On the Mechanism of Reduction by Reduced Nicotinamide Adenine Dinucleotide Dependent Alcohol Dehydrogenase. α-Halo Ketones as Mechanistic Probes[1]

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Received August 10, 1987

α-Haloacetophenones have been used as mechanistic probes for the enzyme (HLADH) controlled reductions by NADH. The cofactor, NADH, itself reduced the α-haloacetophenones and yielded the product diagnostic of free radical reduction, acetophenone. In the presence of NADH/HLADH both the α-fluoro- and α-chloroacetophenones, although they were only slightly reactive with NADH, yielded the hydride reduction products, optically active 1-phenyl-2-haloethanol. The more reactive α-bromoacetophenone was reduced by a radical chain process, which did not involve the enzyme. A halo ketone of intermediate reactivity, α,α-dichloroacetophenone, was reduced by the enzyme to give the products of heterolytic reduction. The suitability of the α-haloacetophenones as diagnostic mechanistic probes for enzyme reactivity and of dihydropyridines as NADH/HLADH models is discussed.

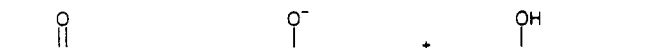
Introduction

Despite the large number of kinetic and structural studies, carried out to elucidate the mechanism of the reactions of NADH-dependent alcohol dehydrogenase, a consensus as to the details of the transformations has not been achieved.[3-6] The key mechanistic question is whether the hydrogen transfer between the coenzyme and the substrate carbonyl occurs as follows: a single-step hydride transfer (–H); a multistep electron transfer–hydrogen atom abstraction (e^-, $H^\bullet$); or, as has recently been suggested,[7] in a three-step sequence, electron transfer, proton transfer, electron transfer (e^-, H^+, e^-).

A large number of studies, using a variety of structurally different 1,4-dihydropyridines, on a variety of substrates, have been carried out to develop efficient NADH mimics. Although enzyme-controlled NADH reductions are catalytic in the cofactor, which is itself catalytically regenerated, the 1,4-dihydropyridines have, in the main, been studied as reagents reacting with substrates in stoichiometric amounts.

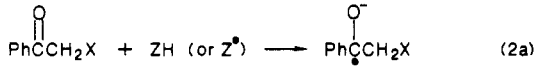
A mechanistic probe has been developed which enables the differentiation between reduction processes which proceed via electron transfer (eq 2) or by hydride transfer (eq 1), i.e., the reduction of an α-haloacetophenone.[1,8,9]

hydride transfer

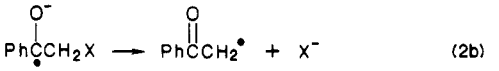


(1)

electron transfer



(2a)



