The Effects of Cyclodextrins on the Reduction of Ninhydrin with N-Alkyl-1, 4-dihydronicotinamides

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The effects of adding various cyclodextrins (α-, β- and γ-cyclodextrin) on the redox reaction between N-alkyl-1, 4-dihydronicotinamide (hydride ion donnor) and ninhydrin (hydride ion acceptor) in aqueous solutions were studied kinetically.

These reactions proceed through the hydride ion donnor-acceptor complex (CT complex) formation without cyclodextrin (CD). The presence of CD especially affects the reduction with N-benzyl-1, 4 -dihydronicotinamide (BNAH). β-CD is most effective for decreasing the reduction rate in three CDs. β-CD inhibited the formation of the CT complex by including BNAH molecule, which caused a decrease in the reaction rate. β-CD also completely inhibited the hydration of BNAH.

γ-CD also included BNAH, however, it had little effect on the reduction rate constant. α-CD had no effect on both the reduction and the hydration. These inhibition behaviors were caused by an inclusion complex formation with CD.

INTRODUCTION

The coenzyme, nicotinamide adenine dinucleotide (NAD^{+}), has received widespread attention. This coenzyme plays an important role in a large number of enzyme catalyzed redox reactions[1]. The characteristic event in these reactions is the stereospecific transfer of a hydride ion from the substrate to the 4 position of the nicotinamide moiety of NAD^{+} (scheme 1) :
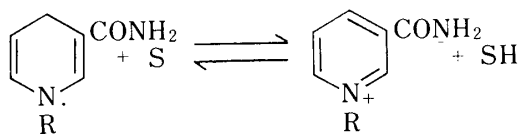
scheme 1

To elucidate and simulate the reaction mecha-

nism of this process, many biomimetic reactions for hydrogen transfer involving various dihydropyridine derivatives have been devised and investigated[2].

CDs are toroidial cyclic oligosaccharides consisting of glucopyranose units which are joined together by a α-1, 4-linkage. α-, β- and γ-CD are composed of six, seven, and eight glucose units, respectively. They provide their central cavity to bind good-sized organic molecules and so-called host-guest complex[3]. Many modified CDs which have reactive sites have been studied as models for enzyme systems[4]. In the enzyme model, this hydrophobic cavity is used to bind the lipophilic part of the substrate molecule[5].

We have previously reported cyclodextrin-nicotinamide derivatives (I and II) as NADH model compounds[6-8], which have a nicotinamide group on the primary hydroxy-side of the β-CD. The corresponding reduced forms can reduce ninhydrin with large rate enhancements. They show enzyme-like

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I   II
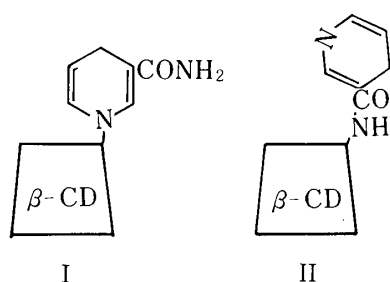
saturation kinetics during the reduction of ninhy-drin which indicates that the reaction involves complex formation. However, in those reports, only how nicotinamide moieties joined with CD activated the reduction had been examined. It had not been studied whether the nicotinamide moieties without a CD ring had reduction reactivity or not, and whether they were able to form the complex with the substrate. Even if the CD do not partici-pate in the reaction as a reactant or particular catalyst, the control of the reaction may be caused by conformational effects. We wish to call these effects "conformational effector" at the molecular level.

We have examined nicotinamide derivatives without a CD ring as a fundamental concept of the NADH enzyme models. In this paper, we wish to report the reduction of ninhydrin with nicotinamide derivatives without a CD ring and the effect of adding parent CD as a "conformational effector" in aqueous solutions.

