Electron Transfer from α-Reduced Nicotinamide Adenine Dinucleotide to Flavoprotein, Cytochromes, and Mixed Function Oxidases of Rat Liver Microsomes*

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SUMMARY

Cytochrome b₅ of rat liver microsomes is reduced by α-NADH; the extent of reduction is equal to that obtained with β-NADH. With both nucleotides, the rates of reduction of cytochrome b₅ are very fast; alternatively, rates of reduction of cytochrome c and dichloroindophenol have been measured. The rates observed with α-NADH are about 10% of the rates observed with β-NADH. Conditions have been established for the measurement of first order kinetic parameters in these reductions: Kₘ of α- and β-NADH are 13.0 and 3.3 μM for cytochrome c reductase activity and 5.1 and 6.7 μM, respectively, for dichloroindophenol reduction. Three lines of evidence suggest that α- and β-NADH may reduce cytochrome c and dichloroindophenol via the same microsomal flavoprotein. (a) There is competition between α- and β-NADH. (b) The relative rates of cytochrome c reduction are diminished equally for α- and β-NADH when the enzyme is inhibited or denatured. (c) The relative rates of reduction of both cytochrome c and dichloroindophenol are increased equally for α- and β-NADH when microsomal cytochrome b₅ reductase is enriched.

Both α- and β-NADH are electron donors to cytochrome P-450 in microsomes. The initial rates of reduction of cytochrome P-450 by α- and β-NADH are equal (K = 0.126 min⁻¹) and considerably slower than reduction by NADPH. In a second, slower phase of cytochrome P-450 reduction, the rate is somewhat less rapid with α-NADH (K = 0.030 min⁻¹) than with β-NADH (K = 0.042 min⁻¹). Compared to β-NADH and NADPH, α-NADH is a more efficacious donor of electrons for microsomal mixed function oxidation of a methyl sterol intermediate of cholesterol biosynthesis. Furthermore, the substrate-independent rate of oxidation of α-NADH is very slow (10% of β-NADH).

To date, only diaphorase-like activities have been reported for oxidation of α-NADH. Although the present report shows clearly that α-NADH may function as an electron donor to microsomal mixed function oxidases, the physiological significance of these findings remains obscure because enzymic reduction of α-NADH has not yet been reported.

Oxidation of α-NADH by broken cell preparations of rat liver is inhibited by cyanide and insensitive to inhibition by Antimycin A, rotenone, and Amytal. Furthermore, at least 50% of the α-NADH-cytochrome c reductase activity of whole homogenate of liver is associated with the microsomal fraction (1). For some time we have been investigating a microsomal mixed function oxidase, methyl sterol oxidase, that accepts electrons from NADH; the oxidase is inhibited by cyanide (2). The multienzymic system is insensitive to inhibition by Antimycin A, rotenone, and Amytal. Accordingly, we substituted α-NADH for β-NADH and observed that methyl sterol oxidase could be supported at maximal rates by the α epimer of NADH as the sole source of exogenous reducing equivalents (see Fig. 1).

In this report we show that in rat liver microsomes electrons are transferred from α-NADH to cytochrome b₅ reductase (Reaction A in Equation 1), from α-NADH to microsomal cytochrome b₅ and P-450 (A and B), and from α-NADH to oxygen and substrate (A, B, and C). Although the principal electron donor to oxidases in microsomes is NADPH, the pathway from NADH also constitutes the over-all reaction (i.e. A, B, C) of a mixed function oxidase (3).

Although microsomal xenobiotic oxidation is supported mainly by NADPH, there is considerable current interest in the pathway of microsomal electron transport from NADH. Recently, Ichikawa and Loehr (4) observed cytochrome b₅-dependent reduction of cytochrome P-450 in subparticles of rabbit liver microsomes. The particles contain cytochrome P-450, but NADPH-cytochrome P-450 reductase had been removed by

1 NADH-cytochrome b₅ reductase (EC 1.6.2.7) is generally measured with an alternate electron acceptor, such as cytochrome c, or oxidation-reduction dyes.
Equation 1. Microsomal electron transport. Proposed by Ichikawa and Loehr (4) and in this report.

