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Oxidation of Cholesterol by a Biomimetic Oxidant, Cetyltrimethylammonium Dichromate

Sabita Patel and B. K. Mishra*

Center of Studies in Surface Science and Technology, Department of Chemistry, Sambalpur University, Jyoti Vihar-768019, Orissa, India

bijaym@hotmail.com

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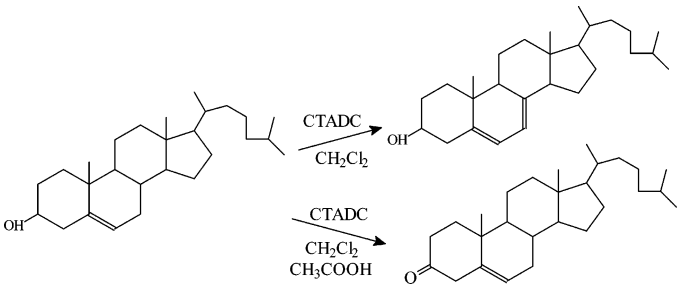

The oxidation of cholesterol by cetyltrimethylammonium dichromate (CTADC) in dichloromethane (DCM) yielded 7-dehydrocholesterol, while with addition of acetic acid in DCM the product was found to be 5-cholesten-3-one. The kinetics of oxidation of cholesterol by CTADC in DCM, in the presence of acid, was investigated with change in [acid], [cholesterol], [CTADC], [surfactant], temperature, and solvents. The reaction was found to be first order with acetic acid and fractional order with CTADC and cholesterol. Michaelis−Menten-type kinetics was observed with respect to cholesterol. The solvent isotope effect was found to be $k(D_2O)/k(H_2O) = 0.72$. The observed experimental data suggest that the reaction occurs in reversed micellar system, akin to an enzymatic environment, and the reaction path involves the intermediate formation of an ester complex, which undergoes decomposition to give the product.

Introduction

As an unsaturated lipid, cholesterol is found in all mammalian cells and lipoproteins. Most of the cellular cholesterols located in the plasma membrane are found to be oriented in such a way that the alcoholic −OH of a cholesterol is exposed to the aqueous system and the other hydrophobic unit is buried in the membrane. Cholesterol is susceptible to spontaneous oxidation, and it yields a variety of oxidized products such as epoxide, peroxides, diols, ketones, dienes, acids, etc.[1] The preponderance of cholesterol in the plasma membrane allows preferential probing of oxidative damage in the concerned compartment.[2] Most of the products are found to be cytotoxic and mutagenic. One of the products, 7-dehydrocholesterol, is reported to be responsible for mental retardation and various congenital abnormalities.[3] The oxidized products of cholesterol are found to affect the molecular order of cell membrane changing its permeability leading to vascular cell injury.[4] The products also lead to change in internalization pathway of endothelin receptor type A.[5] However, most of the oxidized products are due to increase in oxygen functionality. Further, the oxidized products of cholesterol are precursors of many pharmaceutically important products. Cholestenone is a precursor of androsta-1,4-diene-3,17-dione, which can be chemically modified to manufacture oral contraceptives.

In our efforts in exploring some biomimetic oxidants to oxidize organic substrates in organic solvents, we have reported the oxidation behavior of cetyltrimethylammonium permanganate (CTAP),[6] cerate (CTACN),[7] and dichromate (CTADC)[8−10]

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toward various organic substrates. These are inorganic oxidants with an amphipathic organic carrier, cetyltrimethylammonium (CTA^+) ion, to carry the oxidants into the organic (lipid) phase. However, these oxidants are hydrophobic and thus support the existence of a tight ion pair of the cationic carrier and the anionic oxidant counterion in nonpolar medium. In organic solvents, CTAP oxidizes its carrier, CTA^+, in a manner similar to β-oxidation of fatty acids.[6] Other aforesaid oxidants are found to be inert toward their carrier.

