in a total volume of 0.8 ml of buffer C was incubated at 27°C for 2 hours. After this period of time the specific activity had decreased to 3,820 units per mg, and 0.62 umoles of [3H]-NaBH₄ (800 uci per umole) was added. After 10 more minutes, the specific activity was 9 units per mg and the solution was passed into a Biogel P-2 column (1.1 x 36 cm) and eluted with buffer B at 4°C. Absorbance at 278 mHur and 263 mυυρ and radioactivity are plotted versus cumulative elution volume.

Symbols: Δ—Δ, A₂₇₈ (protein); □—□, A₂₆₃ (UDP-hexose); ○—○, radioactivity.

B. The reaction mixture and procedures were similar to Fig. A, except that [3H]-NaBH₄ was added immediately after mixing. The specific activity was 424 units per mg·protein 10 minutes after reduction. Symbols are as in Fig. A.

C. The reaction mixture and procedures were similar to Fig. A, except that epimerase was omitted. Symbols are as in Fig. A.
Figure 26-B.
with the UDP-hexose. The third radioactive band probably represents an artifact since it is also seen in the control experiment (Fig. 26-C).

The peak fractions of UDP-hexose from the 3 columns were concentrated and applied to washed S and S paper, which was developed for 48 hours at 40 using solvent IV. The radioactivity content of the UDP-hexose, obtained from reduction of prolonged incubated mixture of epimerase and UDPG, was over 20 times the radioactivity found in UDP-hexose recovered from the briefly incubated sample. Table 7 is a summary of the radioactivity contents of the UDP-hexoses isolated.

(5) Determination of the position of hydrogen transfer and identification of abortive complex

Similar trapping experiments can be performed by incubating epimerase with tritiated UDPG, containing tritium at either C-3 or C-4, for extended periods followed by reduction with unlabeled NaBH4. The appearance of radioactivity in the NADH would be indicative of which substrate carbon atom donates hydrogen to NADH.

A reaction mixture consisting of epimerase and UDP-[4-3H]-glucose was incubated in bicine buffer for 2 hours. Reduction with NaBH4 was carried out after the specific catalytic activity had decreased to less than 50% of the original. The solution was then passed through a Sephadex G-25 column. The elution profile given in Figure 27 shows that radioactivity was eluted in the same fraction
Table 7

COMPARISON OF THE RADIOACTIVITY FOUND IN THE PROTEIN AND UDP-HEXOSE IN THE TRAPPING EXPERIMENTS OF FIG. 26

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Specific Activity (cpm/umole)</th>
<th>Protein</th>
<th>UDP-hexose</th>
</tr>
</thead>
<tbody>
<tr>
<td>E + UDPG (2 hr.) $^{[3H]}$NaBH$_4$</td>
<td>$5 \times 10^7$</td>
<td>$7.50 \times 10^5$</td>
<td>$7.16 \times 10^5$ $^a$</td>
</tr>
<tr>
<td>E + UDPG (1 min) $^{[3H]}$NaBH$_4$</td>
<td>$8 \times 10^7$</td>
<td>$0.66 \times 10^5$</td>
<td>$0.32 \times 10^5$ $^a$</td>
</tr>
<tr>
<td>UDPG $^{[3H]}$-NaBH$_4$</td>
<td>--</td>
<td>$0.34 \times 10^5$</td>
<td>--</td>
</tr>
</tbody>
</table>

$^a$Normalized data after subtracting the value of the control.
Figure 27. Sephadex G-25 chromatography of epimerase reduced by NaBH₄ after prolonged incubation with UDP-[4-³H]-hexose

The reaction mixture contained 4.95 mg of epimerase (specific activity of 9,310 units per mg), and 4.59 umoles of UDP-[4-³H]glucose (specific activity of 2.5 x 10⁶ cpm per umole) in 1.08 ml of buffer C at 27⁰. After 165 minutes, the specific activity had decreased to 4350 units per mg protein and 2.3 umoles of NaBH₄ were then added in several additions over a period of 100 minutes. The specific activity finally decreased to 811 units per mg protein (8.7%). The reaction mixture was transferred to a Sephadex G-25 column (1.5 x 42 cm) and eluted with buffer B at 4⁰. Fractions of 1.4 ml were collected at 5 minute intervals.

