The Mechanism of Action of the Enzyme Uridine Diphosphoglucose 4-Epimerase

PROOF OF AN OXIDATION-REDUCTION MECHANISM WITH DIRECT TRANSFER OF HYDROGEN BETWEEN SUBSTRATE AND THE B-POSITION OF THE ENZYME-BOUND PYRIDINE NUCLEOTIDE*

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SUMMARY

The properties of the enzyme UDP-glucose 4-epimerase from Escherichia coli have been investigated. Its substrate specificity is comparatively low, it acts on the thymidine, cytidine, and adenine, as well as the uridine diphospho derivatives of glucose. Its specificity toward modifications of carbon 6 of the hexose moiety of the substrate is also low. The formation of NADH on the enzyme in the presence of substrate (WILSON, D. B., AND HOGNESS, D. S., J. Biol. Chem., 239, 2469 (1964)) is less specific for the substrate than is the over-all epimerization. UDP-mannose causes reduction of the enzyme-bound NAD⁺ but is not epimerized.

It has been shown that the epimerization is an oxidation-reduction process involving NAD⁺ as the oxidant and UDP-4-ketoglucose as the enzyme-bound oxidized intermediate. Transfer of the 4-hydrogen from the hexose moiety of UDP-6-deoxyglucose to the B-position of NADH and its transfer back to several nucleotide-4-keto sugars has been demonstrated. The nucleotide-4-keto sugars were synthesized using enzymatic or chemical reactions or both.

The enzyme-bound NAD⁺ is subject to reduction by NaBH₄, and the hydrogen is specifically transferred to the B-side of the nicotinamide ring. When conducted in the presence of substrate, this chemical reduction of NAD⁺ is accompanied by the binding of 1 mole of the substrate and complete loss of enzyme activity. Another method of reducing the enzyme-bound NAD⁺ is to incubate the enzyme with substrate for long periods of time. In addition to the steady state level, there is a slow increase in enzyme-NADH which is accompanied by loss of enzyme activity, strong binding of 1 mole of substrate and incorporation of the 4-hydrogen of the substrate into the B-position of NADH.

The possibility that tryptophan functions as an intermediate hydrogen carrier in this reaction, as it has been proposed to do in several other NAD⁺-linked dehydrogenase reactions (SCHELLENBERG, K. A., J. Biol. Chem., 242, 1815 (1967)), has been investigated. It would appear that there are no intermediate hydrogen carriers involved.

It has been proposed that the interconversion of UDP-glucose and UDP-galactose by the enzyme UDP-glucose 4-epimerase (EC 5.1.3.2) proceeds by an oxidation-reduction reaction at C-4 of the hexose moiety of the substrate, with NAD⁺ serving as the oxidant and UDP-4-ketoglucose as the oxidized intermediate (1, 2). However, although there is evidence that NAD⁺ participates in the reaction (3, 4), there has been no previous evidence for the formation of UDP-4-ketoglucose, either free or as an enzyme-bound intermediate. Recently, we have presented evidence for the presence of a "4-keto intermediate" in the reaction catalyzed by the Escherichia coli enzyme (5).

The purpose of the present communication is to present further evidence for the existence of an oxidation-reduction process in the epimerization catalyzed by the E. coli enzyme, to describe some of its characteristics, and to report investigations on the possible participation of intermediate hydrogen carriers in the process.

MATERIALS AND METHODS

Chemicals—UDP-xylose, UDP-mannose, CDP-glucose, and UMP-morpholidate were from Sigma Chemical Corp. UDP-glucose, GDP-glucose, uridine nucleotides were from P-L Biochemicals. TDP-glucose, UDP-galactose, UDP-[U-¹⁴C]glucose ([U-¹⁴C]glucose, 35 mCi per mmole), and UDP-[¹⁴C]-galactose ([U-¹⁴C]galactose, 5.9 mCi per mmole) were products of Calbiochem. NaBH₄ (5.3 Ci per mmole) was obtained from the Nuclear-Chicago Corp. UDP-fucose was synthesized from fucose-1-P and UMP-morpholidate according to the method of Nordin et al. (6). The 5,5'-dithiobis(2-nitrobenzoic acid) was a product of K and K Laboratories, Inc., and methyl α-quinovoside was from Mann Research Laboratories. CDP-4-keto-6-

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deoxyglucose was prepared from CDP-glucose by the action of the enzyme CDP-glucose oxidoreductase.2

E. coli Strains—The strain B78A,3 a galactose operon constitutive mutant, was grown in the medium described by Wilson and Hogness (4) except that n-fructose was omitted. Growth was carried out in a 10-liter fermenter at 37°, with maximum aeration, until the culture attained maximal optical density at 600 nm. The cells were then harvested in a Sorvall refrigerated centrifuge with a continuous flow head (yield of cells, 18 g per liter of medium).

E. coli strain B (American Type Culture Collection 11303) was grown on Difco bacto-antibiotic medium 3 in a 10-liter fermentor as described by Wang and Gabriel (7). When cell density reached one-half of maximum, the cells were harvested as described above.

Chromatography—All paper chromatograms were developed using the descending technique at room temperature in the following solvents: I, pyridine-ethyl acetate-water (2:5:7) (upper phase) (6); II, absolute ethanol 0.5 M morpholinium tetraborate (pH 8.6)-methyl ethyl ketone (7:3:2) (9); III, absolute ethanol-1 M ammonium acetate (pH 7.5) (3:1) (10); IV, 95% ethano-1 M ammonium acetate (pH 3.8) (7:3) (10). Reducing sugars were detected on the chromatograms by the method of Trevyavan et al. (11), nucleotide sugars by their ultraviolet absorption, and amino acids by the ninhydrin reaction.

Determination of Radioactivity—Chromatograms containing 14C were scanned with a Nuclear-Chicago model 1036 radiochromatogram scanner, those containing tritium with a Packard model 7201 scanner. All liquid scintillation counting was done with a Nuclear-Chicago model 720 series counter. Tritium-containing compounds were dissolved in a scintillation solution consisting of 2 ml of absolute ethanol and 15 ml of reagent grade toluene which contained 4 g of diphenylxazole (PPO) and 0.05 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPPO) per liter of toluene. 14C was counted in 10 ml of a scintillation solution containing 0.05 g of POPPO and 10 g of PPO in 1.5 liter of toluene and 1.0 liter of absolute ethanol. The former system can accommodate 0.03 ml of aqueous solution the latter 0.2 ml.