EXPERIMENTAL

Materials. N-Benzylnicotinamide in reduced form (BNAH) was prepared as follows[10] : A mix-ture of benzyl chloride and nicotinamide was stir-red under nitrogen at 120-130°C for 2 h. Excess benzyl chloride was removed *in vacuo* and the residue was purified by recrystallization from meth-anol. The yield of BNA was 73 %. ^{1}H-NMR(D_2O) δ 6.14(CH_2, 2H), 7.74(benzene ring, 5H), 8.42, 9.18, 9.50 ppm(pyridine ring, 4H) ; IR(KBr) 650, 750, 1440 cm^{-1}. BNA was treated with an aqueous

solution of sodium dithionite in 7.5×10^{-2} M sodium carbonate in an ice bath and protected from room light under a nitrogen atmosphere. After the mixture was stirred for 4 h, the aqueous solution was extracted with chloroform. The organic layer contained only the reduced form (BNAH). Re-crystallization was performed from ethanol. N-Methylnicotinamide (MNA) was obtained by the treatment with methyl iodide and nicotinamide in methanol, and recrystallized from methanol (72 % yield) : ^{1}H-NMR(D_2O) δ 4.6(CH_3, 3H), 8.42, 9.18, 9.50 ppm(pyridine ring, 4H) ; IR(KBr) 1390, 1455 cm^{-1}. The reduction method was the same as in BNAH. The purity of both hydrogenated products was checked by a redox titration.

CD and ninhydrin were purified by recrystalliza-tion from water.

Kinetics. The kinetic experiments for the reduc-tion of ninhydrin were initiated by injecting a 10 μl stock solution of BNAH or MNAH into 3.0 ml of phosphate buffer solution (pH 7.0) containing nin-hydrin and were controlled at 30.0 ± 0.5 °C under a nitrogen atmosphere. The course of the reaction was followed by measuring the absorbance at 360 nm (Fig. 1, Fig. 2). The concentration of CD was in excess over that of BNAH or MNAH and varied from 0 to 1×10^{-2} M. The maximum catalytic rate constants (k_2) and dissociation constants(K_c) between the dihydronicotinamide derivatives and ninhydrin were obtained from Lineweaver-Burk plots. The kinetic experiments for the hydration of BNAH or MNAH were also initiated by inject-ing a stock solution of BNAH or MNAH into 3.0 ml of phosphate buffer solution and measuring the absorbance at 360 nm. The concentration of CD was varied from 0 to 1×10^{-2} M. Dissociation constants (K_{cd}) between CD and BNAH were obtained from a modified Lineweaver-Burk plots (eq. 3) in the reaction and Lineweaver-Burk plots for the hydration.
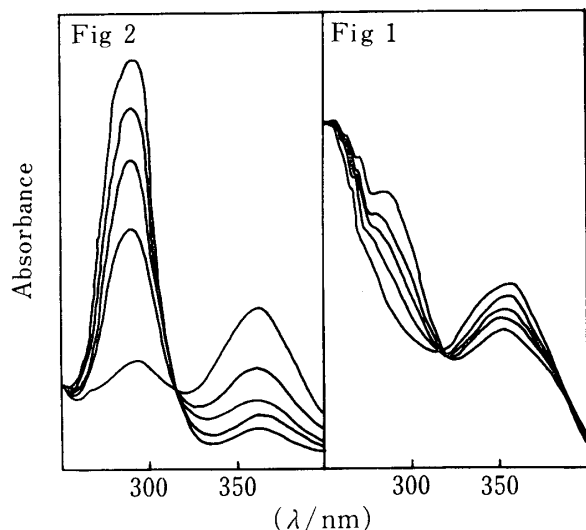
Fig. 1 and Fig. 2. UV spectra of BNAH and MNAH in pH 7.0 phosphate buffer.

RESULTS AND DISCUSSION

Reduction of Ninhydrin with N-Alkyl-1, 4-dihydronicotinamide in Aqueous Solutions.

