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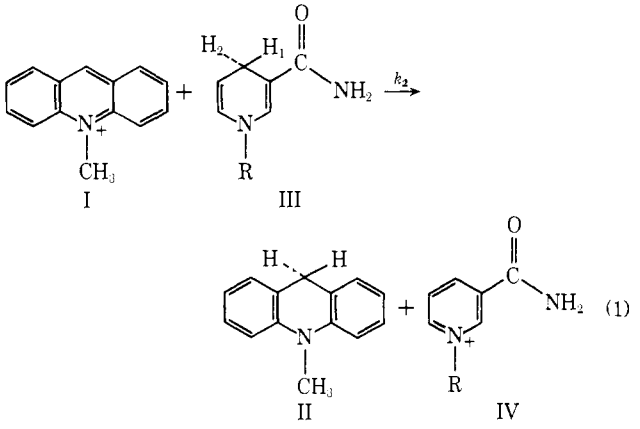
Model Dehydrogenase Reactions. Reduction of *N*-Methylacridinium Ion by Reduced Nicotinamide Adenine Dinucleotide and Its Derivatives

Sir:

Despite the central importance of NADH and NADPH in biochemical oxidation–reductions, relatively few facile nonenzymic reductions by 1,4-dihydronicotinamides are known. "Model" reactions of this type are of potential value in that they may provide helpful clues as to the mechanism of action of NAD^+- and $NADP^+$-dependent dehydrogenases.[1-9] In the present communication we wish to report a new nonenzymic reaction of dihydronicotinamides. Specifically, we have found that *N*-methylacridinium ion (I) is rapidly reduced to *N*-methylacridan (II) by β-NADH and a variety of dihydronicotinamide derivatives at room temperature in essentially quantitative yield (eq 1). Since the reverse reaction has not been observed



(1)

experimentally, the reaction must be strongly thermodynamically favored in the direction written. The reduction of *N*-methylacridinium (I) is considerably more rapid than other nonenzymic transhydrogenation reactions such as the hydrogen exchange between NAD^+ and NADH[10] and the reduction of *N*-benzyl-3-acetylpyridinium chloride[11] and the acetyl analog of NAD^+ by NADH.[12,13]

The second-order rate constants for the reduction of I by a series of dihydronicotinamides are summarized in Table I. Since II and the various oxidized nicotinamides do not absorb strongly above 320 nm, the reaction can be conveniently assayed by following either (a) the disappearance of absorption at 358 nm where I absorbs very intensely ($\epsilon = 2.6 \times 10^4\ M^{-1}\ cm^{-1}$) and the dihydronicotinamides absorb with variable intensities depending on the nature of R; (b) the disappearance of the characteristic absorbance of I in the region of 420 nm; or (c) the disappearance of the intense fluorescence of I at 490 nm. The latter two methods for assaying the reaction are particularly useful when the dihydronicotinamides are present in large excess relative to I.

Table I. Rates of Reduction of *N*-Methylacridinium Ion by a Series of Dihydronicotinamides[a]

Compound	R	k_2, M^{-1} sec^{-1}
β-NADH (IIIa)	ADPR	101.2 ± 2.4[b]
β-NADH (IIIa)	ADPR	98.2 ± 5.1[c]
β-NMNH (IIIb)	Ribose 5-phosphate	41.9 ± 1.6[b]
IIIc′	$CH_3CH_2CH_2$	2040 ± 50[d]
IIIc″	$CH_3CH_2CH_2$; $H_1 = D$	1620 ± 50[d]
IIIc‴	$CH_3CH_2CH_2$; $H_1 = H_2 = D$	1398 ± 60[e]

[a] In these experiments, the concentration of reactants did not exceed 10^{-4} *M*. Under these conditions, the reaction was strictly first order with respect to each component. [b] pH 8.0, 0.1 *M* phosphate buffer; 25°. [c] pH 7.0, 0.1 *M* phosphate buffer; 25°. [d] pH 8.4, 0.01 *M* phosphate buffer; 25°. [e] pH 8.7, 0.01 *M* phosphate buffer; 25°.

The production of II and IV was confirmed by nuclear magnetic resonance and mass spectra, ultraviolet and visible absorption spectra, and thin-layer chromatographic analysis of the products isolated from the reaction mixture. Independently prepared samples of II and the various nicotinamides were used as internal standards in these procedures. Spectral analyses of the reaction mixtures prior to isolation of the products were completely consistent with the stoichiometry indicated in eq 1.