Symbols: 0—0, A₂₇₈ (protein); □—□, A₂₆₃ (UDP-hexose); Δ—Δ, radioactivity.
Figure 27.
Identical experiments were repeated using UDP-[3-3H]-glucose. The specific radioactivity content of the protein isolated from reaction mixture containing UDP-[4-3H]-glucose was significantly larger than that of the UDP-[4-3H]-glucose used, whereas the specific radioactivity content of the protein isolated from solutions containing UDP-[3-3H]-glucose was significantly smaller than that of the UDP-[3-3H]-glucose. A summary of these findings is given in Table 8.

The reduced epimerase recovered from reaction with 3-tritiated substrate was subjected to dissociation by the heating procedure described in Part I. Separation was accomplished by Sephadex G-25 column which is capable of separating UDP-hexose and NADH.

The results reveal 3 peaks (Fig. 28). There was residual radioactivity in the protein fractions, but the NADH released was not radioactive. The fractions corresponding to peak 2 were pooled and purified by paper chromatography using solvent I. Radiochromatographic scanning showed the radioactive area to move with UDPG. Enzymatic assays for both UDPG and UDPGal of the eluate from the paper were positive.

Similar dissociation was performed on the reduced epimerase isolated from reaction with 4-tritiated substrate. Figure 29 shows all the findings of Figure 28 but in addition, the NADH was radioactive.
Table 8

COMPARISON OF RADIOACTIVITY OF PROTEIN AND UDP-HEXOSE IN TRAPPING EXPERIMENTS USING NaBH$_4$

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Specific Activity</th>
<th>$%^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>E + UDP[4-3H]G (2 hr) NaBH$_4$</td>
<td>$2.42 \times 10^6$</td>
<td>110</td>
</tr>
<tr>
<td>E + UDP[3-3H]G (2 hr) NaBH$_4$</td>
<td>$1.55 \times 10^6$</td>
<td>73.3</td>
</tr>
<tr>
<td>E + UDP[3-3H]G (2 hr) NaBH$_4$</td>
<td>$6.70 \times 10^6$</td>
<td>74.6</td>
</tr>
</tbody>
</table>

$\%^a = \frac{\text{specific radioactivity of protein}}{\text{specific radioactivity of UDP-hexose}} \times 100$

The procedures were described in Figure 27.
Figure 28. Dissociation of NADH from reduced epimerase obtained by NaBH₄ reduction of epimerase following prolonged incubation with UDP-[3-³H]-glucose.

Procedures were similar to Figure 10, except that 6 umoles of NADH was added as carrier and the solution had to be concentrated to about 0.5 ml after heating and before passing it into a Sephadex G-25 column.

Symbols: 0—0, A₂₇₈ (protein); □—□, A₃₄₀ (NADH); Δ—Δ, radioactivity.
Figure 28.

[Graph showing absorbance and radioactivity (cpm/ml x 10^-4) against fraction number.]
Figure 29. Dissociation of NADH from reduced epimerase obtained by NaBH₄ reduction of epimerase following prolonged incubation with UDP-[4-³H]-glucose.

Procedures and symbols were as in Figure 28.
Figure 29.

![Graph showing absorbance and radioactivity](image-url)

- **Absorbance**
- **Radioactivity (cpm/ml \times 10^{-4})**

**Y-axis:** Absorbance (0 to 3.0)

**X-axis:** Fraction Number (0 to 60)
Pooled fractions from the third band of Figure 29 were concentrated and purified by paper chromatography. Fluorescent spot was radioactive. This was eluted from the paper and found to contain $2.8 \times 10^4$ cpm. A transfer experiment was done using glutamic acid dehydrogenase system described under methods. The twice crystallized glutamic acid contained $1.9 \times 10^4$ cpm, that is 69% of the radioactivity in the NADH solution was transferred to α-ketoglutarate.

(6) The question of $[^3\text{H}]-\text{NADH}$ dissociation from reduced epimerase complex.

