extensive hydrogen mixing of the 1-butene formed in the coisomerization of *cis*-2-butene-d_0 and *cis*-2-butene-d_8 on the iron film, Touroude and Gault[8] conjectured slow rotation of the half-hydrogenated intermediates along the σ-carbon–metal bond in comparison with the carbon–carbon bond.

For establishing the restricted rotation, however, some direct evidences to exclude the dissociative mechanism should be required. On the MoS_2 catalyst, the hydrogen exchange reaction between (*Z*)-propene-*1*-d_1 and propene-d_6 was performed in the presence of hydrogen. If the dissociative mechanism would participate in the exchange reaction, propene-*1,1*-d_2 and (*Z*)-propene-*1,2*-d_2 should be formed; in contrast with this, the associative mechanism will give the (*E*)-propene-*1,2*-d_2 and the propene-*1,1*-d_2.[6] The result clearly confirmed the pure associative mechanism via the half-hydrogenated species being composed of 70% *n*-propyl and 30% isopropyl species.[9]

Such unusual properties of the σ-alkyl intermediates formed on the MoS_2 catalyst may originate from the 2*H* layer structure (hexagonal) of the MoS_2. The active sites having two degrees of coordinative unsaturation may be on the side of the sandwich-like crystal of MoS_2, and the σ-alkyl intermediates formed on these active sites are strongly inhibited from rotating with respect to the coordination bond.

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UDPgalactose 4-Epimerase Catalyzed Oxygen Dependent Reduction of a Free Radical Substrate Analogue by Two Electron Reducing Agents[1]

Sir:

In the course of our studies on the mechanism of action of *Escherichia coli* UDPgalactose 4-epimerase (E.C. 5.1.3.2), which catalyzes the interconversion of UDPgalactose and UDPglucose, we have discovered a new reaction catalyzed by this enzyme in which NAD^+ mediates the transfer of two electrons from a reducing agent such as $NaBH_4$ or D-glucose to two one-electron acceptors, O_2 and uridine-5′-(2,2,6,6-tetramethyl-4-piperidin-1-oxyl diphosphate) I. I is a stable nitroxide free radical described by Wong and Berliner[2] as a paramagnetic structural analogue of UDP-sugars.

The NAD^+ tightly bound to this enzyme is reversibly reduced to NADH by substrates in the epimerization process,[3] and it can be reduced by $NaBH_4$, $NaBH_3CN$, or any of a variety of sugars including D-glucose in reactions which require or are markedly accelerated by the presence of uridine nucleotides.[4] The resultant epimerase·NADH complexes are inactive and contain tightly bound uridine nucleotide, as indicated in eq 1 for D-glucose and UMP as the reducing system. These reactions involve direct hydrogen transfer.

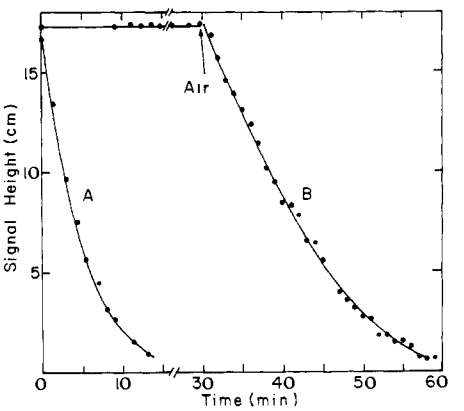
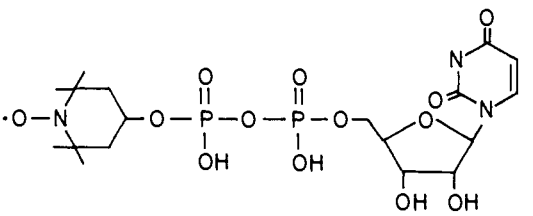


Figure 1. Time course for disappearance of ESR signal of I. In the experiment depicted by curve A the reaction mixture consisted of 3.9 μM I, 0.5 mM $NaBH_4$, and 117 units of UDPgalactose 4-epimerase[3] in 0.2 ml of 0.1 M sodium bicinate buffer at pH 8.5 and ambient temperature. The reaction was initiated at zero time by addition of enzyme. In the experiment depicted by curve B the complete reaction mixture containing 3.5 μM I, 65.6 μM UMP, 90 mM D-glucose, and 585 units of enzyme in 0.2 ml of 0.1 M sodium bicinate at pH 8.5 was prepared in an anaerobic box under N_2 and sealed inside a capillary tube. The capillary was then placed in the cavity of the ESR spectrometer at ambient temperature and the ESR signal monitored. After 30 min the capillary was opened to the atmosphere. Plotted are the ESR signal amplitudes of I measured in a Varian E-4 ESR spectrometer.



I

$$E \cdot NAD^+ + UMP + \text{D-glucose} \rightarrow E \cdot NADH \cdot UMP + \text{D-gluconolactone} + H^+ \quad (1)$$

Free radical I oxidizes epimerase·NADH complexes in an O_2-dependent reaction. When coupled to reduction of epimerase·NAD^+ by $NaBH_4$ or by D-glucose in the presence of UMP the enzyme acts catalytically to destroy the ESR signal of I. Curve A in Figure 1 shows the loss of ESR signal associated with 3.9 μM I in the presence of 0.77 μM enzyme and excess $NaBH_4$. This establishes the catalytic action of epimerase·NAD^+ in the destruction of the free radical. Neither $NaBH_4$ nor NADH alone act on I at rates detected under Figure 1 conditions. Oxygen dependence in the destruction of I is established by curve B in Figure 1, in which the reducing system is D-glucose plus UMP. The loss of ESR signal requires the simultaneous presence of UDPgalactose 4-epimerase, O_2, and either $NaBH_4$ or D-glucose plus UMP. Moreover, it appears to involve binding of I at the active site because the ESR signal of 2,2,6,6-tetramethyl piperidin-1-oxyl-4-ol is stable under the conditions of Figure 1. I is a good competitive reversible inhibitor of the catalytic activity of epimerase·NAD^+, K_I = 0.2 mM.