Enzymes—Lactic dehydrogenase and alcohol dehydrogenase (ammonium sulfate slurries) and glutamic dehydrogenase (glycerol solution) were products of the Boehringer-Mannheim Corp. Enzymes-Lactic dehydrogenase and alcohol dehydrogenase (ammonium sulfate slurries) and glutamic dehydrogenase (glycerol solution) were products of the Boehringer-Mannheim Corp. Venom phosphodiesterase was from the Worthington Biochemical Corp. (potency 0.3, supplier's units) and calf intestinal mucosa alkaline phosphatase (350 i.u. per mg4) was from the Sigma Chemical Co. Beef liver UDP-glucose 4-epimerase was purified from beef liver acetone powder by Steps 1, 3, 4, and 6 of the method of Hogness (4) except that n-fructose was omitted. Growth was carried out in a 10-liter fermenter at 37°, with maximum aeration, until the culture attained maximal optical density at 600 nm. The cells were then harvested in a Sorvall refrigerated centrifuge with a continuous flow head (yield of cells, 18 g per liter of medium).

E. coli UDP-glucose 4-epimerase was purified from E. coli strain B78A through Step VII of the method of Wilson and Hogness (4). The final specific enzyme activity of various preparations based on protein absorbance at 280 nm ranged from 75 to 160 i.u. per 280 nm absorbance unit when assayed at room temperature according to the one step procedure of Imae et al. (12). Electrophoresis on acrylamide gel showed that the most highly purified preparations were nearly homogeneous. They contained a single contaminating band (electrophoresis at pH 8.0) which trailed the main enzyme band and contained less than 10% of the total protein as judged by densitometry. Wilson and Hogness (4) reported that, in the presence of substrate, the E. coli enzyme develops an NADH-like spectrum with an absorption maximum at 345 nm. The various preparations used for the present work were investigated for their ability to form NADH in the presence of substrate. For purposes of quantitation, the difference in absorption at 345 nm and 400 nm was determined in quartz microcuvettes (light path 1 cm) using a Beckman model DU spectrophotometer. Since NADH has no absorption at 400 nm, the difference between these two readings is a good measure of the NADH absorbance. Although all readings were taken against the appropriate protein-buffer blank, it was considered desirable to refer to a base line at 400 nm, especially for reactions of long duration. The majority of enzyme-bound NADH was calculated using the extinction coefficient 6200 (4). The various epimerase preparations used in this work are referred to by number and their 280 nm absorbances due to protein were: No. 2, 10.5; No. 3, 18.5; No. 4, 5.7; No. 6, 7.7; No. 7, 10.4; No. 8, 11.5. The range of the A280 to A400 values for these preparations was from 0.0065 to 0.015 per protein absorbance unit at 280 nm.

Acid Hydrolysis—Nucleotide sugar hydrolysis was carried out by making the solutions 0.1 x in HCl and then heating at 100° for 15 min. This solution was then deionized by pouring over IR-45 (OH- form) and IR-120 (H+ form) ion exchange resin columns before further manipulation.

NaB3H4 Reduction—NaB3H4 (30 μg, 5.3 Ci per mmole) was dissolved in max NaOH and to this solution was added the solution that it was desired to reduce.

Synthesis of 4-Keto-6-deoxyglucose-1-P—TDP-glucose oxidoreductase (1.2 i.u.) was incubated with 27 mg of TDP-glucose in 1 ml of 25 mM Tris-Cl- buffer (pH 8.0) for 12 hours at room temperature. This resulted in the complete conversion of TDP-glucose to TDP-4-ket0-6-deoxyglucose as judged by its 318 nm absorption in 0.1 x NaOH (13). Venom phosphodiesterase (0.7 mg) was then added, the reaction mixture incubated a further 60 min, and then applied to a Dowex 1 (Cl-) column (1 x 7 cm) which was eluted with a 300-ml gradient of 5 to 50 mM HCl (14). The sugar-1-P elutes at approximately 15 mM HCl, well ahead of TMP. The fractions containing the sugar-1-P were pooled and extracted with 3% tri-n-octylamine in chloroform until the aqueous phase was free of chloride ions as determined by AgNO3 test (14). The aqueous solution was then passed through an IR-120 (pyridinium+) column and concentrated to ca. 1 ml. The yield of sugar-1-P was 60% at this point, as judged by 355 nm absorbance in 0.1 x NaOH (15).

Synthesis of UDP-4-keto-6-deoxyglucose—The bis-tri-n-octylammonium salt of 4-keto-6-deoxyglucose-1-P (13 μmoles) was reacted with 35 μmoles of UMP-morpholidate in anhydrous pyridine for 72 hours according to the procedure of Nordin et al. (6). The pyridine was removed from the reaction mixture under vacuum and the residue dissolved in sodium acetate solution which was then extracted with ether (10). The resulting aqueous solution was applied to a column of Dowex 1 (Cl-) (1 x 13 cm) and eluted with a 600-ml linear gradient of 40 to 80

1 The enzyme was a gift from Dr. R. D. Bevill.
2 A gift from Dr. R. M. Kalckar.
3 One International Unit (i.u.) of enzyme activity is the amount of enzyme that will catalyze the conversion of 1 μ mole of substrate per min under the conditions of the assay.
mm LiCl in 3 mm HCl (10). The fractions containing the nu-
cleotide sugar were pooled and passed over an IR-120 (H\(^+\))
column to remove Li\(^+\) and chloride was removed from the eluate
by extraction with tri-n-octylamine as described previously.
Finally the aqueous solution was passed again over an IR-120
(\(H^+\)) column, neutralized with ammonium hydroxide and
concentrated to 1.0 ml volume. The 260 nm absorbance (pH 7)
of this solution indicated a uridine concentration of 2.55 mm, while
the 318 nm absorbance in 0.1 N NaOH indicated a nucleotide-4-
eketo sugar concentration of 2.4 mm (assuming that UDP-4-keto-
6-deoxyglucose has the same extinction as the corresponding
UDP-glucose). The fractions containing the nu-
cleotide sugar were pooled and passed over an IR-120
\((H^+)\) column, neutralized with ammonium hydroxide and
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the 318 nm absorbance in 0.1 N NaOH indicated a nucleotide-4-
eketo sugar concentration of 2.4 mm (assuming that UDP-4-keto-
6-deoxyglucose has the same extinction as the corresponding
thymidine compound (4800)).