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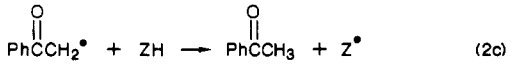
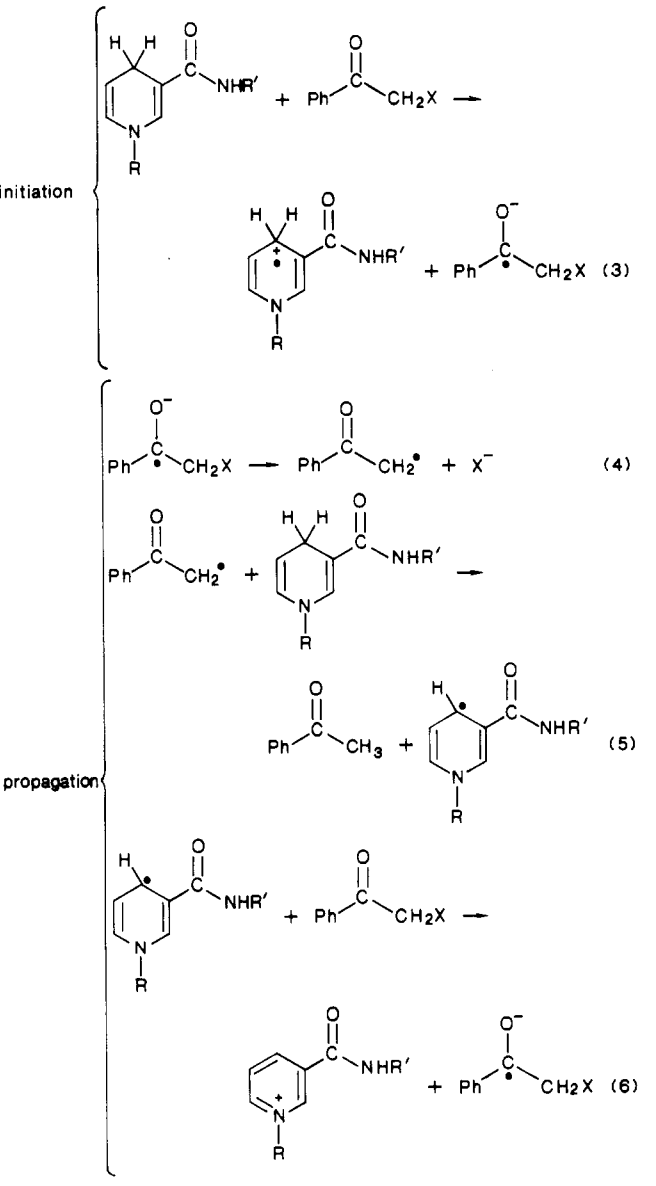


(2c)

Scheme I



Although, for convenience in the electron-transfer process the ketyl has been written as a discrete intermediate, it

(1) Presented in part: Tanner D, D.; Singh, H. K.; Kharrat, A.; Stein, A. R. *Simp. Preprints Div. Pet. Chem.* **1986**, *31* (4), 897.
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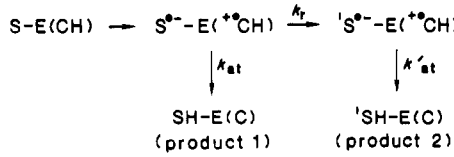
Scheme II



has not been definitely established that dissociative electron capture does not lead directly to the enolyl radical.[10]

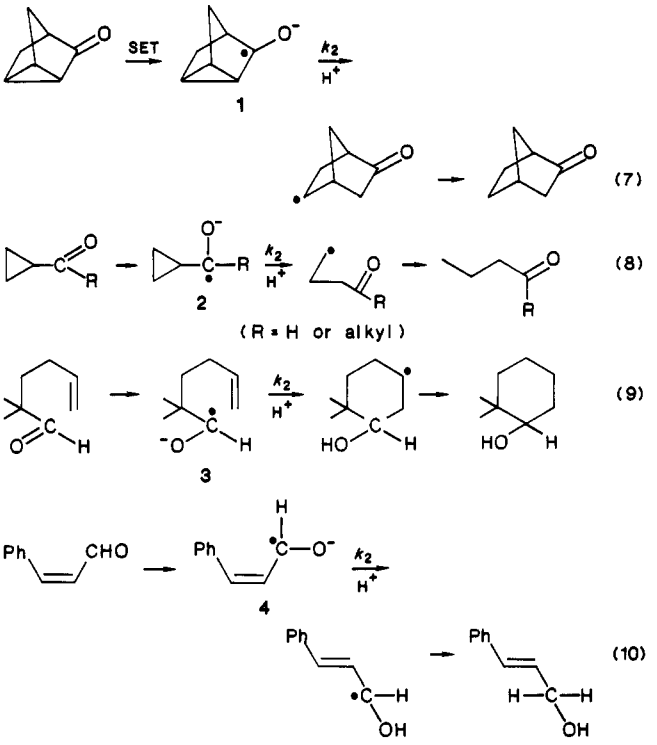
A series of mono-, di-, and trisubstituted acetophenones have been reduced by five common, N-substituted 1,4-dihydronicotinamides. Although different reactivity was observed for combinations of NADH model compounds and halogenated substrates, the reductions, in acetonitrile, were all found to proceed by a free radical chain (see Scheme I).[1,8]

