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Inclusion of Ring A of Cholesterol Inside the β-Cyclodextrin Cavity: Evidence from Oxidation Reactions and Structural Studies

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Abstract. The disposition of cholesterol inside the β-cyclodextrin cavity (β-CD) was deduced from oxidation of cholesterol secondary alcohol groups by $Ca(OCl)_2$ and H_2O_2 in the pyridine–acetic acid system. The amount of cholest-4-ene-3-one formed was found to be proportional to the concentration of β-cyclodextrin, resulting in 56.1% of ketone. The oxidation rate was enhanced by β-cyclodextrin and its methyl, polymer and 1 : 1 copper(II)–β-cyclodextrin derivatives. Detailed investigations involving UV-visible, ^{13}C- and ^{1}H-NMR (T_1, 1D NOE and ROESY) spectroscopic studies were carried out. A binding constant value of 15,385 $\pm$ 1500 M^{-2} was obtained for the 2 : 1 heptakis-2,6-di-*O*-methyl-β-cyclodextrin(DMβ-CD) : cholesterol complex in chloroform from UV studies. Proton and solid state ^{13}C-CP MAS spectra of the β-CD–cholesterol mixture showed large magnitude shifts for the protons from the wider end of the β-CD cavity as well as those of ring A and ring B of cholesterol. Both 1D NOE and ROESY measurements indicated the proximity between ring A and ring B protons of cholesterol and the wider end protons of β-CD and DMβ-CD. Besides, analysis of τ_c, τ_i and τ_m from T_1 measurements showed not only a lowering of rotational motions but a ξ value of 0.016–0.048 for some of the cholesterol protons, typical of a weak complex. Based on these studies, a probable structure for the 2 : 1 complex involving two molecules of β-CD/DMβ-CD was proposed with portions of ring A and ring B being present inside the wider end of the β-CD/DMβ-CD cavity and ring D and the side chain attached at position 17, projecting into the wider end of the second βCD/DMβ-CD molecule.

Key words: β-CD–cholesterol, DMβ-CD–cholesterol, intermolecular NOE.

1. Introduction

Cholesterol-free or low cholesterol foods are very much desired currently, as intake of cholesterol-rich foods results in coronary heart diseases and atherosclerosis [1]. Among several methods available for the removal of cholesterol from milk, egg yolk and animal fats, removal by β-cyclodextrin (β-CD) has recently received much attention [2–4]. β-Cyclodextrin has been shown to form a stable complex with cholesterol (cholest-5-ene-3-β-ol), which is very slightly soluble in water [5]. Though quite an extensive body of literature is available on the inclusion complexation of cholesterol with cyclodextrins [6], exact information on the orientation of a fairly large molecule like cholesterol or any other steroid is not available. The

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present work describes the disposition adopted by cholesterol particularly inside the β-cyclodextrin cavity. Both the reactivity of the included cholesterol and structural features were investigated to arrive at the disposition. Oxidation of the cholesterol secondary alcohol group at position 3 was carried out in the presence of β-CD and its derivatives, namely DMβ-CD and a water insoluble β-CD–epichlorohydrin polymer (β-CD–polymer). Complementary data were obtained from a detailed structural study involving UV-visible, ^{1}H- and ^{13}C-NMR spectroscopic investigations, including 1D NOE, ROESY and T_1 measurements.

2. Materials and Methods

In a typical oxidation reaction, equimolar amounts (2.0 mmoles) of cholesterol (Fluka) and the reagent $Ca(OCl)_2$ (commercial swimming pool bleach with an available chlorine content of 62.8%) along with β-CD (Aldrich) and its derivatives (DMβ-CD and β-CD-polymer prepared from β-CD) [7, 8] in various molar ratios (Table I) were stirred in 20 mL of water or water–benzene (1 : 1) mixture, at 50°C for 6 h. The reaction mixture was acidified and extracted with CH_2Cl_2, dried over anhydrous $MgSO_4$ and concentrated. The products were analysed using a Shimadzu LC 6A HPLC instrument fitted with a μ-porosil silica column, with a time programmed UV detector operating at 233 nm for the first 6 min and at 206 nm for 12 min. Thus, cholest-4-ene-3-one exhibited a retention time of 5.0 min at 233 nm and cholesterol exhibited a retention time of 8.0 min at 206 nm in 2% isopropanol in hexane solvent mixture as the eluent. The flow rate was 1 mL/min of the solvent.

In the case of metal salt oxidation, a mixture of the substrate (3.6 mmoles) and Cu salt (3.6 mmoles) in pyridine (28 mL) and AcOH (5 mL) was stirred at room temperature and to this was added H_2O_2 (13.5 moles) dropwise over a period of 20 min, and the reaction mixture was stirred further at the same temperature for 6 h, neutralized and extracted with CH_2Cl_2. The reaction products were analysed by HPLC as above.

A standard calibration graph was obtained between the amount of cholesterol and cholest-4-ene-3-one injected, against the HPLC peak area, from which the amount of the product formed or unreacted substrate was determined. A value of 63.3% obtained for the control for unreacted cholesterol was considered as 100%, relative to which all the other values were determined. The reaction product was isolated and characterized by its melting point and ^{1}H-NMR spectroscopy.

A Shimadzu UV-240 spectrophotometer was used to study the inclusion complex formation of cholesterol with DMβ-CD.

^{1}H-NMR spectra for structural studies were recorded on a Brüker WH 270 instrument operating at 270 MHz, fitted with a Spectrospin magnet and an Aspect 2000 computer at 20 $\pm$1°C. All signals were referenced to tetramethylsilane (TMS) to within $\pm$0.01 ppm. ^{13}C CP/MAS NMR spectra were recorded at 75 MHz on a Brüker MSL-300 NMR spectrometer operating at room temperature. About 500

Table I. Results of oxidation reactions of cholesterol to cholest-4-ene-3-one.[a]

