Coenzyme Models. Part 35.* Charge Separation on the Micelle Surface as a Strategy to Prove the Multi-step Reaction Mechanism in NADH Model Reductions

Seiji Shinkai,* Takaharu Tsuno, Yukiko Asatani, and Osamu Manabe
Department of Industrial Chemistry, Faculty of Engineering, Nagasaki University, Nagasaki 852, Japan

We have found that (i) the reaction of NADH model compounds with potassium persulphate (PPS) in anionic and non-ionic micelles initiates radical polymerisation of acrylamide and (ii) that reductive desulphonation of 2,4,6-trinitrobenzenesulphonate and 4-carboxy-2,6-dinitrobenzenesulphonate by NADH model compounds in deuterium oxide in the presence of the micelles gives 1,3,5-trinitrobenzene and 3,5-dinitrobenzoic acid, respectively, which contain 3—16% deuterium. The results indicate that the reactions proceed through multi-step hydrogen transfer *via* radical ion-pair intermediates and the micelle is capable of dissociating the radical ion-pairs.

In 1957, Abeles *et al.*[1] proposed, in their pioneering study on the NADH [nicotinamide adenine dinucleotide (reduced form)] model reduction, that hydrogen transfer from NADH model compounds to substrates proceeds through one-step hydride transfer. The hydride transfer mechanism has since been supported by a number of NADH model studies.[2-6] On the other hand, an increasing number of reports that propose multi-step e + H· (or e + H$^+$ + e) transfer have been published. One important piece of evidence for the multi-step hydrogen transfer mechanism is based on the fact that the kinetic isotope effect (k_H/k_D) is significantly smaller than the H/D isotope ratio in the product (Y_H/Y_D).[7-10] Although there are a few examples that show that k_H/k_D is in accord with Y_H/Y_D within experimental errors,[11,12] the results may allow an assumption that there is at least one intermediate along the reduction process. However, the proposal based on the discrepancy between k_H/k_D and Y_H/Y_D is now critically argued: *i.e.*, the discrepancy can be also accommodated by non-productive adduct formation, isotope scrambling, small impurity in deuteriated NADH models, *etc.*[12-15] Therefore, the feasibility of kinetic evidence should be largely discounted. In fact, the first paper concerning the discrepancy between k_H/k_D and Y_H/Y_D by Steffens and Chipman[7] has been corrected by Chipman *et al.*[13] Another important item of evidence for the multi-step mechanism is associated with the observation of e.s.r. signals and the spin-trapping of radical intermediates in the NADH model reduction systems.[16,17] Hood *et al.*[18] described, however, that a free-radical intermediate is highly unlikely and radical species detected by e.s.r. spectroscopy may be artifacts. It seems to us, therefore, that the hydrogen transfer mechanism of the NADH model reductions is in its essential points still equivocal.

The foregoing historical survey suggests that cogent evidence should be sought in the product analysis but not in the simple kinetics. Very recently, MacInnes *et al.*[19] and Chung and Park[20] carried out NADH (and NAD) dependent alcohol dehydrogenase reductions (and oxidations) of several chemically based radical-probe molecules but could not find any indication of the radical intermediates. However, Yasui *et al.*[21] have found that reduction of thiobenzophenone derivatives with NADH model compounds in a solvent containing an *O*-deuteriated alcohol affords products partially deuteriated at their methine positions. The incorporation of solvent proton was rationalised in terms of the multi-step mechanism involving the initial electron transfer. We considered that the incorporation of a solvent proton may be widely observed if one can dissociate the radical ion-pair intermediates that are proposed for the multi-step mechanism. Recently, a technique of charge separation on the micelle surface has been developed through the investigation mimicking the initial step of photosynthesis.[22-24] If the reaction proceeds through one-step hydride transfer, a hydrogen of an NADH model compound must be transferred directly to a substrate (S) under all reaction circumstances. On the other hand, if the reaction proceeds through multi-step hydrogen transfer *via* a radical ion-pair intermediate (NADH$^{+\cdot}$·S$^{-\cdot}$), NADH$^{+\cdot}$ would be tightly associated with the anionic micelle while S$^{-\cdot}$ would be excluded out of the micelle surface owing to electrostatic repulsion between the negative charges. The micelle surface would thus facilitate the dissociation of the radical ion-pair, resulting in 'free' S$^{-\cdot}$, which would initiate radical reactions or pick up solvent protons. In this paper, we have tested the possibility through (i) the polymerisation of a vinyl monomer in the reduction of potassium persulphate (PPS) with NADH model compounds [equation (1)], and (ii) the reductive desulphonation of 2,4,6-trinitrobenzenesulphonate (TNBS) and 4-carboxy-2,6-dinitrobenzenesulphonate (CDNBS) to 1,3,5-trinitrobenzene (TNB) and 3,5-dinitrobenzoic acid, respectively, in D_2O solution [equation (2)].

Results and Discussion

Micellar Effects on the NADH Model Reductions.—NADH model compounds used in the present study are recorded in Table 1 along with their abbreviations. Initially we examined whether the anionic micelle [SDS, (sodium dodecylsulphate)] inhibits the NADH model reductions. If it does, it would be an indication that the micelle surface is capable of mediating the charge separation of fleeting radical ion-pairs. The second-order rate constants determined in an anaerobic aqueous solution are recorded in Table 2. An examination of Table 2 reveals that the NADH model reductions in the presence of the SDS micelle are efficiently suppressed when the substrate is anionic and the NADH model is hydrophobic: for example, the reaction of TNBS with PrNAH is retarded only by 1.6-fold, while that with HxdNAH is retarded 24-fold compared with the non-micellar PrNAH reaction.

Similarly, the reaction of PPS with HxdHAH is retarded 142-fold compared with the non-micellar PrNAH reaction. The inhibitory effect thus suggests that when a high-energy

* Preliminary communications: S. Shinkai, T. Tsuno, and O. Manabe, *J. Chem. Soc., Chem. Commun.*, 1982, 592; S. Shinkai, T. Tsuno, Y. Asatani, and O. Manabe, *Chem. Lett.*, 1982, 1439. Part 34, S. Shinkai, Y. Ishikawa, and O. Manabe, *Bull. Chem. Soc. Jpn.*, in the press.

