Lactate Dehydrogenase-catalyzed Stereospecific Hydrogen Atom Transfer from Reduced Nicotinamide Adenine Dinucleotide to Dicarboxylate Radicals*

PHILLIP C. CHAN AND BENON H. J. BIELSKI

From the Department of Biochemistry, State University of New York Downstate Medical Center, Brooklyn, New York 11203, and Chemistry Department, Brookhaven National Laboratory, Upton, New York 11973

The dicarboxylate radical \( \text{OOC-CH-CH(OH)COO}^- \) was generated in an \( \text{N}_2\text{O} \)-saturated fumarate solution by high energy ionizing radiation. When NADH was present in the solution, product analysis indicated a stoichiometry of 2 molecules of the radical reacted with 1 NADH molecule to form 2 malate and 1 enzymatically active NAD\(^+\) molecules. In a similar experiment using tritium label on position A of NADH, due to an isotope effect, only 10% of the label was transferred to malate: most of the remaining tritium was found in the NAD\(^+\) formed. When lactate dehydrogenase was added, however, no label was detectable in NAD\(^+\), and over 80% of the tritium lost from NADH was found in malate. The stereospecific transfer of the hydrogen atom from lactate dehydrogenase-bound NADH to the dicarboxylate radical suggested that the free radical reaction must have taken place at the active site. The hydrogen atom transfer was inhibited by oxamate. Results from flow experiments in which an irradiated fumarate solution was mixed with a solution of lactate dehydrogenase and NADH are in support of a mechanism in which the hydrogen atom transfer occurs in the first oxidation step.

The reversible oxidation and reduction of the coenzyme nicotinamide adenine dinucleotide involves the transfer of 2 electrons and 1 proton. There are several possibilities for the number and sequence of the transfer steps, e.g. (H\(^-\)); (e, H\(^+\)); (H, e); (e, e, H\(^+\)) and others. The traditional view maintains that an enzyme-catalyzed oxidation of NADH by its specific substrate takes place in a single step by a hydride ion (H\(^-\)) transfer. Alternatively, a two-step mechanism with an obligatory free radical intermediate was first proposed by Michaelis (1). So far, there is still no unequivocal evidence to resolve the controversy between the mechanisms of a hydride transfer and a hydrogen atom transfer in an NAD-dependent enzymatic reaction.

Several laboratories (e.g. Refs. 2–5) have demonstrated the involvement of NAD in various free radical reactions in nonenzymatic systems. Moreover, the existence of an unpaired electron on the pyridine ring in the transient radical state has been well substantiated by spectrophotometric (6) and electron spin resonance (7) studies on radicals derived from NAD and its analogues. There has also been some inferential evidence for enzyme-catalyzed oxidation of NADH with free radical intermediates. Yokota and Yamazaki (8) have proposed a free radical mechanism for the oxidation of NADH and NADPH catalyzed by peroxidase, a system in which the nucleotides served by nonspecific electron donors. In earlier papers (9, 10), we have shown that lactate dehydrogenase was capable of catalyzing oxidation of NADH by superoxide anion radicals generated by either high energy ionizing radiation or the xanthine oxidase system (11), implicating a two-step oxidation of enzyme-bound NADH.

More direct evidence for a free radical reaction taking place at the active site of lactate dehydrogenase is provided in this study by showing a stereospecific hydrogen atom transfer from NADH to dicarboxylate radicals.

MATERIALS AND METHODS

Pig heart lactate dehydrogenase (EC 1.1.1.27) was obtained from Boehringer Mannheim Corp. in 3.2 mM ammonium sulfate suspension. The enzyme was dialyzed overnight against 0.01 M phosphate buffer, pH 7.6, at 4\(^\circ\). The insoluble material was removed by centrifugation. The enzyme concentration was determined at 280 nm, using a molar extinction of 1.97 \( \times 10^4 \) M\(^{-1}\) cm\(^{-1}\) (12). Yeast alcohol dehydrogenase was obtained from Worthington.