In Figure 1 the epimerase·NAD^+ would have been reduced to epimerase·NADH by the reducing systems present, suggesting that the disappearance of the ESR signal resulted

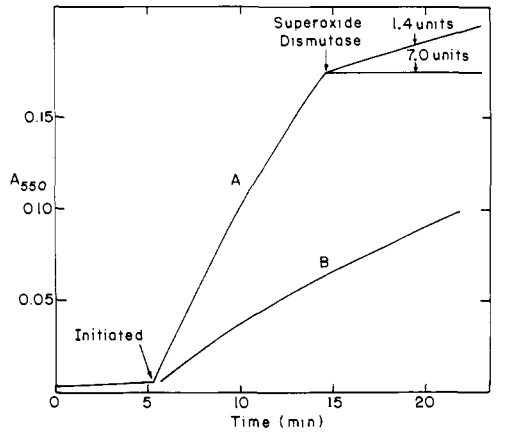


Figure 2. Formation of superoxide during reduction of I by D-glucose. The reaction mixture for curve A consisted of 0.032 mM cytochrome *c*, 0.57 mM I, 0.29 M D-glucose, 1.3 mM UMP, and 1500 units of UDPgalactose 4-epimerase in 0.68 ml of 0.1 M sodium bicinate buffer at pH 8.5. After 14.5 min 1.4 or 7 units of superoxide dismutase (Sigma) were added. The reaction mixture for curve B was identical except for the additional presence of 1.4 units of superoxide dismutase. The reaction could be initiated by the addition of UMP or D-glucose or enzyme or I.

from the oxidation of epimerase·NADH by I. That I actually does oxidize epimerase·NADH was established directly as follows: epimerase·NADH·UMP complexes were prepared by reducing epimerase·NAD^+ with either D-glucose or $NaBH_4$ in the presence of UMP and then separating them from the reducing systems by molecular sieve chromatography. The fluorescence and A_{340} of these complexes were shown to be discharged by I under aerobic conditions. In addition, 1:1 stoichiometry was established by measuring the loss of A_{340} due to epimerase·NADH upon addition of known amounts of I. In two experiments the addition of 4.68 nmol of I to 19.9 nmol of reduced enzyme resulted in decreases in A_{340} equivalent to 5.06 and 4.94 nmol of complex, respectively, which corresponded to molar ratios of 1.08 and 1.06.

One to one stoichiometry together with the oxygen requirement suggested the involvement of oxygen as an electron acceptor and the possibility of the production of its one-electron reduced form, superoxide anion radical. We confirmed this in Figure 2.

Figure 2 shows that a substance capable of reducing cytochrome *c* is produced in solutions containing UDPgalactose 4-epimerase, UMP, D-glucose, and I and that its action is inhibited by 1.4 units of superoxide dismutase and abolished by 7 units. These properties correspond to those of superoxide.[5] We therefore formulate the oxidation of epimerase·NADH complexes by O_2 and I according to eq 1 to 5, in which I is designated I·, to indicate its free radical nature, and its reduced form is I-H.

$$\text{E NADH UMP} + \text{I}\cdot \rightleftharpoons \text{E NADH I}\cdot + \text{UMP} \quad (2)$$

$$\text{E NAD}^+ + \text{I}\cdot \rightleftharpoons \text{E NAD}^+\ \text{I}\cdot \quad (3)$$

$$\text{E NAD}^+\ \text{I}\cdot \xrightarrow{\text{NaBH}_4} \text{E NADH I}\cdot \quad (4)$$

$$\text{E NADH I}\cdot + \text{O}_2 \rightarrow \text{E NAD}^+ + \text{I-H} + \text{O}_2\cdot^- \quad (5)$$

Equations 1, 2, and 5 account for the reduction of I by D-glucose, and eq 3, 4, and 5 account for the reduction by $NaBH_4$. The nucleotide exchange in eq 2 is analogous to that described in earlier work.[4b]

Enzymatic reactions of NAD^+ involve two electron transfer processes usually thought of as concerted transfers of two electrons and a proton, so called "hydride transfers". The possibility that these processes might occur in two one-electron steps, i.e., H· and $1e^-$ transfer in discrete steps, has never been ruled out for enzymatic reactions[6] and has frequently been favorably discussed in the context of model chemical reactions.[7] In work especially pertinent to this study Mauzerall and Westheimer[8a] as well as Schellenberg and Hellerman[8b] have shown that organic free radicals can oxidize dihydropyridines including NADH.

The mechanisms that can be considered for eq 5 include several involving electron transfer from NADH to I and O_2 in one electron steps. For example I may be converted to I-H by abstraction of a hydrogen atom from NADH to form NAD·, which can be expected to react quickly with O_2 to produce superoxide and NAD^+. The reaction of NAD· with O_2 is proposed to be a diffusion controlled chain propagation step in the lactate dehydrogenase catalyzed, superoxide initiated oxidation of NADH by O_2.[9] It is also possible that I and O_2 may react to form a complex which undergoes a two-electron reduction by NADH to form the products of eq 5. Experiments designed to distinguish between these possible mechanisms are in progress.

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