UV spectra of BNAH and MNAH in pH 7.0 phosphate buffer are shown in Figs. 1 and 2, respectively. These spectra show the characteristic peaks around 360 nm for the reduced form of the nicotinamide derivatives. Adding ninhydrin to the solution, dihydronicotinamide moieties with changing nicotinamide moieties, caused a decrease in the absorbance at 360 nm, while the other absorbances increased. In the absence of ninhydrin, the UV spectra changed very slowly. This change was caused by hydration of N-alkyl-1, 4-nicotinamide[11] as shown in scheme 2 :
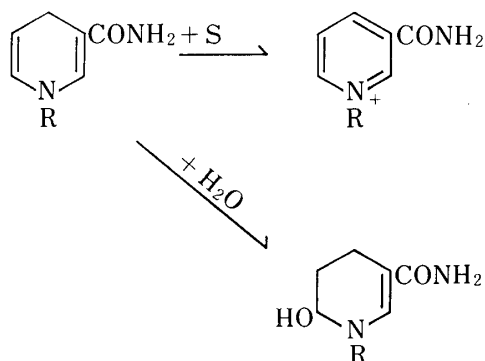
S : a suitable hydride acceptor

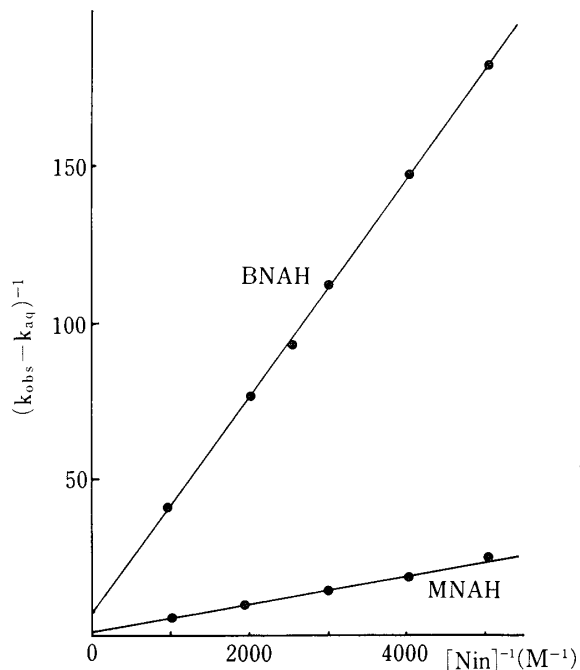The difference between the hydrated form and

Fig. 3. Lineweaver-Burk plots of the reduction with BNAH or MNAH in aqueous solutions.

oxidized form of the nicotinamide derivatives can be observed in the UV spectra. The rate constant of hydration was $3.74 \times 10^{-4} s^{-1}$ and $1.16 \times 10^{-3} s^{-1}$ for BNAH and MNAH, respectively.

Fig. 3 shows the Lineweaver-Burk plots for the reduction of ninhydrin with BNAH or MNAH. The plots were drawn as straight lines. It has previously been reported that the hydride ion transfer in acetonitrile from BNAH to a p-benzoquinone derivative(Q) occurs via the CT complex formed betwen BNAH and Q[12]. In an aqueous solution, the reduction also proceeds through the complex formation between N-alkyl-1, 4-dihydronicotinamide and ninhydrin in a 1 : 1 ratio(scheme 3) :

$$\text{RNAH} + \text{Nin} \underset{\longleftarrow}{\overset{Kc}{\rightleftharpoons}} [\text{RNAH} \cdot \text{Nin}] \xrightarrow{k_2} \text{RNA}^+ + \text{NinH}^-$$

R = benzyl BNAH

= methyl MNAH

scheme 3

The kinetic parameters are shown in Table 1. The Kc value between BNAH and ninhydrin was