NADH and NAD\(^+\) were purchased from Sigma Chemical Co. \(^4\)C-Labeled succinic acid, fumaric acid, malic acid, and lactic acid used as standards for paper and column chromatography were obtained from Amersham/Searle. \(^{3}\)H-Ethanol was supplied by New England Nuclear. All solutions were prepared in triply distilled water.

Preparation of \([\text{A-T}]\text{NADH}\)—NADH labeled with tritium on the A side of C4 of the dihydropyridine ring was prepared by alcohol dehydrogenase-catalyzed oxidation of \([\text{A-T}]\)ethanol with NAD\(^+\) according to the procedure of Rafter and Colowick (13). The product from the preparation was analyzed by paper chromatography. The distribution of its activity is shown in Fig. 1a. About 90% of the activity was located in the NADH band, and the remaining 10% in the NAD\(^+\) band. In order to ascertain the stereospecificity of the label, a sample of the

*This study was carried out, in part, under the auspices of the Energy Research and Development Administration.
same preparation was incubated with lactate dehydrogenase and an excess of pyruvate and chromatographed in the same manner. Fig. 1b shows that over 90% of the activity was transferred to the $R_e$ range of lactate. It is of interest to note that the activity at NAD$^+$ in Fig. 1b is less than half of that in Fig. 1a. This indicates that part of the NAD$^+$ activity in Fig. 1a was probably due to the oxidation of NADH during the chromatography process. This may provide an explanation for the observed decrease in the activity in NAD$^+$ in lactate dehydrogenase-catalyzed transfer of tritium from NADH to malate (see Table III).

The total yield of OH$^-$ in this system is

$$G(\text{OH}^-) = G_{\text{OH}^-} + G_e - 5.5$$

The primary radicals, OH$^-$ and H$_2$, react with fumarate (21, 22) as shown in Reactions 3 and 4, respectively.

$$\text{OH}^- + \text{fumarate} \rightarrow H_2 + \text{fumarate} - \text{malate}$$

The rate constants for these two reactions have been estimated to be greater than $10^6$ mole$^{-1}$ liter$^{-1}$ sec$^{-1}$ (22). Nevertheless, in order to avoid significant extent of direct interaction between the nucleotide and the primary radicals, generation of dicarboxylate radicals in the presence of NADH was always carried out with a large excess of fumarate over NADH.

**Oxidation of NADH by Dicarboxylate Radicals**—Fig. 2 shows that when a N$_2$O-saturated solution containing [fumarate]/[NADH] = 15 was irradiated in a $^{60}$Co source, there was a direct correlation between the oxidation of NADH and the formation of malate and succinate. The mechanism of formation of the major product, malate, may be postulated as in Reactions 5 and 6.

$$I + \text{NADH} \rightarrow \text{NAD}^+ + \text{malate}$$

$$H_2 + I + \text{NAD}^- \rightarrow \text{NAD}^+ + \text{malate}$$

Substitution of II for I in Reactions 5 and 6 would result in the formation of succinate. The G values for the formation of the dicarboxylic acids in Fig. 2 are

$$G(\text{malate})_{\text{ex}} = 4.97; G(\text{succinate})_{\text{ex}} = 0.79$$
The discrepancy in the distribution of the $G$ values (lower value for malate and higher value for succinate) could arise if a small fraction of the hydrated electron reaction with fumarate (in competition with Reaction 2) to form a radical which upon protonation would yield an additional radical in (in competition with Reaction 2) to form a radical which upon protonation would yield an additional radical.

The observed sum of dicarboxylate $G$(malate + succinate)$_{obs}$ = 5.76 is in good agreement (within 5%) with the expected value of $G$(malate + succinate)$_{calc}$ = $G_{H}$ + $G$(OH) = 6.05.