The reaction proceeds by direct hydrogen transfer and is unaffected by reagents known to affect the rates of free-radical reactions. Direct hydrogen transfer was demonstrated in two ways. First, I was reduced with NADH in D_2O and the deuterium content of the resulting *N*-methylacridan was determined from its appearance potential mass spectrum. The intensity of the P + 1 peak (*m/e* 196) for this sample and that for II generated from the oxidation of NADH in H_2O were equal and corresponded to the intensity (15.72%) predicted from the natural isotopic abundance for a parent

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ion with a composition of $C_{14}H_{13}N$.[14] As a further check that no solvent protons or deuterons were incorporated in II and that the reaction proceeds by direct hydrogen transfer, 4-R-NADH-d_1 and IIIc'' were used as reductants for I in H_2O. In each case, the mass spectrum of II isolated from the reaction mixture possessed a more intense P + 1 peak than expected on the basis of the normal isotopic abundance. The free-radical quenching agents, dihydroquinone and 4-*tert*-butylcatechol, do not inhibit the rate of the reduction of I by NADH. When I and NADH are present at concentrations of 4.77×10^{-5} and 2.77×10^{-5} *M*, respectively, neither dihydroquinone nor 4-*tert*-butylcatechol at concentrations of 1×10^{-3} *M* has any significant effect on the second-order rate constant for the reaction. The demonstration of direct hydrogen transfer as well as the insensitivity of the rate to the presence of free-radical quenching agents indicate the reaction proceeds *via* a mechanism formally similar to a hydride transfer mechanism.

Examination of the isotope effects for the reduction of I by the various isotopic forms of *N*-propyldihydronicotinamide (IIIc', IIIc'', and IIIc''') suggests that the reduction of I is not a simple bimolecular process. *N*-Propyldihydronicotinamide instead of NADH was used as the reductant to study isotope effects since the conformation of the coenzyme renders the (*pro-R*)- and (*pro-S*)-hydrogens of the dihydronicotinamide ring chemically nonequivalent.[15,16] Hence, interpretation of the isotope effects would be more complex with NADH than with *N*-propyldihydronicotinamide. If the reduction of I by *N*-propyldihydronicotinamide proceeds by a bimolecular mechanism without the formation of any kinetically significant intermediate, the ratio of undeuterated II (*m/e* 195) to deuterated II (*m/e* 196) obtained after reduction of I with IIIc'' should approximate the primary kinetic isotope effects determined for the reduction of I by IIIc'' and IIIc''' when secondary isotope effects are assumed to be one. Since the isotopic partitioning ratio is 5.4 ± 1.0[17] and the primary kinetic isotope effects obtained by comparing the rates of reduction by IIIc'' and IIIc''' to IIIc' (Table I) are 1.70 ± 0.28 and 1.46 ± 0.10, respectively, a bimolecular mechanism is inconsistent with the experimental data assuming no significant secondary isotope effects.[18] The only way the kinetic isotope effects for IIIc'' can be made to correspond to the isotope partitioning ratio is if the secondary isotope effect for hydrogen transfer is 0.74 ± 0.06.[19] Since secondary isotope effects for reactions which involve conversion of a carbon atom from an sp^3 to an sp^2 hybridization are usually greater than one, a simple bimolecular reaction mechanism is inconsistent with the observed isotope effects.

The divergence of the isotope effects measured kinetically and from product analysis demands the existence of at least one kinetically important intermediate during the course of the reaction whose rate of formation is partially rate limiting. The simplest kinetic scheme possible in this case is indicated in eq 2

$$\text{IIIc}' + \text{I} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{X} \xrightarrow{2k_R} \text{II} + \text{IVc}' \qquad (2)$$