Solvent	Catalyst (mole equivalent)	Unreacted Cholesterol (%)	Product (%) Cholest-4-ene-3-one
Hypochlorite oxidation			
H_2O	None	100.0	0.0
H_2O	β-CD (0.2)	83.4	16.6
H_2O	β-CD (0.4)	72.3	27.7
H_2O	β-CD (0.6)	68.5	31.5
H_2O	β-CD (0.8)	56.2	43.8
H_2O	β-CD (1.0)	43.9	56.1
H_2O	β-CD-poly (1.0)	76.5	23.5
H_2O	DM-β-CD (1.0)	73.2	26.8
CH_3COOH/CH_3CN (2 : 3)	None	40.8	59.2
H_2O–C_6H_6 (1 : 1)	None	97.0	3.0
H_2O–C_6H_6 (1 : 1)	β-CD (1)	88.9	11.1
H_2O–C_6H_6 (1 : 1)	β-CD-poly (1)	89.2	10.8
H_2O–C_6H_6 (1 : 1)	DM-βCD (1)	66.0	34.0
H_2O–C_6H_6 (1 : 1)	CTAB (1)	54.7	45.3
Copper salt oxidaltion			
C_6H_5N	Cu(II)-βCD	65.0	35.0
C_6H_5N	$Cu(OAc)_2$	77.0	23.0
C_6H_5N	$CuSO_4$	84.2	15.8
C_6H_5N	$CuCl_2$	85.7	14.3
C_6H_5N	None	97.1	2.9

[a] Error values in HPLC measurements are of the order ±5%. 2 mmoles of cholesterol and $Ca(OCl)_2$ were employed for the oxidation at 50° C for 6 hrs. Other reaction conditions including those of copper salts are shown in the Experimental Section.

mg of the sample was packed in the rotor which was spun at 2.5–3.5 kHz. High power decoupling was employed to reduce dipolar broadening. Typically 200–500 scans were accumulated and all signals were referenced relative to glycine at 42.1 ppm to within ±0.2 ppm. A Hartman–Hahn contact time of 1 ms was employed with a total recycle time of 6 s. Concentrations used were: cholesterol =0.05 M, DMβ-CD–cholesterol (1 : 1) = 0.03 M (DMβ-CD = 30 mg in 0.5 mL solvent). The mixture for solid-state ^{13}C-NMR was prepared by dissolving 588 mg of β-CD in 10 mL of hot water. 200 mg cholesterol was added to this solution when it readily gave a turbid solution without a precipitate which was lyophilized to obtain the cholesterol–β-CD mixture.

One-dimensional NOE experiments were carried out on the 270 MHz instrument, typically by accumulating 64 scans for off-resonance and the same number of scans for irradiation at a desired position to obtain the difference for each irradiation

experiment. Irradiations were carried out for a duration of about 3 s to ensure that a sufficient amount of NOE built up during each accummulation. T_1 measurements were carried out on the 270 MHz instrument. An inversion recovery method was used, involving a 180°–τ–90° pulse sequence for determining the T_1 values. About 10–15 τ values were used, ranging from 0.1 to 5.0 s to obtain each set of data. A pulse delay equivalent to $5 \times T_1$ was employed between the pulses to allow all the spins to relax completely. If $A\alpha$ and $A\tau$ are the intensities of the signals, at an infinite delay time and for a given value of τ, respectively, $\ln(A\alpha - A\tau)$ plotted against τ values gave a straight line with a negative slope equal to $1/T_1$, from which T_1 values were obtained. The line of best fit was obtained from least-squares analysis from which the T_1 values were determined.

A rotating frame nuclear Overhauser spectrosocpy (ROESY) spectrum was recorded for the 1 : 1 cholesterol–β-CD mixture by dissolving 30 mg in 0.5 mL of DMSO d_6, on a Brüker AM 400 MHz instrument, operating at 20°C. The following pulse sequence was employed [9]. 90°–t_1–[spin-lock]$_{\phi+\pi/2}$ – Acquisition (t_2). Spin locking was carried out with the lock field of the order of one-half of the spectral width (6.279 ppm). A mixing time of 250–300 ms was employed. Spectra were measured in the phase-sensitive mode using TPPI in order to create amplitude modulation in T_1. A spectral width of 2512 Hz was employed. About 256 FIDS of 1024 data points of 64 scans were collected. The data matrices were zero-filled and multiplied in both dimensions with a $\pi/2$ phase-shifted sine function prior to Fourier transformation of the FID.

3. Results and Discussion

3.1. Oxidation of cholesterol

Oxidation of cholesterol with calcium hypochlorite under aqueous conditions was studied. The results obtained are shown in Table I. The hypochlorite oxidation of cholesterol does not proceed in water. Cholesterol is a slightly surface active, non-polar compound that is extremely insoluble in water. It has a critical micellar concentration of 15 μg/litre (38.79 nM) and a maximum aqueous solubility of only 1.8 mg/litre (4.65 μM) [10]. The problem of insolubility can be overcome by using either a suitable co-solvent (CH_3COOH–CH_3CN) or a phase-transfer catalyst (CTAB), which helps in the transfer of substrate from one phase to the other. Since β-CD has the unique property of transferring substrates from an organic to an aqueous phase, the presence of β-CD as an inverse phase transfer catalyst, increased the yield of cholest-4-ene-3-one. No catalytic effect of β-CD in the present reaction could be found. However, the yield increased linearly with increasing β-CD–cholesterol molar ratio. Maximum yield (56.1%) was observed with an equimolar amount of β-CD (to cholesterol). The presence of β-CD also aided in the exclusive formation of cholest-4-ene-3-one and the total disappearance of other oxidised side products, which were observed in other reactions. The yields in the presence of β-CD-polymer and DMβ-CD were also comparable to that of

βCD (0.4 equivalent). Stronger binding between the product (formed inside the β-CD cavity) and the β-CD molecule than that between the substrate and β-CD, may be responsible for facilitating conversion of β-CD equivalent of cholesterol into ketone and the absence of any observed catalytic effect.

The reaction, when carried out in a liquid–liquid two phase system (water–benzene), gave rise to only 3% of the product. However, addition of β-CD and β-CD-polymer, which are not soluble in benzene, but which can solubilize the substrate in water through inclusion complex formation, gave rise to a higher yield than the control (11.1% and 10.8%, respectively). Addition of DMβ-CD, being soluble in both the phases, remarkably increased the yield of the product to 34.0%. The use of CTAB, as PTC, further increased the yield to 45.3%. The use of $CH_3CN : CH_3COOH$ (2 : 3) resulted in a yield of 59.2% of product ketone in the control. The data obtained under various conditions clearly indicated that β-CD and its derivatives, especially DMβ-CD, not only functioned as an inverse phase transfer catalyst but also as a microsolvent mimicking a 2 : 3 mixture of $CH_3CN : CH_3COOH$ solvent system (Table I).