A number of other chemical probes designed to differentiated between a single-step and a multistep reduction have been reported to react with reduced nicotinamide adenine dinucleotide dependent horse liver alcohol dehydrogenase (NADH/HLADH) by a single-step reduction process.[11,12] The chemically based probes all dependent upon the observed presence or absence of a molecular rearrangement. Although potentially diagnostic of a process containing a radical intermediate, all suffer from the same two limitation. First, in an enzyme-controlled reaction the hydrogen atom transfer, k_{at}, may be very much faster than the rate of rearrangewment, k_r, and therefore the product diagnostic of radical rearrangement (product 2, 'SH) may not be observed (see Scheme II). Second, the relative rates of these rearrangements are influenced by stereoelectronic factors which are possibly controlled by the unique topology of the enzyme (coenzyme)–substrate complex (S–E(CH)). The success of the probes is based upon the observation of ring opening of the cyclopropylmethyl radical[12,13] (**1**, **2**), the cyclization of a 5-hexenyl radical (**3**), or the isomerization of a substituted allyl radical (**4**) (eq 7–10). In each of these cases, the relative rates of hydrogen atom transfer vs rearrangement are known to control the ratio of rearranged to unrearranged product.[13–15]

Since our earlier work on the mechanism of the reduction of α-halo ketones[1,8,9] showed that no free radical reduction was observed which did not involve carbon–halogen bond cleavage, the halo ketones are excellent probes to test for the involvement of radical intermediate in NADH/HLADH reductions.

Results

The α-halogenated acetophenones were subjected to reduction by NADH/HLADH (see Table I). The three α-monohaloacetophenones were reduced by the enzyme–coenzyme combination. The fluoro and chloro derivatives yielded halohydrin and only traces of acetophenone (reactions 3 and 6), while α-bromoacetophenone yielded only acetophenone (reactions 12, 13, 17). Small amounts of *m*-dinitrobenzene (DNB) inhibited the reduction of bromoacetophenone by NADH/HLADH (reactions 16, 18) but did not affect the reduction of the fluoro or chloro derivative (reactions 4 and 7). Both the 2-fluoro- and 2-chloro-1-phenylethanol were found to be optically pure, within the detectable limits.



As a reducing agent, without added enzyme, NADH itself gave only traces of acetophenone and little if any halohydrin, with the fluoro- and chloroacetophenones (reaction 1 and 5), while with α-bromoacetophenone reduction produced solely acetophenone (reactions 8–10, 19, 20). The NADH reduction of the bromo ketone was inhibited by DNB (reactions 14, 15, 21, 22).

Since under the reaction conditions, NADH itself was not reactive enough to reduce the monochloro or monofluoro derivatives a more reactive polyhaloacetophenone was subjected to reduction (Table II). With NADH alone, reduction, of α,α-dichloroacetophenone showed dehalogenation and yielded α-chloroacetophenone, small amounts of acetophenone and traces of another compound whose structure is assigned as 1-phenyl-2,2-dichloroethanol (reaction 23). When DNB was added as an inhibitor, only a small amount of the monochloroacetophenone was formed and a 1% yield of the dichlorohydrin was detected (reaction 24). The enzyme-catalyzed reduction (reaction 25) gave essentially the same results as those obtained without the enzyme with the exception that the alcohol from the monochloride was formed. This chlorohydrin was found to be optically pure. When DNB was added to the NADH/HLADH reaction mixture (reaction 26), only traces of the active chlorohydrin are obtained, and a 5.5% yield of an optically active dichloro alcohol is formed.

Discussion

The reduction of the haloacetophenones by the enzyme–coenzyme pair showed conflicting results (see Table I). The α-fluoro- and α-chloroacetophenones, the less reactive ketones, showed only traces of the product, acetophenone, which was formed by the homolytic pathway when the NADH models, N-substituted nicotinamides, were used in their reduction.[8] The enzyme-controlled reductions produced almost exclusively the product halohydrin, formed from a reduction which followed the

(10) Unpublished work from this laboratory. An attempt to observe the intermediacy of the ketyl of the α-haloacetophenones was made by using cyclic voltametry. Only the radical after fragmentation could be detected, and an estimate of the lifetime, $<10^{-5}$ s, of the ketyl is made.