Based on the proposed mechanism, two dicarboxylate radicals are used to oxidize 1 molecule of NADH. Therefore, the expected $G$ value for NADH oxidation should be

$$G(-NADH) = 1/2G$(malate + succinate)$ = 2.9$$

The observed value calculated from the initial rate in Fig. 2 is

$$G(-NADH)_{obs} = 3.1$$

The close agreement of the results suggests that both radicals I and II reacted efficiently with NADH in a ratio of 2:1. The present study, the emphasis is on the major radiation product, malate.

Identification of NAD$^+$ as Product—A solution similar to that described in Fig. 2 was irradiated until over 90% of the absorbance at 340 nm had disappeared. The reaction mixture was then assayed for NAD$^+$ with lactate dehydrogenase and L-lactate. Table II shows that 75% of the observed decrease in absorbance at 340 nm could be accounted for in enzymatically active NAD$^+$. Under optimum conditions with a larger excess of fumarate over NADH and a smaller fraction of NADH oxidized, about 90% of the product could be identified as enzymatically active NAD$^+$. These results are in support of the proposed mechanism in Reactions 5 and 6.

Tritium Transfer from [A-T]NADH to Dicarboxylate Radicals—For the purpose of exploring a direct transfer of a hydrogen atom from NADH to radical I and the stereospecificity of the transfer when catalyzed by an enzyme, [A-T]NADH was used in the experiments summarized in Table III. Incorporation of tritium in the final product, malate, would indicate a direct transfer. When NADH is not bound, an isotope effect would tend to favor the transfer of a hydrogen atom to that of a tritium atom from C4 of the dihydropyridine ring (23); therefore, the reaction in the absence of enzyme would find the tritium preferentially retained in the formed NAD$^+$. In the presence of lactate dehydrogenase, the nucleotide is bound stereospecifically, and hence, the tritium would be incorporated predominantly in malate.

In Experiments 1 and 2 (Table III) [A-T]NADH was added to a system (similar to that described in Fig. 2) to react with I. Analysis of the products in the irradiated solution showed that most of the radioactivity lost from NADH was recovered in NAD$^+$ and with about 10% in malate. The distribution of tritium in the products is consistent with the expected isotope effect upon Reaction 5, when radical I reacts with free [A-T]NADH.

When lactate dehydrogenase was added to the system in Experiments 3 and 4, in contrast to the first two experiments, none of the decrease in the activity in NADH could be detected in NAD$^+$. Most of it was transferred to malate and a small amount to water. These findings show that the transfer of the hydrogen atom from lactate dehydrogenase-bound NADH to radical I was stereospecific.

Table III also indicates that radical I reacted much faster with lactate dehydrogenase-NADH than with the free nucleotide. Although the ratio of free NADH to lactate dehydrogenase-NADH was 4:1 (Experiment 4) based upon $K_{LDH - NADH} = 3.9 \times 10^{-7} \text{M} (24)$

$$LDH - NADH = LDH^+ + NADH$$

radical I reacted predominantly with the bound nucleotide as indicated by the stereospecificity in the tritium transfer.

As discussed earlier, radical II should constitute about 10%
Materials and Methods and compared with the values of the nonirradiated samples.

The reaction mixture from Experiment 4 in Table III was treated with lactate dehydrogenase-bound NADH. To check this supposition, the irradiated sample from the experiment was carried out in a $^{60}$Co source at 23'.

The reaction mixture contained 10 mM fumarate, 1.0 mM phosphate, $[\mathrm{A-T]}\mathrm{NADH}$, and lactate dehydrogenase as indicated, pH 7.6. Irradiation was carried out in a $^{60}$Co source at 23'.

The radioactivity of each component in 0.1 ml of the irradiated solutions was determined according to the procedures described under "Materials and Methods" and compared with the values of the nonirradiated samples.

The numbers within parentheses represent changes of radioactivity as per cent of the decrease in NADH.