The spectra of this compound in both acidic and basic solution were precisely as expected for
UDP-4-keto-6-deoxyglucose, and reduction with NaB\(_3\)H\(_4\) resulted
after hydrolysis.

The enzyme system was used (10). In each case, an identical treatment was carried
out in the absence of epimerase. The sugars were detected using
alkaline silver nitrate and epimerization was detected by the
appearance of the 4-epimeric sugar of the starting compound in
the reaction mixture containing epimerase.

### RESULTS

**Substrate Specificity of UDP-glucose 4-Epimerase**—It has been
reported that the epimerase from *E. coli* will epimerize UDP-
xylose to UDP-L-arabinose, in addition to its action on the
normal substrates (15). We have confirmed this result and
extended these observations to other substances, as shown in
Table I. The specificity of the *E. coli* enzyme is very broad and
extends to substrates with different purine and pyrimidine bases
as well as substances with different modifications at C-6 of the
hexose moiety. Crude preparations of UDP-glucose 4-epi-
merase from yeast have been reported to have a broad specificity
but this was said to be due to the presence of several enzyme
activities (17). Because of the purity of the enzyme preparations
used in this work, it is highly likely that the broad specific-
ity observed is the property of one enzyme.

The enzyme is, however, specific for the configuration of C-2
of the hexose moiety of the substrate, since it fails to epimerize
UDP-mannose. Free energy considerations indicate that the
equilibrium ratio of UDP-mannose to UDP-talose should be in
the region of 24:1 (18). A consideration of the sensitivity of the
technique used to detect talose (Fig. 1) shows that an amount of
talose somewhat less than 5% of the expected equilibrium amount
could be detected. Thus it would appear that the *E. coli* epi-
merase cannot act on UDP-mannose.

**Substrate Specificity for Formation of NADH on Enzyme**—Wil-
son and Hogness (4) have reported that in the presence of UDP-
glucose, 19% of the enzyme-bound NAD\(^+\) becomes reduced.
We have investigated the ability of a number of other substances
to produce this effect (Table II). Of the compounds that re-
resulted in the formation of NADH, all were equally effective with
the exception of UDP-allose which produced about half as much
NADH as the other substances. It is evident that a free 4-hy-
droxy is required for this effect; the uridine dipospho deriva-

### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>International units of enzyme used per micro-mole of substrate</th>
<th>Molarity of substrate</th>
<th>Incubation time</th>
<th>Epimerization(^a)</th>
</tr>
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<tbody>
<tr>
<td>UDP-xylose</td>
<td>1</td>
<td>10</td>
<td>1/2</td>
<td>+</td>
</tr>
<tr>
<td>UDP-fucose</td>
<td>1</td>
<td>10</td>
<td>1/2</td>
<td>+</td>
</tr>
<tr>
<td>UDP-glucose</td>
<td>1</td>
<td>10</td>
<td>1/2</td>
<td>+</td>
</tr>
</tbody>
</table>
| UDP-\(\alpha\)-galacto-hexo-
  dialdose\(^b\)             | 1                                                             | 10                    | 1/2             | +                   |
| UDP-mannose              | 1                                                             | 10                    | 1/2             | +                   |
| TDP-glucose              | 1                                                             | 10                    | 1/2             | -                   |
| CDP-glucose              | 1                                                             | 10                    | 1/2             | +                   |
| ADP-glucose              | 1                                                             | 10                    | 1/2             | +                   |
| GDP-glucose              | 1                                                             | 10                    | 1/2             | +                   |
| Glucose-1-P              | 1                                                             | 50                    | 1/2             | -                   |

\(^{a}\) The symbol "+" indicates that more than 10% of the added
substrate was epimerized. The symbol "-" indicates no detectable
product formed, the limit of detectability being 1% epimeri-
zation of substrate.

\(^{b}\) See Reference 16. The above incubations were carried out at
room temperature in 0.1 m glycine buffer (pH 8.8). At the end of
the incubation, the solution was made 0.3 N in HCl and heated in
a boiling water bath for 15 min. After deionization on IR-120
and IR-45 ion exchange resins and concentration, the compounds
were streaked onto Whatman No. 1 paper and developed in Sol-
vent System I. In each case, an identical treatment was carried
out in the absence of epimerase. The sugars were detected using
alkaline silver nitrate and epimerization was detected by the
appearance of the 4-epimeric sugar of the starting compound in
the reaction mixture containing epimerase.

**Fig. 1.** Reaction of epimerase with UDP-mannose. UDP-
mannose (0.7 pmole) was reacted with 7 i.u. of UDP-glucose 4-epim-
erase in a total volume of 0.55 ml of 0.1 m glycine buffer (pH 8.8)
for 15 hours at room temperature. This mixture was hydrolyzed
with acid, concentrated to 0.4 ml, and chromatographed in solvent
system I for 27 hours. The amounts spotted, from left to right
are: a, 200 nl; b, 10 nl; c, 2 nl of the hydrolysis solution. Standards:
T, talose; M, mannose.
TABLE II
Formation of NADH on epimerase