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Table I. Reduction of α-Haloacetophenones by NADH[a]

run	halo	[NADH]/[ketone]	additives	reactn time, h	product analysis, %		
					$PhCOCH_3$	$PhCHOHCH_2X$	$PhCOCH_2X$
1	fluoro	1.02		170	0.99	<0.005	84.7
2	fluoro	1.02	DNB	170	0.01	<0.005	95.2
3	fluoro	1.02	HLADH	170	0.05	44.1[b]	48.9
4	fluoro	1.02	HLADH, DNB	170	0.005	44.8[b]	49.0
5	chloro	1.27		70	0.08	0.00	91.1
6	chloro	1.25	HLADH	89	0.00	30.5[c]	61.0
7	chloro	1.25	HLADH, DNB	89	0.00	32.2[c]	60.8
8	bromo	1.20		3.4	13.5	0.00	39.3
9	bromo	1.14		23	26.1	0.00	13.1
10	bromo	2.18		23.5	29.7	0.00	1.0
11	bromo[e]	0.00		40	*i*	0.00	62.3
12	bromo	1.20	HLADH[c]	23	16.6	0.00	12.7
13	bromo	1.14	HLADH[d]	23	22.2	0.00	10.5
14	bromo	1.14	DNB	6	≤0.6	0.00	76.2
15	bromo	1.20	DNB	65	≤0.4	0.00	76.1
16	bromo	1.20	HLADH, DNB	24	*i*	0.00	73.0
17	bromo	1.54	HLADH	80	19.9	0.00	0.00
18	bromo[f]	1.54	HLADH, DNB	81	2.0	0.00	0.00
19	bromo[g]	1.23		4.5	15.8	0.00	51.1
20	bromo[h]	1.23		3.75	12.4	0.00	57.7
21	bromo[g]	1.23	DNB	4	0.5	0.00	90.9
22	bromo[h]	1.23	DNB	5	0.3	0.00	88.2

[a] Unless otherwise noted, run in phosphate buffer, pH 7.00, 0.05 M, 20 mL with 1 or 2 mL of HMPA (5% or 10%) added at room temperature in the dark. Solutions were deoxygenated with argon. Product analyses are as percentage of the theoretical amount. [b] $[\alpha]_D^{25}$ 53.2° ($HCCl_3$); ee >95%. [c] $[\alpha]_D^{25}$ −76.4° ($HCCl_3$), −73.9° (hexane); ee >95%. [d] 3.90 mg. [e] 5.10 mg. [f] Normal addition, 10% HMPA. Reaction was run on a mechanical shaker throughout. [g] 5% HMPA; some bromoacetophenone separates on addition. [h] 25% HMPA; no phase separation. [i] Trace.

Table II. Reduction of α,α-Dichloroacetophenone by NADH[a]

run	[NADH]/[ketone]	additives	reactn time, h	product analysis, %			
				$PhCOCH_3$	$PhCHOHCH_2Cl$	$PhCOCH_2Cl$	$PhCHOHCHCl_2$[b]
23	1.14		71	0.16	0.0	26.0	*e*
24	1.14	DNB	73	0.0	0.0	2.4	1.0
25	1.14	HLADH	72	0.04	5.7[c]	17.2	1.0[d]
26	1.14	HLADH, DNB	75	0.0	0.3[c]	1.3	5.5[d]

[a] $PhCOCHCl_2$ in 1.0 or 2.0 mL of HMPA solution (odd and even numbered runs, respectively) was added to 20 mL of phosphate buffer (pH 7.00, 0.05 M) containing NADH and HLADH (if any). DNB (if any) was added in 0.07 mL of HMPA solution. Three-day runs were shaken throughout, others periodically. Yields are as percentages of the theoretical amount. [b] GC FTIR shows OH (3620 cm^{-1}), no C=O. GC–MS was identical with that of an authentic sample. Since standards were not available, quantities are approximate. [c] Calculated, $[\alpha]^{25}_D$ = −86° (chloroform). [d] Calculated, $[\alpha]^{25}_D$ = −29° (chloroform).