where X designates the intermediate whose precise nature cannot be deduced from the data presently available. Steffens and Chipman[6b] have reported isotope effects for the reduction of trifluoroacetophenone by IIIc' and IIIc'' similar to those reported here for the reduction of I. They have proposed a kinetic scheme similar to that indicated in eq 2 and have suggested that the intermediate, X, is a noncovalent complex, possibly of a charge-transfer nature, which forms prior to hydrogen transfer. Although this type of an intermediate is consistent with the observed isotope effects, the value of k_1 that can be estimated for the formation of the complex composed of III and either I or trifluoroacetophenone would be of the order of magnitude of 10^3 M^{-1} sec^{-1} or 10^{-3} M^{-1} sec^{-1}, respectively. Since these rate constants are substantially lower than the rates of formation of stacked dimers of aromatic compounds such as proflavine, where the rate of dimerization is 7.9×10^8 M^{-1} sec^{-1},[20] either the charge transfer or noncovalent nature of these compounds can be questioned or more than one noncovalent complex exists on the reaction pathway. Some support for charge-transfer intermediates comes from recent kinetic studies on the reduction of a series of flavine derivatives by NADH and *N*-propyldihydronicotinamide which have indicated that noncovalent complex formation prior to hydrogen transfer may take place during this reaction.[21]

Additional data will be necessary before the generality of obligatory intermediate formation in nonenzymic dihydronicotinamide reductions becomes clear. Yet for the reaction systems discussed here, and possibly for the zinc ion catalyzed reduction of 1,10-phenanthroline-2-carboxaldehyde by *N*-propyldihydro-

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(17) The *N*-methylacridan product analyzed for deuterium was isolated from a 100-ml reaction mixture composed of 4.4×10^{-4} *M* I and 4.3×10^{-4} *M* IIIc'' which had been allowed to react for 4 hr. The reaction mixture was extracted with three 25-ml aliquots of reagent grade chloroform which were pooled and twice washed with 10-ml aliquots of water. After the chloroform was dried, the solution was evaporated to dryness and the resulting *N*-methylacridan was analyzed for deuterium by mass spectrometry. All determinations of the isotopic composition of *N*-methylacridan were performed at the appearance potential of the P − 1 (*m/e* 194) peak of undeuterated *N*-methylacridan.

(18) For a strict bimolecular reaction, the isotopic composition of II produced by reduction with IIIc'' is given by II(*m/e* 195)/II*(*m/e* 196) = $k_{H'}/k_D$, where II*(*m/e* 196) is the observed intensity minus the intensity due to the isotopic abundance of the *m/e* 195 peak, $k_{H'}$ is the rate of hydrogen transfer from C-4 which is bound to one hydrogen and one deuterium, and k_D is the rate of deuterium transfer from C-4 which is bound to one hydrogen atom and one deuterium atom. As pointed out by Steffens and Chipman,[6b] a primary isotope effect for dihydronicotinamide reduction can be obtained from kinetic experiments using the monodeuterated dihydronicotinamide, IIIc'', since $k_2^{IIIc'}/k_2^{IIIc''} = 2k_H/(k_{H'} + k_D) = (2k_H/k_{H'})/[1 + (k_D/k_{H'})]$ where $k_2^{IIIc'}$ and $k_2^{IIIc''}$ are experimentally determined second-order rate constants, k_H is the rate of hydrogen transfer from C-4 bound to two hydrogen atoms, and $k_H/k_{H'}$ is the secondary isotope effect for hydrogen transfer. For a strict bimolecular reaction, comparison of the observed second-order rate constants for the reduction by dideuterio- (IIIc''') and dihydro- (IIIc') nicotinamide yields the following simple relationship: $k_2^{IIIc'}/k_2^{IIIc'''} = k_H/k_{D'}$ where $k_{D'}$ is the rate of deuterium transfer from a C-4 bound to two deuterium atoms.

(19) In order for the kinetic isotope effects for the dideuterionicotinamide (IIIc''') to be consistent with the isotope partitioning ratio, a bimolecular mechanism requires that the product, $(k_H/k_{H'})(k_D/k_{D'})$, be equal to 0.272. But since the data for the monodeuterated derivative demand that $k_H/k_{H'}$ be 0.74, $k_H/k_{H'}$ must be significantly greater than $k_D/k_{D'}$. This appears to be unlikely and serves as added evidence against a simple bimolecular mechanism.

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nicotinamide,[22] some type of intermediate seems essential. Since *N*-propyldihydronicotinamide reduces I roughly 50,000 times faster than trifluoroacetophenone,[6b] intermediate formation is apparently important for a wide range of reaction rates. A more complete description of the chemical nature of these intermediates should provide a better understanding of the mechanism of catalysis of NAD^+ and $NADP^+$-dependent dehydrogenases.