During reductive inactivation of epimerase by substrates, the appearance of UDP-4-ketoglucose has been proven. UDP-4-ketoglucose could be an intermediate in the catalysis or the produce of an abortive hydrogen transfer. One possible cause of activity loss and appearance of UDP-4-ketoglucose in a form reducible by NaBH$_4$ could be spontaneous dissociation of NADH from the complex, leaving an apoenzyme in a reaction mixture which continues to show an increase in $A_{345}$ due to the formation of free NADH. UDP-4-ketoglucose, in this event, would either be enzyme-bound or free, but susceptible to NaBH$_4$ reduction in either case because of its relatively long lifetime, NADH being absent from the enzyme.

This possibility was tested by inactivating epimerase with UDP-[4-$^3\text{H}$]-glucose for 2 hours, adding NADH carrier to the solution,
then separating NADH from protein by gel filtration. There was no spontaneous dissociation of \(^{3}H\)-NADH from the complex because no radioactivity was associated with NADH as shown in Figure 30. The specific enzyme activity decreased from 8,610 to 2,990 units per mg after 2 hours of incubation. It became 6,180 after passage through the gel column, indicating that the reductive inactivation of epimerase by UDPG is a reversible process.

\[(7) \text{Exchange of unlabeled UDP-glucose with bound E\cdot NADH \cdot UDP-}
\[\text{[U-}^{14}\text{C]}\text{-hexose}\]

An abortive complex formed during substrate inactivation of the epimerase was identified to be a reduced epimerase-substrate complex (74). This suggested that there may be exchange of free substrate with the enzyme-bound keto-intermediate. A simultaneous exchange of the free substrate for the bound one in the abortive complex is also very likely. Unlabeled UDPG was combined with E\cdot NADH \cdot UDP-[U-}^{14}\text{C]}\text{-glucose and NaBH}_4. After 15 minutes of incubation, the compounds were separated by gel column. Identical experiments were performed using the complex alone with UDPG omitted.

In the reaction mixture containing free UDPG, 90\% of the radioactivity was detected in the UDP-hexose fractions and only 10\% of the radioactivity remained in the protein fractions, indicating exchange between free and enzyme-bound substrates. When free substrate was not added, the complex only lost 1.7\% of the total radioactivity in the small molecular weight fractions and it
Figure 30. Sephadex G-25 chromatography of epimerase, UDP-hexose and NADH.

The reaction mixture consisted of 1.22 mg of epimerase (specific activity of 8,610 units per mg), 2.5 umoles of UDP-[4-\(^{3}\)H]-glucose (specific activity of 1.32 x 10\(^{6}\) cpm per umole) and 100 umoles of sodium bicinate buffer pH 8.5 in a total volume of 1.0 ml. After 2 hours of incubation at 27\(^{\circ}\), the specific activity fell to 2,990 units per mg. NADH (1.0 umole) was added immediately before the solution was placed into a Sephadex G-25 column (1.5 x 42 cm), and eluted with buffer B. Fractions of 0.95 ml were collected at 5 minute intervals at 4\(^{\circ}\).

Symbols: 0——0, \(A_{278}\) (protein); □——□, \(A_{283}\) (UDP-hexose); 
\(\Delta\)  \(\Delta\), \(A_{340}\) (NADH); 0——0,■——■, \(\Delta——\Delta\), corresponding radioactivity.
Figure 30.
retained 80.5% of the original radioactivity (Fig. 31, A and B).

A summary of the findings is shown in Table 9. The reduced enzyme-substrate complex prepared had a specific radioactivity content nearly the same as that of the UDP-hexose used, indicating that only one mole of substrate is bound to every mole of protein. Nelsestuen and Kirkwood suggested that the abortive complex contains both the keto-intermediate and the substrate (74).

(8) **Intermolecular hydrogen transfer**

This experiment was designed to determine if there is exchange between free and bound keto-intermediates or between free keto-intermediate and bound substrate or vice versa. Basically it is a test of whether UDP-4-ketosugars can be free intermediates in the catalytic reaction.

