Enzymatic Synthesis of Cytidine Diphosphate 3,6-Dideoxyhexoses

VIII. STUDIES OF THE PROPERTIES OF E3 AND ITS ROLE IN THE FORMATION OF CYTIDINE DIPHOSPHATE 4-KETO-3,6-DIDEOXYGLUCOSE*

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SUMMARY

Enzyme E3, one of the two enzymes required to promote the formation of CDP-4-keto-3,6-dideoxyglucose from CDP-4-keto-6-deoxyglucose, is thought to catalyze the NADPH requiring reduction in which the 3-deoxy group is actually formed from the intermediate discussed in the preceding paper. One mole of NAD(P)H was oxidized for every mole of sugar reduced when the reaction was carried out in an argon atmosphere. Experiments with both A- and B-[4-3H]NADPH have shown that there is no direct hydride transfer from NADPH to either the sugar being reduced or to pyridoxamine 5'-phosphate, a participant in the reaction. On the other hand, using tritiated water 1 atom of hydrogen is incorporated into the CDP-4-keto-3,6-dideoxyglucose formed.

E3 alone also catalyzes the oxidation of NAD(P)H although there is no chromophoric group such as a flavin associated with the enzyme. However, added FMN or 2,6-dichlorophenolindophenol greatly enhances the rate of NAD(P)H oxidation by E3.

The formation of CDP-4-keto-3,6-dideoxyglucose from CDP-4-keto-6-deoxyglucose in Pasteurella pseudotuberculosis type V is catalyzed by two enzymes, E1 and E3 (1), both of which have been purified to homogeneity (2). NAD(P)H is the reducing agent, and pyridoxamine 5'-phosphate is a required cofactor (3). In the preceding paper, it was shown that the probable role of E1 in the reaction is to catalyze the formation of an enzyme bound Schiff base-Δ-3,4-glucoseen adduct between pyridoxamine 5'-phosphate and CDP 4 keto 6 deoxyglucose. The intermediate was termed E1-pyridoxamine 5'-phosphate-X (see Fig. 10).

Previous work (2) had revealed that E3, by itself, had the ability to oxidize NAD(P)H, and it was therefore suggested that the physiological role of the enzyme is to carry out the actual reductive part of the reaction producing CDP-4-keto-3,6-dideoxyglucose. In the present paper, experiments are presented which attempt to define more completely, the mechanism of action of E3. Studies designed to localize the actual site and mechanism of reduction are described and the properties of E3 as a NAD(P)H oxidase are examined.

MATERIALS AND METHODS

CDP-d-glucose, glucose 6-phosphate, α-phenanthroline, diethyldithiocarbamate acid, NAD, NADH, NADP, NADPH, FMN, FAD, ATP, diethyldithiocarbamate, glutathione, N-ethylmaleimide, cytochrome c, 2,6-dichlorophenolindophenol, triphenyltetrazolium chloride, glucose 6-phosphate dehydrogenase, isocitrate dehydrogenase, and glutathione reductase were purchased from the Sigma Chemical Co. $\text{H}_2\text{O}$, 18 Ci per mole; Na$^{18}$H$_4$, 200 mCi per mmole; α-[H]$\text{H}^3$glucose, 5.15 Ci per mmole; and Aquasol liquid scintillation mixture were obtained from New England Nuclear. CDP-$\text{n}-[\text{U}-\text{14C}]$glucose, 50 mCi per mmole, was obtained from International Chemical and Nuclear. CDP-4-keto-6-deoxy-α-glucose, both labeled with 14C and unlabeled, CDP-glucose oxidoreductase, and enzymes E1 (through Step 6) and E3 were prepared as previously described (2). $\alpha$-[2-H]Iscocitric acid, 20 mCi per mmole, was prepared according to the method of Lowenstein (4).

Protein Determinations—Protein content was determined by the method of Lowry et al. (5).

Radioactivity Measurements—Radioactivity was determined by using standard liquid scintillation techniques using 10 ml of Aquasol as the counting medium. From 100 to 500 μl of an aqueous solution containing the radioactivity was counted.