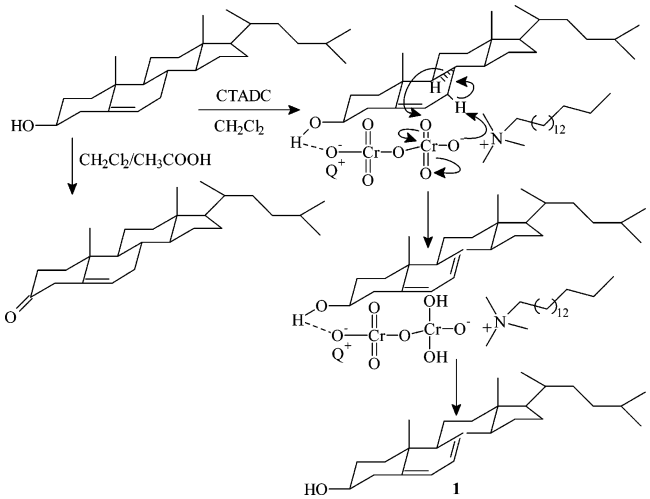
Chromium oxidants with lipopathic carriers are well established. Quaternary ammonium ions, e.g., pyridinium and quinolinium ions, with halochromate as the counterions are reported to be mild oxidants for organic substrates. Pyridinium chlorochromate,[11,12] pyridinium dichromate,[12] and pyridinium fluorochromate[13] have been used in the allylic oxidation of cholesterol. We have used CTAP for oxidation of cholesterol to yield a diol at the double bond.[14] Recently, we have synthesized CTADC from cetyltrimethylammonium bromide and potassium dichromate and have investigated its oxidation behavior of the oxidant on various organic substrates.[8−10] CTADC is highly soluble in organic solvents and does not dissociate in the medium. It does not contain any acidic proton, and without acid it shows bizarre behavior. Aromatic amines and thiols give rise to coupled products,[8] and aldoximes produce nitriles.[10] However, in the presence of acid, it shows normal oxidation behavior of Cr(VI).

Herein, we have made an attempt to investigate the biomimetic oxidation activities of CTADC toward cholesterol. To achieve the objectives, the oxidation products were characterized, and kinetics were run in media with varied polarities and also in microheterogeneous systems generated from the presence of cationic surfactant, CTAB (cetyltrimethylammonium bromide), anionic surfactant, SDS (sodium dodecyl sulfate), and nonionic surfactant, Triton X-100 (isooctylphenoxypolyoxyetanol), at different concentrations. The kinetic model that mimics the biological system was used to analyze the resultant data. By varying [substrate], [acid], and [CTADC] in the reaction process and from the solvent isotope effect, a suitable mechanism for the reaction was proposed. The thermodynamics of the reaction was also analyzed by running the kinetics at various temperature.

Results and Discussion

Reaction in the Absence of Acid. When cholesterol was refluxed with CTADC in DCM for 6 h, a product separated through chromatotron was isolated and was identified as 7-dehydrocholesterol (**1**) from its ^{13}C NMR, ^1H NMR, and FAB-MS spectral characteristics. The introduction of the double bond by a dehydrogenation process at C_7-C_8, thus, is carried out by CTADC. Earlier dehydrogenation of cholestanol has been achieved by remote functionalization, wherein the free radical is involved for abstraction of hydrogen. Breslow[15] has attached