A stopped flow apparatus was modified to allow one of the solutions containing fumarate and $\mathrm{N}_2\mathrm{O}$ to be exposed to a Van de Graaff and determined from a calibration curve without lactate dehydrogenase and NADH. The experimental procedure and analysis of tritium in malate were described under "Materials and Methods."

![Graph](http://www.jbc.org/)

**Fig. 3.** Radioactivity distribution of non-nucleotide products from Table IV, Experiment 4. An irradiated sample from the experiment was treated with Norit to remove the nucleotides and then chromatographed in 8-ml fractions. Each fraction was evaporated to dryness and then taken up with 0.7 ml of water, of which an aliquot of 0.5 ml was counted in a scintillation counter.

**TABLE III**

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>LDH (µM)</th>
<th>Dose (krad)</th>
<th>$[\mathrm{A-T]}\mathrm{NADH}$ before irradiation (µM)</th>
<th>Change in radioactivity* (cpm in 0.1 ml solution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>26.4</td>
<td>160</td>
<td>$\Delta$NADH $-$155 $+$1100(71.5)$^b$ $+$45 (2.9)$^b$ $+$240(13.2)$^b$</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>21.6</td>
<td>75</td>
<td>$-$2731 $+$2330(84.3) 0 $+$231(8.5)</td>
</tr>
<tr>
<td>3</td>
<td>1.3</td>
<td>15.0</td>
<td>50</td>
<td>$-$3024 $-$70 $+$530(17.5) $+$2490(82.2)</td>
</tr>
<tr>
<td>4</td>
<td>3.8</td>
<td>21.6</td>
<td>85</td>
<td>$-$3870 $-$155 $+$85 (2.1) $+$3370(84.8)</td>
</tr>
</tbody>
</table>

*The radioactivity of each component in 0.1 ml of the irradiated solutions was determined according to the procedures described under "Materials and Methods" and compared with the values of the nonirradiated samples.

**TABLE IV**

**Lactate dehydrogenase-catalyzed tritium transfer in flow experiments**

The solution for irradiation contained 40 µM fumarate, 24 mM $\mathrm{N}_2\mathrm{O}$, and 1 mM phosphate, pH 7.5. The nonirradiated solution contained lactate dehydrogenase as indicated, 60 µM $[\mathrm{A-T]}\mathrm{NADH}$ (6.4 x $10^4$ cpm/µmol), and 10 mM phosphate, pH 7.5. In Experiment 1, 2.0 mM oxamate was added in the nonirradiated solution. Equal volumes of the solutions were mixed in a modified stopped flow apparatus as described earlier (17). The concentration of $I$ was regulated by varying the energy input in the Van de Graaff and determined from a calibration curve without lactate dehydrogenase and NADH. The experimental procedure and analysis of tritium in malate were described under "Materials and Methods."

**TABLE IV**

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>LDH (µM)</th>
<th>Dissolved gas in LDR-NADH solution</th>
<th>Concentration of $\text{I}^\text{a}$ mixing (µM)</th>
<th>T transfer to malate (equiv. value of [A-TNADH])</th>
<th>($b/a$) x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28</td>
<td>$\mathrm{N}_2$</td>
<td>0.56</td>
<td>0.38</td>
<td>68</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td>$\mathrm{N}_2$</td>
<td>0.27</td>
<td>0.19</td>
<td>70</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>Air</td>
<td>3.50</td>
<td>2.35</td>
<td>67</td>
</tr>
<tr>
<td>4</td>
<td>28</td>
<td>Air</td>
<td>1.88</td>
<td>1.18</td>
<td>63</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td>Air</td>
<td>0.68</td>
<td>0.54</td>
<td>79</td>
</tr>
</tbody>
</table>