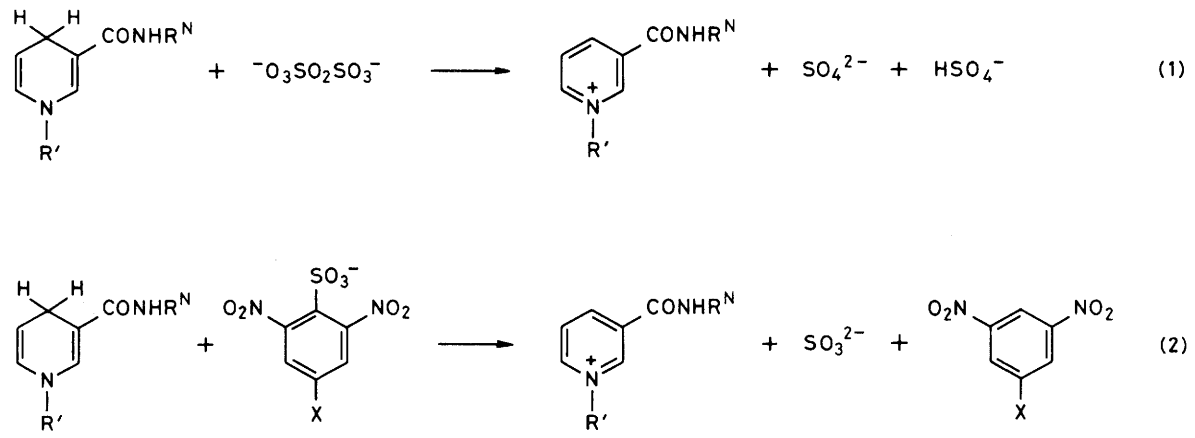
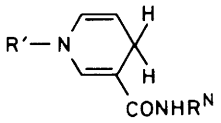


Table 1. Abbreviations of NADH model compounds



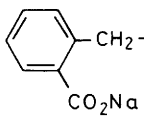
Abbreviation	R′	R^N
PrNAH	$Me(CH_2)_2$—	H—
HxdNAH	$Me(CH_2)_{15}$—	H—
BzlNAH	$PhCH_2$—	H—
N-DodBzlNAH	$PhCH_2$—	$Me(CH_2)_{11}$—
N-Dod-2-CarbBzlNAH		$Me(CH_2)_{11}$—

Table 2. Second-order rate constants for the NADH model reductions

NADH model	Substrate	Additive [concentration (mM)]	k_2/M^{-1} s^{-1}
PrNAH	TNBS	None	14.9 [a]
PrNAH	TNBS	SDS (10)	9.31 [a]
HxdNAH	TNBS	SDS (10)	0.619 [a]
PrNAH	PPS	None	23.2 [b]
PrNAH	PPS	SDS (20)	9.20 [b]
HxdNAH	PPS	SDS (20)	0.163 [b]

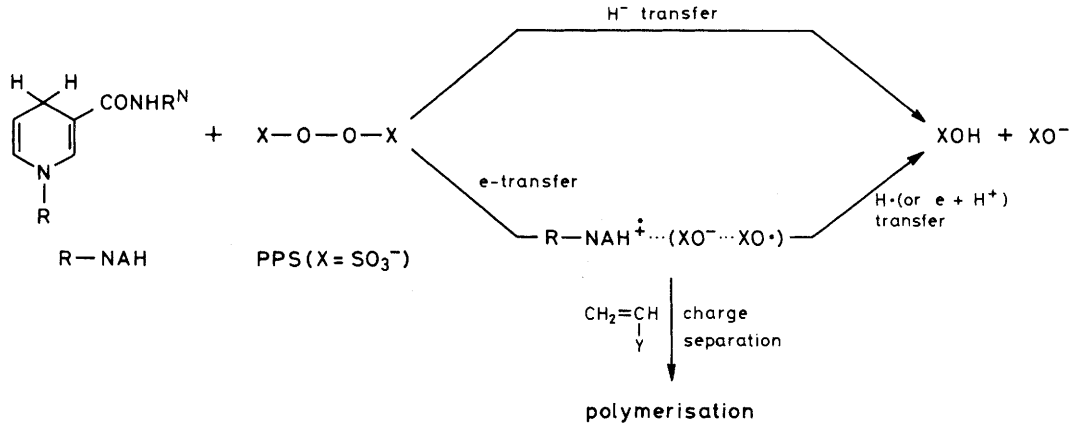
[a] 35 °C, pH 8.2 with 0.03M phosphate buffer. [b] Apparent second-order rate constants are recorded here (see Experimental section): 40 °C, pH 9.05 with 0.02M borate buffer.

ion-pair ($NADH^{+\cdot}\cdot S^{-\cdot}$) is formed, $NADH^{+\cdot}$ (in particular, hydrophobic $NADH^{+\cdot}$) is tightly bound to the micelle phase while the $S^{-\cdot}$ (in particular, hydrophilic $S^{-\cdot}$) is repelled into the bulk water.

Radical Polymerisation of Acrylamide Initiated by Reduction of PPS by NADH Model Compounds.—The reaction of peroxides with NADH model compounds has been examined by Huyser and his co-workers.[25,26] They proposed a radical chain mechanism. In 1971, Wang *et al.*[27] considered that no radical species would be produced if the reduction proceeds through one-step hydride transfer, whereas the radical polymerisation would be initiated if the reduction really proceeds through multi-step electron transfer. In other words, the radical polymerisation may be a useful probe for distinguishing the reduction mechanisms. However, their attempt ended in a failure. They thus elucidated that the radical intermediate (*e.g.*, $NADH^{+\cdot}$) afforded from NADH model compounds would also act as a radical scavenger. If this rationale is reasonable, the system would initiate the radical polymerisation when the radical ion-pair intermediate is successfully dissociated into the 'free' radical species. Thus, we carried out the polymerisation of acrylamide in the presence of 1,4-dihydronicotinamides as the NADH models, PPS as an electron acceptor, and surfactant micelles as charge-separating media (Scheme 1). The results are recorded in Table 3.