Formation of CDP-4-keto-3,6-dideoxy-d-glucose—Assay for E1 and E3—As described previously (2), CDP-4-keto-6-deoxy-d-[U-14C]glucose, 50 mCi per mmole, pyridoxamine phosphate, NAD(P)H, E1, and E3 were incubated together for the desired time at 30°C. The mixture was then spotted on a 400-μm thin layer of MN-300 cellulose and chromatographed in isobutyric acid-1 m NH$_4$OH (5:3). The radioactive spot corresponding to the product was located, scraped off of the plate, and counted.

Assay for NAD(P)H Oxidase Activity—In a total volume of 450 μl of 0.65 m potassium phosphate buffer, pH 7.5, were combined NAD(P)H, 90 nmole, and the desired amount of E3. The decrease in A$_{340}$ was monitored as a function of time. For this reaction, E$_{480}$ = 6200 m$^{-1}$cm$^{-1}$. In certain cases, the following electron acceptors were added to the reaction mixture: FMN, 0.01 mm; cytochrome c, 0.1 mm; or DCPIP, 0.1 mm. When a flavin was

* The abbreviations used are: DCPIP, 2,6-dichlorophenolindophenol; NEM, N-ethylmaleimide.
used, the reaction was monitored at 340 nm. When cytochrome c was used, the reduction of the dye was followed directly ($B_{100} = 27,400 \text{ cm}^{-1} \cdot \text{m}^{-1}$). This was also the case for DCPIP ($B_{500} = 20,000 \text{ cm}^{-1} \cdot \text{m}^{-1}$).

$D-[1-3H]Glucose 6-phosphate$ - This compound was prepared by the action of hexokinase on $D-[1-3H]glucose$, 5 Ci per mmole, in the presence of ATP and MgCl$_2$. The product was isolated by chromatography on Bio-Rad AG 1X4-C anion exchange resin in a voltage gradient of HCl (0 to 0.6 M). The HCl was removed by lyophilization, and the residue was dissolved in water and neutralized to pH 7.0 with NaOH.

A $[4-3H]NADPH$ - In 15 ml of 0.06 M Tris-HCl buffer, pH 8.0, were combined NADP, 4 moles; $1-[2-3H]$isocitric acid (20 mCi per mmole), 5.5 moles of the correct isomer; MgCl$_2$, 90 moles; and 40 units of purified isocitrate dehydrogenase. The solution was incubated for 30 min at 35$^\circ$ and then boiled for 1 min. To the mixture was then added 1 ml of water, and the resulting solution was applied to the top of a 2.5-ml column of AG 1X4-C anion exchange resin. The column was washed with 20 ml of water and then eluted with 20 ml of 0.75 M NaCl in 0.01 M Tris-HCl buffer, pH 8.0. The resulting solution was concentrated in a vacuum to about 1 ml. The specific activity of the material was 20 mCi per mmole.

B $[4-3H]NADPH$ - This compound was synthesized by the same method used for the A isomer except that $D-[1-3H]glucose$ 6-phosphate, 0.9 mCi per mmole, was substituted for the isocitrate, and glucose 6-phosphate dehydrogenase was substituted for isocitrate dehydrogenase. The NADPH isolated had a specific activity of 25 mCi per mmole.