Unlabeled UDP-xylose was incubated with UDP-[4-3H]-hexose and a large amount of epimerase for extended periods. Under the conditions used a large degree of substrate-induced reductive inactivation occurred, that is, a large amount of abortive complex, if it exists at all, was present. Upon separation of the sugar nucleotides as their corresponding simple sugars, pentoses were analyzed for the presence of tritium.

Figures 32-A and B show the progressive labeling of both pentoses. The sum of radioactivity in 2 samplings was calculated to correspond to 0.07 umole of D-xylose and 0.06 umole of L-arabinose, using the original radioactivity of the UDP-[4-3H]-glucose. The
Figure 31. Exchange of unlabeled UDPG with bound UDP-hexose in E·NADH·UDP-[U-14C]-hexose.

A. The formation and isolation of E·NADH·UDP-[U-14C]-hexose was as described in Figure 29 but utilized UDP-[U-14C] glucose. Into a solution containing 2.72 mg of the reduced epimerase complex (specific activity of $1.2 \times 10^6$ cpm per umole), was added 5.06 umoles of unlabeled UDPG to a total volume of 2.18 ml. After 15 minutes of incubation at 27°, it was transferred into a Sephadex G-25 column (1.5 x 42 cm) and eluted with buffer B at 4°. Fractions of 0.8 ml were collected at 5 minute intervals.

Symbols: 0—0, $A_{278}$ (protein); □—□, $A_{263}$ (UDP-hexose); Δ---Δ, radioactivity.

B. A 1.8 ml (1.31 mg) of reduced enzyme-substrate complex was placed into a Sephadex G-25 column. The column was of the same size, eluted with the same buffer. Fractions of 1.3 ml were collected at 5 minute intervals. Symbols were identical to Figure A.
Figure 31 - A

![Graph showing absorbance and radioactivity against fraction number](image-url)
Table 9

SUMMARY OF RADIOACTIVITY OF PROTEIN AND UDP-HEXOSE FROM EXPERIMENTS ON EXCHANGE OF UNLABELED UDPG WITH BOUND E-NADH-UDP-[U-\(^{14}\)C]-HEXOSE

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Specific Activity (cpm/umole)</th>
<th>Protein</th>
<th>UDP-hexose</th>
<th>%</th>
<th>Calculated</th>
<th>Experimental</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Calculated</td>
<td>Experimental</td>
<td></td>
<td>Calculated</td>
<td>Experimental</td>
<td></td>
</tr>
<tr>
<td>UDP-[U-(^{14})C]-glucose</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td></td>
<td>1.15 x 10^6</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>E + UDP-[U-(^{14})C]glucose → E'</td>
<td>1.15 x 10^6</td>
<td>1.20 x 10^6</td>
<td>105</td>
<td>1.15 x 10^6</td>
<td>1.15 x 10^6</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>E' + UDPG</td>
<td>1.20 x 10^6</td>
<td>0.11 x 10^6</td>
<td>10.3</td>
<td>6.61 x 10^3</td>
<td>6.10 x 10^3</td>
<td>92.5</td>
<td></td>
</tr>
<tr>
<td>E'</td>
<td>1.20 x 10^6</td>
<td>0.97 x 10^6</td>
<td>80.5</td>
<td>1.20 x 10^6</td>
<td>2.5 x 10^4</td>
<td>1.7^a</td>
<td></td>
</tr>
</tbody>
</table>

^a The total radioactivity in the region of small molecular weight fractions, therefore it represents the total amount of UDP-hexose dissociated from E'.

^b \(\% = \frac{\text{experimental specific radioactivity}}{\text{calculated specific radioactivity}} \times 100\)

The procedure was as in Figure 31.
Figure 32. UDP-galactose-4-epimerase catalyzed intermolecular transfer of tritium from UDP-[4-3H] hexoses to UDP-pentoses.