Based on the kinetic measurements, we confirmed that the reaction of NADH model compounds and PPS is fairly fast and even in the slowest case (*i.e.*, HxdNAH in the SDS micelle) the reaction is completed in 2 h. The control experiments in the absence of NADH model compounds established that the polymerisation of acrylamide by PPS at 35 °C does not take place for 8 h in SDS and for 4 h in Brij-35 (Table 3, entries 2 and 9). The reaction of PrNAH with PPS in the absence of surfactant gave only a trace amount (less than 1%) of polyacrylamide. On the other hand, the same reaction in the presence of the SDS micelle gave the polymer in 40—83% yields. The average degree of polymerisation of the polymer (entry 5), which was determined by measuring terminal potassium ions by atomic absorption spectroscopy, was 1 270. The result strongly suggests that the fleeting ion-pair, $PrNAH^{+\cdot}\cdot PPS^{-\cdot}$, is dissociated by the anionic micelle, resulting in the radical polymerisation of acrylamide by $PPS^{-\cdot}$ in the bulk water phase.

Unexpectedly, the reaction of the HxdNAH with PPS in the SDS micelle could not initiate the polymerisation.[28] We previously conducted the polymerisation at 40 °C for 2 h. The kinetic measurements suggest that the reaction time is too long; we could not always obtain reproducible results, probably due, for example, to the acid-catalysed decomposition of NADH model compounds in the SDS micelle, and slow thermal decomposition of PPS. The difference is probably explicable in that the SDS micelle inhibits the reaction of HxNAH with PPS so strongly that the rate of the production of the radical initiator ($SO_4^{-\cdot}$) is too slow to maintain the continuous radical polymerisation. Also, the acid-



Scheme 1.

Table 3. Polymerisation of acrylamide initiated by potassium persulphate (PPS) and NADH models [a]

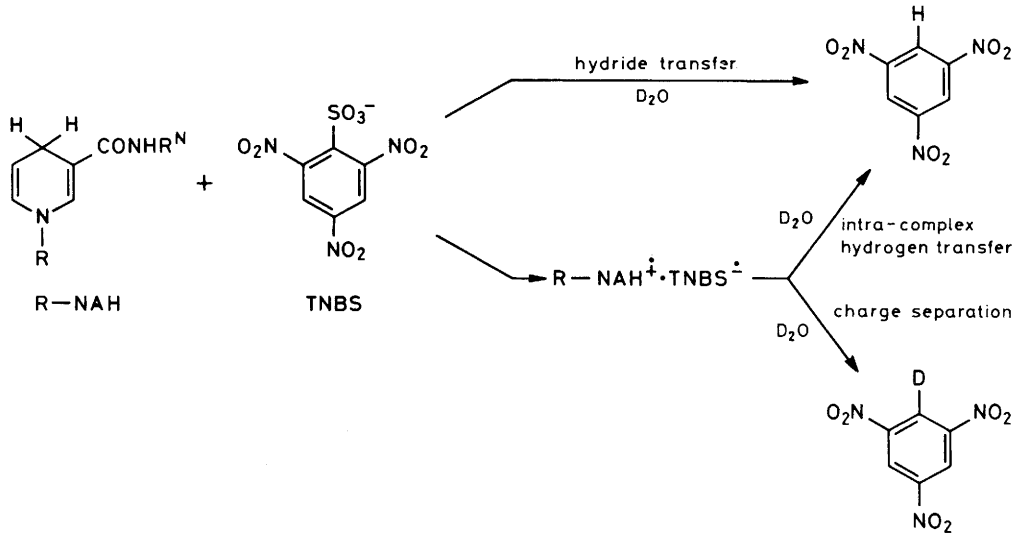
Entry	NADH model	Additive [concentration (mM)]	Reaction time (h)	Polymer yield (%)
1	None	None	1—8	0
2	None	SDS (30)	1—8	0
3	PrNAH	None	2	trace (<1%)
4	PrNAH	SDS (30)	2	40—46
5	PrNAH	SDS (30)	4	83
6	HxdNAH	SDS (30)	1—8	0
7	None	Brij-35 (15) + SDS (15)	2	0
8	HxdNAH	Brij-35 (15) + SDS (15)	2	79
9	None	Brij-35 (30)	2—4	0
10	PrNAH	Brij-35 (30)	2	72
11	HxdNAH	Brij-35 (30)	2	69
12	HxdNAH	Brij-35 (30)	4	83

[a] 35 °C, pH 9.1 with 0.02M borate buffer, ethanol 2% v/v, [NADH model] = [PPS] = 1.0×10^{-3}M, [acrylamide] = 1.0×10^{-1}M.

catalysed decomposition of HxdNAH, which is efficiently catalysed by anionic micelles,[29,30] must be taken into consideration. We thus tried to weaken the inhibitory effect of the SDS micelle by diluting with a non-ionic surfactant, Brij-35 (entries 7 and 8). As expected, the reaction of HxdNAH and PPS gave the polymer in 79% yield in the presence of equimolar amounts of SDS and Brij-35. We subsequently found, however, that the polymerisation easily takes place in the presence of the single micelle of Brij-35 (entries 10—12).