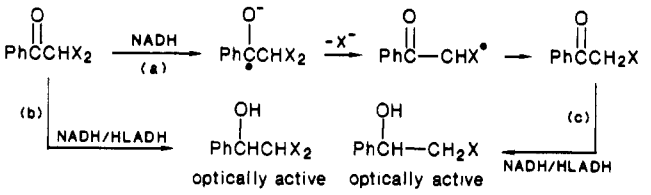
heterolytic, one-step, hydride-transfer process. Both of the substrates yielded only traces of acetophenone, the formation of which apparently was inhibited by small amounts of DNB when NADH was used in the absence of the enzyme. The more reactive α-bromoacetophenone, on the other hand, gave only acetophenone in the presence or absence of the enzyme.

DNB inhibited the formation of acetophenone but did not hinder the enzyme-controlled fromation of the halohydrins. The enzyme-controlled reduction yielded optically pure (within the experimental limits) (−)-2-chloro- or (+)-2-fluoro-1-phenylethanol.

The reactive substrate, α-bromoacetophenone, apparently reacts with NADH by a rapid free radical chain process external to the enzyme; either the bromo ketone is not bound by the enzyme and/or the enzyme cannot compete with a rapid free radical chain process. In the presence of the enzyme and DNB, although the external radical chain process is inhibited, the bromohydrin was not formed at a rate fast enough to detect under our reaction conditions.[16]

To test the proposal that the less reactive substrates, the fluoro or chloro ketones, are not reactive enough to be

Scheme III



reduced by a radical chain process by NADH and that the competitive enzyme-controlled reduction takes place to yield optically active halohydrin, a substrate of intermediate reactivity, α,α-dichloroacetophenone, was allowed to react with NADH. The dehalogenated product expected from the free radical chain process, α-chloroacetophenone, was formed along with traces of acetophenone. The radical reduction products could be inhibited by added DNB. The enzyme-catalyzed reduction, NADH/HLADH, yielded monochloro ketone, optically active chlorohydrin, and small amounts of optically active 2,2-dichloro-1-phenylethanol. The reaction process can be understood on the basis of the proposal that NADH itself will not be reactive enough to reduce the α-chloroacetophenone by a radical chain process but will be able to reduce the dichloride by this process and that the enzyme-controlled reaction is only competitive with the radical chain when the substrate is only moderately reactive. With these processes, the products and their stereochemistry are rationalized and

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give a consistent picture (see Scheme III). When DNB was added to the enzyme controlled reduction, path a was inhibited and path b becomes more competitive. Both process c and b, the enzyme-controlled reductions, yield the products ascribed to heterolytic reduction.

Conclusions

The results reported in this paper, together with our earlier results[1,8] for 1,4-dihydro-*N*-benzylnicotinamide, a commonly used NADH model, show that α-haloacetophenones are in fact good model substrates for NADH reductions. Both the dihydronicotinamide, in solvents such as acetonitrile, and reduced NADH, in aqueous solution, reduce α-haloacetophenone, especially the bromo substrate, via a free radical pathway with formation of acetophenone through halogen loss. The enzyme-mediated NADH reactions, however, do not give the products of the radical reaction; instead of acetophenone, the optically pure halohydrin is formed.

Since the products formed from the enzyme-controlled reaction are the products of heterolytic reduction, it is attractive to conclude that the enzymatic reductions proceed by a hydride-transfer process. Two caveats must be attached to this conclusion. First, if the reductions are homolytic and an electron-transfer process is involved, it is not dissociative, and the homolytic hydrogen atom transfer is faster than fragmentation. Alternatively, fragmentation may be inhibited in the bound state due to the topology of the enzyme, and elimination takes place more slowly than atom transfer. The latter proposal is not so attractive since α-fluoroacetophenone yields the fluorohydrin, and the fluorine atom has been used effectively to mimic the steric requirements of a hydrogen atom in a number of enzyme-controlled processes.[17]