SCHEME 1



a benzophenone moiety to position 3α of a steroid molecule for dehydrogenation at different positions under various experimental conditions. A *m*-dichloroiodobenzoyl group has been used by Breslow et al.[16] for dehydrogenation at C_9-C_{11} of cholestanol. In the present case, the reaction process may be initiated with an association of the 3-OH group with the chromate ion of CTADC, and subsequent reaction takes place at an equidistant site of the active center of the reagent at the cholesterol nucleus (Scheme 1). The secondary overlap of π-orbitals of cholesterol at the C_5-C_6 position with that of Cr═O may assist the system in achieving proper orientation. This type of noncovalent bond interaction for remote functionalization has also been proposed for double-bond insertion in the sterol system by benzophenone derivatives, where electrostatic interactions or hydrogen bonding between the two substrates are sufficient to bring the two molecules close enough together for bond insertion and hydrogen abstraction.[15] Dehydrogenation of organic molecules due to Cr(VI) oxidation is not new. Zhu and Okamura have reviewed the synthesis of calciferol (vitamin D), wherein there is a report on formation of a dehydrogenated product from cholesteryl acetate derivative by using Cr(VI).[17] In a four-step reaction process a double bond is introduced at the C-7 position of the B ring of the cholesteryl unit. The oxidative couplings of amines and thiols by CTADC to yield corresponding diazo and disulfide derivatives[8] have also been proposed to be a dehydrogenation coupling process. The dehydrogenation, in the present case, may occur through a seven-membered cyclic transition state involving a change of oxidation state of Cr(VI) to Cr(IV) (Scheme 1).

The three-dimensional structure of cholesterol oxidase (ChOX) from *Brevibacterium sterolicum* has been solved, and it is proposed that the enzyme's catalytic site is formed by a hydrophobic cavity where the sterol binds and interacts with the flavin of FAD.[18] Flavin adenosine dinucleotide (FAD) is known to be a dehydrogenating agent and is responsible for dehydrogenation at vicinal sites during β-oxidation of fatty acids.[19] In analogy to ChOX, the CTA^+ has an affinity to the

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TABLE 1. Effect of [Chol], [CTADC], and [Acetic Acid] on the Oxidation of Cholesterol by CTADC at 300 K

[CTADC] × 10^4 (M)	[Chol] (M)	[acetic acid] (M)	$k_{obs} \times 10^3$ (s^{-1})
0.47	0.05	3.24	3.934
0.94	0.05	3.24	3.255
1.89	0.05	3.24	2.28[a]
2.83	0.05	3.24	1.973
4.70	0.05	3.24	1.457
1.89	0.002	3.24	0.326
1.89	0.01	3.24	0.844
1.89	0.02	3.24	1.274
1.89	0.08	3.24	2.959
1.89	0.05	0.81	0.557
1.89	0.05	1.62	1.179
1.89	0.05	6.48	4.479
1.89	0.05	8.1	5.197

[a] $10^3 k_{obs}$ at 298, 303, and 308 K were found to be 2.073, 2.43, and 3.197 s^{-1} respectively.

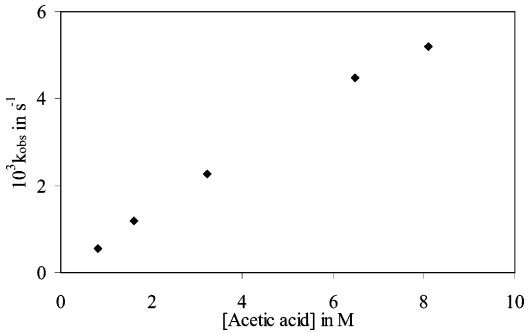

FIGURE 1. Plot of $10^3 k_{obs}$ vs [acetic acid] in the oxidation reaction of CTADC with cholesterol at 300 K.

electron-rich site of cholesterol, i.e., 3-hydroxy and $\Delta^{5,6}$ π-electron clouds, and as a consequence, the tightly bound dichromate to the quaternary ammonium ion remains close to the reaction site, i.e., C_7-C_8, and thus is responsible for the dehydrogenation process.