Experiments on effect of oxamate

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>LDH (µM)</th>
<th>Dissolved gas in LDR-NADH solution</th>
<th>Concentration of $\text{I}^\text{a}$ mixing (µM)</th>
<th>T transfer to malate (equiv. value of [A-TNADH])</th>
<th>($b/a$) x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>6</td>
<td>Air</td>
<td>0.60</td>
<td>0.35</td>
<td>59</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>Air</td>
<td>0.60</td>
<td>0.06</td>
<td>10</td>
</tr>
</tbody>
</table>

A choice between the two alternative mechanisms can be determined experimentally: tritium from $[\mathrm{A-T]}\mathrm{NADH}$ should be transferred in the first oxidation step in Reactions 5 and 6 and in the second oxidation step in Reactions 8, 9, and 10.

A stopped flow apparatus was modified to allow one of the solutions containing fumarate and $\mathrm{N}_2\mathrm{O}$ to be exposed to a Van de Graaff-generated electron beam and then mixed rapidly with a lactate dehydrogenase-NADH solution, saturated with either $\mathrm{N}_2$ or air. Since $I$ is the common oxidant in both steps, Experiments 1 to 5 in Table IV, were designed to have conditions favorable for $I$ to react only in the first step and only with lactate dehydrogenase-bound NADH. There was always a large excess of lactate dehydrogenase-NADH relative to the other reactants in the flow cell.

Mechanism of Two-Step Oxidation—Although the absorption spectrum of the free radical of NAD has been known for several years (6), the molecular structure of the radical is still not well defined. The commonly used symbol of NAD· implies the transfer of a hydrogen atom from NADH in the first oxidation step. Alternatively, NADH· has also been postulated as a free radical intermediate (3, 6, 25), suggesting a loss of 1 electron from NADH in the first step. The mechanism for the interaction between radical I and NADH in Reactions 5 and 6 is in accordance with the first alternative. A similar mechanism with an ionic nucleotide intermediate can be represented in Reactions 8, 9, and 10:

$\text{I} + \text{NADH} \rightarrow \text{NADH}^+ + \text{I}^-$  

$\text{H}^+ + \text{I}^- \rightarrow \text{malate}$  

$\text{I} + \text{NADH}^+ \rightarrow \text{NAD}^+ + \text{malate}$
concentration of I, and only a trace amount of the nucleotide was in the unbound state. Under such conditions, even if there was still a small fraction of I used in the second oxidation step, variation of the ratio of [I]:[LDH-NADH] should influence the ratio of I used in two different steps, i.e. the greater the ratio of [I]:[LDH-NADH] the greater should be the fraction of I used in the second oxidation step.

The anaerobic experiments (Experiments 1 and 2 in Table IV) were carried out with a 2-fold concentration difference of radical I. The ratio of tritium transferred to the amount of radical I added did not show a significant difference, thus suggesting that most of the dicarboxylate radicals were used up in the first oxidation step.

In the aerobic experiments (Experiments 3 through 5), the LDH-[A-T]NADH-containing solution was air-saturated, so that after mixing, the final oxygen concentration was kept constant at 0.12 mM. Through controlled variation of the radical I concentration, the effective ratio of [O_2]/[I] could be varied from 40 to 200. As can be seen in Table IV, a 5-fold difference in the concentration of radical I did not affect significantly the tritium transfer ratio; in general, it remained in the same range as in the anaerobic system. It has been well established that while molecular oxygen does not react to any significant extent with NADH, it oxidizes the NADH radical to NAD^+ at a diffusion-controlled rate (26). Hence, by maintaining a relatively high ratio of [O_2]:[I], the second oxidation step by radical I can be effectively eliminated. Therefore, these particular experiments strongly support the mechanism in which the hydrogen atom transfer takes place in the first oxidation step.