The reaction mixture consisted of 4.5 umoles of UDP-[4-3H]-hexose (specific activity of $1.31 \times 10^5$ cpm per umole), 4.5 umoles of UDP-xylose and 300 umoles of sodium bicinate buffer pH 8.5, in a total volume of 1.28 ml. A sample of 0.43 ml was taken at 0 time and 1.3 ml (0.1 umole) of epimerase was added to give a total volume of 2.16 ml. The reaction mixture was incubated at 27°. Samples of 1.08 ml were removed at 10 hours and another sample at 20 hours. Each sample (except sample 1) was placed into a 100° bath for 1 min to denature the protein and was centrifuged twice with washing of the precipitate. Volume of the supernatant was measured and enough HCl was added to make a 0.1 N solution with pH adjusted to below 2. The sample was again placed back into a 100° bath for 15 minutes, mixed bed resin (Dowex-1-CO₃⁻, Dowex-50-H⁺) was added and the solution was thoroughly mixed. It was filtered, washed, concentrated, spotted on Schleicher and Schuell paper and developed with solvent II for 24 hours at 4°. The solvent separated galactose, xylose with glucose and arabinose traveling together. The sugars were identified by radioachromatographic scanner and AgNO₃ staining (89). The spots corresponding to xylose and glucose-arabinose were cut, eluted and rechromatographed using solvent III.
at room temperature for 24 hours. This further purified xylose and also separated glucose and arabinose. The areas corresponding to each sugar were cut into strips of approximately 1 cm. The strips were soaked overnight in 1 ml of water in sealed scintillation vials. Fifteen ml of scintillation fluid was added and vials were placed to count in the scintillation counter. The radioactivity was plotted against distance from the origin. Part A gives data on xylose and Part B gives data on glucose + arabinose. The open bars are data obtained at 20 hours, shaded bars at 10 hours, and cross-hatched bars at zero time.
Figure 32-B

Radioactivity (cpm)

Distance from Origin (cm)

Glucose

Arabinose
total radioactivity content of the pentoses was 4.3% that of the
hexose. At 10 hours of incubation, the combined amount of
radioactive arabinose and xylose was 0.06 umole and at 20 hours, it
was 0.075 umole. The residual specific catalytic activity of
epimerase was 3,470 units per mg. This represents the existence of
36% oxidized active enzyme and it corresponds to the formation of
0.032 umole of inactive enzyme. The total enzyme in the reaction
mixture was 0.1 umole and one half was removed in each sampling. The
ratio of radioactive pentoses to inactive enzymes was 2.34. Assuming
that half of the abortive complex was E-NADH-UDP-hexose, then the
ratio would be double, indicating that the pento-intermediate
returned to the inactive complex more than once.
DISCUSSION

Substrate-dependent reduction of epimerase-bound NAD$^+$ was first observed by Wilson and Hogness in an equilibrium mixture of UDPGal and epimerase (34). Our data have shown that when substrate was allowed to react with epimerase for extended periods, there was progressive reduction of enzyme with concomitant loss of catalytic activity. A control experiment showed that enzyme is stable in the absence of substrate under otherwise identical conditions (75). The process is reversible since nearly all the catalytic activity was recovered when the solution was passed through a Sephadex G-25 column. This makes the dissociation inactivation of epimerase dimer very unlikely. The degree of inactivation corresponded to the amount of reduction indicating that both were the results of a single process.

The possibility that initial binding of substrate to the enzyme causes a conformational change with subsequent change in catalytic properties did not appear to be of importance. UMP was capable of causing slight reduction in activity, possibly by binding to the active site and inducing conformational change. However, the effect was small and reversible. Pyridine nucleotide accelerated the UDPG-induced inactivation probably through an independent process. Commercial NAD$^+$ is contaminated with ADP-ribose, which has been
reported to be an irreversible inhibitor (77). Apparently it irreversibly reduces enzyme-NAD complex to enzyme-NADH-ADP-keto-ribose complex. The effect of mercaptoethanol is opposite that of NAD$^+$. The mechanism is difficult to interpret.