<table>
<thead>
<tr>
<th>Enzyme preparation used</th>
<th>Compound tested</th>
<th>Substrate concentration</th>
<th>NADH formed as a percentage of that formed in the presence of UDP-glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-8 UDP-glucose</td>
<td></td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>5 UDP-allose(^a)</td>
<td></td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>7 UDP-allose(^a)</td>
<td></td>
<td>5.5</td>
<td>100</td>
</tr>
<tr>
<td>5 UDP-mannose</td>
<td></td>
<td>1.1</td>
<td>100</td>
</tr>
<tr>
<td>4 UDP-xylose</td>
<td></td>
<td>2.0</td>
<td>100</td>
</tr>
<tr>
<td>5 UDP-fucose</td>
<td></td>
<td>0.8</td>
<td>100</td>
</tr>
<tr>
<td>5 TDP-glucose</td>
<td></td>
<td>0.8</td>
<td>100</td>
</tr>
<tr>
<td>7 TDP-glucose</td>
<td></td>
<td>5.3</td>
<td>100</td>
</tr>
<tr>
<td>5 UDP 3-O-methylglucose(^a)</td>
<td></td>
<td>0.62</td>
<td>100</td>
</tr>
<tr>
<td>7 UTP</td>
<td></td>
<td>6.0</td>
<td>100</td>
</tr>
<tr>
<td>7 UDP</td>
<td></td>
<td>5.5</td>
<td>100</td>
</tr>
<tr>
<td>7 UMP</td>
<td></td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>7 Glucose-1-P</td>
<td></td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>7 Glucose 1 P + UMP</td>
<td></td>
<td>150</td>
<td>100</td>
</tr>
<tr>
<td>7 UMP-morpholidate</td>
<td></td>
<td>36</td>
<td>100</td>
</tr>
<tr>
<td>7 UDP-glucuronic acid</td>
<td></td>
<td>37</td>
<td>100</td>
</tr>
<tr>
<td>8 UDP 4-deoxyglucose(^c)</td>
<td></td>
<td>1.4</td>
<td>100</td>
</tr>
<tr>
<td>8 UDP 4-keto-6-deoxyglucose</td>
<td></td>
<td>0.55</td>
<td>100</td>
</tr>
<tr>
<td>5 UDP 4-O-methylglucose(^c)</td>
<td></td>
<td>1.0</td>
<td>100</td>
</tr>
</tbody>
</table>

\(^a\) A gift from Dr. D. K. Peterson.
\(^b\) A gift from Dr. W. L. Salo.

Fig. 2. NaBH\(_4\) reduction of epimerase in the absence of UDP-glucose. Three experimental cuvettes and a blank cuvette each contained 140 \(\mu\)l of epimerase preparation 8 and 10 \(\mu\)l of 2 \(M\) glycine buffer (pH 8.8). At zero time, 10 \(\mu\)l of 0.60 \(M\) NaBH\(_4\) were added to the three reaction cuvettes and 10 \(\mu\)l of water to the blank. At 14 min, 2 \(\mu\)l of 5 \(M\) acetate were added to each cuvette. At 15 min, 2 \(\mu\)l of 5 \(M\) TDP-glucose were added to Cuvette 2, 2 \(\mu\)l of 5 \(M\) TDP-4-keto-6-deoxyglucose were added to Cuvette 1 and to the blank. The absorbance at 345 and 400 nm was followed in all three cuvettes. □-□, Cuvette 1; △-△, Cuvette 2; ○-○, Cuvette 3.

Fig. 3. NaBH\(_4\) reduction of epimerase in the presence of UDP-glucose. Two cuvettes were added 150 \(\mu\)l of epimerase preparation 8 and 10 \(\mu\)l of 1 \(M\) glycine buffer (pH 8.8). To the reaction cuvette were added 20 \(\mu\)l of 10 \(M\) UDP-glucose and to the blank 20 \(\mu\)l of water. At zero time, 10 \(\mu\)l of 60 \(M\) NaBH\(_4\) were added to the reaction cuvette and 10 \(\mu\)l of water to the blank. The absorbance at 345 nm was monitored and at 120 min 2 \(\mu\)l of 10 \(M\) TDP-4-keto-6-deoxyglucose were added to each cuvette. The enzyme activity was determined by the methods described under "Materials and Methods."
excess of 20 hours. UDP-mannose and UTP were also tested and found very effective in stabilizing the NADH produced by NaBH₄ reduction. The enzyme activity of the reduced epimerase is only 7% of the original activity and TDP-4-keto-6-deoxyglucose restores 93% of the original activity (Fig. 3).

Site of Reduction by NaBH₄—In order to study the site of this reduction, use was made of NaBH₄. An epimerase preparation was reduced in the presence of UDP-glucose and dialyzed to remove all exchangeable tritium. The product was then heated in a boiling water bath to release pyridine nucleotides from the enzyme. The resulting nucleotides were separated on a DEAE-cellulose column (Fig. 4A) and the large peak of radioactivity eluting with NADH indicates that NAD⁺ is a major site of the reduction brought about by NaBH₄. Parallel experiments were done in which the enzyme was reduced in the absence of UDP-glucose and allowed to undergo spontaneous oxidation. In this case there is no radioactivity associated with either NAD⁺ or NADH, indicating that both chemical reduction and spontaneous reduction were specific for the same side of the nicotinamide ring of NAD⁺.

In order to establish the absolute stereospecificity of the reaction, the following experiments were performed. A portion of the NADH from the column discussed in Fig. 4A (1.15 × 10⁶ cpm) was reacted with alcohol dehydrogenase and acetaldehyde (an A-specific dehydrogenase, see Reference 20) in 1 ml of a solution that was 18 mM in potassium phosphate buffer (pH 6.5). When the 340 nm absorbance indicated the complete oxidation of NADH, the volatile material from the reaction mixture was collected and found to contain only 550 cpm of tritium, indicating that less than 5% of the tritium was located on the A-side of NADH. Reaction of a second portion of the NADH solution was carried out with glutamic dehydrogenase (a B-specific dehydrogenase, see Reference 20) and α-ketoglutarate in the column buffer. Chromatography of this reaction mixture on paper using Solvent System IV for 30 hours separated glutamate from NADH and NAD⁺. The glutamate and pyridine nucleotide areas were counted separately and 76% of the tritium was found in glutamate. Less than 5% of the tritium chromatographed as glutamate in a control reaction which contained no glutamic dehydrogenase. It is evident from these experiments that reduction of epimerase-bound NAD⁺ with NaBH₄ is specific for the B-side of the nicotinamide ring, as is the spontaneous reoxidation process.