Table 1. Kinetic parameters in the reduction [a]

nicotinamide derivatives	$10^4 k_{aq}$[b] (s^{-1})	$10^4 Kc$ (M)	$10^2 k_2$ (s^{-1})	$10 k_2/Kc$
BNAH	3.76	46.5	12.8	2.75
MNAH	11.6	42.6	92.3	21.7
nicotinamide-CD (I)[6]	—[c]	21.0	2.0	0.99
nicotinamide-CD (II)[7]	—[c]	106	145	13.7

a) [ninhydrin] $=2.0\sim10.0\times10^{-4}$M, pH7.0 phosphate buffer,
b) k_{aq} is the rate constant of hydration of nicotinamide derivatives according to scheme 2.
c) not reported

almost the same as that between MNAH and ninhydrin. The values were half to that of I[6] and two times to that of II[7]. It seems that the driving force of complex formation was due to the nicotinamide moiety and that the role of the CD cavity was recognition of the guest molecules.

The k_2 value for MNAH was 7 times larger than that for BNAH which caused the difference in the overall rate constant (k_2/Kc).

The Effects of Adding CDs on the Reduction with Nicotinamide Derivatives. Table 2 shows the effect of α-, β- and γ-CD on the rate constants of the reduction of ninhydrin with BNAH and MNAH. The effect of adding CD on the rate constant was not observed using MNAH as a catalyst. When using BNAH as a catalyst, β- and γ-CD had a significant effect on the reduction. These inhibition effects seemed to be caused by a complex formation between CD and ninhydrin or nicotinamide derivatives(RNAH). If the effect of adding CD is caused by the interaction between CD and ninhydrin, the differences between MNAH and BNAH should not be observed. So, these inhibition effects were caused by a complex formation between CD and BNAH.

Table 2. The effects on the rate constants of reduction of ninhydrin with nicotinamide derivatives

CD \ RNAH	$10^3 k_{obs}$ (s^{-1})[a]	
	MNAH	BNAH
In the absence of CD	95.0	10.52
α-CD	—[b]	8.48
β-CD	101	3.73
γ-CD	98.7	7.05

a) [ninhydrin] $=5.00\times10^{-4}$M, [CD] $=1.00\times10^{-3}$M, [MNAH] $=$ [BNMA] $=5.00\times10^{-5}$M, temperature : 30.0 ± 0.5 °C
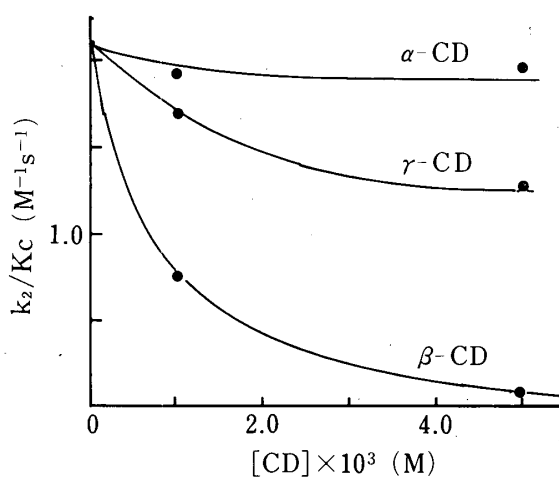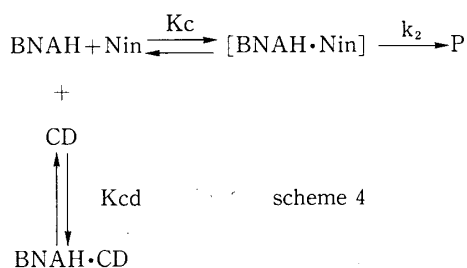b) not examined.

Fig. 4. Inhibition effect for reduction with BNAH by adding three CDs.

As is shown in Fig. 4, adding an excess β-CD (1000 equivalent of substrate) caused complete inhibition of the reduction. Adding an excess γ-CD indicated about 60 % decrease in the rate constant and adding α-CD did not have much effect on the reduction rate constants. The effect of CD depended to the cavity size of the CD which indicated that the effect was due to the complex formation with CDs.