The foregoing polymerisation data suggest that the radical polymerisation is due to the charge separation of $NAD^{+\cdot}$·$PPS^{-\cdot}$ at the micelle surface followed by the initiation of the radical chain reaction by $SO_4^{-\cdot}$ repelled into the bulk-water phase. It has been established in the polymer-chemistry field that a number of amines facilitate the decomposition of peroxides through redox-type one-electron transfer. A similar one-electron transfer would take place from NADH models to peroxides,[25,26] but in the absence of the micelles the initial electron transfer to afford $NADH^{+\cdot}$·$PPS^{-\cdot}$ would be followed immediately by the H·(or e + H^+) transfer as an intra-complex ($NADH^{+\cdot}$ . . . $SO_4^{-\cdot}$ . . . SO_4^{2-}) step. As a result, $SO_4^{-\cdot}$ cannot attack the vinyl monomer even though it is formed transiently in the ion-pair complex. In the presence of the SDS micelle, $NADH^{+\cdot}$ species would be efficiently bound to the anionic micelle phase, whereas hydrophilic $SO_4^{-\cdot}$ is efficiently repelled from the anionic micelle surface into the bulk aqueous phase. This charge separation is the primary role of the SDS micelle. Furthermore, polyacrylamide is soluble only in water, so that the polymer would be scarcely partitioned to the micelle phase. In other words, the growing chain would be kept separated from the $NADH^{+\cdot}$ species in the micelle phase. This is the secondary role of the SDS micelle. The roles of the Brij-35 micelle may be similarly explained: $NADH^{+\cdot}$ is a relatively hydrophobic cation allowing hydrophobic binding to the non-ionic micelle, whereas $SO_4^{-\cdot}$ is a quite hydrophilic anion. Hence, although no charge repulsion (or charge attraction) effect exists in this system, the charge separation may be achieved based on the difference in the hydrophobic characteristics.

Hydrogen Transfer Reaction from NADH Models to TNBS and CDNBS in Deuterium Oxide.—Reductive desulphonation of TNBS and CDNBS by NADH model compounds has been reported by Kurz and Frieden,[3,31] and Brown and Fisher.[32] The substituent effect reported therein suggests that substantial charge transfer occurs in the formation of the activated complex of this model dehydrogenase reaction and the hydride mechanism is considered favourably. If the reaction proceeds through one-step hydride transfer, a hydrogen atom of the NADH model compound must be transferred directly to the substrate (S) under all reaction conditions. On the other hand, if the reaction proceeds through multi-step hydrogen transfer *via* a radical ion-pair intermediate ($NADH^{+\cdot}$·$S^{-\cdot}$), $S^{-\cdot}$ has a chance to pick up a solvent proton resulting in the incorporation of the solvent proton into the final product. The possibility is further increased when the ion-pair is separated on the micelle surface. We tested the possibility through the reductive desulphonation of TNBS and CDNBS by



Scheme 2.

Table 4. Contents of deuterium in 1,3,5-trinitrobenzene (TNB) recovered from the reaction of 1,3,5-trinitrobenzenesulphonate (TNBS) and NADH models [a]

Entry	NADH model	Additive [concentration (mM)]	$(M^+ + 1)/M^+$ (%)	Deuteriated TNB (%)
1	PrNAH	None	9.2 ± 0.3	1.3
2	PrNAH	SDS (30)	15.9 ± 0.4	8.0
3	PrNAH [b]	SDS (30)	13.3 ± 0.4	5.4
4	PrNAH [c]	SDS (30)	14.9 ± 0.5	7.0
5	HxdNAH	SDS (30)	13.0 ± 0.5	5.1
6	HxdNAH	Brij-35 (20)	10.0 ± 0.5	2.1
7	HxdNAH	CTAB (30)	9.5 ± 0.5	1.6
8	BzlNAH	None	8.3 ± 0.3	0 (<0.4)
9	BzlNAH	SDS (30)	8.3 ± 0.3	0 (<0.4)
10	*N*-DodBzlNAH	SDS (30)	13.5 ± 0.5	5.6
11	*N*-DodBzlNAH	Brij-35 (20)	10.0 ± 0.5	2.1
12	*N*-DodBzlNAH	$2C_{12}SO_3Na$ (1.0) [d]	12.4 ± 0.5	4.5
13	*N*-Dod-2-CarbBzlNAH	SDS (30)	19.9 ± 0.4	12.0
14	*N*-Dod-2-CarbBzlNAH	Brij-35 (30)	8.8 ± 0.3	0.9
15	Polymeric model	None	24.0 ± 0.5	16.1

[a] 30—35 °C, pH 8.2 with 0.02M *N*-ethylmorpholine or 0.03M phosphate, 2 h in an N_2 stream, 99.9% D_2O, [NADH model] = 1.4×10^{-2}M, [TNBS] = 2.8×10^{-2}M. [b] In the dark. [c] Under irradiation by a 200 W tungsten lamp. [d] $Me(CH_2)_{11}O_2CCH_2CH(SO_3Na)CO_2(CH_2)_{11}Me$.

NADH model compounds in D_2O (99.9% purity) at 30—35 °C under anaerobic conditions (Scheme 2).

First, we corroborated that the desulphonation products, 1,3,5-trinitrobenzene (TNB) and 3,5-dinitrobenzoic acid, do not exchange the protons with solvent (D_2O) proton in the absence and in the presence of the micelles used in the present study. We also corroborated that the exchange does not occur in the presence of poly(styrene-*p*-sulphonate). This is a control experiment for entry 15 in Table 4. The content of deuterium was determined by mass spectroscopy. TNB was determined directly, whereas 3,5-dinitrobenzoic acid was determined as its methyl ester derivative. The method was guaranteed by calibration with n.m.r. (see Experimental section). The results are summarised in Tables 4 and 5.

The TNB recovered after 2 h from the reaction with BzlNAH in D_2O solution gave a ratio of $M^+ + 1$ to M^+ ions in the mass spectrum of 8.3 ± 0.3 (entry 8, Table 4), which is in good accord with the ratio calculated from the natural abundance value (7.9%). The reaction with PrNAH allowed the slight incorporation of deuterium (1.3%, entry 1, Table 4). These data show that the hydrogen atom is transferred almost directly from the NADH model compounds to TNBS in the absence of the micelles. In the presence of the SDS micelle, the incorporation of deuterium was not observed for the reaction with BzlNAH, but a significant amount of deuterium (8.0%) was detected in TNB recovered from the reaction with hydrophobic NADH models, HxdNAH and *N*-DodBzlNAH, in the SDS micelle. Similarly, Brij-35 and $2C_{12}SO_3Na$ (membrane-forming surfactant, see footnote to Table 4) [33] were effective, but CTAB (hexadecyltrimethylammonium bromide) was not. We also examined the effect of light (entries 3 and 4), but the content of deuterium in TNB was not largely affected by photoirradiation. These findings clearly indicate that the fleeting radical ion-pair is separated, although partially, by the micelle surface (Figure).