Experimental Procedure

Tissue and Enzyme Preparations—Male, adult rats of the Sprague-Dawley strain were killed by decapitation. Liver was perfused in situ with 0.25 M sucrose solution. Liver was then removed and homogenized in 3 volumes of potassium phosphate buffer (pH 7.4 and containing 1 mM glutathione). Mitochondria, cell debris, and nuclei were removed by centrifugation at 10,000 \( \times g \) for 20 min. The resulting supernatant fraction was centrifuged again at 105,000 \( \times g \) for 1 hour. The resulting microsomal pellets were washed one time with fresh buffer and collected by centrifugation. The microsomes were diluted with buffer to a concentration of 10 to 20 mg of protein per ml. (Mitochondrial contamination was judged to be less than 50%). Protein was assayed by the method of Lowry et al. (5).

In some experiments microsomes were treated with Subtilisin VII as described previously (6). The resulting preparation of microsomal subparticles was essentially free of NADPH-cytochrome b₅ reductase and cytochrome P-450 as described previously (6). The detergent-isolated enzyme contains residual detergent, an alternate procedure for obtaining the NADPH-cytochrome b₅ reductase by treatment of rat liver microsomes with very small quantities of phospholipase A under mild conditions has been used in this laboratory (11).

Assays—Cytochrome P-450 was assayed by the method of Omura and Sato (12), and cytochrome b₅ was assayed as described previously (13).

NADH-dichloroindophenol reductase was measured at 25° by recording the change in absorbance at 600 nm of a 1.0-ml solution containing the following: 45 nmoles of 2,6-dichloroindophenol; 70 nmoles of either \( \alpha \)- or \( \beta \)-NADH; 100 \( \mu \)moles of phosphate buffer (pH 7.4); and approximately 40 \( \mu \)g of microsomal protein. A molar extinction coefficient of 21 \( \times 10^3 \) liters mole\(^{-1}\) cm\(^{-1}\) was used (14). Dicumarol was added to eliminate possible contribution of adsorbed soluble diaphorase (1); no effect of Dicumarol was observed.

NADH-cytochrome c reductase was measured similarly by substituting 30 nmoles of cytochrome c for DCIP\(^{2+}\); the change in absorbance was measured at 550 nm. A difference molar extinction coefficient for reduced minus oxidized cytochrome c of 18.5 liters mole\(^{-1}\) cm\(^{-1}\) was used (15). Substrate-independent oxidation of NADH was measured at 25° as changes in absorbance at 340 nm for \( \beta \)-NADH (16) and at 344 nm for \( \alpha \)-NADH (17). Molar extinction coefficients of 6.22 \( \times 10^3 \) for \( \beta \)-NADH and 5.6 \( \times 10^3 \) liters mole\(^{-1}\) cm\(^{-1}\) for \( \alpha \)-NADH were used. Reaction vessels contained 100 nmoles of NADH; 100 nmoles of CN-, when added; and 100 \( \mu \)moles of phosphate buffer (pH 7.4) in 1.0 ml final volume.

An aerobic reduction of cytochrome P-450 was measured at 25° in Thunberg anaerobic cuvettes that contained, in 3 ml: either 420 nmoles of \( \alpha \)-NADH, 360 nmoles of \( \beta \)-NADH, or 510 nmoles of NADPH; approximately 6.0 to 6.3 mg of protein; 60 mg of glucose; 1 mg of glucose oxidase; and 300 \( \mu \)moles of phosphate buffer (pH 7.4). The cuvettes were sealed with paraffin, and the gas phase was exchanged with 100% CO by several cycles of evacuation and filling. The reaction was started by addition of reduced pyridine nucleotide from the side arm. Changes in the absorbance measured at 450 and 490 nm were measured in the dual wave length mode of a model 356 Perkin-Elmer spectrophotometer.

