# INTERACTIONS OF METHYL-β-CYCLODEXTRIN WITH ADENINE AND PYRIDINE/NICOTINAMIDE DERIVATIVES AS DETERMINED BY COMPETITIVE FLUORESCENCE METHODS

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## ABSTRACT

*In comparison with β-cyclodextrin, methyl-β-cyclodextrin was more effective in binding TNS, as indicated by the higher fluorescence intensity enhancement and the higher association constant for the formation of the inclusion compound. Using a competitive fluorescence technique with the dye TNS, adenine and pyridine derivatives were observed to be relatively poor ligands, whereas, $N^1$-alkylnicotinamide chlorides formed inclusion compounds much more effectively with methyl-β-cyclodextrin. The effectiveness of binding of $N^1$-alkylnicotinamide chlorides increased as a function of the size of the alkyl substituent, with a positive chainlength effect being observed between the $N^1$-heptyl and $N^1$-dodecyl derivatives. The importance of nonpolar interactions in the binding of these derivatives was evident by the observed change in free energy per methylene unit of -0.548 kcal/mole.*

## INTRODUCTION

Cyclodextrins (Cds) are cyclic D-glucopyranoses bonded through α-1,4-glucosidic linkages. Three major cyclodextrins, referred to as α-, β-, and γ-forms (6,7,and 8 glucose units, respectively), are produced from starch by bacterial cyclodextrin glycosyltransferase (CGTase).[1] Different ratios of cyclodextrin products are formed from CGTases of several organisms reported.[2-5] One of the most noteworthy properties of cyclodextrins is the ability to form inclusion complexes with a variety of organic and inorganic compounds which are trapped within their hydrophobic cavities and stabilized by non-covalent interactions such as hydrophobic, Van der Waals, and hydrogen bonding.[6-7] Modification of natural cyclodextrins by chemical or enzymatic methods gives rise to several forms of modified cyclodextrins with a wide range of desired characteristics.[8] This leads to extensive applications of cyclodextrins in various fields of industry ranging from food, pharmaceutical, cosmetics and toiletries, agrochemical, chemical technology, and plastics.[9-10]

Inclusion complex formation has long been an important area in cyclodextrin research. A considerable number of potential inclusion guest molecules of varying size and hydrophobicity have been studied in an attempt to elucidate further desired properties of guest molecules and to produce complexes for a variety of applications. In this respect, Kondo, *et al.*[11] found that 6-*p*-toluidinylnapthalene-2-sulfonate (TNS) exhibits remarkable fluorescence enhancement in the presence of cyclodextrins, and established a method using TNS as a probe for monitoring hydrolysis of cyclodextrins by amylases. Another application reported by Demont, *et al.*,[12] involves the study of potential guest molecules capable of competing with TNS for occupancy within the cyclodextrin cavity and therefore, allowing for the determination of binding constants for guest-cyclodextrins complexes through spectrofluorometric measurements.

The current study was designed to apply competitive fluorescence techniques for the investigation of potential ligands for competition with TNS in forming inclusion compounds with suitable cyclodextrins. Association constants for cyclodextrin inclusion compounds with TNS and with ligands such as adenine, pyridine, and $N^1$-alkylnicotinamide derivatives will be determined and compared.

## MATERIALS AND METHODS

### Materials

TNS (potassium salt), β-cyclodextrin, methyl-β-cyclodextrin, NAD, and all adenine derivatives were purchased from Sigma. $N^1$-Benzyl-3-acetylpyridinium chloride and $N^1$-alkylnicotinamide chlorides were prepared as previously described.[13-14]

### Methods

*TNS Titration* : The fluorescence titration of TNS with cyclodextrins was carried out at constant concentrations of TNS. Reaction mixtures (2 ml) contained 10 μM TNS in 0.1 M sodium bicarbonate buffer, pH 9.0 and concentrations of cyclodextrins varied from 0.025 to 0.5 mM. Relative fluorescence intensity of TNS-cyclodextrin complexes was measured with excitation at 366 nm and emission at 460 nm.[11] Association constants for TNS-cyclodextrin complexes were determined from the equation :

$$\Delta F = F_{max}\, C/(K_a^{-1} + C) \quad \text{..... ( a )}$$

where $\Delta F$ = fluorescence enhancement upon complex formation

$F_{max}$ = maximum fluorescence when all TNS are included in cyclodextrin (a constant)