It is interesting that the reduction with PrNAH results in a deeper deuterium incorporation than that with BzlNAH, for a similar tendency has been reported by Yasui *et al.* for the reduction of thiobenzophenone.[21] If the reaction proceeds according to the multi-step mechanism, the degree of deuterium incorporation reflects the rate ratio of the initial electron transfer to the subsequent proton transfer or to the

Table 5. Contents of deuterium in methyl 3,5-dinitrobenzoate derived from 3,5-dinitrobenzoic acid that is produced by the reaction of 4-carboxy-2,6-dinitrobenzenesulphonate (CDNBS) and NADH models [a]

Entry	NADH model	Additive [concentration (mM)]	$(M^+ + 1)/M^+$ (%)	Deuteriated methyl 3,5-dinitrobenzoate (%)
1	PrNAH	None	10.5 ± 0.4	0 (<1)
2	PrNAH	SDS (30)	13.5 ± 0.3	3.8
3	HxdNAH	SDS (30)	14.0 ± 0.4	4.3
4	BzlNAH	None	10.2 ± 0.2	0 (<1)
5	*N*-DodBzlNAH [b]	SDS (30)	13.2 ± 0.3	3.5
6	Polymeric model [b]	None	15.0 ± 0.5	5.3

[a] The reaction conditions are recorded in a footnote to Table 4. [b] 4 h.

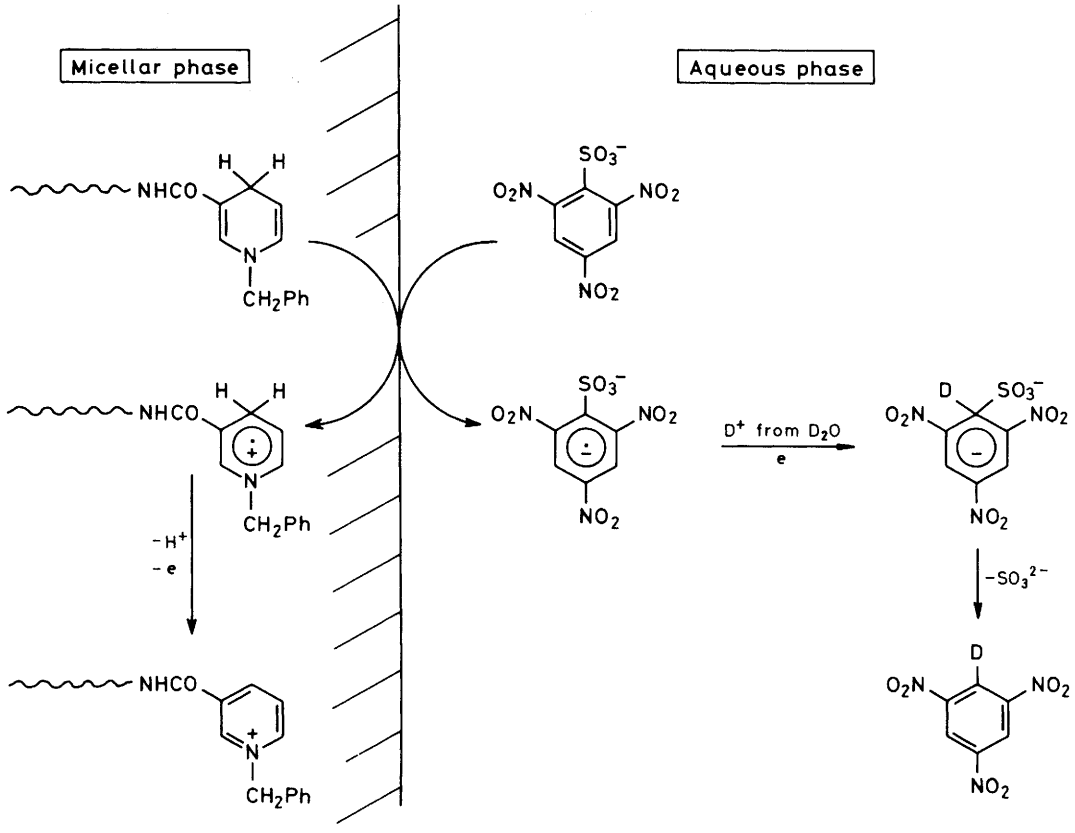
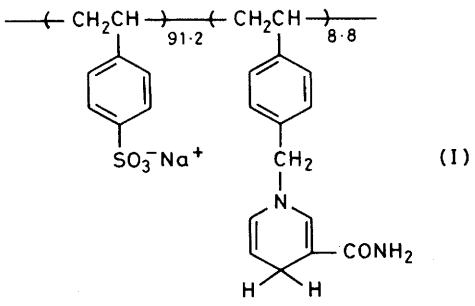


Figure 1. Schematic representation of the charge separation of a radical ion-pair formed from *N*-DodBzlNAH and TNBS

micelle-mediated charge-separation. Obviously, PrNAH has electron-releasing power stronger than the BzlNAH, while the reverse is true in the subsequent intra-complex proton transfer. As a result, the radical ion-pair intermediate formed between PrNAH and TNBS has more chance to undergo charge separation than that formed between BzlNAH and TNBS.