Because either commercial \( \alpha \)-NADH may contain small amounts of \( \beta \)-NADH or microsomes may contain small amounts of endogenous \( \beta \)-NADH (18), lactate dehydrogenase (0.02 mg) and 2 \( \mu \)moles of pyruvate were added for each milliliter of solution to catalyze oxidation of contaminating \( \beta \)-NADH.

Methyl sterol oxidase was measured with 4,4-dimethyl-5α-cholest-7-en-3β-ol as described previously (11).

Materials—NADPH (Lot 20619), \( \beta \)-NADH\(^{2+}\) (Lot 100235), isocitrate dehydrogenase (Lot 200233), lactate dehydrogenase (Lot 985-82), and glucose oxidase (Lot 100498) were purchased from

\(^2\) The abbreviation used is: DCIP, dichloroindophenol.
FIG. 2. Reduction of microsomal cytochrome b5 by α- and β-NADH. Rat liver microsomes (0.77 mg of protein per ml) were suspended in 1.0 ml of 0.1 M phosphate buffer, and the solutions were divided between two cuvettes. The spectrum was scanned after addition of 70 nmol of either α-NADH or β-NADH to the sample cuvette.

TABLE I

Microsomal enzymic activities observed with α-NADH and β-NADH

Assays were carried out as described under "Experimental Procedures." The number of separate samples is indicated in parentheses. Standard deviations are indicated.

| Enzymic activity assayed | Enzyme velocity measured with |
|-------------------------|-------------------------------|
|                         | α-NADH | β-NADH |
| NADH-cytochrome c reductase | 109.6 ± 25.1 (5) | 987.8 ± 207.6 (5) |
| NADH DCIP reductase | 61.6 ± 3.1 (3) | 506.3 ± 19.7 (3) |
| NADH oxidation* | 0.88 ± 0.27 (5) | 9.5 ± 0.75 (5) |
| NADH oxidation + 0.1 mM CN− | 0.76 ± 0.01 (3) | 0.78 ± 0.07 (3) |

* Also referred to in the text as substrate-independent oxidation of NADH by oxygen.

Calbiochem. α-NADH (Lot 110C-7050), α-NADH+ (Lot 28B-7720), β-NADH (Lot 32C-6090), cytochrome c (type VI, Lot 32C-7390), Antimycin A (type III, Lot 59B-0430), rotenone (grade 11), Triton X-100, 2,6-dichlorophenol, pyruvate, p-chloromercuriphenylsulfonic acid, and deoxycholate were purchased from Sigma and used without further purification.

Dicumarol and L-isocitrate were purchased from Nutritional Biochemicals Corp. The nonionic detergents, Lubrol WX (13) and Triton WR-1339 (18), were purchased from ICI Organics and Ruper Chemical Co., respectively.

**RESULTS**

Reduction of Microsomal Cytochrome b5 and Other Electron Acceptors by α-NADH—In an unpublished observation, Okamoto et al. (1) indicated that microsomal cytochrome b5 may be reduced by α-NADH. The purpose of this subsection is to establish that both α-NADH and β-NADH may supply reducing equivalents for reduction of microsomal cytochrome b5, and that other electron acceptors, such as cytochrome c and DCIP, may be used to study reaction velocities. Then, appropriate conditions were established to measure initial kinetic parameters with both α- and β-NADH as the source of reducing equivalents and cytochrome c and DCIP as electron acceptors.