$C$ = total concentration of cyclodextrin

$K_a$ = association constant of TNS-cyclodextrin complex

using nonlinear least-squares regression analysis of the EZ-FIT V 1.1 Computer Program.[15]

*Competitive Binding Assay* : Potential guest molecules were added to reaction mixtures to compete with TNS for occupancy within the cyclodextrin cavity as described by Demont, *et al.*[12] Reaction mixtures (2 ml) contained 10 μM TNS, two different concentrations of the guest molecule, and methyl-β-cyclodextrin varied from 0.025 to 0.2 mM. In studies of adenine and pyridine derivatives as guest molecules, reactions were carried out in 0.05 M Tris-HCl buffer, pH 8.0. There was no significant difference in the fluorescence titration of TNS at pH 8.0 compared to the previous titration at pH 9.0 in bicarbonate buffer. Association constants for methyl-β-cyclodextrin - guest complexes were determined from the equation :

$$\Delta F = F_{max}\, C/([K_a^{-1}\,(1 + G\,K_{\acute{a}}^{-1})] + C) \quad \text{..... ( b )}$$

where $\Delta F$, $F_{max}$, $C$, $K_a$, are as in equation (a) and

$G$ = concentration of guest molecules

$K_{\acute{a}}$ = association constant of guest-cyclodextrin complex

using nonlinear least-squares regression analysis through the competitive inhibition model of the same software program.[15]

*Fluorescence measurements* : All measurements were performed on a Perkin-Elmer 650-40 spectrofluorometer.

## RESULTS

### Interactions of Cyclodextrins with TNS

The formation of inclusion compounds between TNS and two cyclodextrins was studied under current conditions in order to determine association constants ($K_a$) for comparison with the binding of other ligands. The effect of various concentrations of cyclodextrins on the fluorescence intensity of TNS is shown in Figure 1. The more effective binding of TNS with methyl-β-cyclodextrin was indicated by the higher fluorescence intensity enhancement observed compared to that obtained with the TNS complex with β-cyclodextrin. The $K_a$ value for the methyl-β-cyclodextrin-TNS complex was approximately five-times higher than that observed for the β-cyclodextrin-TNS complex (Table 1).

### Interactions of Methyl-β-Cyclodextrin with Adenine and Pyridine Derivatives

A number of adenine and pyridine derivatives were studied as potential guest molecules for the formation of inclusion compounds with cyclodextrins. The effectiveness of complex formation by these ligands was evaluated by measuring the competition between the ligands and TNS for inclusion into cyclodextrins, as indicated by the decrease in fluorescence enhancement of the TNS-methyl-β-cyclodextrin complex in the presence of the ligands. The competitive interaction observed for $N^1$-benzyl-3-acetylpyridinium chloride with methyl-β-cyclodextrin, shown in the plot of 1/fluorescence enhancement versus 1/methyl-β-cyclodextrin concentration (Fig. 2) is characteristic of the data obtained for all of the ligands studied. $K_a$ values obtained are listed in Table 1. Of the ligands studied, the most effective, $N^1$-benzyl-3-acetylpyridinium chloride was still approximately 70-times less effective than TNS in binding.

### Interactions of Methyl-β-Cyclodextrin with $N^1$-Alkylnicotinamide Chlorides

$N^1$-Alkylnicotinamide chlorides of varying chainlength were studied as potential ligands for competition with TNS in forming inclusion compounds with methyl-β-cyclodextrin. The competitive interaction for $N^1$-decylnicotinamide chloride shown in Figure 3 is typical of those obtained with all of the quaternary nicotinamide derivatives studied. The $K_a$ values obtained in these studies are listed in Table 2. The most effective competing ligand was $N^1$-cetylnicotinamide chloride with a $K_a$ value of 45.45 $mM^{-1}$. The smaller chainlength derivatives, $N^1$-butyl and $N^1$-pentylnicotinamide chlorides were the least effective ligands exhibiting $K_a$ values of 0.1 $mM^{-1}$. A positive chainlength effect was observed in the binding of $N^1$-alkylnicotinamide chlorides to methyl-β-cyclodextrin between the $N^1$-heptyl and $N^1$-dodecyl derivatives. The linear relationship between the logarithms of the association constants and the number of methylene groups of the $N^1$-alkylnicotinamide chlorides is shown in Figure 4. The fact that the association constant for the $N^1$-benzylnicotinamide chloride was essentially the same as those determined for the hexyl and heptyl derivatives would indicate that the aromatic ring system does not provide for a more effective interaction with this cyclodextrin.