The Calculation of Inhibition Constant Kcd of β-CD. From these results, it was suggested that

CD acted as an antagonist as shown in scheme 4 :

$$BNAH + Nin \underset{\longleftarrow}{\overset{Kc}{\longrightarrow}} [BNAH \cdot Nin] \overset{k_2}{\longrightarrow} P$$

$$+$$

$$CD$$

$$\updownarrow \quad Kcd \qquad \qquad scheme\ 4$$

$$BNAH \cdot CD$$

If CD acts as scheme 4, the analysis of the complex formation between CD and BNAH and between BNAH and ninhydrin could be performed using the equations shown below ;

$$\frac{d[P]}{dt} = k_2\ [BNAH \cdot Nin] = k_{obs}[BNAH]_0 \qquad eq.1$$

where [BNAH·Nin] is the concentration of the complex and $[BNAH]_0$ is the concentration at initial stage of BNAH. If $[CD]_0 >> [BNAH]_0$ and $[Nin]_0 >> [BNAH]_0$, eq.1 generates the following equation ;

$$k_{obs}[BNAH]_0 = \frac{k_2[BNAH]_0\ [Nin]_0}{Kc(1+Kcd[CD]_0) + [Nin]_0}$$

$$eq.2$$

where Kcd is the dissociation constant between CD and BNAH. When eq.2 was reciprocated, eq.2 changes the following equation :

$$\frac{1}{k_{obs}} = \frac{Kc(1+Kcd[CD]_0)}{k_2[Nin]_0} + \frac{1}{k_2} \qquad eq.3$$

This equation was the same as the Lineweaver-Burk plot if $Kc(1+Kcd[CD]_0)$ replaces Kc. Thus, k_2 and $Kc(1+Kcd[CD]_0)$ can be obtained by plotting the reciprocal of k_{obs} against the reciprocal of the ninhydrin concentration. Kcd and Kc can also be obtained by plotting $[CD]_0$ against $Kc(1+Kcd$ $[CD]_0)$. Even in the presence of excess β-CD, the plots were drawn as straight line (Fig.5). The intercept values were all the same which means the k_2 value of the various concentrations were almost the same. The plots of the concentration of CD versus the $Kc(1+Kcd[CD]_0)$ as caluculated from Fig. 5 is shown in Fig. 6. The plots were drawn as a
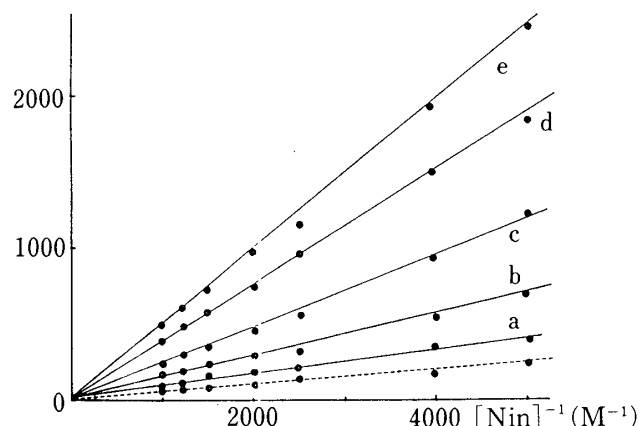

Fig. 5. The dependency of Lineweaver-Burk plots on the concentration of β-CD ; (······) for the plots in the absence of β-CD, [β-CD] are varied 5×10^{-4} M (a), 1×10^{-3} M (b), 2×10^{-3} M (c), 4×10^{-3} M (d) and 5×10^{-3} M (e).
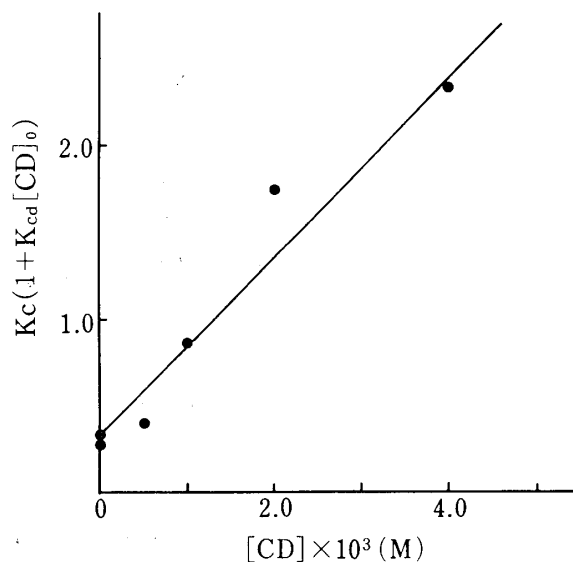