Addition of α-NADH to a suspension of rat liver microsomes yielded an immeasurably rapid reduction of cytochrome b5 (Fig. 2). The extent of reduction achieved was equal for the two epimers of NADH: 0.504 ± 0.015 (S.D.) and 0.517 ± 0.026 (S.D.) nmol per mg of protein for α- and β-NADH, respectively. Although the rates of α-NADH oxidation using oxygen as the terminal electron acceptor were more rapid, in the presence of 0.1 mM CN− the rates of oxidation were equal for the two epimers (Table I). The rate of reduction of cytochrome c by α-NADH was also very rapid (Table I). The velocity was about 11% of the rate measured with β-NADH. Similarly, the rate of reduction of DCIP by α-NADH was about 12% of the rate observed with β-NADH. Although the rates of β-NADH oxidation using oxygen as the terminal electron acceptor were more rapid, in the presence of 0.1 mM CN− the rates of oxidation were equal for the two epimers (Table I). Thus, severe rate limitations exist in the latter process in contrast to cytochrome c reduction to the extent that the reaction velocities of α- and β-NADH oxidations were indistinguishable. Neither 10 μM rotenone, 10 μM Dicumarol, nor 0.1 mM CN− inhibited NADH-cytochrome c reductase in these preparations. Furthermore, addition of pyruvate and lactate dehydrogenase (see "Experimental Procedure") did not alter rates of α-NADH-dependent processes. Finally, addition
Fig. 4. Lineweaver-Burk plots of effect of concentrations of \( \alpha \)- and \( \beta \)-NADH on cytochrome c and DCIP reductase assays. Initial rates were measured as shown (Fig. 3). \( A \), the concentration of cytochrome c was 30 \( \mu \)M. For \( \alpha \)- and \( \beta \)-NADH, 0.025 and 0.010 mg of protein per ml was used, respectively. \( B \), the concentration of DCIP was 45 \( \mu \)M. For \( \alpha \)- and \( \beta \)-NADH, 0.04 and 0.02 mg of protein per ml was used, respectively.

of pyruvate and lactate dehydrogenase resulted in no measurable change in absorbance at 340 nm when \( \alpha \)-NADH was used. Thus, no \( \beta \)-NADH was present in the samples of \( \alpha \)-NADH.

The initial rates of reduction of cytochrome c and DCIP were relatively constant during the first 40 s for both electron acceptors (Fig. 3). The rate was considerably faster with \( \beta \)-NADH. Similarly, additions of various amounts of either electron donors or electron acceptors resulted in usual saturation kinetic data, shown in Fig. 4. From Fig. 4A, values of 13.0 and 3.3 \( \mu \)M were calculated for the \( K_m \) of \( \alpha \)- and \( \beta \)-NADH, respectively, in the cytochrome c reductase assay. Similarly, the \( K_m \) for \( \alpha \) and \( \beta \)-NADH in NADH-DCIP reductase was 5.1 and 6.7 \( \mu \)M, respectively (Fig. 4B). The kinetic constants (graphs not shown) for the acceptors were 0.5 and 1.5 \( \mu \)M (\( \alpha \)-NADH) and 5.0 and 20.0 \( \mu \)M (\( \beta \)-NADH) for cytochrome c and DCIP, respectively. Thus, artificial electron acceptors, as well as cytochrome \( b_5 \), may be reduced effectively by \( \alpha \)-NADH in the microsomal system. Furthermore, rates of reduction can be measured accurately since conditions used yielded first order kinetic data for analysis. Establishment of these conditions was essential for the investigation of competition between \( \alpha \)- and \( \beta \)-NADH described below.

**Competition between \( \alpha \)- and \( \beta \)-NADH**—Either one or more than one flavoprotein of microsomes may accept electrons from the NADHs (see Equation 1). To distinguish between these two possibilities, three kinds of experimental evidence are reported in this section: the kinetic results showing competition between \( \alpha \)- and \( \beta \)-NADH; the effects of enzyme destruction on ratios of activity measured separately with \( \alpha \)- and \( \beta \)-NADH; and the effects of enzyme enrichment on ratios of activity measured separately with \( \alpha \)- and \( \beta \)-NADH.