We considered that the dissociation of the ion-pair may be further facilitated by employing an anionic NADH model compound, because the cationic charge formed on the dihydropyridine moiety is neutralised by the intramolecular anionic group. We thus synthesized *N*-Dod-2-CarbBzlNAH. The reduction by *N*-Dod-2-CarbBzlNAH in the SDS micelle (entry 13, Table 4) resulted in the incorporation of 12.0% deuterium, which is much higher than that of the non-ionic counterpart, *N*-DodBzlNAH (5.6%). This may be due to the effect of the intramolecular charge-neutralisation, as we expected, but also may be explained by the stabilisation of the radical ion-pair intermediate, which enhances the probability of the charge separation.

The hydrophobic core of micelles may not be prerequisite to accomplishing the charge separation in some cases. If so, anionic polyelectrolytes immobilising an NADH model compound may also be useful as a modified charge-separating medium. We thus synthesized a polymeric NADH model by copolymerisation of 1-(4′-vinylbenzyl)nicotinamide chloride and sodium styrene-*p*-sulphonate (I). Surprisingly, the reduction with the polymeric NADH model provided the highest value of deuterium incorporation (16.1%). The result implies that the polyelectrolyte has a relatively high charge density by which the efficiency of the charge separation is primarily governed.



(I)

In the reduction of TNBS by NADH models, the substrate is mono-anionic in the ground state and becomes di-anionic in the transition state where it accepts one electron from NADH models. The situation is important in order to achieve the charge-separation because the di-anionic transition-state species ($TNBS^{-\cdot}$) is more efficiently excluded out of the surface of the SDS micelle than the mono-anionic ground-state species (TNBS). It occurred to us that the di-anionic substrate, which becomes tri-anionic in the transition state, may be more advantageous to the charge separation. We thus used CDNBS as a substrate and determined the deuterium content in 3,5-dinitrobenzoic acid as its methyl ester derivative. The results (Table 5) were disappointing, however. The highest value of the deuterium incorporation was again observed for the polymeric NADH model, but it was only 5.3%. Conceivably, the monoanionic ground-state species, which becomes di-anionic at the transition state, is more advantageous than the di-anionic ground-state species.

Conclusion

The foregoing results indicate that the micelle surface, as well as the polyelectrolyte chain, is capable of separating the radical ion-pair intermediate $NADH^{+\cdot}{\cdot\cdot}S^{-\cdot}$. The $S^{-\cdot}$ dissociated from $NADH^{+\cdot}$ can pick up a solvent proton or initiate radical polymerisation. These results strongly support the involvement of the multi-step hydrogen-transfer mechanism: that is, there is a route to products that is not *via* a hydride transfer. The relatively low deuterium incorporation in the dihydronicotinamide reduction of TNBS and CDNBS could be rationalised in two ways: (i) the hydride-transfer mechanism competes successfully with the multi-step mechanism *via* a radical ion-pair or (ii) the reaction proceeds exclusively through the multi-step mechanism but the intra-complex hydrogen transfer ($NADH^{+\cdot}{\cdot\cdot}TNBS^{-\cdot} \longrightarrow NAD^{\cdot}{\cdot\cdot}TNBSH^{\cdot}$) occurs more efficiently than the charge separation. Although further investigation is needed to provide a clear answer to this question, it seems rather unlikely to us that two different mechanisms coexist in one NADH model reduction system. We thus consider, preferably, that the reaction proceeds exclusively through the multi-step mechanism.

As described in the introductory section, NADH model reduction appears to be a simple hydride transfer in some cases. It is also true, however, that it can act as one-electron donor to a number of oxidants (peroxides, halogenoalkanes, nitroalkanes, haemin, thioketone, *etc.*).[21,34–37] It thus seems very difficult to explain all the NADH model reductions by a single mechanism. The equivocal behaviours of NADH model reductions remind us of the relation between S_N2 and S_{RN} mechanisms: *i.e.*, it may appear like a multi-step hydrogen transfer when it is not fully stabilised. The stability of the radical intermediates depends on the nature of the substrates. We are now interested in the application of the charge-separation technique to the NADH model reduction of carbonyl substrates, which is more closely related to alcohol dehydrogenase reactions. However, the charge separation of this system would be more difficult because one can expect that the intermediate complexes with carbonyl substrates are not so stabilised as those with TNBS or CDNBS. In conclusion, we would like to consider that NADH model reduction is described, basically, in terms of the multi-step mechanism and in one extreme case where the pathway has no stabilised intermediate appears, apparently, as the hydride mechanism.

Experimental

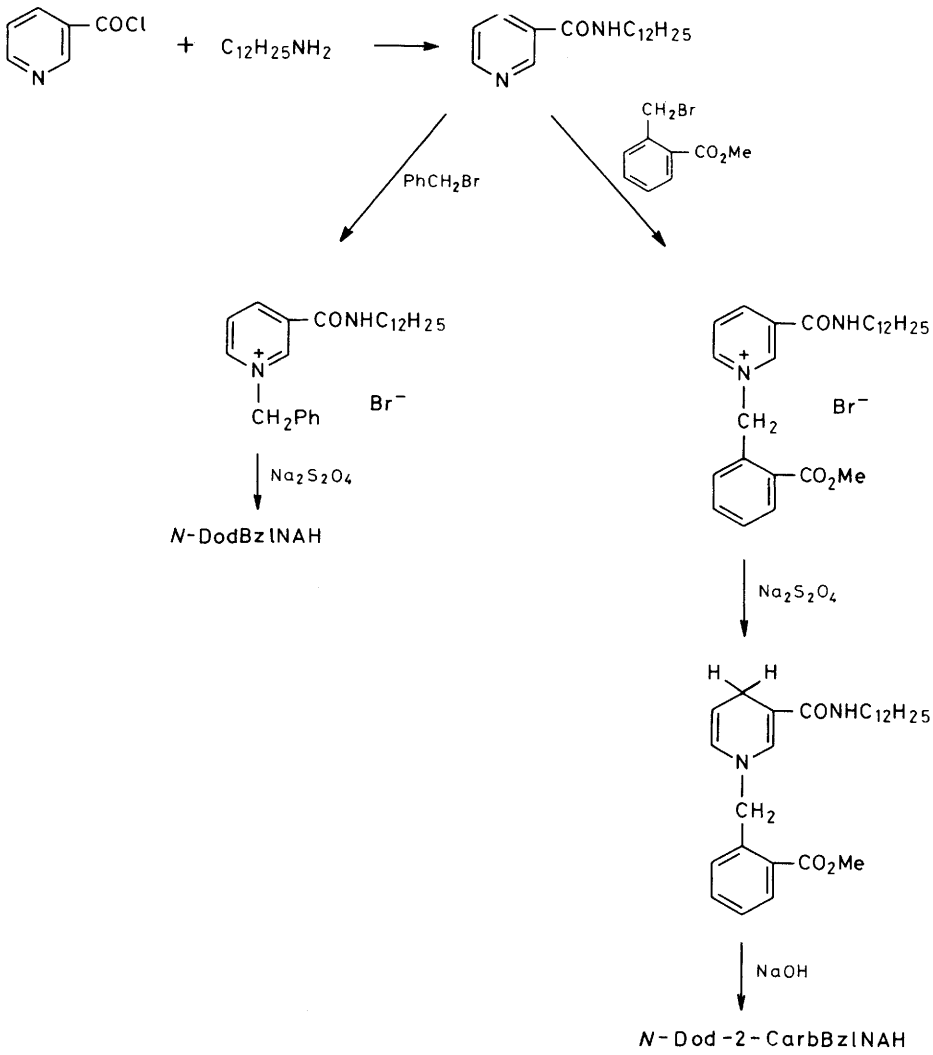
NADH Model Compounds.—PrNAH and BzlNAH were prepared according to the methods described in the literature.[38,39]