Experimental Section

Materials. Yeast β-nicotinamide adenine dinucleotide, reduced form as the sodium salt, NADH, and horse liver alcohol dehydrogenase, HLADH (Sigma Chemical Company) were used without further purification. The α-haloacetophenones, *m*-dinitrobenzene, and di-*tert*-butylbenzene, the latter used as an internal standard for GLPC analysis, were prepared and/or purified as previously described.[9]

α,α-Dichloroacetophenone (Aldrich) was purified by twice fractionally freezing—about 30–40%—and decanting the liquid. The resultant material, mp 21 °C, was >99.7% by GLPC. 2-Bromo-1-phenylethanol was made from styrene by using *N*-bromosuccinamide in DMSO containing an excess of H_2O; bp 110–111 °C (2 mmHg), 99.7% by GLPC.[18]

2,2-Dichloro-1-phenylethanol was prepared by the lithium aluminum hydride reduction of α,α-dichloroacetophenone (THF). IR (vapor) 3620 (OH). GC/MS (EI), *m/z* (relative intensity) 107 (100), 79 (52), 77 (28). ^{1}H NMR (80 MHz, $CDCl_3$) δ 3.07 (s, 1 H, OH, 4.92 (1 H, d, J = 1.5 Hz), 5.80 (1 H, d, J = 1.5 Hz), 7.4 (m, 5 H).

Phosphate buffer, 0.05 M, pH 7.00 (Fisher Scientific), was used directly. Ethanol, 95%, was redistilled, and hexamethylphosphoramide, HMPA, was vacuum-distilled and stored over molecular sieves. Other solvents were reagent or Analar grade and used directly.

Argon (Linde) was deoxygenated by the Meites method using the vanadyl–vanadate couple.[19]

General Procedure for NADH Reductions. HLADH, if any, usually 6 ± 2 mg, was weighed directly into the 30-mL hypo-vials used for all reactions. The NADH, typically 0.170 g, 2.20×10^{-4} mol per reaction, was either weighed directly into the viral or, if a number of reactions were to be started at once, into a volumetric flask of appropriate size. Phosphate buffer (20 mL), pH 7.00, 0.05 M, or if the NADH was dissolved in the buffer the solution (20 mL) was added and the vial sealed with a butyl rubber septum. With two hypodermic needles, deoxygenated argon was bubbled through the solution for 10–15 minutes before the substrate, approximately 2.0×10^{-4} mol, as 1 or 2 mL of HMPA or of ethanol solution was added via the Ar inlet needle. Dinitrobenzene, if any, about 2–2.5 mg, $(1.2–1.5) \times 10^{-5}$ mol, was generally added separately in 0.1 mL of HMPA but was sometimes dissolved in the substrate solution. Argon bubbling was continued for another 5–10 min, and the vial was foil wrapped and stored in a dark cupboard with occasional shaking for the requisite time unless otherwise noted.

With four stacked separatory funnels, the aqueous reaction mixture was extracted with four 25–30-mL portions of ether. Typically 15 mg of *p*-di-*tert*-butylbenzene was added as an internal standard to the top funnel. The ethereal phases were washed three of four times with 30-mL portions of saturated brine, then dried over Na_2SO_4 overnight, decanted, and carefully concentrated on a rotatory evaporator, and the organic residue was taken up in 1 or 2 mL of $HCCl_3$ for chromatographic analysis.

Before each set of reactions was run the activity of the NADH/HLADH was assayed by reproducting the previously reported reduction of *trans*-cinnamaldehyde.[12]

Gas–Liquid-Phase Chromatography. Separations of α-bromoacetophenone from 2-bromo-1-phenylethanol and of acetophenone from 1-phenylethanol and styrene oxide were problematic; of the many phases tried, only dexsil-300, a 50-ft open tubular column, gave satisfactory separations. For mono- and dichloroacetophenone substrates, OV101 was best, but separation of chlorohydrin from α-chloroacetophenone was not quite complete even with a 20-ft packed column. For the monofluoro compound, only an FFAP column (16 ft) proved satisfactory.