Oxidation was also attempted with H_2O_2 in pyridine–acetic acid in the presence of a 1 : 1 copper complex of β-CD (Cu(II)–β-CD), $Cu(OAc)_2$, $CuSO_4$ and $CuCl_2$. The results are shown in Table I. The reaction in the presence of Cu(II)–β-CD exhibited a yield of 35.0% of cholest-4-ene-3-one, compared to those of $Cu(OAC)_2$ (23.0%), $CuSO_4$ (15.8%), $CuCl_2$ (14.3%) and control (2.9%). Although copper in Cu(II)–β-CD was shown to be present in the middle of the cavity [11], the greater proportion of cholest-4-ene-3-one obtained in the presence of Cu(II)–β-CD probably indicated that ring A containing the hydroxyl group of cholesterol was included inside the cavity to facilitate oxidation by pyridine-*N*-oxide [12, 13], produced by the action of peracetic acid (formed in situ by H_2O_2 and acetic acid) on pyridine.

Little is known about the structure and the relative orientation of cholesterol within the β-CD cavity. Hence, ^{1}H- and ^{13}C-NMR, one-dimensional difference nuclear Overhauser enhancement (1D NOE), rotating-frame nuclear Overhauser enhancement spectroscopy (ROSEY) and proton spin-lattice relaxation time (T_1) measurements, along with UV-visible spectroscopic studies, were carried out to arrive at the disposition of cholesterol inside β-cyclodextrin cavity.

3.2. NMR SPECTROSCOPY

The differences in chemical shift values observed between free cholesterol and β-CD, in both ^{1}H- as well as ^{13}C-NMR are shown in Tables II and III. The assignments were made from ^{13}C-NMR measurements of ApSimon *et al.* [14] and selective 2D Distortionless Enhancement by Polarization Transfer (DEPT) ^{1}H–^{13}C heteronuclear shift correlation spectra of cholesterol [15–18]. The ^{1}H and ^{13}C assignments of some quarternary and CH_2 atoms may be interchangeable among themselves. Due to poor solubility of the cholesterol–β-CD complex in water,

solid-state ^{13}C-CP-MAS (cross polarisation-magic angle spinning) NMR spectra were recorded for both cholesterol and the mixture. ^{1}H-NMR spectra of β-CD, cholesterol and the mixture were recorded in DMSO d_6, although DMSO d_6 was considered as a decomplexant. Table III gives the data for the DMβ-CD–cholesterol mixture.

From Table II it can be seen that the clearly observable ring B carbon (C-6) of cholesterol is affected, which showed a maximum downfield shift of 2.9 ppm, while C-9 (0.6 ppm), C-16 (0.6 ppm) and C-19 (0.5 ppm) showed reasonable upfield shifts. All the β-CD carbons showed downfield shifts in the order of 3.7–5.6 ppm, indicating inclusion affecting the β-CD carbon signals.

The ^{1}H-NMR spectrum of the β-CD–cholesterol mixture in DMSO d_6 showed smaller differences in the order of 0.01–0.08 ppm, which included ring A and B protons namely 6-H (0.05 ppm), 3-H (0.02 ppm), H-4α, H-4β, 3-OH (0.08 ppm), H-7β (0.03 ppm) and 26-H, 27-H (0.02 ppm, Figure 1). Here also, β-CD signals showed shifts of the order of 0.01–0.09 ppm with the maximum upfield shifts observed for the secondary hydroxyl function (2-OH 0.07 ppm and 3-OH 0.06 ppm) at the wider end of the β-CD cavity and 0.09 ppm downfield shift for H-3. This indicated that inclusion may be through the wider end, since the narrower end signals showed smaller shifts than those mentioned above. The five methyl group signals of cholesterol showed shifts in the order of 0.02–0.04 ppm.

The spectra of cholesterol and those of its DMβ-CD mixture in $CDCl_3$ produced 0.1 ppm difference for the 3-H proton of ring A while the other protons of ring A and ring B showed smaller shifts (0.01–0.02 ppm) (Table III). DMβ-CD signals showed smaller shifts in $CDCl_3$ (0.01–0.7 ppm).

In all these cases, although the other ring protons were not satisfactorily detected, the effects noticed for the protons in ring A and ring B were larger than those of other signals, i.e. both ring A and ring B were affected to a greater degree than the remaining portion of the cholesterol molecule on inclusion. Further confirmation of this disposition was arrived at from the following NMR spectroscopic studies.

3.3. One-dimensional difference nuclear Overhauser enhancement and ROESY measurements

One-dimensional difference NOE spectroscopy was carried out to generate information regarding the proximity between cholesterol and DMβ-CD in $CDCl_3$. Both positive and negative enhancements were observed for some cholesterol protons when DMβ-CD protons were irradiated and vice-versa (Figures 2 and 3, Table IV). Maximum effects were observed for some ring A (H-1, H-4α, β) ring B (6-H, H-7β) and other cholesterol protons (19-H, 18-H), when protons from the wider end of the DMβ-CD cavity, namely H-3, 3-OH and H-2, were irradiated. The reverse was also found to be true. The effect on the ring A and ring B protons, when protons from the middle and narrower ends of the DMβ-CD cavity (H-1, H-4, H-5 and H-6a&b) were irradiated was found to be very small. This clearly indicated

Table II. ^{13}C- and ^{1}H-NMR chemical shift values for free and complexed cholesterol with β-CD.[a]