With a low concentration of \( \alpha \)-NADH added to all samples (16.7 \( \mu \)M \( \alpha \)-NADH), saturation of DCIP reductase by \( \beta \)-NADH was essentially normal, and there was no strong indication of competition (Fig. 5A). However, \( K_m \) for \( \alpha \)- and \( \beta \)-NADH were too similar for observation of a marked effect with DCIP as the electron acceptor (see Fig. 4B). The competitive effect of
including a much higher concentration of \(\alpha\text{-NADH} (94 \mu M)\) in the assay of \(\beta\text{-NADH}-cytochrome c\) reductase was more pronounced (Fig. 5B). The velocity of cytochrome c reductase at saturation of \(\beta\text{-NADH}\) was maximal whether or not \(\alpha\text{-NADH}\) was added. In addition, the data were analyzed according to the method described by Dixon and Webb (19) for two substrates.\(^3\) For each concentration of \(\beta\text{-NADH}\), the value of \(K_m\) for \(\alpha\text{-NADH}\) was calculated. A relatively constant value was obtained, 13.3 \(\mu M\), which was essentially identical with the value of \(K_m\) for \(\alpha\text{-NADH}\) obtained directly from double reciprocal plots (13.0 \(\mu M\) in Fig. 4A). Conversely, a fully additive effect \(\text{(i.e. no competition)}\) was observed when microsomal \(\text{NADPH-cytochrome c reductase was measured in the presence of \(\alpha\text{-NADH}\) (Fig. 5C)}\). Since electron transfer from \(\text{NADPH to cytochrome c is catalyzed by a different flavoprotein (3) of microsomes, the additive rather than the competitive effect was expected.}\

The effects of different destructive treatments on rates of \(\text{NADH-cytochrome c reductase activities were measured with \(\alpha\text{- and \(\beta\text{-NADH. Several different treatments were used, including effects of different concentrations of both ionic and non-}\)ionic detergents, p-chloromercuriphenylsulfonic acid, and heat denaturation (Fig. 6). Parallel responses were observed between \(\alpha\text{- and \(\beta\text{-NADH-dependent cytochrome c reductase activities. For example, Lubrol WX was inhibitory at all concentrations tested, but treatment with Triton X-100, deoxycholate, and even p-chloromercuriphenylsulfonic acid led to modest increases in activity at low concentrations. Triton WR-1339 was not toxic, which has been observed repeatedly (18). Even with slow heat denaturation, parallel changes were observed. The only exception was stimulation of \(\alpha\text{-NADH-dependent reduction by intermediate concentrations of Triton X-100, which are just sufficient to clarify the microsomal suspension. Thus, with the relatively minor exception that may result from a physical change in vesicles, decays of enzymic activity were equal and parallel for reactions supported by \(\alpha\text{- and \(\beta\text{-NADH.}\\n\n\text{The \(\text{NADH-cytochrome b}_6\) reductase of liver microsomes was obtained by various treatments (Table II). Particles similar to those obtained by partial proteolysis with trypsin (4) and supernatant fraction from phospholipase A treatment (11) contain the enzyme (Column I, Table II), but cytochrome \(b_6\) is removed somewhat by each of these treatments. Thus, reduction of cytochrome \(c\) by electron transfer from cytochrome \(b_6\) was diminished (Column II). Furthermore, with enrichment of the reductase, the ratio of activity measured with DCIP and cytochrome \(c\) as the terminal electron receptors remained essentially equal for \(\alpha\text{- and \(\beta\text{-NADH (Columns III and IV, Table II). Thus, with greater than 40-fold enrichment of the enzyme (from 268 to 11,700 nmoles per min per mg of protein; Table II), as well as with destruction of the enzyme (Fig. 6), parallel changes were observed. The change in ratio of rates measured with each acceptor (Columns V and VI) were parallel, too. However, since the ratio changes, the properties of the reductase may be altered. For example, the detergent-solubilized enzyme still contained substantial amounts of residual detergent, and the enzyme tended to aggregate.}\(^4\)\n
\(^3\) The following values were substituted into the equation given for catalysis of two substrates: \(K_m(\beta\text{-NADH}) = 3.3 \mu M; V_{\max} (\alpha\text{-NADH}) = 75.3 \text{nmoles per min}; V_{\max} (\beta\text{-NADH}) = 678.8 \text{nmoles per min. In the data shown as Fig. 5B, the concentration of \(\alpha\text{-NADH} \text{used was 94 \(\mu M.}\)

\(^4\) H. E. Seifried and J. L. Gaylor, unpublished results.