HxdNAH was prepared by the reduction of 1-hexadecylnicotinamide iodide. 1-Hexadecylnicotinamide iodide (2.0 g, 4.2 mmol) and triethylbenzylammonium chloride (4.0 g, 18 mmol) were dissolved in 200 ml of a mixed solvent of ethanol–water (1 : 2 v/v). The solution was stirred, efficiently, under a stream of N_2. To the solution, heated to 40—45 °C, were added, dropwise, 50 ml of an aqueous solution containing sodium dithionite (14.6 g, 86 mmol) and sodium carbonate (7.50 g, 74 mmol). The progress of the reaction was monitored by a t.l.c. method [silica gel, ethyl acetate–methanol (1 : 1)]. The reaction was stopped after 5 h. The aqueous solution was extracted with chloroform, the chloroform layer being subsequently washed five times with water containing $NaHCO_3$. The chloroform solution was dried over sodium sulphate and then concentrated *in vacuo*. The residue (HxdNAH) was a yellow oil at room temperature and solidified in a refrigerator (m.p. *ca.* 5 °C); yield 55%; one spot on t.l.c.; δ(Me_2SO-d_6) 0.84 (t, Me), 1.1—1.3 (m, $C_{14}H_{28}$), 2.91 (t, NCH_2), 3.20 (d, 4-CH_2), 4.50 (m, 5-H), 5.70 (d, 6-H), 6.35 (s, NH_2), and 6.75 (s, 2-H).

N-DodBzlNAH and *N*-Dod-2-CarbBzlNAH were synthesized according to the reaction sequence in Scheme 3.

N^3-Dodecylnicotinamide was prepared from nicotinoyl chloride and dodecylamine in pyridine; m.p. 74—76 °C (lit.,[40] 63—64.5 °C), yield 97%. Subsequently, N^3-dodecylnicotinamide (2.11 g, 7.26 mmol) was treated with benzyl bromide (5.36 g, 31.3 mmol) in 5 ml of methanol (40 h reflux). The solution was concentrated to dryness, the residue being recrystallised from acetone; N^3-dodecyl-1-benzylnicotinamide bromide, m.p. 79—81 °C, yield 30%. N^3-Dodecyl-1-benzylnicotinamide bromide was reduced to *N*-DodBzlNAH by sodium dithionite in a manner similar to that used for the preparation of HxdNAH; yellow oil (m.p. *ca.* 5 °C), yield 45%; one spot on t.l.c.; δ(Me_2SO-d_6) 0.90 (t, Me), 1.1—1.4 (m, $C_{10}H_{20}$), 2.70 (d, 4-CH_2), 3.30 (m, N^1CH_2 and N^3CH_2), 4.40 (m, 5-H), 5.35 (d, 6-H), and 7.21 (m, Ph and 2-H).

Similarly, treatment of N^3-dodecylnicotinamide with methyl *o*-(α-bromo)toluate [41] gave N^3-dodecyl-1-(2′-methoxycarbonylbenzyl)nicotinamide bromide in 76% yield, m.p. 76—78 °C (Found: C, 62.3; H, 7.7; N, 5.5. $C_{27}H_{39}BrN_2O_3$ requires C, 62.4; H, 7.6; N, 5.4%); δ(Me_2SO-d_6) 0.89 (t, Me), 1.1—1.3 (m, $C_{10}H_{20}$), 3.41 (t, N^3CH_2), 3.82 (s, CO_2Me), 6.30 (s, N^1CH_2), 7.5—8.4 (m, Ph), 9.05 (m, 4- and 5-H), 9.30 (d, 6-H), and 10.00 (s, 2-H). N^3-Dodecyl-1-(2′-methoxycarbonylbenzyl)nicotinamide bromide was reduced to N^3-dodecyl-1-(2′-methoxycarbonylbenzyl)-1,4-dihydronicotinamide by sodium dithionite in a manner similar to that used for the preparation of HxdNAH; m.p. *ca.* 30 °C; yield 79%; one spot on t.l.c.; δ(Me_2SO-d_6) 0.88 (t, CH_3), 1.1—1.3 (m, $C_{10}H_{20}$), 3.20 (d and t, 4-CH_2 and N^3CH_2, respectively), 3.87 (s, CO_2Me), 4.64 (s, N^1CH_2), 5.2 (m, 5-H), 5.70 (d, 6-H), 7.00 (s, 2-H), and 7.2–7.8 (m, Ph). Finally, the 1,4-dihydronicotinamide was hydrolysed to *N*-Dod-2-CarbBzlNAH by NaOH



Scheme 3.

in methanol–water (5 : 3 v/v) (40—45 °C, 6 h). The sodium salt of *N*-Dod-2-CarbBzlNAH was recovered as a precipitate on the addition of NaCl; m.p. 86—90 °C; yield 78%; one spot on t.l.c.; δ(Me_2SO-d_6) 0.94 (t, Me), 1.6 (m, $C_{10}H_{20}$), 3.03 (d, 4-CH_2), 3.31 (t, N^3CH_2), 4.70 (s, N^1CH_2), 5.22 (m, 5-H), 5.90 (d, 6-H), 6.95 (s, 2-H), 7.0—7.8 (m, Ph).