Standard mixtures of those components of the reaction product mixtures that were available, together with the internal standard, *p*-di-*tert*-butylbenzene, were used to obtain "correction factors" for quantization of the reaction products. Analyses were reproducable to within a few percent.

The identities of the species responsible for GLPC signals were confirmed by comparison of retention times of and by "spiking" with known compounds. For at least one reaction with each substrate, GLPC–FTIR was used to confirm the identities of substrates and products, again by comparison to the spectra of known materials. GLPC–MS was also used for some cases (see tables).

Optical Activities and Enantiomeric Purities. Chloroform solutions of the reaction products containing halohydrins or other potentially optically active alcohols were measured on a Perkin-Elmer Model 241 spectropolarimeter. Product mixtures containing no alcohol were inactive; those containing alcohol formed by enzyme-mediated reduction of the carbonyl showed moderate to fairly large negative rotation (see tables).

Since our $C_6H_5CH(OH)CH_2Cl$ gave $[\alpha]^{25}_D$ −73.9° (*c* 1.1, hexane) vs the literature value of −47.8°,[20] the optical purity of our material was determined by NMR using the chiral shift reagent tris-[((trifluoromethyl)hydroxymethylene)-*d*-camphorato]europium-(III). Only one enantiomer, (−), was detectable when less than 5% of the other should have been detected.

The fluorohydrin, $C_6H_5CH(OH)CH_2F$, obtain from the enzymatic-controlled reductions with and without added DNB (Table I, reactions 3 and 4) gave optically pure material, $[\alpha]^{23}_D$ 53.2° ($HCCl_3$). The optical purity was determined to be >95% ee by using the chiral shift reagent, tris[((trifluoromethyl)hydroxymethylene)-*d*-camphorato]europium(III). Only one enantiomer could be detected.

Control Experiments. Reactions of substrate and acetophenone (24.7 mg, 2.06×10^{-4} mol) or 2-bromo-1-phenylethanol (39.3 mg, 1.96×10^{-4} mol) with HLADH (3.31 mg) and without and with *m*-dinitrobenzene (3.31 mg, 1.97×10^{-5} mol) and without were run in the usual fashion in 20 mL of buffer containing NADH

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(177.0 mg, 2.28×10^{-4} mol) with 1 mL of HMPA added.

From runs of 4 and 24 h, recoveries of acetophenone ranged from 95.5% to 101.5% with at most a trace, <0.4%, of 1-phenylethanol from the 24-h runs containing HLADH, with or without DNB.

For the bromohydrin, 89 ± 10% of the starting material was present. Drying over K_2CO_3 instead of Na_2SO_4 for 40–44 h before GLPC analysis gave 34–44% styrene oxide. Longer exposure to K_2CO_3 increased the conversion. The corresponding chlorohydrin formed styrene oxide at a much slower rate. The mono- and trifluoro analogs did not. Even the bromohydrin gave, at most, traces of styrene oxide after prolonged storage over Na_2SO_4.

To test the extraction procedure used, a reaction mixture containing substrate and NADH in 20 mL of phosphate buffer and 1 mL of HMPA were extracted immediately after substrate addition by the method described above. Quantitative or near quantitative recoveries of substrate was achieved for 2-bromoacetophenone, 2-chloroacetophenone, 2,2-dichloroacetophenone, and 2-fluoroacetophenone.

Acknowledgment. We thank the Natural Sciences and Engineering Research Council of Canada and the University of Alberta for their generous support of this work. We also thank Professor R. S. Brown for many helpful discussions during the course of this work.

Registry No. HLADH, 9031-72-5; NADH, 58-68-4; α-fluoroacetophenone, 450-95-3; α-chloroacetophenone, 532-27-4; α-bromoacetophenone, 70-11-1; α,α-dichloroacetophenone, 2648-61-5; 2,2-dichloro-1-phenylethanol, 2612-36-4.