If the NaBH₄-reduced epimerase is treated with TDP-4-keto-6-deoxyglucose there is quantitative transfer of tritium from NADH to the TDP-sugar (Fig. 4B) and it was established, that, after hydrolysis of the TDP-sugar, all of the tritium chromatographed in Solvent System I as either quinovose or fucose. Chromatography of this reaction mixture on paper using Solvent System IV for 30 hours separated glutamate from NADH and NAD⁺. The glutamate and pyridine nucleotide areas were counted separately and 76% of the tritium was found in glutamate. Less than 5% of the tritium chromatographed as glutamate in a control reaction which contained no glutamic dehydrogenase. It is evident from these experiments that reduction of epimerase-bound NAD⁺ with NaBH₄ is specific for the B-side of the nicotinamide ring, as is the spontaneous reoxidation process.

Location of Tritium in 6-Deoxyhexoses—A chemical degradation was performed to firmly establish the location of the tritium in the TDP-sugars from the experiment described in Fig. 4B. The acid hydrolysate of the TDP-sugars was chromatographed in Solvent System I and the fucose and quinovose areas of the chromatograms eluted.

The quinovose was dried and reacted in 10 ml of 1% anhydrous methanolic HCl for 3 hours at 75°C in a sealed tube. The reaction mixture was then neutralized with NaHCO₃, filtered, and concentrated under vacuum. The entire mixture was transferred to Whatman No. 1 paper and developed in Solvent System I for 9 hours. A radioactivity scan showed a single peak chromatographing with authentic methyl-β-quinovoside which chromatographs well ahead of quinovose in this solvent. The methyl-β-quinovoside was eluted from the paper and diluted with cold methyl-β-quinovoside to a final specific activity of 2.79 × 10⁶ cpm per pmole. Degradation of this methyl-β-quinovoside was carried out according to the methods of Bevill et al. (21) (periodate oxidation followed by NaBH₄ reduction and hydrolysis). Carbon 3 is isolated as ammonium formate and purified by sublimation, carbon atoms 1 and 2 are isolated as the methone of glycolaldehyde and carbon atoms 4, 5, and 6 are isolated as propylene glycol (21). The propylene glycol was oxidized with periodic acid (1 mole per mole of methyl-β-quinovoside) and the reaction mixture was neutralized with BaCO₃ filtered, and ethanol added to a final concentration of 50%. Dime done (5,5-dimethyl-1,3-cyclohexanedione) was added (2 moles per mole of methyl-β-quinovoside) and the methone of formaldehyde crystallized out of the solution. It was recrystallized from 50% ethanol, m.p. 190-191°C. Formaldehyde is considerably more reactive toward dimedone than is acetaldehyde, the other product of this periodate oxidation. This results in a clean-cut separation of the two aldehydes at this point.

The radioactivity of the hydrogens attached to carbon atoms 5 and 6 of the hexoses was determined by degrading the fucose sample. The labeled fucose was diluted with cold fucose to a final specific activity of 2.8 × 10⁶ cpm per pmole and this material was oxidized with periodic acid (5 moles per mole of fucose). This procedure produces acetaldehyde from carbon atoms 5 and 6 and formate from carbon atoms 1 through 4. The oxidation reaction mixture was neutralized with BaCO₃ filtered, and ethanol was added to a final concentration of 50%. Dime done...
was added (2 moles per mole of fucose) and the methione of acetaldehyde crystallized out of the reaction mixture. It was recrystallized from 50% ethanol, m.p. 132 134°.

A summary of the results of this degradation (Table III) clearly establishes that the tritium is entirely located at carbon 4 of the hexose molecules. Consequently it is clear that in the oxidation and reactivation of NaBH₄-reduced epimerase, the B-hydrogen from NADH is transferred to carbon 4 of the 4-keto sugar resulting in a mixture of the 4-epimers of the sugar.

Specificity of Oxidation Reaction—Several derivatives of 4-keto-6-deoxyglucose were tested for their ability to reoxidize NaBH₄-reduced epimerase in order to determine if the reoxidation reaction showed the characteristics of enzyme specificity. Using a competitive reaction technique, the following comparative activities were obtained, normalized on a value of 100 for the reduction of NADH resulting in a mixture of the 4-epimers of the sugar.

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of the epimerase has been studied. The results (Table IV) show that, in both cases, for every molecule of NADH formed on the enzyme there is 1 molecule of the substrate bound to the enzyme sufficiently firmly to resist dialysis. This substrate can, however, be released from the enzyme by dialysis against 8 m urea, thus establishing that it is not covalently bonded. The enzyme-substrate bond is also released by heating at 100° and chromatographs identically with UDP-galactose and UDP-glucose in Solvent Systems II, III, and IV, thus demonstrating that there is no change in the substrate during binding. Acid hydrolysis of this bound substrate produced only labeled glucose and galactose (chromatography in Solvent System I) and it is thus clear that the substrate is not bound in the "inactive intermediate" as UDP-4-ketoglucose but as the normal substrates UDP-glucose and UDP-galactose.

Formation of "Inactive Intermediate" in Presence of UDP-[4-3H]hexose—UDP-[4-3H]quinovose (in mixture with UDP-[4-3H]fucose) was produced by reduction of UDP-4-keto-6-deoxyglucose with NaBH₄. The labeled nucleoside diphosphate sugar was purified by successive chromatography in Solvents III and IV. The material eluted from the final paper chromatographic step had a specific activity of 1.2 x 10⁹ cpm per pmole on the basis of uridine absorption at 260 nm. Hydrolysates of this material showed the presence of only two radioactive compounds which chromatographed identically with fucose and quinovose in Solvent System I.

The "inactive intermediate" was formed using the radioactive nucleotide as described in Fig. 7. The specific activity of the nondialyzable tritium on the basis of enzyme-bound NADH absorption at 345 nm was 2.5 x 10⁹ cpm per pmole of NADH, which is very close to twice the specific activity of the nucleotide sugar. Chromatography, following denaturation of the protein, revealed radioactivity in the compounds NADH, UDP-fucose, and UDP-quinovose (Fig. 7B). If the enzyme is treated with TDP-4-keto-6-deoxyglucose prior to denaturation, the radioactivity associated with NADH disappears and reappears associated with TDP-6-deoxyhexoses (Fig. 7A). Reaction of the NADH produced in the reaction described in Fig. 7B with A- and B-specific dehydrogenases, showed the coenzyme to be labeled on the B-side of the ring (Fig. 8). The radioactivity associated with the dialyzed enzyme is distributed evenly between original substrate molecules and NADH (or the other compounds to which it was subsequently transferred) (Figs. 7 and 8) establishing that the NADH has the same specific activity as the original substrate.