The polymeric NADH model compound was prepared by copolymerisation of 1-(4′-vinylbenzyl)nicotinamide chloride (0.55 g, 2.0 mmol) [42] and sodium styrene-*p*-sulphonate (8.3 g, 40 mmol) followed by the reduction with sodium dithionite. The polymerisation was carried out in aqueous solution with potassium persulphate (0.11 g, 0.40 mmol) as initiator at 70 °C for 26 h in an N_2-flushed ampoule. The polymer was recovered as a precipitate by pouring into excess of methanol, yield 56%. The content of the NAD model unit was estimated to be 8.8 mol% from elemental analysis. The polymer (1.0 g) was dissolved in 500 ml of water. The solution was stirred efficiently under a stream of N_2 and an aqueous solution containing sodium carbonate (0.88 g) and sodium dithionite (0.40 g, 2.3 mmol) was added dropwise. After 2 h, the polymer was recovered as a precipitate by adding NaCl. Assuming the molar absorptivity of the 1,4-dihydronicotinamide ($\lambda_{max.}$ in water, 350 nm) to be 7 000 l mol^{-1} cm^{-1}, 85% of the NAD model unit was converted into the 1,4-dihydronicotinamide unit.

Other Materials.—CTAB, SDS, and acrylamide were recrystallised from ethanol before use. Brij-35 was purchased from Wako Pure Chem. Co. Ltd. and used without further purification. CDNBS was prepared according to the method of Kurz and Frieden.[31]

Reaction Methods and Product Analyses.—A typical method for the reaction of NADH models with TNBS is as follows. An NADH model compound was dissolved in a degassed buffered heavy water (99.9% purity) solution (containing surfactant if necessary). The solution was stirred at 30–35 °C under a stream of N_2 and then TNBS was added. The reaction was usually continued for 2 h and the solution was extracted with chloroform. TNB was isolated by the t.l.c. method from the chloroform solution: the yields of TNB determined by g.l.c. were 50—65%, on the basis of NADH model compounds. On the other hand, the yields of the oxidised NAD model compounds, which were determined by analysing the aqueous solution with a high-speed t.l.c. scanner (Shimadzu CS-920), were 96—98%. Further details of the reaction method are recorded in a footnote to Table 4.

The method for the reaction of NADH models with CDNBS is similar to that described above, but the isolation method is somewhat different. After the reaction, the solution was adjusted to pH 1 with concentrated HCl and then extracted

with chloroform. Concentration of the chloroform solution gave a yellow solid. The residual solid was dissolved in methanol and the solution was evaporated *in vacuo* to dryness. This operation was repeated. The residue was then taken up in anhydrous ether and treated with diazomethane. Finally, the product was purified as methyl 3,5-dinitrobenzoate by the t.l.c. method.

The deuterium content in TNB and methyl 3,5-dinitrobenzoate was estimated by mass spectroscopy: the natural abundance values $[(M^+ + 1)/M^+]$ are 7.91 and 9.74%, respectively. To confirm the method, we prepared trinitrobenzene containing various amounts of deuterium by reduction of TNBS with mono- and partially di-deuteriated BzlNAH and compared the mass spectral data with the n.m.r. data. The two methods provided an identical result, within 5% relative error.

The polymerisation of acrylamide was carried out using an ampoule with a side-arm with a valve-cock. Acrylamide and PPS were dissolved in a buffered aqueous solution (containing surfactant if necessary). The ampoule was sealed by a stopper and immersed in a dry ice–methanol bath. The ampoule was flushed with nitrogen through the side-arm by the freezing and thawing method. The ampoule was then immersed in a water bath thermostatted at 30 ± 0.2 °C. Under a stream of N_2 from the side-arm the stopper was opened and a methanol solution containing an NADH model compound was added. After sealing the ampoule by the stopper and the valve-cock in the side-arm, the polymerisation was continued for the appropriate time. The aqueous solution was poured into excess of methanol and the polymer (when it precipitated) was recovered. After drying *in vacuo*, the yields of the polymer were determined. The oxidised NAD model compounds were detected in the aqueous solution in 92—97% yields.

Kinetic Measurements.—The kinetic measurements were carried out spectrophotometrically in a thermostatted cell-holder by monitoring the disappearance of the absorption band of NADH model compounds. The reactions were carried out anaerobically in a modified Thunberg cuvette. The reaction of NADH model compounds (5.00×10^{-5}M) with TNBS (0.500—5.0mM) at 35 ± 0.1 °C satisfied the pseudo-first-order equation in the presence of excess of TNBS, and the pseudo-first-order rate constants (k_1') were proportional to the TNBS concentration. Thus, the second-order rate constants (k_2) for this reaction were calculated from the slopes of the k_1' *versus* [TNBS] plots. On the other hand, the disappearance of NADH model compounds (5.00×10^{-5}M) in the reaction with PPS (0.500—5.0 mM) at 40 ± 0.1 °C did not fully satisfy the pseudo-first-order equation. The apparent first-order rate constants (k_{app}) were thus approximated by the initial slopes. The k_{app} values were proportional to the PPS concentration, so that the apparent second-order rate constants were determined from the slopes of the k_{app} *versus* [PPS] plots.

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