Reaction in the Presence of Acid. Under reflux conditions, the solution of CTADC and cholesterol in 20% acetic acid of DCM became green. The completion of the reaction was ascertained by monitoring TLC of the reaction mixture. The product was obtained from the green reaction mixture as a white crystal, which was characterized from its IR and NMR (H and ^{13}C) spectral data and the melting point to be 5-cholesten-3-one (**2**). The results were compared with the data of authentic samples. The formation of keto functionality may be explained by assuming the formation of an acid dichromate. In presence of acetic acid, the dichromate ion becomes free from the grasp of the quaternary onium ion due to the change in polarity of the medium and also the probable substitution of onium ion by proton of acetic acid. It becomes easier to abstract the α-proton of cholesterol yielding cholestenone. To obtain further support for this, the reaction kinetics of the oxidation reaction was monitored in the presence of acid, and the kinetic data are tabulated in Table 1.

Without acid, the reaction became too slow to measure. The linear plot of [acid] vs rate constant (Figure 1) was found to pass through the origin, substantiating almost no reaction with acid. The rate of reaction was found to be first order with respect to acid.

The stoichiometry of the reaction was found to be 2:3 for Cr(VI) and cholesterol. However, in the earlier case, i.e., without acetic acid, 1 mol of cholesterol reacts with 1 mol of CTADC

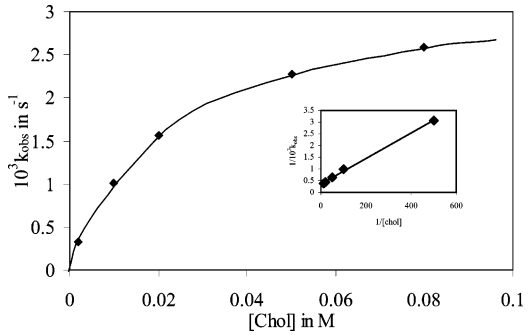

FIGURE 2. Plot of $10^3 k_{obs}$ vs [Chol] in the oxidation reaction of CTADC with cholesterol at 300 K.

yielding 1 mol of 7-dehydrocholesterol and 1 mol of Cr(IV). The existence of Cr(IV) as the reduced state in oxidation of benzyl alcohol by quinolinium fluorochromate has also been reported by Dave et al.[20] Cr(IV) further changes to Cr(III) by a disproportionation reaction in a sequential manner. The existence of Cr(III) in the product mixture was established from the absorption maximum at 580 nm.[21] However, the change in the intensity at 580 nm was not reliable to study the rate of formation of Cr(III).

$$\mathrm{Cr(IV) + Cr(VI) \rightarrow 2Cr(V)}$$
$$\mathrm{Cr(V) \rightarrow Cr(III) + 2e}$$

The reaction rate was found to increase with an increase in the concentration of cholesterol achieving a plateau at higher concentration. Thus, the oxidation of cholesterol exhibited normal Michaelis−Menten kinetics with a maximum value of k_{obs} at 3.3×10^{-3} s^{-1} (Figure 2). The steady-state dissociation constant of the oxidant−substrate complex known as the Michaelis−Menten constant, K_m ($= (k_{-1} + k_2)/k_{+1}$), was obtained as 24.0×10^{-3} M. While investigating the effect of water pool in a reversed micelle on oxidation of cholesterol by cholesterol oxidase in a CTAB−octane/hexanol system, Gupte et al. obtained a K_m value of 2.408×10^{-3} M.[22] By using a Lineweaver−Burk-type double-reciprocal equation (eq 5), the binding constant K ($= k_{+1}/k_{-1}$) and k_2 are obtained as 63.3 L mol^{-1} and 2.893×10^{-3} s^{-1}, respectively The corresponding double-reciprocal curve is shown in the inset of Figure 2. From K (63.3 L mol^{-1}) and K_m and k_2 values the k_{+1} and k_{-1} were calculated to be 358.2×10^{-3} and 5.597×10^{-3} s^{-1} respectively.