Fig. 6. Modified Lineweaver-Burk plots.

straight line and the value of Kc which was caluculated from Fig. 7 was same as the value of Kc which was described in Table 1. The mechanism of inhibition with β-CD on the reduction was the same as shown in scheme 4. The value of Kcd was 6.6×10^{-4} M.

In the presence of γ-CD, the plots of $1/k_{obs}$ versus the reciprocal of the concentration of ninhydrin were drawn as straight lines, but the plots of Kc $(1+Kcd[CD]_0)$ versus the concentration of CD was not as a straight line. The k_2 values calculated from the former plots (according to eq.3) were not concerted with that without CDs. The mechanism of inhibition with γ-CD on the reduction was not as shown scheme 4. It seems that the cavity size of γ

-CD is too large to inhibit the approach of ninhydrin molecules by a 1 : 1 complexation with BNAH.

The Effects of Adding CD on the Hydration of Dihydronicotinmides.

To support above suggestion, the effects of adding CDs on the hydration reactions were examined. BNAH changes to the hydrated form slowly in an aqueous solution as indicated in scheme 2. This reaction rate was inhibited by adding β- or γ-CD, but not inhibited by adding α-CD. Even if 200 times excess of α-CD was added, the rate constant was the same as that without CD. The dissociation constant (K_{cd}) between CD and BNAH and the maximum rate constant (k') were determined according to the equation:

$$BNAH + CD \underset{\xrightarrow{\hspace{1cm}}}{\overset{K_{cd}}{\rightleftharpoons}} BNAH\ CD$$

$$k_{aq} \searrow \qquad \downarrow k'$$

$$BNAH_{aq}$$
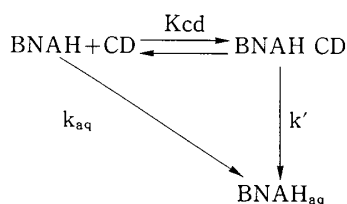
$$\frac{1}{k_{obs} - k_{aq}} = \frac{K_{cd}}{(k_{aq} - k')\,[CD]_0} + \frac{1}{k_{aq} - k'} \qquad eq.4$$

In the presence of various concentrations of β-CD, the plots were drawn as a straight line (Fig. 7). The K_{cd} between β-CD and BNAH was 8.50×10^{-4} M, which is in good conformity to the value calculated from the reduction rate constant. The k' value was $0.00\ s^{-1}$. On the other hand, the K_{cd} between γ-CD and BNAH was 2.97×10^{-2} M (30 times larger than the value in β-CD). Moreover, the k' value was $1.80 \times 10^{-4} s^{-1}$ ($ca.$ 40 % of the k_{aq} value). The above results indicate that β-CD includes one BNAH tightly protecting the double bond between the C-5 and C-6 positions on the pyridine ring. γ-CD was able to include one BNAH, however, the cavity size so large that the inclusion was loose and the hydration could not be completely protected. The cavity size α-CD was too small to include BNAH.
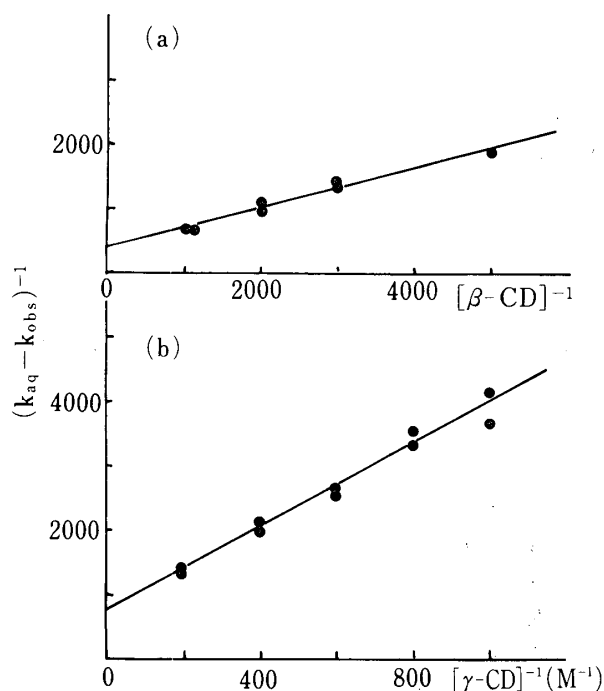