**Attempts to Demonstrate Direct Hydride Transfer from NADH to C$_5$ of Sugar** — One method of reduction of the E1-pyridoxamine 5'-phosphate-substrate complex alluded to in the preceding paper and shown in Fig. 10 is the direct transfer of a hydride ion from NADH to C$_5$ of the sugar. However, repeated attempts to demonstrate this, using specifically labeled A and B $[4-3H]$NADPH, were completely unsuccessful; no H$^-$ from either compound was incorporated into the product, the CDP-4-keto-3,6-dideoxyglucose (Figs. 2 and 3). As can be seen from these figures, there was a significant increase in the amount of $[4-3H]$NADPH after the reaction when $[4-3H]$NADPH labeled on either the A or B side is used as a substrate, a result which implies that E3 can utilize either side of the dihydroxypropylene ring of NADPH. As a control, the same experiment was performed with glutathione reductase, an enzyme known to be specific for the B side of the dihydroxypropylene ring. In this case, only $[4-3H]$glucose 6-phosphate was incorporated into the product, GDP-4-keto-3,6-dideoxyglucose.
exchange was occurring with the C₄ proton of the sugar or with
insulin-like control would seem to rule out the possibility that nonspecific tritium
incorporation into CDP-4-keto-3,6-dideoxyglucose. Since the specific activity of the solvent hydrogens was
measured at 0.5 hours at 25°C. At the end of this period, 15 ml of the reaction mixture was withdrawn and analyzed for CDP-4-keto-3,6-dideoxyglucose formation as described in the text. The remainder was combined with 4.5 ml of water containing 0.75 mg of NADP. The solution was put on a 2-ml column of AG 1X4-Cl⁻ anion exchange resin and washed with 31 ml of water and 20 ml of 0.09 M NaCl with 2 mM Tris-HCl, pH 7.5. A linear gradient of 100 ml between 0.1 M NaCl in 2 mM Tris-HCl, pH 7.5, and 0.4 M NaCl with 2 mM Tris-HCl, pH 7.5, was applied to the top of the column, and 1.5 ml fractions were collected. Aliquots of 200 μl of each fraction were analyzed for ³H and ¹⁴C content by liquid scintillation analysis. The position of CDP-4-keto-3,6-dideoxyglucose is shown by the ¹⁴C line (▲ ▲). The tritium profile produced after the reaction studied is shown by (O — O). The tritium profile obtained with an identical experiment except that the enzymes were omitted is shown by (● — ●). The position of NADP is indicated by the arrow. A small amount of [³H]NADP is present before the enzyme reaction occurs, and results from decomposition of the [³H]NADP. [³H]NADP is eluted from the column at about 0.5 M NaCl. In the presence of both enzymes, the reaction proceeded to greater than 95% completion.

NADP⁺ was produced in the reaction when A-[⁴-³H]NADPH was used as would be expected. This lack of stereospecificity toward NADPH exhibited by E3 is shared by only an extremely small number of enzymes, one of which is lipoic dehydrogenase ("diaphorase"), a flavoprotein (6).

Incorporation of Tritium from [³H₂O into CDP-4-keto-3,6-dideoxyglucose during Its Formation—In the presence of tritated water, 18 Cl per mmole, there was significant incorporation of tritium from the solvent into CDP-4-keto-3,6-dideoxyglucose (Fig. 4). A total of 41,000 cpm of tritiated product was formed in 205,000 dpm, 0.093 μCi of ³H (counting efficiency was 20%). In the reaction, 43 nmoles of product were formed; the specific activity of the product was thus 2.2 mCi of ³H per mmole of sugar. Since the specific activity of the solvent hydrogens was 9 Cl per mole atom, 0.24 mole of tritium was incorporated per mole of sugar. Assuming a solvent isotope effect of 3 to 4, this value indicates that 1 mole of solvent hydrogen was incorporated per mole of sugar reduced. No such incorporation was evident when E3 was eliminated from the reaction mixture. This control would seem to rule out the possibility that nonspecific tritium exchange was occurring with the C₄ proton of the sugar or with

Fig. 2. Attempt to incorporate tritium from A-[⁴-³H]NADPH into CDP-4-keto-3,6-dideoxyglucose during its synthesis. In a volume of 130 ml of 0.05 M potassium phosphate buffer, pH 7.5, were combined A-[⁴-³H]NADPH (20 mCi per mmole), 25 nmoles; CDP-4-keto-6-deoxy-[U-¹⁴C]glucose (18.5 mCi per mmole), 13.2 nmoles; pyridoxamine phosphate, 1.3 nmoles; E1, 20 μg; and E3, 7 μg. The mixture was incubated for 1.5 hours at 25°C. At the end of this period, 15 ml of the reaction mixture was withdrawn and analyzed for CDP-4-keto-3,6-dideoxyglucose formation as described in the text. The remainder was combined with 4.5 ml of water containing 0.75 mg of NADP. The solution was put on a 2-ml column of AG 1X4-Cl⁻ anion exchange resin and washed with 31 ml of water and 20 ml of 0.08 M NaCl in 0.01 M potassium phosphate buffer. Fractions of 0.75 ml were collected and 200 μl of each fraction were analyzed for ³H and ¹¹C. An identical experiment was performed with the omission of E3 as a control. Results are expressed as counts per min of tritium; reaction (O — O); control (● — ●). The position of the peak fraction of CDP-4-keto-3,6-dideoxyglucose is indicated by the arrow.