*Effect of Oxamate on Tritium Transfer*—In order to verify further the hypothesis that the tritium transfer reaction takes place at the active site of lactate dehydrogenase, a specific inhibitor known to bind to the substrate site was tested. Oxamate has been shown to be a competitive inhibitor for the lactate dehydrogenase-catalyzed oxidation of NADH by pyruvate (27). The presence of high concentrations (mM range) of oxamate in a reaction mixture during irradiation would tend to complicate the system. Therefore, the flow system was used for studying the effect of oxamate on the tritium transfer reaction.

In Experiments 6 and 7 in Table IV, the concentration of NADH was kept at 60 μM, while the lactate dehydrogenase concentration was decreased to 6 μM, so that only about one-third of the nucleotide was present as lactate dehydrogenase-NADII. In Experiment 6, in the absence of oxamate, the ratio of tritium transfer to the concentration of added radical I was 59%. In Experiment 7, in which 2 mM oxamate was added to the nonirradiated lactate dehydrogenase and NADH solution, the ratio of tritium transfer was decreased to 10%. As expected from earlier results in Table III, radical I reacted mainly with lactate dehydrogenase-bound NADH in Experiment 6, involving a stereospecific transfer of tritium. On the other hand, since the substrate sites in Experiment 7 were occupied by oxamate, I could only react with the unbound NADH. In the latter case, as discussed earlier, the isotope effect favored the transfer of a hydrogen atom in preference to a tritium atom from C4 of the dihydropyridine ring.

The tritium transfer ratio in Experiment 6 was somewhat lower than that in Experiments 1 to 5. This was most likely due to the fact that a lower concentration of lactate dehydrogenase-NADH in the former scavenged the dicarboxylate radical I less effectively.

**DISCUSSION**

Earlier reports (9, 10) have demonstrated that lactate dehydrogenase is capable of catalyzing a free radical chain oxidation of NADH initiated by O_2 and propagated by O_2. Since in the chain oxidation process, the hydrogen atom released from NADH cannot be distinguished from that of the solvent, the system does not lend itself to hydrogen atom transfer studies. Therefore, in this study, dicarboxylate radicals were used as hydrogen atom acceptors for a more definitive investigation.

Experiments in the absence of enzyme show that with suitable concentration ratios of the reactants, ionizing radiation can be successfully used to generate OH radical adducts of fumarate (radical I), which reacts with NADH to form malate and enzymatically active NAD^+. The observed stoichiometry of this reaction indicates that for every 2 molecules of malate formed, 1 NADH molecule was oxidized, which suggests that a nucleotide radical intermediate was formed during the over-all oxidation process.

Studies by Sarma and Kaplan (28) have shown that free NADH exists in solution in two helical configurations which are in equilibrium with the co-planar form, and therefore, both hydrogen atoms on the C4 of the dihydropyridine ring are susceptible to attack by reactants. Labels in these 2 hydrogen atoms are subject to an isotope effect (23, 28). Our experiments in Table III show that when radical I reacted with unbound [A-T]NADH, the label was preferentially retained in the oxidation product NAD^+. The observed isotope effect for tritium was as expected higher than the reported effect for deuterium (23, 28).

A different mechanism of tritium transfer, however, was found in the presence of lactate dehydrogenase. When the nucleotide was bound to the enzyme, as was demonstrated in the analysis of the preparation of [A-T]NADH (Fig. 1), all of the label was found on the A side of the dihydropyridine ring, the accessible side (29, 30). The interaction between I and bound nucleotide should, hence, result in a transfer of all of the label to the radical. Analysis of the products showed that over 80% of the tritium lost from LDH-[A-T]NADH was found in malate, and none remained in NAD^+. This strongly indicated that the transfer of the label from the nucleotide to the dicarboxylate radical must have taken place right at the active site of lactate dehydrogenase. This postulation was further supported by oxamate inhibition of the tritium transfer reaction.