$$\mathrm{cholesterol + CTADC} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{[complex]} \quad (1)$$

$$\mathrm{[complex]} \xrightarrow{k_2} \mathrm{products} \quad (2)$$

Thus, by applying the steady-state approximation[23]

$$\mathrm{rate} = -\frac{\mathrm{d[Complex]}}{\mathrm{d}t} = \frac{Kk_2[\mathrm{cholesterol}][\mathrm{CTADC}]}{1 + K[\mathrm{cholesterol}]} \quad (3)$$

$$-\frac{\mathrm{d[complex]}}{\mathrm{d}t} \times \frac{1}{[\mathrm{CTADC}]} = k_{obs} = \frac{Kk_2[\mathrm{cholesterol}]}{1 + K[\mathrm{cholesterol}]} \quad (4)$$

$$\frac{1}{k_{obs}} = \frac{1}{k_2 K[\mathrm{cholesterol}]} + \frac{1}{k_2} \quad (5)$$

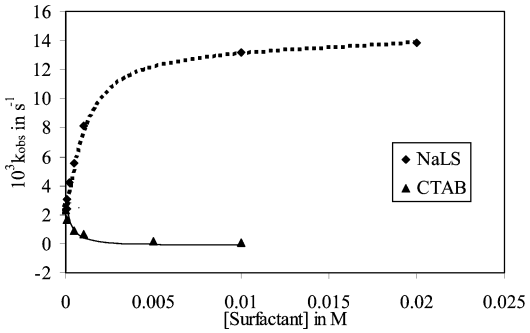

FIGURE 3. Effect of surfactant in the oxidation reaction of cholesterol with CTADC.

With increasing concentration of CTADC, the rate constant was found to decrease nonlinearly in a concave fashion (Table 1). The decrease in rate constant with increase in [CTADC] may be ascribed to the formation of reversed micelle wherein the dichromate ion is enveloped by CTA^+ and cholesterol is more partitioned to bulk DCM. Thus, the effective concentration of cholesterol at proximity of dichromate decreases. CTAB forms reversed micelles in DCM[24] and catalyzes various chemical reactions by partitioning the reagent and the substrate. When CTAB was added to the reaction mixture, the rate constant was found to decrease sharply with increasing [CTAB]. Above a concentration of 5×10^{-3}M of CTAB, the rate constant almost levels up around 1×10^{-4} s^{-1} (Figure 3). This observation also corroborates the earlier argument on partition of the oxidant and substrate in two different subphases. Assuming the rate decrease is due only to the partition effect, for complete entrapment of a dichromate (2×10^{-4} M) by CTA^+ (5×10^{-3} M) it requires a composition of 1:25 of CTADC/CTAB. From the logarithmic plot of rate constant and [CTADC], the order of reaction is found to be 0.432, indicating a complex mechanism being operated during the reaction process.

In presence of SDS, the dichromate ions become free from the engulfment of the reversed micelle due to the anionic amphiphile and thus is more available to cholesterol for oxidation. With increasing [SDS], the rate constant experiences a plateau above a concentration of 0.002 M. Addition of TX-100 did not exhibit any significant change in the rate constant.

When the reaction was monitored in various solvents with different polarity, the rate was found to change significantly (Table 2). Interestingly, both bulk parameters and polarity parameters of the solvents were found to exhibit specific trends in the reactivity.

When the log *k* values were plotted against polarity parameters such as dielectric constant (ϵ), dipole moment (μ), solvent polarity (π^*),[25] anion-solvating power of the solvent (A), and cation-solvating power of the solvent (B),[26] an ordination between two classes of solvents was experienced, each class contributing differently to the reaction system. Benzene, toluene, DCM, and chloroform formed a group, while the rest of the solvents (tetrahydrofuran, dimethylformamide, ethyl acetate, and acetone) formed another group. In the former group, the change in the polarity of the solvent did not have a significant effect on the rate, while with other solvents a decreasing trend in reactivity with increasing polarity scale was observed. When log *k* was plotted against β (hydrogen bond acceptor basicity)[25] and log *P* (partition coefficient)[27] values separately, straight lines with negative and positive slopes were observed. The resulting regression equation considering both the parameters is presented in eq 6.