The reductive inactivation of epimerase by substrates can be understood on the basis of the following scheme:

\[
\begin{align*}
& \text{E-NAD} \quad \text{SH} \\
& \text{E-NAD} \quad \text{PH} \\
& \text{E-NADH} \quad \text{SH} \\
& \text{E-NADH} \quad \text{PH} \\
\end{align*}
\]

The normal catalytic pathway involves complexes I to IV. During epimerization III is reduced, however, it is transient in existence and is easily reactivated by intermediate X to form either complex II or IV. Therefore, it cannot account for the progressive inactivation. Substrate inactivation of epimerase is a slow gradual process. The time required for inactivation is so long that it is necessary to postulate the presence of another inactive reduced enzyme. Formation of E-NADH due to spontaneous dissociation of keto-intermediate is not likely because of the known instability of E-NADH. Spontaneous dissociation of NADH from complex III did not occur. Formation of complex V, the abortive complex, is the actual cause of progressive reductive suppression of epimerase activity. We were able to identify that complex V is really a
reduced enzyme-substrate complex in which one mole of substrate is bound to every mole of reduced enzyme. The detection of tritium in the UDP-hexose recovered in the delayed trapping experiments using $[^3H]\text{-NaBH}_4$ suggests that free keto-intermediate is released from complex III in exchange for substrate and that the latter binds to E-NADH to form the abortive complex V. The longer the duration of incubation of epimerase and substrate, the larger are the concentrations of both free keto-intermediate and abortive complex V, and the greater is the degree of reduction and inactivation until equilibrium is achieved. Maitra and Ankel in simultaneous independent $[^3H]\text{-NaBH}_4$ trapping experiments have shown the UDP-hexose to be 4-tritiated UDPG and UDPGal (80). The free keto-intermediate trapped chemically is therefore UDP-4-ketoglucose, which is most likely the intermediate of the epimerization.

Immediately after mixing enzyme and substrate, a majority of enzyme is in the form of complexes II and IV with small amounts of complex III and negligible amounts of complex V. When this solution was reduced with $[^3H]\text{-NaBH}_4$, the reduced epimerase, but not the substrates, contained large amounts of radioactivity. In the later stage of incubation, there was progressive increase in complex V with depletion of complexes I and III, which are the active reducible species. Reduction by $[^3H]\text{-NaBH}_4$ at this stage would result in less labeling of the enzyme since the majority of enzyme was in the abortive complex form. Substantial amounts of radioactivity were then found in UDP-hexoses, since the concentration of
the accumulated keto-intermediate was considerable.

The reductive inactivation shows a biphasic curve, characterized by a rapid burst and followed by a slow phase. In the first rapid phase, there was formation of both complexes III and V. The second phase was slow and may take hours or even days to reach equilibrium. The fact that substrate-induced reductive inactivation is not complete but reaches equilibrium is explained by the back reaction due to the accumulation of keto-intermediate. When its concentration has reached a critical level, it can recombine with complex V to regenerate complex III. This releases substrate and reactivates the enzyme. In other words, the interconversion of complexes III and V is detectably reversible. There is exchange of keto-intermediate with bound substrate and vice versa. This is consistent with our findings which also establish exchange of free substrate for bound substrate in E-NADH·SH using UDP-[U-14C]-glucose.

The correctness of this interpretation was established by permitting complexes III and V to interconvert for a long time in the presence of two substrates. Intermolecular hydrogen transfer was easily detected when unlabeled UDP-xylose was allowed to react with enzyme and UDP-[4-3H]-hexose. There was formation of 2 kinds of abortive complexes and intermediates. The reactivation of UDP-hexose complex V by a free UDP-4-ketopentose intermediate explained the appearance of tritium in the UDP-pentose. The
experiment did not exclude the possibility of exchange of free intermediate for a bound one which would lead to the same results and is subject to similar interpretation.

The maximum concentration of keto-intermediate is that of the enzyme used in the reaction and it was less than $10^{-5}$M. This concentration is small compared to that of the substrate, which was $10^{-3}$M, yet the keto-intermediate was able to compete with substrate for the reduced enzyme. The affinity of the reduced epimerase for the intermediate is 100 fold greater than its affinity for the substrate. The dissociation constant of the reduced enzyme for UDPG is small as shown by the very tight binding of substrate by E-NADH. The dissociation constant for intermediate must be smaller, showing the tightness of binding of keto-intermediate to the reduced enzyme during catalysis.