^{13}C-NMR (solid state)				^{1}H-NMR (DMSO-d_6/$CDCl_3$)			
Carbon signal	Chemical shift (ppm)		Shift in (ppm)	Proton signal	Chemical shift (ppm)		Shift in (ppm)
	Free	Complex			Free	Complex	
				β-CD			
β-CD				2-OH	5.80	5.73	+0.07
C-1	98.5	104.1	−5.6	3-OH	5.74	5.68	+0.06
C-4	77.4	82.3	−4.9	H-1	4.83	4.82	+0.01
C-2,3,5	69.3	73.0	−3.7	6-OH	4.45	4.48	−0.03
C-6	56.3	60.6	−4.3	H-5, H-6a&b	3.60	3.66	−0.06
				H-4	3.60	3.58	+0.02
Cholesterol[a]				H-3	3.31	3.40	−0.09
C-5	142.0	d	–	H-2	3.31	3.34	−0.03
C-6	122.7	125.6	−2.9	Cholesterol[c]			
C-3	71.6	e	–	6-H	5.32	5.27	+0.05
C-17	57.9	57.8	+0.1	3-H	3.48	3.46	+0.02
C-9,14	51.3	50.7	+0.6	H-4α, H-7β, 3-OH	2.20	2.12	+0.08
C-4,7,13	42.5	42.6	−0.1				
C-16	40.6	40.0	+0.6	H-7β	1.96	1.93	+0.03
C-1,2,10,20	37.1	37.1	0	25-H	1.87	–	–
C-8,15	32.4	32.5	−0.1	H-1β, H-11α, H-12α	1.80	1.78	+0.02
C-11,12,25	29.8	28.7	+1.1	H-24α	1.71	–	–
C-15,22	24.5	24.5	0				
C-23,24,26,27	23.0	22.7	+0.3	8-H, H-15α, 20-H	1.40	–	–
C-19,21	20.6	20.1	+0.5	H-22β	1.37	–	–
C-18	12.0	12.0	0	H-1α	1.36	–	–
				H-2αβ, H-15β	1.29	–	–
				H-11β, H-12β	1.27	–	–
				9-H	1.21	–	–
				19-H	0.99	0.95	+0.04
				26-H, 27-H	0.92	0.90	+0.02
				21-H	0.84	0.87	−0.03
				18-H	0.68	0.64	+0.04

[a] ^{1}H and ^{13}C NNR assignments were based on those of Reference [7]. 10 mg cholesterol and 30 mg β-CD/DMCD in 0.5 mL of the solvent.
[b] +ve and −ve indicate upfield and downfield shift, respectively.
[c] Some CH_2 and quarternary carbon assignments are interchangeable.
[d] Could not be detected.
[e] Merged with β-CD signals.

Table III. T_1 values of cholesterol and local motional analysis in cholesterol – DM β-CD mixture in $CDCl_3$.

Signal	Chemical shift (ppm)		T_1 values (s)		Enhancement	T_1	τ_c ($\times 10^{-13}$ s)		τ_i ($\times 10^{-13}$ s)		τ_c/τ_m
	Free	Complex	Free	Complex	in T_1 (%)	(complex) (s)	Free	Complex	Free	Complex	(complex)
Cholesterol–DMβ-CD											
6-H	5.33	5.32	1.58	2.22	+40.04	2.22	2.13	1.52	0.0005	a	0.01
3-OH	5.05	5.05	–	0.75	–	–	–	–	–	–	–
H-1	4.96	4.94	–	0.36	–	–	–	–	–	–	–
H-3	3.90	3.90	–	1.49	–	–	–	–	–	–	–
3-H	3.51	3.61	1.56	0.66	−57.77	0.66	2.17	5.13	0.034	3.12	0.038
5-H, 6-Hab	3.68	3.61	–	–	–	–	–	–	–	–	–
2-OCH_3	3.60										
H-4	3.44	3.37	–	0.67	–	–	–	–	–	–	–
6-OCH^3	3.37										
H-2	3.25	3.23	–	0.47	–	–	–	–	–	–	–
H-4α, H-4β	2.25	2.26	1.49	2.07	+38.87	2.07	2.27	1.63	0.14	b	0.01
H-7β	1.98	1.97	1.69	0.51	−69.26	0.52	1.99	6.50	b	4.58	0.05
3-OH	1.72	1.75	0.35	1.54	+342.81	1.54	9.73	2.20	8.19	0.07	0.02
19-H	0.99	0.98	1.53	0.68	−55.23	0.68	2.21	4.94	0.079	2.91	0.04
21-H1, 26-H-	0.90	0.88	1.65	1.26	−23.57	1.26	2.04	2.67	b	0.55	0.02
27-H	0.84	0.83	1.33	1.60	+20.86	1.60	2.55	2.11	0.42	b	0.015
H-18	0.66	0.65	1.49	0.71	−52.29	0.71	2.27	4.76	0.14	2.72	0.035

[a] Some peaks were composite of signals from different groups. Concentrations same as in Table II.
[b] Could not be determined satisfactorily.

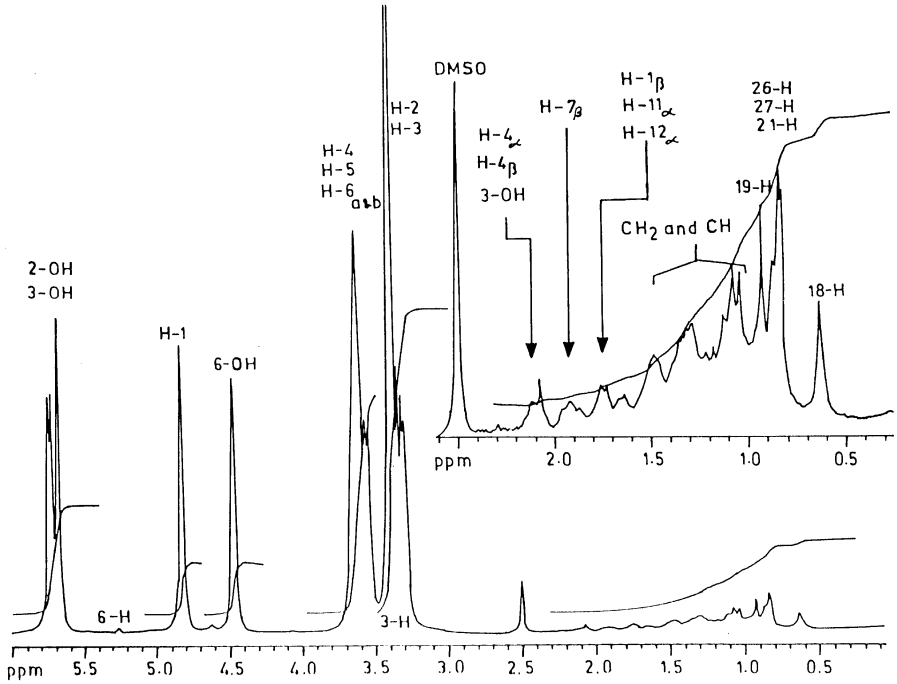

Figure 1. 270 MHz ^{1}H-NMR spectrum of 50 mg of the β-CD–cholesterol (1 : 1) mixture in DMSO d_6. The inset shows the expanded region between 0–2.5 ppm showing cholesterol signals.