Fig. 3. Attempt to incorporate tritium from B-[⁴-³H]NADPH into CDP-4-keto-3,6-dideoxyglucose during the synthesis. The procedure used was identical to that described in Fig. 2 except that B-[⁴-³H]NADPH, 25 mCi per mmole, was used. The position of CDP-4-keto-3,6-dideoxyglucose is shown by the ¹⁴C line (▲ ▲). The tritium profile produced in the complete reaction mixture is represented by (O — O). Tritium from the control with E3 omitted is shown by (● — ●). Tritium marks the position of NADP. The small amount of [³H]NADP present in the mixture before the reaction is the result of decomposition of the [³H]NADPH. In the presence of both enzymes, the reaction proceeded to greater than 95% completion as judged by thin layer chromatography analysis of the CDP-4-keto-3,6-dideoxy-¹⁴C-glucose formed.

Fig. 4. Incorporation of tritium from [³H₂O into CDP-4-keto-3,6-dideoxyglucose during its synthesis. Into the bottom of a tube were placed 48 nmoles of CDP-4-keto-6-deoxy-[U-¹⁴C]glucose, 45,000 cpm; pyridoxamine 5'-phosphate, 1 n mole; glucose 6-phosphate, 0.4 μmole; MgCl₂, 1.25 μmoles, and enzyme E1, 15 μg; in 100 μl of 0.03 M potassium phosphate buffer, pH 7.5. The contents of the tube were quickly frozen and lyophilized to dryness. Into the tube were then placed in 100 μl of [³H₂O, 18 Cl per mole; NADPH, 25 nmoles; glucose 6-phosphate dehydrogenase, 4 units; and E3, 9 μg. The tube was evacuated for 30 s with a water aspirator and incubated for 2 hours at 30°C. The [³H₂O was removed by vacuum distillation, 100 μl of water were added and again removed by vacuum distillation. This procedure was repeated once more. Finally, 100 μl of water were again added, 10 μl of the solution were withdrawn for analysis of CDP-4-keto-3,6-dideoxyglucose formation, and the remainder was put on a 1-ml column of AG IX4-Cl⁻ anion exchange resin. The column was washed with 30 ml of 0.03 M of water and 20 ml of 0.08 M NaCl in 0.01 M potassium phosphate buffer, pH 7.5. To the column was then applied a 50-ml linear gradient between 0.1 M NaCl and 0.2 M NaCl in the same buffer. Fractions of 0.75 ml were collected and 200 μl of each fraction were analyzed for ³H and ¹⁴C. An identical experiment was performed with the omission of E3 as a control. Results are expressed as counts per min of tritium; reaction (O — O); control (● — ●). The position of the peak fraction of CDP-4-keto-3,6-dideoxyglucose is indicated by the arrow.
the original C₃ proton. It thus seems that the hydrogen introduced at C₃ by the reductive part of the reaction is derived from the solvent. It was previously reported (7) that tritium was not incorporated from solvent into the sugar. This discrepancy might result from the difference in experimental procedures used in purifying the CDP-4-keto-3,6-dideoxyglucose for tritium analysis.

Attempt to Demonstrate Direct Hydride Transfer from NADPH to C₃-Methylene Carbon Atom of 5'-Pyridoxamine 5'-Phosphate—An alternative reduction mechanism is the reduction by NADPH at the methylene carbon at C₃ of pyridoxamine 5'-phosphate while it is part of the pyridoxamine 5'-phosphate-Schiff base-Δ-3,4-glucoseen intermediate (see Fig. 10, Part II). In an experiment designed to test this possibility (see "Materials and Methods"), E₁ and CDP-4-keto-6-deoxyglucose were present in stoichiometric amounts so that each reaction center would undergo no more than one turnover; pyridoxamine 5'-phosphate was present in a 100-fold excess. When the pyridoxamine 5'-phosphate was isolated following the reaction, the total radioactivity isolated with pyridoxamine 5'-phosphate was 3,527 cpm (this value is found after subtracting a blank of 6,084 cpm obtained in the absence of E₁ from a value of 9,954 cpm obtained with the complete reaction mixture). The value of 3,527 cpm is only 2.5% of the amount expected, 142,000 cpm (counting efficiency = 28%), if direct hydride transfer had occurred at pyridoxamine 5'-phosphate during the reaction. The expected value takes into consideration a hypothetical isotope effect of 100 representing a factor of 10 from the transfer of tritium from [l-3H]glucose 6-phosphate to NADP and another factor of 10 for the transfer of tritium from NADPH to pyridoxamine 5'-phosphate. Since the ratio of NADP to CDP-4-keto-6-deoxyglucose was 1:5, tritium would be expected to be transferred from glucose 6-phosphate to both sides of the dihydro-pryidine ring of NADPH after the first 20% of the over-all reaction had occurred.