Thus the formation of the "inactive intermediate" is a complex reaction which results in the following: (a) the specific activity of the NADH formed is identical with the specific activity of the substrate (UDP-[4-3H]hexose), (b) the "inactive intermediate" formed has twice the specific activity, based on NADH content, as does the starting substrate (UDP-[4-3H]hexose) and, (c) 1 molecule of substrate is bound per molecule of NADH formed on the enzyme (Table IV). A situation consistent with these observations is one in which a substrate molecule contributes its 4-hydrogen to reduce enzyme-bound NAD⁺ and a second substrate molecule is bound by the enzyme sufficiently firmly to resist dialysis.

While heat denaturation of the NaBH₄-reduced inactive enzyme, or of the inactive intermediate, resulted in release of enzyme-bound NADH as reported here, attempts to release the NADH formed on the initial contact of the enzyme with the substrate failed. This might be due to the enzyme being selectively denatured in its nonreduced state.

### Table IV

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<th>Enzyme preparation used</th>
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<tr>
<td>Reduced enzyme</td>
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<td>Inactive intermediate</td>
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<td>Non-dialyzable ³⁴C</td>
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### Enzyme Preparation

Epimerase preparation 7 (0.3 ml) was incubated with 20 μl of 2 m glycine buffer (pH 8.8) and 30 μl of 3.3 m UDP-[4-3H]-quinovose (1.20 x 10⁶ dpm per pmole) for 24 hours. This preparation was then dialyzed for 24 hours against a total of 10⁵ volumes of 10 mK₂HPO₄. It is a radioactivity scan of 15 μl of the resulting solution chromatographed in Solvent System III, after denaturation by boiling. The two peaks located between the UDP-glucose and the TDP-glucose spots are, proceeding from the origin, UDP-fucose and UDP-quinovose. The radioactive scan of 20 μl of the above enzyme solution to which had been added to 2 μl of 10 m TDP-4-keto-6-deoxyglucose before denaturation of the enzyme (chromatography in Solvent III). The two radioactivity peaks chromatographing just ahead of the TDP-glucose correspond to TDP-fucose and TDP-quinovose produced by reaction of NADH-enzyme with TDP-4-keto-6-deoxyglucose.

**Tritium Incorporation into Enzyme Preparation**—In order to check the possibility of an intermediate hydrogen carrier between the substrate and NADH, such as has been proposed by Schellen-
Reduced epimerase described in Fig. 4 was dialyzed against 8 M synthetic indolenine has proven readily reducible by NaBH₄ such a moiety would incorporate tritium upon reduction with NaBH₄. A tryptophan standard showed considerable retention under these conditions. Thus it would appear that indole rings are not the site of tritium incorporation into the protein. Further work will be required to establish the location of this nondialyzable tritium which was introduced by NaBH₄.

Role of Enzyme Sulfhydryl Groups—The role of sulfhydryl groups in the activity of UDP-glucose 4-epimerase is interesting, more particularly in view of Schellenberg’s proposal that oxidized tryptophan in NAD⁺-linked dehydrogenase is stabilized by sulfhydryl groups (24). Wilson and Hogness have reported that the epimerase contains two sulfhydryl groups per subunit, one of which reacts rapidly with Ellman’s reagent (5,5’-dithiobis-(2-nitrobenzoic acid) (29)) and the other of which reacts more slowly (30). As seen in Fig. 9, there are indeed two separable rates of reaction with Ellman’s reagent. The protein concentration of the reaction mixture described in Fig. 9 was determined using the biuret procedure (31), with bovine serum albumin as the standard. From this value, and the concentration of sulfhydryl groups calculated from the 412 nm absorbance (29) of the 200-min sample in Fig. 9, the number of sulfhydryl groups was calculated to be 2.0 ± 0.1 per molecular weight of 40,000 which agrees with the reported value (30). It is evident from Fig. 9 that the enzyme activity is affected by titration of the first sulfhydryl group but not by titration of the second. The maximum inhibition in any case was in the region of 50% and is completely reversible, all of the activity being restored by dialysis against 1 mM 2-mercaptoethanol.

While one literature report claims that there is no inhibition of the E. coli epimerase with a different sulfhydryl reagent than that used here (12), other workers have found that titration of the sulfhydryls will affect the enzyme by dissociating the subunits (32). The conditions that produce this effect were not reported, however.

Action of Epimerase on Mixtures of Substrates—It has been reported recently that the epimerization of UDP-glucose involves intramolecular hydrogen transfer (33). We have independently observed the same phenomenon with the E. coli epimerase utilizing a different set of reactions. UDP-[4-3H]quinovose, prepared as described above, and a large excess of UDP glucose were incubated with the E. coli epimerase under conditions that brought both epimerization reactions to equilibrium. Since both of these substrates are acted upon by the enzyme, an intermolecular hydrogen transfer would result in a large transfer of tritium from UDP-quinovose to UDP-glucose. Upon hydrolysis and chromatography of the liberated sugars in Solvent System I, all of the tritium was associated with the 6-deoxyhexoses with no transfer to either glucose or galactose. Thus the hydrogen transfer is, indeed, intramolecular.

Through the use of a similar approach, it has been possible to show that enzyme that becomes reduced during the normal course of the epimerization reaction will not react with nucleotide 4-keto sugars in the manner already described for the NaBH₄-reduced epimerase (Figs. 2 and 8) and for the “inactive intermediate” (Fig. 5). In a typical experiment, UDP-[4-3H]quinovose (5.3 × 10⁶ cpm), 0.2 μmole of TDP-4-keto-6-deoxyglucose, and 100 i.u. of epimerase were incubated in 0.19 ml of 0.1 m glycine buffer (pH 8.8) for 5 hours at room temperature. Chromatography of the products in Solvent System III revealed all of...
the radioactivity was associated with UDP-6-deoxyhexoses and none with the TDP-6-deoxyhexoses. If oxidation of the enzyme-bound NADH by TDP-4-keto-6-deoxyhexulose had taken place, TDP-hexoses labeled in position 4 would obviously have been produced.