inclusion of ring A and ring B through the wider end of the β-CD cavity. While irradiating, positive and negative signs for NOE from different groups of cholesterol and DMβ-CD were observed, indicating different correlation times (τ_c) being operative. Positive and negative effects for NOE indicated either $\omega^2\tau_c^2 \ll 1$ (when the maximum NOE observed will be +0.5) or $\omega^2\tau_c^2 \gg 1$ (when the maximum NOE will be -1) for the extreme motional narrowing condition, respectively. Also, the 6-H proton of cholesterol exhibited a higher positive value 69.9% ($>$50%) when H-2 for β-CD was irradiated which probably arose due to an error in measurement of less intense protons like 6-H compared to β-CD. The variation in the extent of the observed effects clearly depicted the proximity between certain groups of cholesterol and DMβ-CD including different signs for NOE intensities indicating that the observed effects were due to NOE rather than spin-diffusion.

On the 270 MHz instrument, which represents a value for ω = 1.696 $\times$ 10^9 rad s^{-1}, a non-NOE condition $\omega\tau_c \simeq 1$, is very much possible provided τ_c for the cholesterol–DMβ-CD mixture is of the order of 1 $\times$ 10^{-9} s. However, this was not found to be so (see section on T_1 measurements). To rule out the effects of spin-diffusion and viscosity completely, a ROESY spectrum was recorded for the cholesterol–β-CD mixture in DMSO d_6.

Table IV. Percentage of NOE values from 1D NOE experiment in DMβ-CD–cholesterol mixture in $CDCl_3$.[a]

Group irradiated		Group affected[b]												
DMβ-CD	Cholesterol	DMβ-CD					Cholesterol							
	ppm	5.0	3.9	3.6	3.4	3.2	5.3	2.2	2.0	1.8	1.0	0.9	0.8	0.6
	Signal	3-OH 1-H	H-3 H-6a,b 2-OCH_3	5-H H-4	6-OCH_3	H-2	6-H	H-4α, H-4β	H-7β,	H-1, H-11α, H-12α	H-23	19-H 19-H	21-H 21-H	18-H 26-H 27-H
3-OH, H-1		–	–	−0.15	−0.1	5.9	–	–	–	−5.2	−0.16	−0.13	−0.61	−1.2
H-3		–	–	0.55	0.45	11.3	18.9	–	–	–	0.16	0.56	0.04	0.89
H-2		4.9	2.9	0.2	0.03	–	69.9	–	–	–	−0.56	0.83	−0.61	−0.89
	6-H	−3.7	2.9	−0.05	−0.1	–	–	30.9	22.2	5.6	−0.04	−0.04	0.06	−0.89
	H-4α, H-4β	−1.5	1.3	−0.15	−0.1	−3.3	73.3	–	–	−7.0	−0.08	0.42	−0.31	–
	H-1β, H-12α, H-11α	–	11.7	0.35	0.1	5.3	38.9	–	–	–	−0.16	−0.28	0.20	0.89
	18-H	−2.2	−0.7	−0.3	−0.3	−0.7	–	–	–	–	–	–	–	–
	21-H, 26-H 27-H	−3.3	–	0.07	0.08	0.7	–	–	–	–	–	–	–	−3.1
	19-H	6.4	2.6	1.1	0.5	−1.1	–	–	–	–	–	–	–	−4.7
	H-23	−2.5	−0.2	−0.1	−0.2	−0.5	–	–	–	–	–	−1.7	–	–

[a] Both inter as well as intranuclear NOE values are shown. Concentrations same as in Table II.
[b] Negative NOE are indicated by (−) sign.

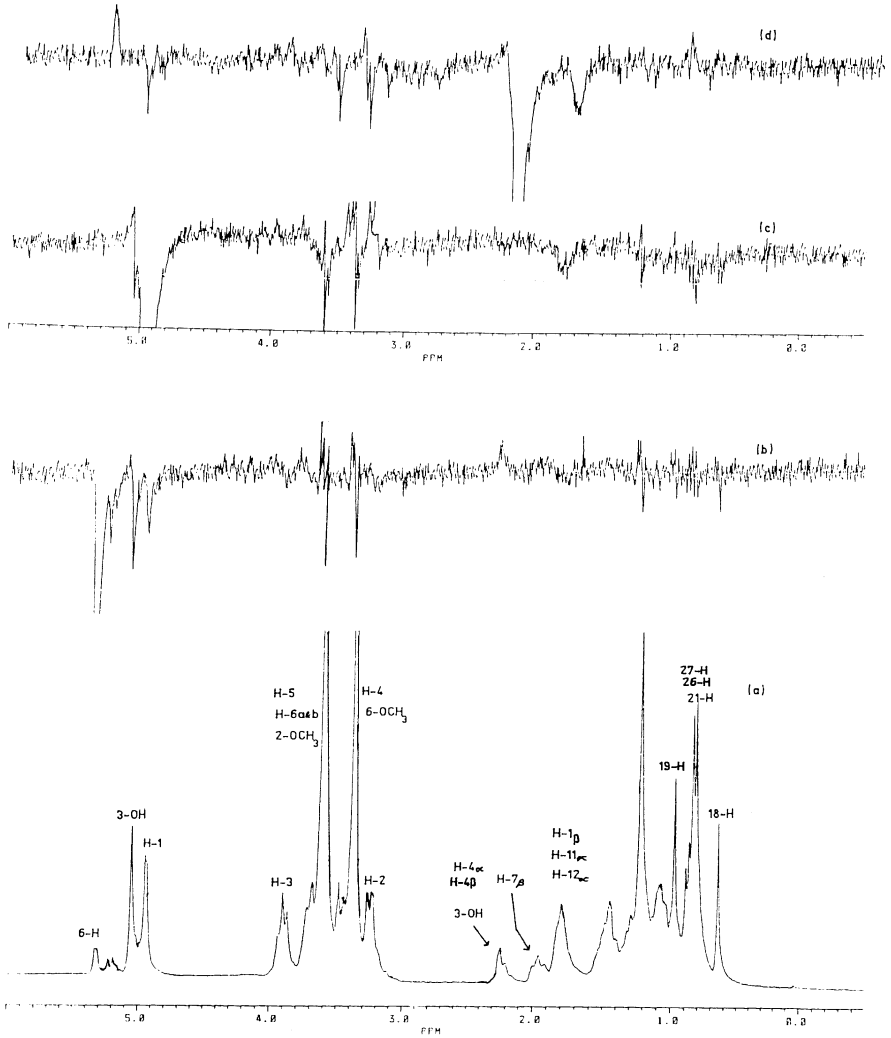

Figure 2. 1D NOE spectra of the cholesterol–DMβ-CD mixture (10 mg cholesterol and 30 mg DMβ-CD in 0.5 mL $CDCl_3$ at 20°C). Irradiated at (a) off-resonance; (b) 6-H; (c) H-1, 3-OH; (d) H-4α, H-4β.