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**Fig. 6.** Effect of various destructive treatments upon \(\text{NADH-cytochrome c reductase activity in microsomes. Microsomes (0.01 to 0.05 mg of protein per ml) were suspended in buffer with 30 \(\mu M\) of cytochrome \(c,\) and the suspensions were divided between cuvettes. The indicated amount of detergent or other substance was added, and the mixtures were incubated for 2 min at 25°. The reaction was initiated by the addition of 70 nmoles of either \(\alpha\text{- or \(\beta\text{-NADH. For heat denaturation, microsomes were suspended in buffer, and the mixture was maintained at 50° with gentle stirring for the indicated length of time. Samples were withdrawn and cooled rapidly to 25° for enzyme assay.}**

**Substitution of \(\alpha\text{-NADH for \(\beta\text{-NADH in Microsomal Oxidases.}** —Since \(\alpha\text{-NADH can serve as a source of reducing equivalents for microsomal cytochrome \(b_6\) reductase, it follows that \(\alpha\text{-NADH could be substituted for \(\beta\text{-NADH in \(\beta\text{-NADH-dependent microsomal oxidases. According to electron transfer from \(\alpha\text{-NADH and \(\beta\text{-NADH to two terminal oxidases was investigated: cytochrome P-450, which is the terminal oxidase of drug and xenobiotic metabolism (20); and methyl sterol oxidase, which is one component of the multi-enzymic microsomal system of cholesterol biosynthesis (21).}**

Cytochrome P-450 was reduced very rapidly by \(\text{NADPH (Fig. 7A). Under these anaerobic conditions, the extent of reduction of cytochrome P-450 was about equal to that obtained with \(\text{NADPH when microsomes were incubated for several minutes with either \(\alpha\text{-NADH or \(\beta\text{-NADH (Fig. 7A).}**

When experiments were carried out very carefully, we observed that the rate of reduction of cytochrome P-450 was clearly biphasic with either \(\alpha\text{- or \(\beta\text{-NADH as the source of reducing equivalents (Fig. 7B). In the rapid phase of reaction the rates were identical with \(\alpha\text{- and \(\beta\text{-NADH, but a difference was noted in the second phase when \(\alpha\text{-NADH was substituted for \(\beta\text{-NADH. The component reduced in the rapid phase was equivalent to 30 to 40% of the total cytochrome P-450, which is very similar to the extent of \(\beta\text{-NADH-dependent cytochrome P-450 reduction reported by Ichikawa and Leech (4), but, unfortunately, their results were reported for only the first few minutes of reduction (\text{i.e. the rapid phase of} k = 0.126 \text{min}^{-1}).**

For assay of methyl sterol oxidase, the microsomes were first treated with phospholipase A to remove contributions of en-
cy- and \( \beta \)-NADH may serve as electron donors to the same in vivo. Because the evidence supports the suggestion that both obvious since enzymic reduction of cytochrome P-450 (Fig. 7), and it functions as \( \beta \) epimer.5

Upon substrate addition could not be measured directly with the use of alternate electron acceptors, such as oxidation-reduction dyes and cytochrome c (I), oxidation of \( \alpha \)-NADH by microsomal flavoprotein, cytochrome \( \beta \) reductase (Table II; Figs. 5 and 6), transhydrogenation from \( \beta \)-NADH to \( \alpha \)-NAD+ may be possible. For example, transhydrogenation from NADH to NADPH has been shown for the same microsomal cytochrome \( \beta \) reductase (23). Under conditions used in this work, however, we were unable to detect formation of \( \alpha \)-NADH by the analogous process.6 Thus, since reduction of the same flavoprotein by \( \alpha \) and \( \beta \)-NADH was indicated by several lines of evidence, transhydrogenation would have been expected.