**Action of Beef Liver Epimerase on Nucleotide-4-Keto Sugars**—Since beef liver epimerase requires added NAD+ for maximal activity and is inhibited by NADH (2), it is of interest to see if it can catalyze the oxidation of added NADH at the expense of the reduction of UDP-4-keto-6-deoxyhexulose. In order to check this possibility, 68 i.u. of beef liver epimerase in 0.75 ml of 0.1 mM NADH (0.1 M glycine buffer, pH 8.8) were added to 50 ml of 2.5 mM UDP-4-keto-6-deoxyhexulose and the 340 nm absorbance monitored against that of an NADH-buffer-enzyme blank. Less than 0.02 absorbance change was observed over a period of 10 min, indicating that the enzyme has less than 0.5% of the activity in this oxidation-reduction process than it has in catalyzing the epimerization. TDP-4-keto-6-deoxyhexulose (0.38 mM) produced no detectable oxidation of NADH under the same conditions. These results can be explained on a number of bases. The beef liver enzyme may have a high specificity toward the hydroxymethyl group at carbon 6 of the hexose. The order of binding of substrates may be such that pyridine nucleotides are bound first and that when NADH is bound the enzyme is rendered incapable of binding with nucleotide sugar derivatives. This would also explain the inhibitory effect of NADH on normal enzyme function. Finally, the dissociation of pyridine nucleotides from the enzyme may be too slow to allow an observable change in 340 nm absorbance during the time of the experiment.

**DISCUSSION**

A summary of the reactions we have observed with E. coli UDP-glucose 4-epimerase is shown in Fig. 10. Those reactions designated by numbers are involved in the normal epimerization process; those reactions designated by letters are ones which we have observed and utilized in our work on the mechanism of the epimerization. We regard these as parts of the normal mechanism that have been caused to function in isolation by the experimental techniques that we have developed. For a complete epimerization to occur, the reaction must proceed by Steps 1, 2, and 3 followed by reactions analogous to 2 and 1, but associated with the 4-epimer of the starting substrate.

Reaction 1 is the formation of an enzyme-substrate complex in which the substrate is bonded in a nonequivalent manner. The dissociation of the substrate in the reverse reaction is entirely dependent on the oxidation state of the pyridine nucleotide as seen in part reaction 2, where reduction of the enzyme-bound NAD+ produces an enzyme capable of tightly binding substrate. This binding would, perhaps, be expected to be associated with large changes in the secondary and tertiary structure of the enzyme; indeed other workers have reported large changes in the optical rotatory dispersion and circular dichroism of yeast epimerase in the presence of substrate analogues (34). The binding of UMP by yeast epimerase in a manner that is possibly similar to that reported here has been observed previously (35). When the enzyme is reduced by borohydride in the absence of the substrate (part reaction 3), there is no possibility that the secondary and tertiary structural changes, due to substrate binding, can take place. It is interesting to note that the NADH in this form of the enzyme is subject to spontaneous oxidation (part reaction a) which may well be a consequence of the exposure of the NADH by the unfavorable structural situation. This form of the enzyme is capable of reacting with the substrate to give rise to the form in which the NADH is stable (part reaction c). The observation that TDP-glucose does not fully stabilize the NADH produced by part reaction 6 may be due to its having a poor "fit" on the enzyme, thus it may slowly dissociate and expose the NADH to spontaneous oxidation.

**Reaction 2 of** Fig. 10 is the oxidation of position 4 of the substrate in which there is transfer of the 4-hydrogen to the B-side of NAD+. The details of this reaction are inferred from part reaction 6, where hydrogen from position 4 of the substrate was found to be transferred to the B-side of NAD+ in the formation of the "inactive intermediate." The reverse of Reaction 2 is inferred from part reactions d and e in which it was demonstrated that NADH-containing epimerase transfers hydrogen from the B-side of NADH to nucleotide diphosphate-4-keto sugars and results in the reduction of the 4-keto group to a 4-hydroxyl. The part reaction e makes plain that there are two binding sides on the enzyme, one of which (in the specific case of this reaction) binds the substrate and other binds the added nucleotide-4-keto sugar. This follows from the fact that enzyme containing both firmly bound substrate and NADH reacts very rapidly with added nucleotide-4-keto sugars (Fig. 3). The two sites may be a reflection of the fact that the enzyme consists of two identical subunits (30).

The epimerase appears to be one of a growing list of enzymes which can be described as "suicidal" (36, 37). The observed details of this inactivation are that one substrate molecule causes the reduction of the enzyme-bound NAD+ and, as a closely related event, a second substrate molecule is taken onto the enzyme and is bound sufficiently strongly to resist dialysis. The first molecule of substrate bound, which is the one that reduces the enzyme, is not released as a nucleotide-4-keto sugar, since it would then reoxidize the "inactive intermediate" (part reaction 6, Fig. 10) and the inactive intermediate would not accumulate. It is to be expected, on the basis of model studies, that the postulated intermediate, UDP-4-ketoglucose, would be unstable in solution (38) and if it should slowly dissociate from the enzyme.

We have observed that the oxidation of NADH-containing epimerase by nucleotide-4-keto sugars proceeds in a nearly stoichiometric fashion when the nucleotide-4-keto sugar is limiting (0.7 mole of NADH per mole of nucleotide-4-keto sugar).
or decompose while attached to the enzyme surface, that particular enzyme molecule would be left "stranded" in the reduced and inactive state. This would leave the site available to nucleotide-4-keto sugars added to the medium which would reoxidize the NADH and reactivate the enzyme. It is worth noting that any cyclic process of the type postulated to occur on the epimerase would become inactivated with time unless all of the intermediates involved had half-lives which were long in comparison with the time over which the enzyme's action could be observed.

Reaction 3 in Fig. 10 is a change in the relative positions of the nucleotide sugar and pyridine nucleotide which allows the reduction of the 4-keto sugar to the 4-epimer of the starting compound. Since the hydrogen transfer is truly intramolecular, some alteration in the relative positions of these molecules must occur (33). The existence of Reaction 3 is also supported by the observation that UDP-mannose readily produces NADH but is not epimerized. If Reaction 3 is specific for the configuration at carbon 2 of the hexose moiety, then this observation is completely accounted for.