Attempt to Trap Covalent E₃-Pyridoxamine 5'-Phosphate-Substrate Complex—If the active sulfhydryl group of E₃ (2) was added covalently to the C₂ position of the sugar by a Michael-addition while it was involved in the Schiff base-Δ-3,4-glucoseen intermediate (Fig. 10, Part I; B is the sulfhydryl), addition of NaBH₄ and omission of NADPH from the reaction mixture might result in the reduction of the Schiff base by the borohydride, thus causing an irreversible bond formation between the sulfhydryl group and C₅. When such an experiment was attempted (see "Materials and Methods"), using 14C-labeled substrate, the radioactivity found in the total reaction mixture was no greater than that found when either E₃ or NaBH₄ was omitted from the reaction. Thus, a covalent attachment between E₃ and CDP-4-keto-6-deoxyglucose could not be demonstrated.

Properties of Enzyme E₃

Coincidence of Sugar Reductase and NADH Oxidase Activities—E₃ (Step 6) was subjected to DEAE-cellulose chromatography at pH 5.8 as previously described (2). Aliquots of each fraction were tested for NADH oxidase and sugar reductase activities, and the results are presented in Fig. 5. The two activities were found to be coincidental on the column.

Effect of NEM on NADH Oxidase Activity of E₃—Preincubation of E₃ with 0.02 mM NEM resulted in the loss of 85% of the NADH oxidase activity of the enzyme (Fig. 6). Previous work (7) has shown that under the same conditions used here, the sugar reductase activity of the enzyme is inhibited to the same extent.
stimulation of the formation of CDP-4-keto-3,6-dideoxyglucose by the addition of FAD to the reaction mixture; they further reported that there was no FAD-induced stimulation of the conversion of CDP-4-keto-3,6-dideoxyglucose to CDP-3,6-dideoxyhexose. In the present work, using highly purified enzymes, both FAD and FMN markedly inhibited the formation of CDP-4-keto-3,6-dideoxyglucose from CDP-4-keto-6-deoxyglucose when added in concentrations of 10-4 M and higher (Table I). Perhaps in the crude system, the flavin acts by inhibiting some enzyme activity that would normally degrade the nucleotide sugar substrate. In any case, using purified enzymes, there is no flavin-catalyzed stimulation of the E1, E3 system.

In an effort to explain the inhibition of sugar reduction by flavin coenzymes, it was hypothesized that they may be acting by binding to E3 and uncoupling NADH oxidation from sugar reduction. To test this possibility, the effect of various electron acceptors on the E3-catalyzed rate of NADH oxidation was examined. Both FMN and DCPIP could be utilized by the enzyme as electron acceptors (Table II); on the other hand, cytochrome c and triphenyltetrazolium chloride were ineffective in this role. When the mode of action of FMN catalysis was examined in greater detail (Fig. 7), it was demonstrated that the interaction of FMN with E3 obeyed saturation kinetics. The K for FMN was found to be 2.7 x 10^-6 M.

Gonzalez-Porque and Strominger (2) had reported previously that addition of a cofactor solution isolated from a cell extract containing pyridoxamine 5'-phosphate resulted in a 2-fold stimulation of the rate of NADH oxidation by E3. In the present study, however, when a pure commercial preparation of the coenzyme was used, no stimulation of NADH oxidation was observed. Based on the results found here, the stimulation by cofactor solution previously reported was probably due to the presence of a small amount of some electron acceptor in the solution.

Since FMN was shown to bind to the enzyme, a spectrum was taken of a mixture of FMN and E3 in an argon atmosphere. No decrease in the flavin peak at 450 nm was noted, however. Since E3 has a single active sulfhydryl group (2), it was thought that this residue might form a covalent bond with the flavin according to the mechanism suggested by Brown and Hamilton (9). The formation of a covalent adduct at the C1a or N5 position of the flavin ring has often resulted in the disappearance of the 450-nm peak (10).