## DISCUSSION

The formation of an inclusion compound between TNS and methyl-β-cyclodextrin is accompanied by a considerable fluorescence enhancement. At 0.1 mM methyl-β-cyclodextrin a 20-fold fluorescence enhancement of TNS was observed (Fig. 1). In comparison, the fluorescence enhancement of TNS with 0.1 mM β-cyclodextrin was only approximately 3-fold. In addition to the higher fluorescence enhancement, the association constant for the methyl-β-cyclodextrin-TNS complex was also essentially 5-times higher than that observed for the β-

Table 1. Association Constants for Cyclodextrin Inclusion Compounds with TNS and Adenine and Pyridine Derivatives

| **Inclusion Complex[a]** | **$K_a$ ($mM^{-1}$)** |
|---|---|
| β-CD/TNS | 2.27 ± 0.16 |
| MCD/TNS | 11.49 ± 1.38 |
| MCD/Adenosine | 0.04 ± 0.007 |
| MCD/Adenosine 5α-carboxylic acid | 0.03 ± 0.004 |
| MCD/5'-AMP | 0.02 ± 0.003 |
| MCD/β-NAD | 0.11 ± 0.024 |
| MCD/$N^1$-Benzyl-3-acetylpyridinium chloride | 0.15 ± 0.017 |

[a]Abbreviations used: β-CD, β-cyclodextrin; MCD, methyl-β-cyclodextrin

Table 2. Association Constants for Methyl-β-cyclodextrin Inclusion Compounds with $N^1$-Alkylnicotinamide Chlorides

| **Inclusion Complex[a]** | **$K_a$ ($mM^{-1}$)** |
|---|---|
| MCD/$N^1$-Butylnicotinamide Chloride | 0.10 ± 0.013 |
| MCD/$N^1$-Pentylnicotinamide Chloride | 0.09 ± 0.017 |
| MCD/$N^1$-Hexylnicotinamide Chloride | 0.15 ± 0.015 |
| MCD/$N^1$-Heptylnicotinamide Chloride | 0.15 ± 0.024 |
| MCD/$N^1$-Octylnicotinamide Chloride | 0.43 ± 0.040 |
| MCD/$N^1$-Nonylnicotinamide Chloride | 1.09 ± 0.10 |
| MCD/$N^1$-Decylnicotinamide Chloride | 3.52 ± 0.18 |
| MCD/$N^1$-Undecylnicotinamide Chloride | 6.71 ± 0.59 |
| MCD/$N^1$-Dodecylnicotinamide Chloride | 15.38 ± 1.83 |
| MCD/$N^1$-Cetylnicotinamide Chloride | 45.45 ± 7.27 |
| MCD/$N^1$-Benzylnicotinamide Chloride | 0.12 ± 0.013 |