TABLE 2. Observed Rate Constants for the Oxidation Reaction of Cholesterol in Various Organic Solvents at 300 K[a]

solvent	$k_{obs} \times 10^3$ (s^{-1})	solvent	$k_{obs} \times 10^3$ (s^{-1})
benzene	3.792	ethyl acetate	0.672
toluene	3.036	acetone	0.315
chloroform	2.441	tetrahydrofuran	0.234
dichloromethane	2.28	*N*,*N*-dimethylformamide	0.0844

[a] [Chol] = 0.05 M, [CTADC] = 1.89×10^{-4} M, and [acetic acid] = 3.24 M.

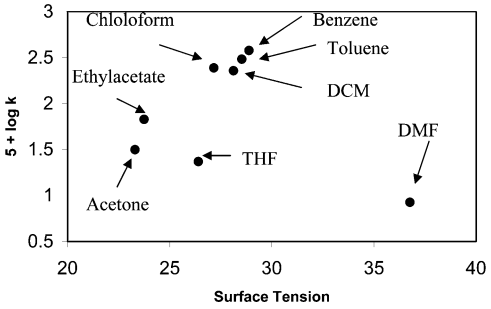

FIGURE 4. Plots of 5 + log *k* vs surface tension of the solvents for the oxidation of cholesterol with CTADC.

$$\log k = 0.215(\pm 0.093) \log p - 0.98(\pm 0.528)\beta - 2.899(\pm 0.231) \quad R = 0.9798, F = 48.13 \quad (6)$$

This result suggests the existence of a relatively less polar transition state than the reacting species. Thus, a mechanism of the oxidation process can be proposed wherein the anionic dichromate forms a neutral ester as the intermediate which, during α-hydrogen abstraction, forms a more nonpolar cyclic transition state. On the contrary, Banerji[28] have observed a rate acceleration in Cr(VI) oxidation of organic sulfides using pyridinium fluorochromate due to an increase in polarity of the medium, and have assigned the results to the increased polarity in the transition state.

Further, the correlation of the reactivity with the bulk parameters such as surface tension (γ) and viscosity (η) of the solvents were analyzed. In this case also, the plots demarcated the two classes of solvents with two opposite trends (Figure 4). In the nonpolar solvents, there was an increase in the rate constant with an increase in viscosity or surface tension, while

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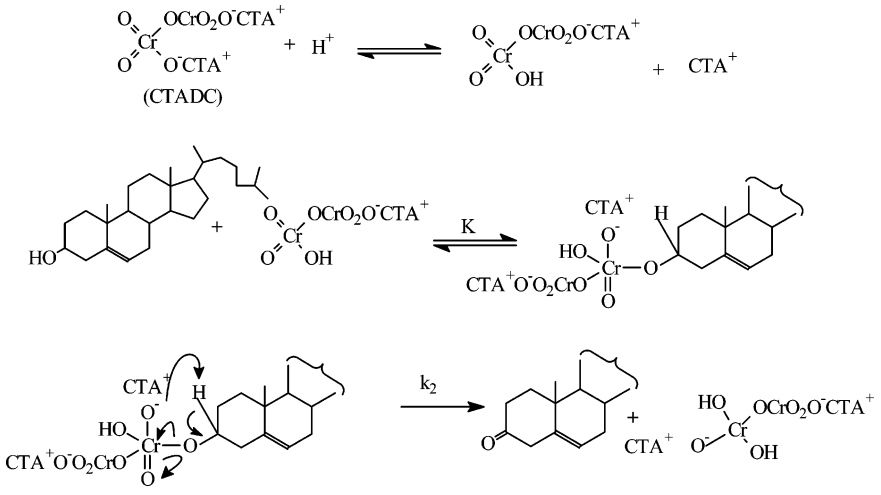
SCHEME 2



the variation in the bulk properties for relatively polar solvents experienced a decrease in the rate constants.