Inhibition of Sugar Reduction by o-Phenanthroline—As seen in Table III, inclusion of 2 mM o-phenanthroline in the reaction mixture caused an 85% inhibition of sugar reduction. 8-Hydroxyquinoline-5-sulfonic acid at the same concentration inhibited the reaction as well but to a much smaller extent. Neither α,α-bipyridyl, diethyldithiocarbamic acid, nor EDTA produced any detectable inhibition of the synthesis of CDP-4-keto-3,6-dideoxyglucose. When three small compounds known for their ability to complex with the IIB metals (11), cyanide, azide, and sulfide all at 10 mM concentrations, were tried as inhibitors, no inhibition was detected. A concentration curve for o-phenanthroline inhibition of sugar reduction is shown in Fig. 8. For

### Table I

| Flavin | Concentration (mM) | Product formed (cpm) |
|--------|--------------------|----------------------|
| FAD    | 0.01               | 9137                 |
|        | 0.10               | 4253                 |
|        | 1.00               | 631                  |
| FMN    | 0.01               | 8331                 |
|        | 0.10               | 3943                 |
|        | 1.00               | 341                  |
| None   |                    | 9438                 |

### Table II

| Acceptor | Concentration (mM) | Relative rate |
|----------|--------------------|---------------|
| Oxygen   | ?                  | 1             |
| FMN      | 0.01               | 31.8          |
| 2,6-Dichlorophenolindophenol | 0.10 | 128.0 |
| Triphenyltetrazolium chloride | 0.10 | 0 |
| Cytochrome c | 0.10 | 0 |

*a This value is arrived at by taking the difference between the rates of NADH oxidation in the presence and absence of FMN.

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**Fig. 7.** Effect of FMN on the NAD(P)H oxidase activity of E3. A, Lineweaver-Burk plot of the relationship of NADH oxidation and FMN concentration. B, velocity data from which the plot in A was derived.
In the experiment presented, 50% inhibition occurred at about $7 \times 10^{-4} \text{M}$. Attempts were made to locate the site of inhibition by o-phenanthroline. As shown in Fig. 9, this compound had little if any effect on the rate of NADH oxidation by E3. Furthermore, attempts to inactivate either E1 or E3 by dialysis against o-phenanthroline were unsuccessful. Finally, of five different E1 plus E3 preparations used in this study, one was found which, when used in the production of CDP-4-keto-3,6-dideoxyglucose, was not subject to inhibition by o-phenanthroline. Although o-phenanthroline inhibition is usually thought to indicate the presence of a metal in the system, the failure of other metal chelators to inhibit the E1, E3 system, in addition to the failure of dialysis against o-phenanthroline to inhibit suggests that o-phenanthroline may be working in the sugar reduction system by some chaotropic or competitive binding mechanism rather than by metal complexation.

**TABLE III**

| Chelator                        | Activity |
|--------------------------------|----------|
| None                           | 100      |
| $\alpha, \alpha$ -Dipyridyl     | 100      |
| 8-Hydroxyquinoline-5sulfonic acid | 64       |
| o-Phenanthroline                | 21       |
| EDTA                            | 100      |
| Diethylthiocarbamic acid        | 100      |

**DISCUSSION**

The mechanism of reduction of the sugar remains only partially solved. As shown in this paper, only 1 mole of NADH is required to reduce a mole of CDP-4-keto-6-dideoxyglucose. Using tritium transfer techniques, direct hydride transfer from [3H]-NADPH to the sugar could not be demonstrated. If such a transfer did occur, there are two reasons why it might remain undetected. First, the presence of an unusually large kinetic isotope effect might cause the total incorporation to be so small that it would remain unnoticed under the conditions of the experiment. Second, as seen in Fig. 10, after the hypothetical addition of a hydride ion to C2 of the sugar had occurred, a structure involving a Schiff base adjacent to the protons in question would remain; this Schiff base, because of its property as an electron sink, might then facilitate exchange of the C2 protons with the solvent before hydrolysis of the Schiff base occurred. This would imply that if the reaction were carried out in tritiated water, tritium should be incorporated from the solvent into the sugar. In the preceding paper, it was demonstrated that the C2 proton originally on the substrate remained attached to the product after the reduction had occurred. This finding does not entirely eliminate the exchange mechanism just proposed since the proton introduced into the sugar by the reduction might be in a more favorable steric configuration to undergo exchange than the proton originally on the substrate.