It has been shown that the formation of NADH on the enzyme is largely independent of temperature. If it is assumed that Reaction 2 (Fig. 10) is a single equilibrium and not a complex reaction, it follows that the enthalpy change for this oxidation-reduction reaction is close to zero. At substrate concentrations approaching saturation, all of the enzyme molecules will be in one of the two forms characteristic of Equilibrium 2. Since under these conditions 10% of the enzyme-bound NAD+ is reduced (4), the equilibrium constant for this reaction is 0.23, which corresponds to a $\Delta G$ of about 1 kcal per mole and a $\Delta S$ of $-3.5$ entropy unit. Although these calculations involve several assumptions, it would appear that the large change in the substrate brought about by Reaction 2 involves a surprisingly small change in entropy.

It would be well, at this point, to make some comparison between the work reported here on the E. coli epimerase and some of the work reported by the group of Kaledkar on the epimerase from yeast. These workers have observed that the incubation of the yeast epimerase with a nucleoside monophosphate and a free sugar results in an inactivation of the enzyme and a reduction of the enzyme-bound NAD+ which is very reminiscent of the production of the "inactive intermediate" described above (see Reference 39 for a recent paper by the Kaledkar group on this subject). Any comparison between these processes involves the concept that the yeast and the coli enzymes proceed by either identical, or at least highly similar, mechanisms. While it would appear not unlikely that such could be the case, it is evident that the present feeling in the Kaledkar group is that there are very considerable differences in the way the enzymes from the two sources operate (see the discussion section of Reference 39 for a point-by-point comparison). In a very recent paper, Davis and Glaser (40) have shown that it is possible to repeat the observations of Kaledkar and coworkers with the coli epimerase, and they propose that this reduction reaction be regarded as a model for a part of the coli epimerase mechanism. It is our feeling that, until the claimed differences in the literature between the yeast and coli enzymes are resolved, it is probably not profitable to make close comparisons between observations on the yeast system and those reported here. It is, of course, valid to make comparisons between our results and those reported by Davis and Glaser on the reduction of the coli enzyme by 5'-UMP and glucose. Such comparisons appear to us to indicate that one should be cautious in assuming that the reaction observed by Davis and Glaser represents a close model of a part of the epimerase action. We have observed a direct transfer of the 4-hydrogen from a normal substrate to the enzyme-bound NAD+; they observe no transfer whatever between the 4-hydrogen of glucose and the enzyme-bound NAD+. Further they measured the kinetic isotope effect involved in the reduction of NAD+ using glucose-3d, glucose-4d, and glucose-5d. They found no isotope effect whatever with the 4- and 5-deuterio compounds but there was a marked isotope effect with 3-deuterio-glucose. This indicates that in the model system it is the 3-hydroxyl of the free glucose that is oxidized to provide hydrogen for the reduction of the enzyme-bound NAD+, while it is evident from the data presented here that the formation of inactive intermediate involves reduction of the NAD+ at the expense of the oxidation of the 4-hydroxyl of the enzyme's substrate.

The evidence presented here establishes that the E. coli epimerase is a very unusual dehydrogenase which is completely cyclic in its action. This unusual property makes it useful in studying the general properties of dehydrogenation processes. Schellenberg (22-25) has proposed that tryptophan side chains may be involved as intermediate hydrogen carriers in dehydrogenase enzymes. In his model the first step is an interaction between NAD+ and a tryptophan residue within the enzyme molecule to produce NADH and an oxidized tryptophan (indolenine) moiety. The second step involves the oxidation of the substrate by the indolenine moiety. Schellenberg has proposed that the indolenine is stabilized by formation of an adduct with a cysteine residue (26).

If this mechanism were operative in the case of UDP-glucose 4-epimerase it is clear that the 4-hydrogen of the substrate would be transferred to an indolenine moiety and back to the substrate with no possibility of transfer to a pyridine nucleotide. The fact that tritium from UDP [4-3H]quinovose was transferred to NAD+ with none being found in the protein when the "inactive intermediate" is formed (Fig. 7, protein remains at the origin in this system) allows the Schellenberg mechanism to be ruled out for this particular "dehydrogenase." Transfer of the B-hydrogen from enzyme-bound NADH to position 4 of the nucleotide-4-keto sugar also rules out this mechanism.

The possibility exists, however, that the enzyme contains indolenine moieties in its "resting" state (absence of substrate). If this were so, added substrate would reduce the indolenine residues which in turn would be reoxidized by NAD+ and the result would be net transfer of substrate 4-hydrogen to pyridine nucleotide. The possible existence of indolenine moieties in the "resting" enzyme was investigated by reduction of the enzyme with NaBH₄. Although NaBH₄ readily reduces indolenes (26), reduction of the enzyme with NaBH₄ did not result in incorporation of detectable quantities of tritium into tryptophan. In addition, titration of the free sulphydryl groups did not inhibit the enzyme to the extent anticipated from the proposed model (23). Thus the presence of indolenine moieties in UDP-glucose 4-epimerase is quite unlikely and tryptophan, therefore, does not appear to participate as a hydrogen carrier in this particular "dehydrogenase" reaction.

Another enzyme for which these same arguments can be made is TDP-glucose oxidoreductase. This enzyme also catalyzes an intramolecular hydrogen transfer (41). It has been reported that tritium from the substrate is transferred to NAD+ (42),
just as we have reported here for the epimerase, and thus the participation of tryptophan as an intermediate hydrogen carrier can be ruled out in this case also. The existence of "indolenine-" moieties in this enzyme, however, has not been investigated.

It is of interest to note that the three enzymes which have been reported to transfer substrate hydrogen to tryptophan all show A-specificity toward NAD+ (22-25), whereas the three enzymes which have been reported not to perform such a transfer show B-specificity (see also Reference 43). It will be interesting to see if this pattern persists as more evidence becomes available.

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The Mechanism of Action of the Enzyme Uridine Diphosphoglucose 4-Epimerase: PROOF OF AN OXIDATION-REDUCTION MECHANISM WITH DIRECT TRANSFER OF HYDROGEN BETWEEN SUBSTRATE AND THE B-POSITION OF THE ENZYME-BOUND PYRIDINE NUCLEOTIDE

Gary L. Nelsestuen and Samuel Kirkwood


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