[a]Abbreviation used: MCD, methyl-β-cyclodextrin

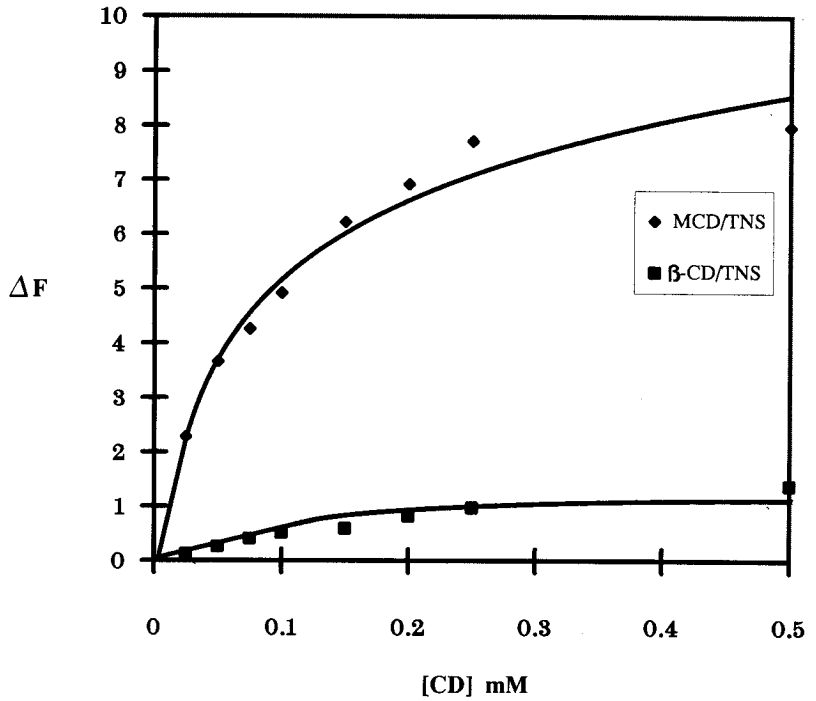


**Fig. 1.** Fluorescence Intensity of TNS as a function of increasing β- and methyl-β-cyclodextrin concentrations. TNS concentration was 10 μM in 0.1 M sodium bicarbonate buffer, pH 9.0. Fluorescence measurements were performed with excitation at 366 nm and emission at 460 nm. β-CD/TNS (■ – ■); methyl-β-CD/TNS (◆ – ◆)

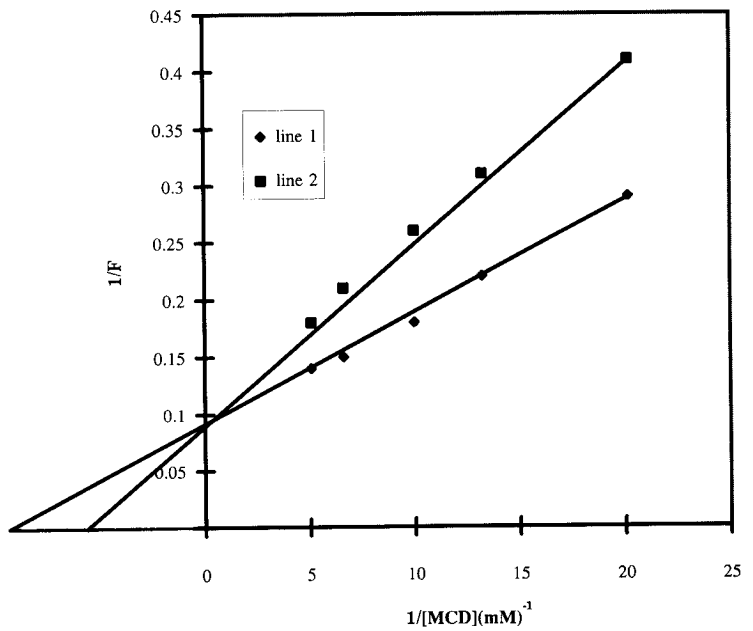


**Fig. 2.** Competition between N[1]-benzyl-3-acetylpyridinium chloride and TNS for occupancy in methyl-β-cyclodextrin. Methyl-β-cyclodextrin concentration was varied from 0.05 to 0.2 mM. Reaction mixtures were as described under Methods. The concentrations of N[1]-benzyl-3-acetylpyridinium chloride used were: Line 1, none (◆ – ◆); line 2, 5 mM. (■ – ■)