Three rather independent lines of evidence (Table II; Figs. 5 and 6) support the conclusion that the same flavoprotein may accept electrons from \( \alpha \) and \( \beta \)-NADH. However, demonstration of \( \alpha \)-NADH-dependent reduction of cytochrome \( \beta \) reductase will have to be carried out with enzyme purified to homogeneity without either undergoing partial proteolysis or with residual detergent. Until this is achieved, the alternate proposal that microsomes contain more than one NADH-selective flavoprotein capable of reducing cytochrome \( \beta \) must be considered (24).

The metabolic significance of the NADH-dependent pathways of microsomal electron transport is just now emerging. Other workers reported a synergistic effect of NADH on NADPH reduction of cytochrome P-450 (25). More recently, Ishikawa and Loehr (4) observed that cytochrome P-450 could be reduced by a NADH-dependent system in rat liver microsomes from which cytochrome \( \beta \) and the NADPH-specific flavoprotein were removed. In the scheme of electron flow from pyridine nucleotide to cytochrome P-450 postulated by Ishikawa and Loehr, cytochrome \( \beta \) is indicated as an alternate electron acceptor for electrons from cytochrome \( \beta \) reductase. This conclusion is consistent with our results (6, 26) and with the suggestion that, although cytochrome \( \beta \) may be in oxidation-reduction equilibrium, it may not be an obligatory electron carrier from pyridine nucleotide to cytochrome P-450 and the terminal oxidases.

An experimental approach to the study of the contribution of NADH to microsomal mixed function oxidases is now possible with the use of \( \alpha \)-NADH. Since increments of \( \alpha \)-NADH oxidation rates due to addition of substrate can be observed because the basal rate of oxidation of \( \alpha \)-NADH in the absence of substrate is very slow (Table I), participation of the NADH pathway in mixed function oxidases may be studied without resorting to partial proteolysis (4, 6), detergent treatment (13, 26), or other chemical and physical means that have been used to remove the cytochrome \( \beta \) from the multi-enzymic system. Furthermore, neither \( \alpha \)-NAD+ nor \( \alpha \)-NADH produced inhibition of methyl

\[ \text{Table II} \]

Comparison of activities with \( \alpha \)-NADH and \( \beta \)-NADH and cytochrome \( \beta \) reductase prepared by different methods

| Enzyme source | I \( \beta \)-NADH-DCIP reductase | II \( \beta \)-NADH-cytochrome c reductase | III \( \alpha \)-NADH | IV \( \beta \)-NADH | V DCIP | VI Cytochrome c oxidase |
|---------------|-------------------------------|--------------------------------|----------------|----------------|--------|-----------------|
| Microsomes    | 500                           | 988                           | 0.56           | 0.51           | 0.12   | 0.11           |
| Subtilisin-digested particles | 268                           | 332                           | 0.51           | 0.81           | 0.23   | 0.38           |
| Supernatant fraction from phospholipase A treatment*  | 500                           | 100                           | 1.35           | 2.19           | 0.34   | 0.41           |
| Detergent-isolated reductaseb      | 2,420                         | 106                           | 32.6           | 22.8           | 0.36   | 0.25           |
| Proteolytic-isolated reductaseb | 11,700                        | 195                           | 57.4           | 59.8           | 0.48   | 0.50           |

* Low and variable concentrations of cytochrome \( \beta \) (see Refs. 6 and 11).

b No measurable amount of cytochrome \( \beta \) (see Refs. 7 and 0).