Our data from the experiments using tritiated substrate have established unequivocally that hydrogen transfers from C-4 of the pyranose ring to NAD$^+$ with the formation of NADH and UDP-4-keto-glucose.
SUMMARY

Uridine diphosphate-galactose-4-epimerase (EC 5.1.3.2) catalyzes the interconversion of UDPG and UDPGal. The _E. coli_ enzyme possesses non-covalently bound NAD⁺ which does not dissociate even on extensive purification. Epimerization is an oxidation-reduction process in which hydrogen is transferred from substrates to NAD⁺ with the formation of NADH and an intermediate shown here to be a UDP-4-ketosugar. This is followed by reoxidation of NADH and concomitant reduction of the UDP-4-ketosugar to form products.

Reduction of epimerase by NaBH₄ alone results in the formation of E⁺NADH which undergoes spontaneous reoxidation to regenerate native enzyme. However, in the presence of UDPG, reduction by [³H]NaBH₄ produces a stable E⁺NAD³H⁺UDP-hexose complex in which NADH is radioactive but UDP-hexose is not. On heat dissociation of this complex, [³H]-NADH was released with 90% of the tritium at the B side of the dihydropyridine ring; UDP-hexoses released contained little or no radioactivity.

In UMP-dependent reductive inactivation by [⁴-³H]-glucose or [³-³H]-glucose, no tritium can be detected either in enzyme bound NADH or in the protein itself. These findings are at variance with a recent claim that hydrogen is transferred from carbon-3 of glucose,
as detected by an apparent kinetic isotope effect for the reaction of glucose-3-d\textsubscript{1} (Davis, L., and Glaser, L., BBRC \textsuperscript{43}, 1429 (1971)).

Slow autooxidation of E-NADH-UDP-hexose complex occurs under special conditions. Processes causing dissociation of substrates, for example multiple passage through gel columns, prolonged dialysis, and aging, lead to spontaneous reoxidation. Autooxidation is prevented by the presence of UDPG.

Reduced inactive E-NADH-UDP-hexose complex can be reactivated by the presence of cyclohexanone. The reactivation is slow, incomplete, and dependent on cyclohexanone concentration. Initially, cyclohexanone apparently triggers dissociation of UDP-hexoses from the complex by partially denaturating the protein. This permits E-NADH to reoxidize spontaneously, a process which is prevented by UMP. Cyclohexanone-induced reoxidation of E-NAD\textsuperscript{3}H-UDP-hexose complex produces free tritiated NAD\textsuperscript{+} and tritiated water but no tritiated cyclohexanol. It is possible that reoxidation is mediated by an election acceptor on the protein, perhaps a disulfide group.

Both UDPG and UDP-xylose are capable of reductively inactivating epimerase. The process is biphasic, characterized by a burst of activity loss and $A_{645}$ increase, followed by a slower process. The initial burst approaches an equilibrium position in which about 30\% of the activity remains and NAD\textsuperscript{+} is not fully reduced. The amount of NADH formed corresponds to the loss of enzyme
activity. Progressive inactivation results from the formation of E-NADH-UDP-sugar with dissociation of UDP-ketosugar. This interpretation is supported by the following experimental findings, which also establish that the intermediate released is UDP-4-ketosugar. UDP-hexoses tightly bound to E-NADH-UDP-hexose readily exchange with free UDP-glucose. UDP-4-ketoglucose can be trapped by $[^3H]$-NaBH$_4$ once E-NADH-UDP-hexose has formed but not before. In similar delayed trapping experiments utilizing NaBH$_4$ and tritiated substrates, tritium is transferred to NAD$^+$ by UDP-[4-$^3$H]glucose but not by UDP-[3-$^3$H]glucose. Since the initial burst approaches an equilibrium, the postulated replacement of UDP-keto-hexose intermediate by substrates is detectably reversible, and UDP-keto-hexose is a free intermediate under these conditions. That this interpretation is correct is established by experiments which show that, upon prolonged exposure of this enzyme to a mixture of UDP-[4-$^3$H]-hexose and UDP-xylose, a known substrate, tritium can be detected in pentoses. Therefore, hydrogen transfer under these conditions is partially intermolecular, although under normal conditions it is intramolecular. These findings establish that UDP-4-ketoglucose and UDP-4-ketoxylose are free intermediates under these conditions.
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