A mechanism consistent with the available evidence for the lactate dehydrogenase-catalyzed oxidation of NADH by radical I may be postulated as follows:

I + LDH-NADH → malate + LDH-NAD⁻ (11)

H⁺ + I + LDH-NAD⁺ → malate + LDH-NAD⁺ (12)

LDH-NAD⁺ ↔ LDH + NAD⁺ (13)

The sequence of this mechanism is supported by a series of flow experiments in which radical I, produced by ionizing radiation in one solution, was rapidly mixed with a nonirradiated solution containing NADH and lactate dehydrogenase in large excess. The extent of tritium transfer was similar under aerobic and anaerobic conditions.

In the aerobic experiments, molecular oxygen is known to oxidize the NAD⁺ radical to the corresponding NAD⁺ at rates
of $10^8 - 10^{10}$ m$^{-1}$ s$^{-1}$ (2, 26), and, therefore, should have effectively eliminated the second oxidation step

$$\text{NAD}^+ + O_2 \rightarrow \text{NAD}^+ + O_2^-. \quad (14)$$

$$\text{LDH-NAD}^+ + O_2 \rightarrow \text{LDH-NAD}^+ + O_2^- \quad (15)$$

by the $I$ radical. Thus, the observed transfer of tritium to malate in these experiments is consistent with the postulated hypothesis that the hydrogen atom transfer occurred in the first oxidation step in Reaction 11.

In the anaerobic experiments, most of the $I$ radicals were consumed in Reaction 11, while a smaller fraction underwent reaction 12. The remaining enzyme-bound NAD$^+$ radicals probably dissociated and dimerized in Reactions 16 and 17 (6, 31)

$$\text{LDH-NAD}^+ \rightarrow \text{LDH} + \text{NAD}^+ \quad (16)$$

$$\text{NAD}^+ + \text{NAD}^+ \rightarrow (\text{NAD})_2 \quad (17)$$

In order to have a direct transfer of a hydrogen atom from NADH to $I$ at the active site, the radical must be able to fit into the proper position in lactate dehydrogenase-NADH binary complex. In a fluorescence spectrophotometric study, Winer and Schwert (32) found that L-lactate could bind to lactate dehydrogenase-NADH to form a ternary complex and to cause a marked increase in its fluorescent emission. Among various $\alpha$-hydroxyl carboxylic acids tested, L-malate and some others were detected to have a similar fluorescence-enhancing effect as L-lactate. They postulated that malate, having part of its molecular structure similar to that of lactate, was capable of substituting lactate to bind at the same site to form a highly fluorescent ternary complex (32). In the same manner, radical $I$ may also be expected to approach the active site of the lactate dehydrogenase-NADH complex. Thus, the proximity of the unpaired electron on C3 of $I$ to the dihydropyridine ring could attract the susceptible hydrogen (33) from the bound NADH.

In an electron paramagnetic resonance study on various substituted pyridine radicals, Neta (7) observed a rapid protonation of these species in neutral and alkaline solutions. Since the observed protonation was most pronounced with radicals which were not substituted on the ring nitrogen, there is no certainty whether lactate dehydrogenase-NAD$^+$ may undergo a similar protonation reaction before reacting with a second radical $I$ in Reaction 12.

Although some details of the over-all mechanism are still uncertain, the stereospecific transfer of a hydrogen atom from NADH to a free radical in this study strongly implicates the existence of an enzyme-bound nucleotide radical as an intermediate in the oxidation process.

**Acknowledgment**—We thank Dr. Leo Kesner for helpful discussions on analytical procedures and for performing analyses in the organic acid analyzer, and Mr. Robert Kindya for his skilled technical assistance. Thanks are also due to Dr. R. Holroyd for some stimulating discussions and his constructive criticism of this manuscript.

**REFERENCES**

Lactate dehydrogenase-catalyzed stereospecific hydrogen atom transfer from reduced nicotinamide adenine dinucleotide to dicarboxylate radicals.

P C Chan and B H Bielski


Access the most updated version of this article at http://www.jbc.org/content/250/18/7266

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/18/7266.full.html#ref-list-1