The result shown in Fig. 4 clearly indicates that tritium is incorporated from the solvent into the sugar during reduction. If
the observed lack of a direct hydride transfer to the sugar is accepted as fact, the solvent incorporation experiment confirms this result. An alternative mechanism, originally suggested by Pape and Strominger (7), is that the sulphydryl group of E3 adds to C5 of the sugar in some intermediate form. Reduction could then be imagined as occurring at the sulfur atom causing an electron pair to transfer to the sugar and pick up a proton from the solvent. As related earlier, however, such an E3-substrate complex could not be detected.

As seen in Fig. 10, another alternative reduction mechanism that would result in the incorporation of tritium from the solvent into the sugar is the attack of a hydride ion from NADPH on the methylene carbon atom of pyridoxamine 5'-phosphate while it was taking part in the Schiff base intermediate. Again, experiments designed to test this possibility were completely negative. Isotope effects should not have been a problem here considering the extremely high specific activity of the starting material used. Furthermore, the problem of "postreduction" exchange should not exist since there was only one turnover of each active site of E1 and a 100-fold excess of pyridoxamine 5'-phosphate to substrate. A mechanism of reduction involving hydride attack at the Schiff base bond has recently been shown by Shinkai and Bruice (12) to be quite feasible. Using an aqueous system, they were able to show the nonenzymic catalyzed reduction of pyridoxal phosphate by an analogue of 1,4-dihydriopyridine in an aqueous system. On chemical grounds, this reduction might be expected to be even faster if the aldehyde were replaced by the corresponding Schiff base.

The possibility exists that a previously undetected group on one of the enzymes is the direct target of reduction by NADPH, and that the hydrogen on the enzyme, derived from NADPH, was exchanged with solvent protons before it was transferred to the sugar. Such a mechanism has been seen with proteins using flavins or disulfides as intermediate acceptors. However, solutions of both E1 and E3 are colorless and have no detectable spectra at wavelengths higher than 350 nm. Furthermore, there is only 1 mole of cysteine per mole of E3.

Other types of reduction mechanisms involving the participation of an enzyme-bound metal or organic free radical would also result in the incorporation of solvent tritium into the sugar upon reduction. As mentioned before, the reduction of CDP-4-keto-6-deoxyglucose was found to be inhibited under certain conditions by o-phenanthroline. Because of the failure of other chelators to produce this inhibition as well as the failure of dialysis of the enzymes against o-phenanthroline to produce inhibition, it would seem that neither E1 nor E3 is a metalloenzyme. However, this question can be solved, ultimately, only by isolating large quantities of each enzyme and doing a chemical analysis for the presence of a metal. Although there is no evidence for the involvement of a protein-bound organic free radical in the E1-E3 system, such a species has recently been identified in the Escherichia coli ribonucleotide reductase system (13, 14). The chemical nature of this species, however, has yet to be ascertained, although the presence of enzyme-bound iron is required for its stabilization.

The mechanism by which E3 acts as a NAD(P)H oxidase also remains unsolved. The enzyme clearly exhibits the ability to oxidize NADH in the presence of oxygen without the participation of an added intermediate electron acceptor. Since a chromophoric residue is not associated with the enzyme during its purification (no absorption greater than 320 nm (2)), it would seem that E3 is possibly the only non-flavin containing diaphorase so far described (15). That the NADH oxidase activity seen in E3 preparations is due to the presence of E3, itself, is indicated by three findings. First, as shown in previous work (2), this activity was seen with enzyme judged to be homogeneous by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Second, both the sugar reductase and NAD(P)H oxidase activities appear to be coincidental during DEAE-cellulose chromatography at pH 5.8, the final step in the purification of the protein. Finally, NEM was found to inhibit both the NAD(P)H oxidase and sugar reductase activities.

E3 has been shown to be able to transfer electrons to two externally added electron acceptors, FMN and DCPIP. Both of these have in common a Schiff base type of structure to which electrons from NADH could be added directly. This Schiff base structure is also found in the enzyme-bound pyridoxamine 5'-phosphate-sugar intermediate involved in sugar reduction. It would seem that the mechanisms involved in NADH oxidation and sugar reduction are intimately connected. The solution of one of these mechanisms would thus be of great help in unraveling the mystery of the other.

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