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Large Polar Effects in the Oxidation of Hexadecanoic (Palmitic) Acid by Nitric Acid

Sir:

Free radicals are noted for their insensitivity to polar effects. For example, photochlorination of 1-chlorobutane with Cl_2 at 68° gave equal rates of attack on C_2 and C_3,[1] and photochlorination of octanoic acid with Cl_2 or *t*-BuOCl in CCl_4 gave comparable amounts of attack on C_4–C_7.[2] This subject has been treated in reviews.[3–5] The major exceptions are reactions involving nitrogen cation (aminium) radicals.[6,7] These exhibit large polar effects. Typical is the 80% selectivity for ω-1 photochlorination in C_6–C_8 acids[7] and esters[6] and the >90% ω-1 selectivity found in C_6–C_8 alcohols.[7,8]

Another type of free radical reaction has now been found which shows large polar effects. This is the nitric acid oxidation of hexadecanoic acid, Table I. At low conversion (6%), 77% of the diacids are C_{10}–C_{15} showing a high selectivity for attack at positions remote from the carboxyl group. As the oxidation progresses, the longer diacids cleave in the middle to produce two molecules of shorter diacids so that the distribution of diacids shifts toward C_4–C_8 (Table I) and obscures the initial high selectivity for remote attack.

The selectivity for remote attack accounts for the facts that little CO_2 or acetic acid is produced and that the net weight of isolated acids increases as the reaction progresses, Table I. It also accounts for the fact that the rate of disappearance of dibasic acids increases with chain length on treatment with 62% HNO_3.[9] The rate constants (in min^{-1}) were $<10^{-5}$ for C_4–C_6 and 2×10^{-4}, 7×10^{-4}, 3×10^{-3}, and 2×10^{-2} for C_7–C_{10}. The data in Table I also show that the longer chain diacids are selectively oxidized.

Table I. Relative Yields of Dicarboxylic Acids from the Oxidation of 2.56 g of Hexadecanoic Acid with 30 ml of 70% HNO_3 at 90°

Time, hr	4		24	120
Acid product, g	2.60		2.68	2.90
% $C_{15}H_{31}COOH$ reacted	6		80	100

No. of carbons in dicarboxylic acid	Relative yields 4 hr EGSS-X	4 hr SE-30	24 hr EGSS-X	120 hr EGSS-X
4	0	0	3	8
5	3	0	4	16
6	4	0	7	24
7	4	1	12	26
8	(5)[a]	4	20	16
9	7	8	20	8
10	13	11	14	2
11	13	14	9	0
12	15	17	6	0
13	16	(17)[a]	3	0
14	11	17	2	0
15	9	(11)[b]	0	0
16	0	0	0	0

[a] The value is a mean of the preceding and following value. Direct measurement was prevented because of overlap with unidentified band (total band area 31). Value was estimated from data on EGSS-X column.

The increase in weight in going from reactant to products makes this an attractive method for the production of C_5–C_8 diacids. On the basis that the distribution at 6% conversion indicates the initial cleavage and given the distribution at 120 hr, the theoretical yield of diacids is 3.0 g. This is in good agreement with the 2.9 g isolated and further shows the absence of oxidations other than the remote oxidation pattern described.

For the early stages (6% conversion), there was concern that keto acids were present in the products (hydroxy acids were unlikely because hydroxy compounds instantly produce copious NO_2 on contact with 70% HNO_3). The general agreement between gc analyses on the polar EGSS-X and the nonpolar SE-30 columns (Table I) indicated that keto esters were not a major problem. In agreement, an infrared spectrum showed a keto band at 1410 cm^{-1} that was only about 5% of the area of the ester carbonyl band at 1440 cm^{-1}. However, a band which is suspected of being due to a keto ester appeared between C_9 and C_{10} on the EGSS-X column and coincided with the C_{15} band on the SE-30 column. A similar pattern was found in model studies on the oxidation of 12-hydroxystearic acid with 70% HNO_3 at 90°.

The preliminary results are reported now because of (1) the industrial importance of oxidizing fatty acids to long chain diacids and (2) the unusually large polar effects found for this free radical reaction.

There is no direct evidence identifying the attacking radical in the HNO_3 oxidation, although the abundance

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