The observed ROESY spectrum of the mixture is shown in Figure 4. Several prominent cross-peaks involving exchange processes connected with the hydroxyl protons of β-CD (2-OH, 3-OH and 6-OH) and the small amount of water protons present in the solvent were observed. Significant cross-peaks were observed between the cholesterol 19-H protons and the H-3 and H-2 protons of β-CD, the H-4α and H-4β protons of cholesterol and the H-1 proton of β-CD, respectively. This clearly indicated that the NOE effects observed between some cholesterol and β-CD protons described above were devoid of spin-diffusion. The less intense

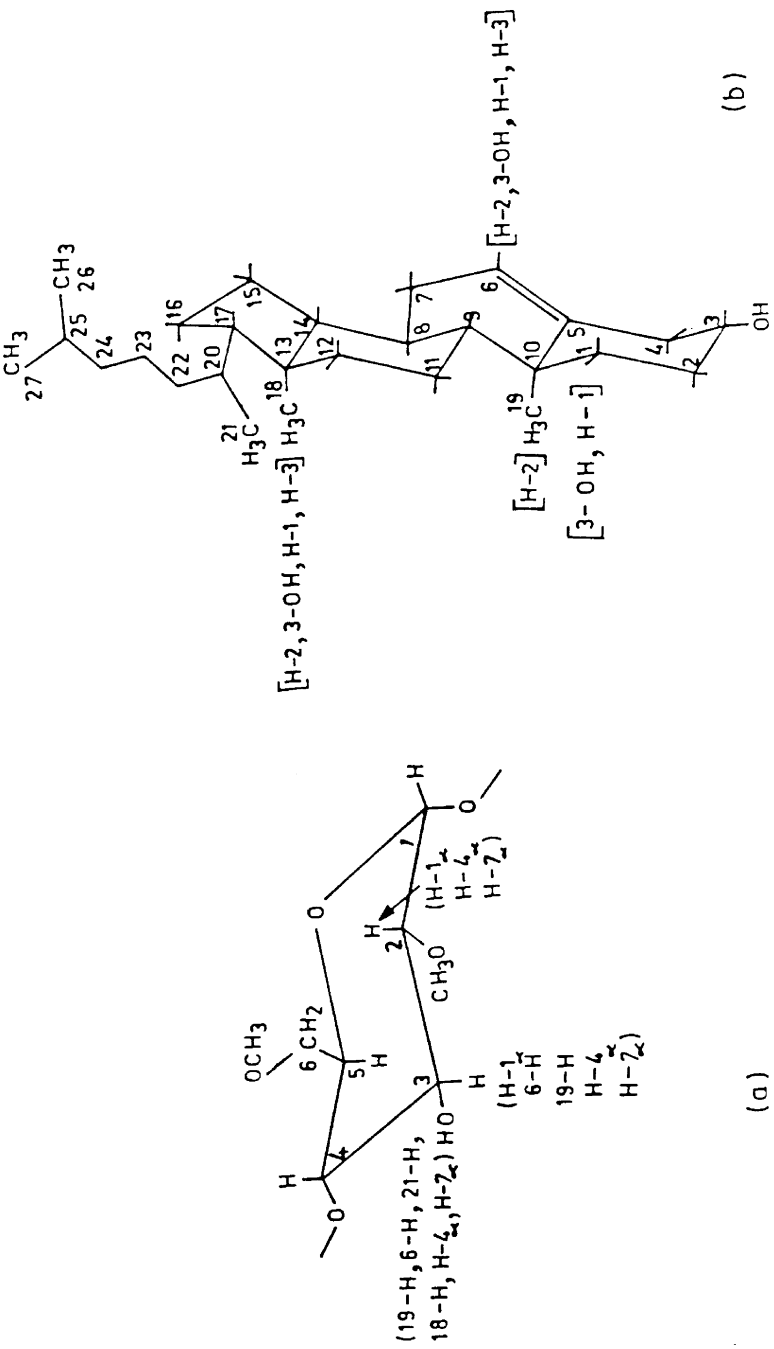

Figure 3. ID NOE connectivities of the DMβ-CD–cholesterol mixture. (a) DMβ-CD glucose unit (b) Cholesterol. The values in square and plain brackets represent the signals which on irradiation affected that particular cholesterol or DMβ-CD signals respectively. In cases where effects of irradiation of more than one signal were noticed, the order of the signals from left to right indicate the decrease in order of magnitude of percentage NOE. Only those irradiations which showed an effect greater than 0.8% are shown. Intramolecular NOE connectivities are not shown. Only intermolecular values are indicated.