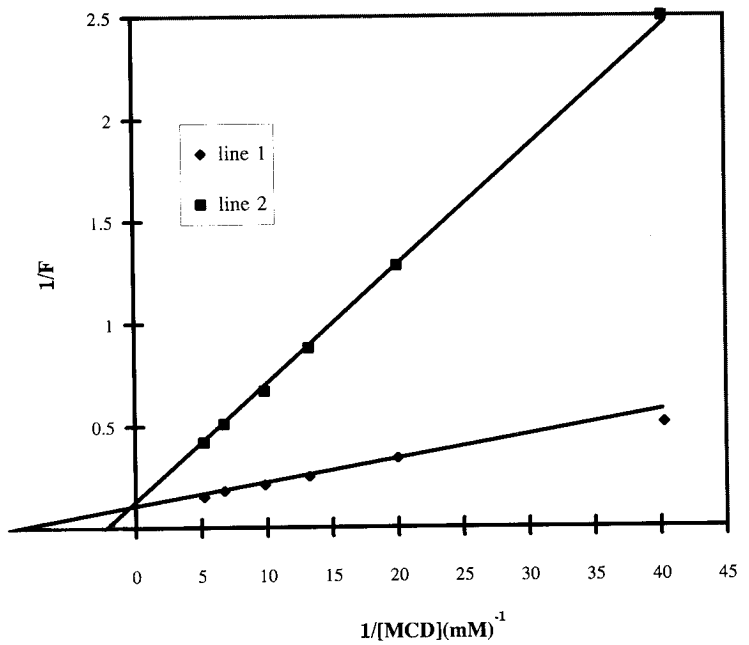


**Fig. 3.** Competition between $N^1$-decylnicotinamide chloride and TNS for occupancy in methyl-β-cyclodextrin. Methyl-β-cyclodextrin concentration was varied from 0.025 to 0.2 mM. Reaction mixtures were as described under Methods. The concentrations of $N^1$-decyl- nicotinamide chloride used were: Line 1, none (◆ – ◆); line 2, 1.25 mM. (■ – ■)

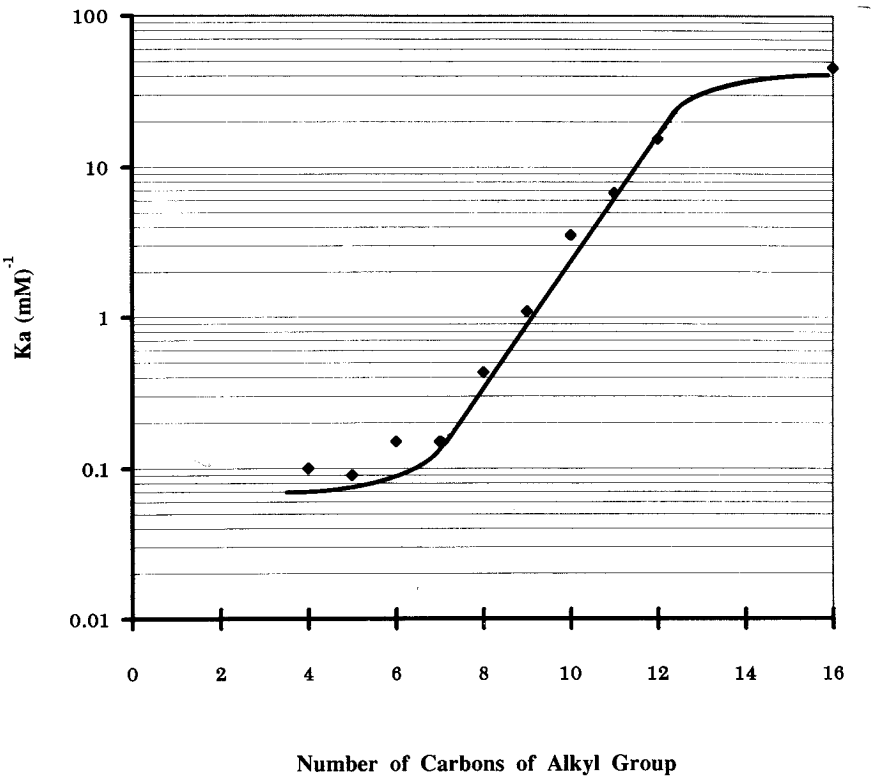


**Fig. 4.** The relationship of the logarithm of the association constant to the chainlength of the alkyl substituent of the $N^1$-alkylnicotinamide derivatives.

cyclodextrin-TNS complex (Table 1). Demont, *et al.*[12] reported the binding of TNS to dimethyl-β-cyclodextrin to be about 3-times higher than the binding of TNS to β-cyclodextrin or to hydroxypropyl-β-cyclodextrin, while binding to the maltosyl derivative was even less effective. No data for the binding of TNS to methyl-β-cyclodextrin was reported in their study. The methyl derivative with a degree of substitution of 10.5-14.7 used in the current study may have cavity dimensions slightly difference from the dimethyl derivative.[16] The steric hindrance due to the substituent groups should be less in the methyl-β-cyclodextrin allowing for a more effective interaction with TNS than in the case of the dimethyl derivative. On the basis of the large fluorescence enhancement and the better binding of TNS to methyl-β-cyclodextrin, this interaction was chosen for competitive studies to measure association constants for the binding of other ligands of interest.