To date, essentially diaphorase-like activities have been reported for enzymic oxidation of \( \alpha \)-NADH. That is, with the use of alternate electron acceptors, such as oxidation-reduction dyes and cytochrome c (I), oxidation of \( \alpha \)-NADH by microsomal enzymes had been observed, but no metabolic functions could be ascribed to the enzymic oxidation. In the present report, we have shown that \( \alpha \)-NADH may serve as electron donor to cytochrome \( \beta \), which is a natural electron acceptor in microsomes (Fig. 2). Furthermore, \( \alpha \)-NADH is an efficacious electron donor for reduction of cytochrome P-450 (Fig. 7), and it functions as an electron donor to microsomal mixed function oxidation of a cholesterol biosynthetic intermediate (Fig. 8). However, the physiologic significance of these latter observations is still not obvious since enzymic reduction of \( \alpha \)-NAD+ would have to occur in vivo. Because the evidence supports the suggestion that both \( \alpha \) and \( \beta \)-NADH may serve as electron donors to the same

\[ \text{Unpublished observations, this laboratory.} \]
REDUCTION OF P-450 IN MICROSOMES
BY PYRIDINE NUCLEOTIDES

under CO
pH 7.4

0 15 30
TIME (min)

BIPHASIC REDUCTION OF
P-450 BY NADH

= 0.126 min⁻¹

k = 0.030 min⁻¹

= 0.042 min⁻¹

FIG. 7. Time course of reduction of cytochrome P-450 by NADPH, α-NADH, or β-NADH. Assay of reduction of cytochrome P-450 was carried out as described under "Experimental Procedure." In B, the total content of cytochrome P-450 was measured after 30 min of enzymic reduction by the addition of dithionite to the sample cuvette (12). Values were then expressed as the percentage of unreduced P-450 following incubation with α-NADH (●—●) and β-NADH (○—○). Calculated rate constants are indicated on the figure.

sterol oxidase when added at concentrations that were inhibitory for β-NADH and NADPH (Fig. 8). Indeed, maximal oxidase rates were obtained with α-NADH. Thus, with α-NADH as the electron donor to the NADH-dependent process, significance of the reduction of cytochrome P-450 (Fig. 7) by NADH and rates of NADH-dependent drug oxidation (20) can be studied directly in untreated microsomes.

Finally, others have suggested that the methemoglobin reductase of erythrocytes is very similar to cytochrome b5 reductase of tissue microsomes (27–29). We studied substitution of α-NADH for β-NADH and found that methemoglobin was not reduced by either isomer when incubated with intact microsomal particles. In addition, cytochrome b5 is reduced by incubation of the erythrocyte enzyme with α-NADH. These results are consistent with the observations of Hara and Minakami (29) who reported no reduction of methemoglobin by β-NADH unless microsomes were first disrupted by detergent treatment. Subsequent work with α-NADH in this enzymic process may facilitate the study of methemoglobin reductase in erythrocytes in a manner analogous to that described above for the study of NADH contributing to the microsomal system. Similarly, subsequent work with α-NADH in microsomal ethanol oxidation described recently by Okamoto (30) may facilitate resolution of the controversy over the significance of this oxidative process.

The recent suggestion of Jacobson et al. (31) that α-NADH(H) may be produced as an artifact of isolation of β-NADH(H) and not naturally occurring may be particularly interesting. However, the use of the α-NADH epimer for the purposes described above is as a model compound substituted for β-NADH. Thus, our emphasis does not rest on the arguments for or against natural occurrence.

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Fig. 8. Effect of concentration of reduced pyridine nucleotides on methyl sterol oxidase activity. Methyl sterol oxidase activity was assayed with phospholipase A-treated microsomes as described previously (11) with 100 nmoles of substrate and approximately 5 mg of microsomal protein in a final volume of 2.0 ml. Each incubation flask contained 0.5 μmole of β-NADH in addition to the indicated amounts of reduced pyridine nucleotides. Pyruvate and lactate dehydrogenase were omitted from the samples incubated with β-NADH. Results are the average of duplicate samples from two separate incubations. ●—●, α-NADH; ○—○, β-NADH; □—□, NADPH.
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