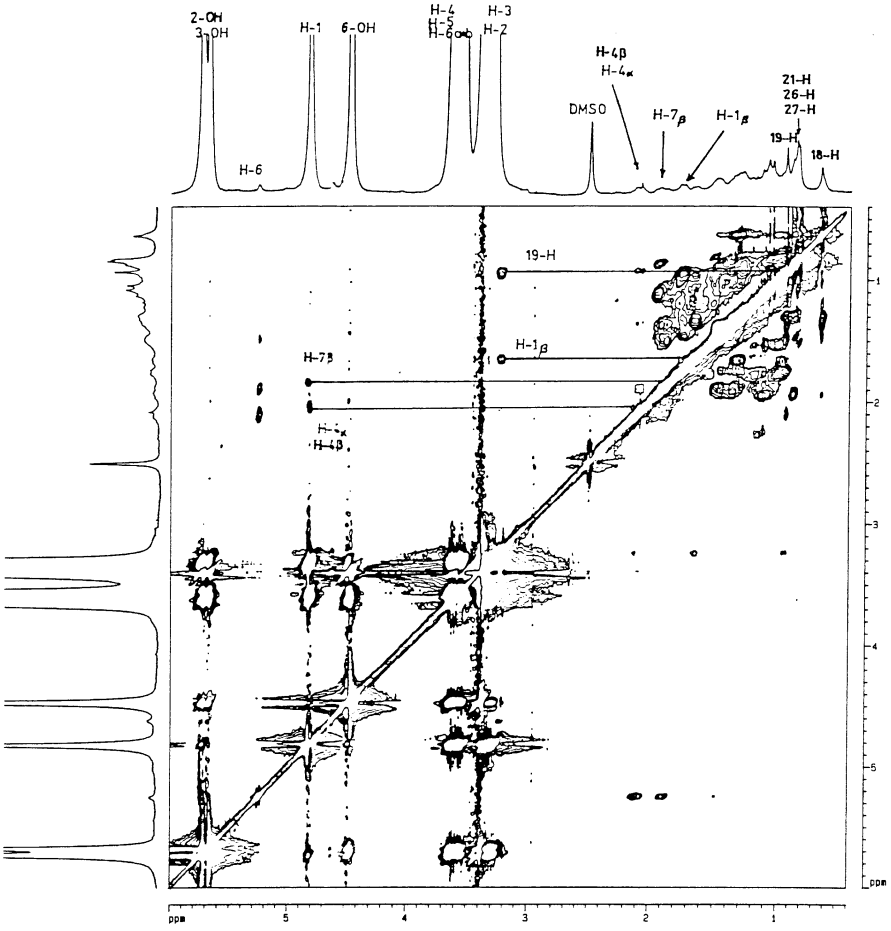

Figure 4. 400 MHz ROESY spectrum of the β-CD–cholesterol mixture in DMSO d_6. Intermolecular connectivities are shown in the diagram. Concentrations used are as mentioned in Figure 2. While the left hand side (to the reader) of the diagonal represent ROESY connectivities those on the right hand side refer to NOE connectivities.

ROESY cross-peaks and very few intermolecular connectivities observed may be due to very weak complexation between cholesterol and β-CD in DMSO d_6.

3.4. SPIN-LATTICE RELAXATION TIME (T_1) MEASUREMENTS

There was a reduction in the T_1 values of certain protons of cholesterol in the presence of DMβ-CD (in $CDCl_3$). However, certain protons also showed an enhancement in T_1 values (Table III). The groups in the vicinity of ring A and ring B of cholesterol, namely H-4α, H-4β (39%), H-7β ($-$69%), 3-OH (very high), 18-H ($-$52%), 26-H, 27-H ($-$24%), 6-H (40%), 3-H ($-$58%), 19-H ($-$55%) and 21-H (21%) were affected along with methyl groups. The largest positive effect observed

was for 3-OH followed by 6-H > H-4α > H-4β. The other protons such as 3-H, 19-H, 27-H and 18-H along with some CH and CH_2 groups from ring C and ring D and the side chain also showed a decrease in T_1 values. The decrease in T_1 indicated that the relative mobilities of groups were reduced due to complexation. The highest reduction in T_1 observed with 3-H of cholesterol indicated that this 3-OH of cholesterol might be hydrogen bonded to the protons in the narrower end of β-CD.

The observation of a definite NOE indicated that the main contribution to spin-lattice relaxation time-measurements was dipole–dipole interaction. Hence, assuming that the observed T_1 was solely due to T_{1DD}, local motions of the β-CD embedded cholesterol could be evaluated as described elsewhere [19, 20].

From the relation

$$\frac{1}{T_{1\,\text{obs}}} = \frac{\alpha}{T_{1\,\text{complex}}} + \frac{(1-\alpha)}{T_{1\,\text{free}}}$$

$T_{1\,\text{complex}}$ can be evaluated from $T_{1\,\text{obs}}$ and α, the fraction of cholesterol bound, which again can be evaluated from the binding constant value.

For the extreme motional narrowing condition,

$$\omega^2 T_c^2 \ll 1,$$

$$\frac{1}{T_{1DD}} = 2r^4\hbar^2 I(I+1)\tau_c/r^6$$

enables evaluation of τ_c, the correlation time of the cholesterol – β-CD complex. The overall rotational time for cholesterol (τ_m) and the internal rotational time for the groups of cholesterol (τ_i) can also be determined from τ_c by employing the treatment of Brevard *et al.* [21].

$$\tau_c = A\tau_m + (B+C)(1/\tau_m + 1/\tau_i)^{-1}$$

where A, B and C are constants arrived at from θ, the angle between the relaxation vector of the main field gradient (cholesterol protons) and the principal axis of rotation of the cholesterol molecule, which is the same as the β-CD axis.

A τ_m value of 13.65×10^{-12} s was assumed for a molecule like cholesterol and the values of τ_c and τ_i are shown in Table III. It can be seen that τ_c for various protons of cholesterol in the mixture is of the order of 10^{-13} s and the values are higher than those for free cholesterol. For some protons the values were also lower. For τ_c values of 10^{-13} s, $\omega^2\tau_c^2 \ll 1$ which once again reinforced the assumption that the observed NOE are free from spin-diffusion effects. The increase in τ_c for the mixture is suggestive of a molecule with a larger size due to inclusion. The internal rotation time (τ_i) increased for various groups of cholesterol due to complexation. A 91-fold increase in τ_i was noticed for 3-H, the largest for any

proton dealt with in cholesterol, a 37-fold increase for 19-H and a 20-fold increase for 18-H. Evaluation of τ_m and τ_i also aided in the determination of ξ (τ_c/τ_m) the coupling coefficient between the two rotamers – β-CD and cholesterol. For the various groups of cholesterol studied, values of ξ were in the order of 0.016–0.048 implying that the complex formed between cholesterol and β-CD was very weak, although UV studies gave a value of 15 385 $\pm$ 1500 M^{-2} for the 2 : 1 complex.

3.5. UV-VISIBLE SPECTROSCOPIC STUDY

Due to the poor solubility of the β-CD–cholesterol complex and its potential to self assemble in water, the UV-visible spectroscopic study was carried out with DMβ-CD in chloroform. Cholesterol showed an absorption band at λ_{max} 241 nm (ϵ = 8917) in chloroform. To a solution of cholesterol (1.12×10^{-4} M) in chloroform, DMβ-CD (1.11×10^{-3} M) in the above cholesterol solution (to avoid the dilution effect) was added in increasing amounts. Both hyperchromicity in the absorption band as well as a very slight bathochromic shift (1 nm) were observed. Isosbestic points were observed at 238 and 250 nm, indicating that more than one species (including free cholesterol) was present in the solution. The values at which isosbestic points were observed also indicated that the complexation affected the vicinity of the olefinic portion significantly.