The association constants for methyl-β-cyclodextrin complexes with adenine nucleosides, adenine nucleotides and pyridine derivatives were determined through competitive fluorescence assays. Although the binding of adenosine derivatives was relatively poor, the binding of adenosine was somewhat better than that observed with adenosine-5'-carboxylic acid and the mononucleotide, 5'-AMP. This would be expected due to the negatively charged groups on the carboxylate derivative and the nucleotide. In fact, the 5'-AMP with two negative charges at the pH studied, was less effective than the carboxylate derivative having only one negative charge. The dinucleotide, NAD, unexpectedly exhibited a $K_a$ value approximately 5-times higher than that determined for 5'-AMP It might be considered that the better binding of NAD may reflect properties due to a folded conformation of this dinucleotide molecule. However, a more plausable interpretation may involve a direct interaction between NAD and TNS, such that the NAD induced decrease in the fluorescence enhancement of TNS in the presence of methyl-β-cyclodextrin could be due to a direct interaction of NAD with TNS rather than NAD competing with TNS for inclusion into the cyclodextrin. This latter interpretation has some support from the observation that the fluorescence of TNS is slightly increased in the presence of NAD and the absence of the cyclodextrin (data not shown). The direct interaction of NAD with aromatic compounds was demonstrated in early studies of charge transfer complexes between NAD and indole derivatives.[17]

The formation of inclusion compounds between methyl-β-cyclodextrin and $N^1$-alkylnicotinamide chlorides was significantly influenced by nonpolar interactions of the alkyl substituents of these compounds. Several studies of other guest ligands and using other techniques[18-19] have reported relationships of binding and hydrophobicity with cyclodextrins. Using the competitive fluorescence technique with the dye, TNS applied in the current study, $N^1$-alkylnicotinamide chlorides were shown to be relatively effective ligands for the formation of inclusion compounds with methyl-β-cyclodextrin. The effectiveness of binding of these compounds increased as a function of the size of the alkyl substituent, with a positive chainlength effect being observed between the $N^1$-heptyl and $N^1$-dodecyl derivative (Fig. 4). The smaller alkyl derivatives, the butyl, pentyl, and hexyl compounds exhibited relatively poor binding which may reflect the closer polar environment of the positively charged pyridinium ring nitrogen. From the linear relationship in Figure 4, a change in free energy per methylene group ($\Delta\Delta G/"CH_2"$) of -0.548 kcal/mole was determined. This value lies well within the range suggested for chain-chain interactions through dispersion forces.[20] Similar positive chain-length effects were observed previously[14,21-23] in the binding of $N^1$-alkylnicotinamide chlorides as co-enzyme-competitive inhibitors of several dehydrogenases with $\Delta\Delta G/"CH_2"$ values in the range of -0.4 to -0.6 kcal/mole. The linear positive chainlength effe ct observed in the interactions of the nicotinamide derivatives with methyl-β-cyclodextrin (Fig. 4) could not be

extended to include the larger 16-carbon chain, $N^1$-cetylnicotinamide chloride. Although the cetyl derivative was the most effective ligand of the series, the deviation from linearity would suggest anticipated unfavorable steric interactions with this larger ligand.

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## บทคัดย่อ

เมทธิล-เบตา-ไซโคลเดกซ์ทรินมีประสิทธิภาพในการจับกับ TNS ดีกว่าเบตา-ไซโคลเดกซ์ทริน เมื่อเปรียบเทียบการเพิ่มความเข้มของแสงฟลูออเรสเซนส์และค่าคงที่แอสโซซิเอชันในการสร้างสารประกอบเชิงซ้อนที่สูงกว่า และจากการใช้เทคนิคคอมเพททิทีฟฟลูออเรสเซนส์ โดยการแย่ง TNS จับเมทธิล-เบตา-ไซโคลเดกซ์ทริน พบว่าอนุพันธ์ของอะดีนีนและไพริดีนเป็นลิแกนด์ที่ไม่ดีนัก ขณะที่สารในกลุ่มอนุพันธ์ของนิโคตินาไมด์สร้างสารประกอบกับไซโคลเดกซ์ทรินได้ดีกว่ามาก และความสามารถในการจับระหว่างแอลคิล-นิโคตินาไมด์กับไซโคลเดกซ์ทริน เพิ่มเป็นสัดส่วนโดยตรงกับขนาดของจำนวนคาร์บอนในสายโซ่แอลคิลตั้งแต่อนุพันธ์เฮพติลถึงโดเดซิล ข้อมูลนี้แสดงความสำคัญของปฏิสัมพันธ์นอนโพลาร์ในการยึดจับดังกล่าว โดยค่าพลังงานอิสระต่อกลุ่มเมทธิลลีนเท่ากับ -0.548 กิโลแคลอรีต่อโมล