The changes in entropy, enthalpy, and the free energy of activation were calculated for the oxidation of cholesterol ([Cholesterol] = 0.05 M) with CTADC ([CTADC] = 1.89×10^{-4}) in the presence of 3.24 M acetic acid by using the Eyring equation and are found to be -208.1 J K^{-1} mol^{-1}, 26.368 kJ mol^{-1}, and 89.392 kJ mol^{-1}, respectively. A high negative value in $\Delta S^{\ddagger}$ supports the proposal of involvement of a cyclic transition state during H-abstraction from the substrate.

From the above findings a tentative mechanism has been proposed (Scheme 2) wherein the CTADC equilibrates with acetic acid to form the protonated dichromate, which subsequently reacts with cholesterol giving rise to a dichromate ester. The proximal oxygen anion of the dichromate abstracts the α-hydrogen leading to formation of cholestenone.

To have more insight into the reaction mechanism, the solvent isotope effect was investigated using 1:1 mixtures of H_2O/acetic acid and D_2O/acetic acid. Acetic acid was equilibrated with H_2O and D_2O separately for 3 h before its use in the reaction process. The solvent isotope effect $k(H_2O)/k(D_2O)$ was found to be $1.35 \times 10^{-4}/1.87 \times 10^{-4} = 0.72$. When a preequilibrium protonation is involved, the acid-catalyzed rate is faster in D_2O than in H_2O.[29] The trend, $k(D_2O) > k(H_2O)$, indicates that the hydroxyl group is not involved in either preequilibrium or in the rate-determining step. Further, significant changes due to change in solvent on the rates preclude the possibility of breaking an O−H bond in the rate-determining step and thus support the formation of the dichromate ester in the reaction process.

Experimental Section

Materials. Cetyltrimethylammonium dichromate (CTADC) was prepared by the method reported earlier,[8,9] and its purity was checked by estimating Cr(VI) iodometrically.[30] Cholesterol was purified by recrystallization from alcohol. Glacial acetic acid was used as a source of hydrogen ion and was used without further purification. The organic solvents were purified by standard methods.[31] The surfactants cetyltrimethylammonium bromide (CTAB) and sodium dodecyl sulfate (SDS) were purified by recrystallization from methanol solution, and their purity was checked from physical constants.

Kinetic Measurements. The oxidation of cholesterol by CTADC in the presence of acetic acid was studied in different solvents and surfactant systems. The temperature in the reaction cell was controlled by circulating water by using an INSREF thermostat within a temperature fluctuation of ±0.1 K. The reactions were performed under pseudo-first-order conditions by keeping a large excess (100 times or more) of the cholesterol with respect to CTADC. The rate of disappearance of the Cr(VI) species was followed spectrophotometrically by monitoring the absorption band at 350 nm from which the first-order rate constant, k_{obs}, was obtained from the linear ($r = 0.99$) plot of log[CTADC] against time for up to 75% completion of the reaction. The values reported are the average of at least duplicate runs and were reproducible within ±4% error. Some of the kinetics were run under nitrogen atmosphere, and the data were found to be almost the same (within an error of 2%) as those under normal atmospheric conditions. Hence, most of the reactions were carried out without a nitrogen environment.

Product Analysis. After keeping the reaction mixture of CTADC, cholesterol, and acetic acid in proper composition for 12 h in DCM, the volume of the reaction mixture was reduced to 30% under vacuum. The mixture was kept at 5 °C for 12 h. A white crystal separated out, which was recrystallized from its alcohol solution. The IR and NMR spectral analyses of the product agreed well with those of 3-cholestenone.

Stoichiometry. The stoichiometry of the reaction was determined by performing the experiment at 298 K, under the conditions of [CTADC] ≈ [cholesterol] at varying cholesterol concentrations. The disappearance of Cr(VI) was followed until the absorbance values became constant. The [CTADC] was estimated after 48 h. A stoichiometry ratio, Δ[CTADC]/Δ[cholesterol] ≈ 0.35, was observed, which confirmed a 1:3 CTADC/cholesterol relationship.

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