A plot of ΔA (the difference in absorbance between free and complexed cholesterol) against [DMβ-CD]/[cholesterol] exhibited two inflections (Figure 5a) corresponding to both 2 : 1 as well as 1 : 1 complexes (DMβ-CD : cholesterol). The binding constant value was evaluated by Scatchard analysis by plotting $\Delta A/(\Delta A_{max} - \Delta A)$ [DMβ-CD] (ratio of fraction bound to total substrate concentration) against $\Delta A/\Delta A_{max} \cdot \rho$ (fraction bound) (Figure 5b). A binding constant value of 15 385 $\pm$ 1500 M^{-2} for the 2 : 1 complex was evaluated from the initial slope equal to $-1/K_D$, the binding constant value. This value corresponds to a free energy value (ΔG) of -23.85 KJ $mole^{-1}$.

The foregoing structural and reactivity studies, suggest that the cholesterol molecule is embedded inside the β-CD cavity (Figure 6). UV studies showed that a 2 : 1 (DMβ-CD–cholesterol) complex was possible at lower concentration of DMβ-CD. Since the cholesterol molecule is almost linear, both ends of cholesterol, namely ring A and ring B on one extreme and ring D and the side chain on the other, may be included inside the wider end of two DMβ-CD molecules with wider ends of both the DMβ-CD molecules facing each other. It is not certain whether unmodified β-CD also forms a 2 : 1 complex with cholesterol or not. It is probable that it also forms predominantly a 2 : 1 complex at a lower ratio of β-CD, similar to DMβ-CD. Although ring A has been shown to be buried inside the cavity of one of the host molecules, the extent to which ring B is inserted into the cavity is not entirely evident from these studies. It is possible that more than half of ring B is present inside the cavity. The extent of protrusion of ring B inside the β-CD cavity may vary beween β-CD and DMβ-CD. Similarly, the extent of

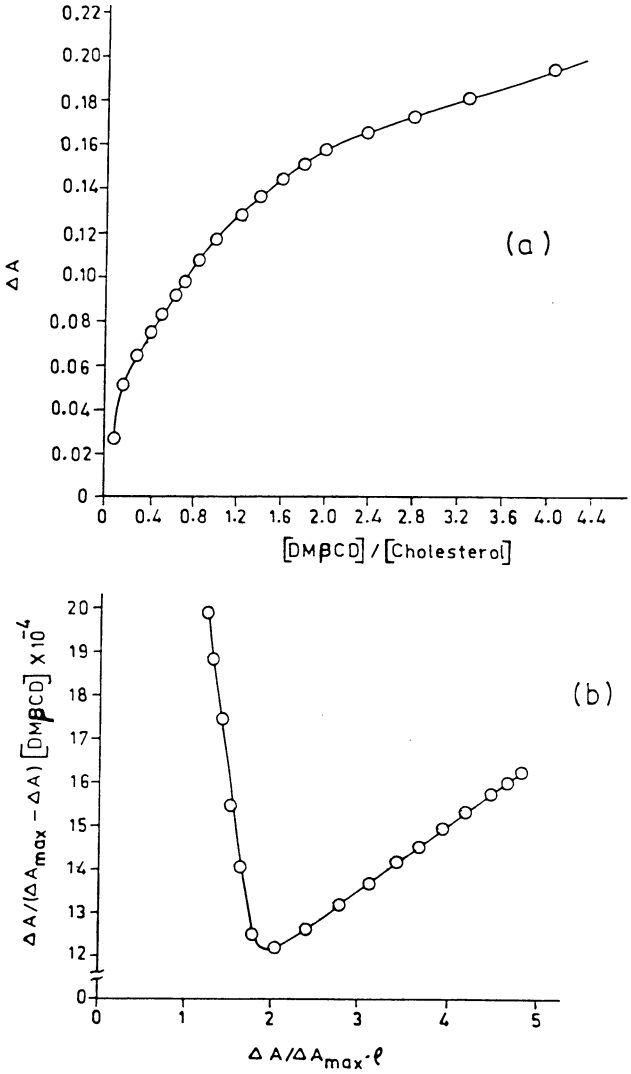

Figure 5. Determination of (a) the stoichiometry and (b) the binding constant value for the DMβ-CD–cholesterol mixture in $CHCl_3$ (λ_{max} = 241 nm). [Cholesterol] = 1.12×10^{-4} M; DMβ-CD] = 1.11×10^{-3} M. The data were an average from three independent experiments.

protrusion of ring C, D and the side chain portion inside the β-CD/DMβ-CD cavity is also not clear. However, based on the magnitudes of differences observed in the NMR parameters, it can be surmised that the observed differences are due to the difference in polarity of the two ends of the cholesterol molecule. While ring A and B can be considered to be slightly more polar, ring D and the side chain portion are less polar. Hence, coupled with the extent of protrusion inside the β-CD/DMβ-CD

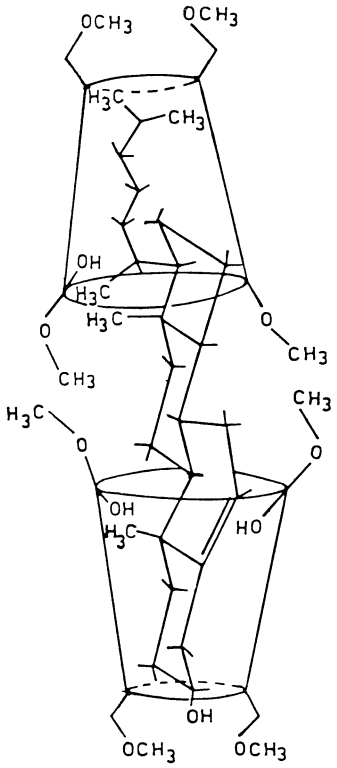

Figure 6. Disposition of cholesterol inside the DMβ-CD cavity for the 2 : 1 complex as deduced fom UV, 1D NOE, ROESY and T_1 experiments.

cavities, the observed differences have been distinctly brought out by the NMR experiments.

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