Substrate-Specific Reduction Mechanisms for NADH Models. Reduction of *N*-Methylacridinium Iodide and α,α,α-Trifluoroacetophenone[1]

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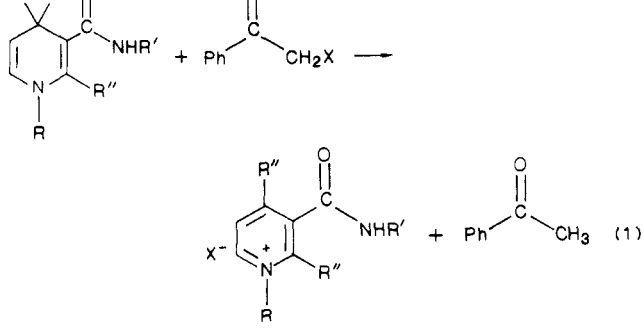
Received September 4, 1987

The reduction of α,α,α-trifluoroacetophenone by five N-substituted dihydronicotinamides (DHNAs) proceeds by a free radical chain process initiated by single electron transfer (SET). In dry acetonitrile the chain, whose propagation steps contain a SET–hydrogen atom transfer sequence, could be inhibited with *m*-dinitrobenzene or initiated by AIBN. Under the same reduction conditions methylacridinium iodide does not undergo homolytic chain reduction. The reduction of methylacridinium iodide by all five of the DHNAs followed clean second-order kinetics which were consistent with a bimolecular hydride transfer. Although a chain reaction involving solely cross termination can also follow second-order kinetics, no evidence could be obtained for either chain inhibition or initiation. The reduction mechanism followed by the NADH models appears to be substrate-specific and results reached from such model studies must be evaluated with some reservation.

Introduction

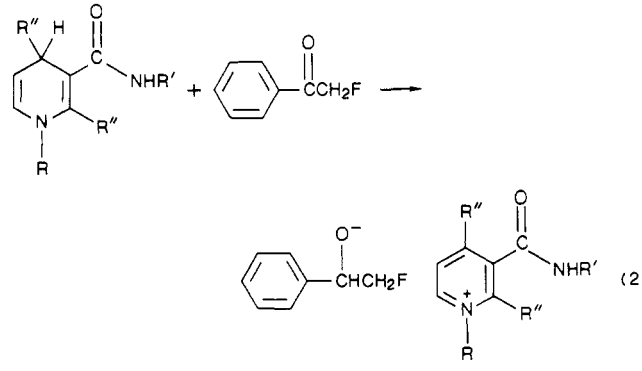
Recently[3] we have published the results of a study of the reduction of α-fluoro-, α-chloro-, and α-bromoacetophenone with four *N*-substituted-1,4-dihydronicotinamides (DHNAs), I–IV; eq 1. The reactivity of the DHNAs varied



(1)

I, R = C_6H_4; R′ = H; R″ = H
II, R = $C_6H_5CH_2$; R′ = H; R″ = H
III, R = *n*-C_3H_7; R′ = H; R″ = H
IV, R = *n*-C_3H_7; R′ = $PhCH(CH_3)$; R″ = H
V, R = *n*-C_3H_7; R′ = $PhCH(CH_3)$; R″ = CH_3

with substituents R, R′, and R″ and followed the order that depended upon the reagents ability to support a positive charge: IV > III > II > I. The mechanistic pathway followed for the reductions was shown to be a free radical chain reaction that involved an electron-transfer step both in its initiation and its propagation sequence. In the polar solvent, methanol, a small amount of hydride-transfer reduction takes place in competition with homolytic reduction. The heterolytic reduction showed some substrate specificity in that only α-fluoroacetophenone, the least reactive of the substrates, showed this competitive behavior (eq 2). The same reduction by two competing processes



(2)

is observed when the substrates, the chloride or fluoride, are reduced by triphenyltin hydride.[4,5] It was apparent that the donor–acceptor ability of the substrates was the factor that determined the dominance of one or the other mechanism.[3-5]

(1) Presented in part at the 192nd National Meeting of the American Chemical Society, Anaheim, CA, September 1986; PETR, Preprints, 1986, *31*, 897.
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0022-3263/88/1953-1646$01.50/0