Fig. 7. Lineweaver-Burk plots in the hydration of BNAH in the presence of β-CD(a) and γ-CD (b)

CONCLUSIONS

The reduction reaction with dihydropyridine derivatives in an aqueous solution also proceeds via 1 : 1 complex formation between the dihydropyridine derivative and ninhydrin. The formation constants were comparable with the formation constant between nicotinamide-CD and ninhydrin which had been previously reported. It appears that the rule of the nicotinamide group was not only to act as a reactive site but also a complex formation site, even in nicotinamide-CD (I of II) systems. In order to simulate NADH coenzyme reaction, it is neccessary to study about nicotinamide derivatives not as reactive groups but as a hydride ion donnors with ability of complex formation. CD inhibits both the reduction and hydration. The effect depends on the ring size of CD and all effects are caused by a 1 : 1 complex formation. β-CD is most effective, γ-CD has moderate influence and α-CD has no effect on both reactions. These inhibitions seem to be caused by the inclusion of the benzyl moiety of BNAH.

This is the one example that CD acts as "comformational effector". β-CD indicates only an inhibition in these reaction system. But the results of γ-CD make it possible to use the γ-CD cavity as a "micro-reactor" in which two different kind of molecules such as a catalyst and a substrate can cooperate and react effectively. In this system, the acceleration was not observed. A better choice of substrate will make this system more effective.

REFERENCES

1) H. Sund, *"Pyridine Nucleotide-Dependent Dehydrogenases"*, W. de Gruyter & Co.; Berlin West Germany.

2) R. J. Kill and D. A. Widdowson, in *"Bioorganic Chemistry"*, ed. E. E. van Tamelen, Academic Press, London, 1978, vol IV. p. 239 and reference therein.

3) *"Host-Guest Chemistry"* ed. T. Osa, Kyoritsu-Shuppan, Tokyo, (1979).

4) A. P. Croft and R. A. Bartsh, *Tetrahedron,* **39** 1417 (1983).

5) M. L. Bender and M. Komiyama, *"Cyclodextrin Chemistry"*, Springer, Berlin, (1978).

6) M. Kojima, F. Toda and K. Hattori, *J.C.S. Perkin I,* 1647 (1981).

7) S. Tsurumi, Master thesis dedicated to the Faculty of Engineering, Tokyo Institute of Polytechnics, (1982).

8) K. Hattori and F. Toda, *J. Syn. Org. Chem., Japan,* **40** 1180, (1982)

9) K. Takahashi, Doctral thesis dedicated to Pharmaceutical Institute, Tohoku University, (1986).

10) P. Karrer and F. J. Stare, *Helv. Chim.,* **20** 418 (1937).

11) Y. Choi Chung, T. Ikeda, R. Kojin, H. Ikeda and F. Toda, Abstracts of 50th Conference of J.C.S., **II** 854 (1985)

12) S. Fukuzumi, N. Nishizawa and T. Tanaka, *Chem. Lett.,* **1983**, 1755.