# Interaction of Acridine Orange with Bile Salts: A Spectroscopic Approach

*A Dissertation*
*Submitted for the partial fulfilment*

*FOR THE DEGREE OF*
**MASTER OF SCIENCE IN CHEMISTRY**

*Under The Academic Autonomy*

**NATIONAL INSTITUTE OF TECHNOLOGY ROURKELA**

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# CERTIFICATE

This is to certify that the dissertation entitled **"**Interaction of Acridine Orange with Bile Salts: A Spectroscopic Approach**"** being submitted by Miss Suchismita Subadini (Roll no: 412CY2027) to the Department of Chemistry, National Institute of Technology, Rourkela, Odisha, for the award of the degree of Master of Science is a record of bonafide research carried out by her under my supervision and guidance. To the best of my knowledge, the matter embodied in the dissertation has not been submitted to any other University / Institute for the award of any Degree or Diploma.

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# ACKNOWLEDGEMENT

At the very first, I take this great opportunity to express my best regards and sincere thanks from the deepest chord of my heart profound to my supervisor Dr. Usharani Subuddhi, Department of Chemistry, NIT Rourkela, for her scholastic guidance, brilliant suggestions and inspiration throughout the course of investigation.

I heartily owe my sincere gratitude & thankfulness to Dr. Niranjan Panda, HOD of Department of Chemistry, NIT Rourkela, for giving me the opportunity to do the project work in the institute and use the departmental facilities.

Specially, I would like to thank Ms. Smruti Snigdha Mishra, Ph. D Scholar, Department of Chemistry, for her overall guidance, valuable suggestions and endless help in doing the experimental work and preparing the thesis.

It is my pleasure to thank Ms. Subhraseema Das, Ph. D scholar, Department of Chemistry, for her co-operation and suggestions throughout the entire period of the project. I would also like to thank my lab mate Mr. Rajkishore Mallik for encouraging and supporting me during the project work.

Lastly, I express my heartfelt devotion to my beloved parents and my elder brother for their blessings, love, affection & incessant inspiration.

**Suchismita Subadini**

# 1. Introduction

## 1.1 Dyes

Dyes are unsaturated organic substances that absorb in the visible range of light (400-700nm). The presence of colour imparting chromophoric groups and the acid or basic auxochromic groups are responsible for their dyeing ability [1]. Apart from their wide spectrum of applications in dyeing processes as textile dyeing, photography and printing ink dyes *etc.* they also have significant contributions in the field of analytical chemistry, and biochemistry [2-4].

## 1.2 Acridine orange (AO)

Acridine orange (N,N,N′,N′-tetramethylacridine-3,6-diamine hydrochloride) is a cationic dye which is readily soluble in water [Fig.1.1].

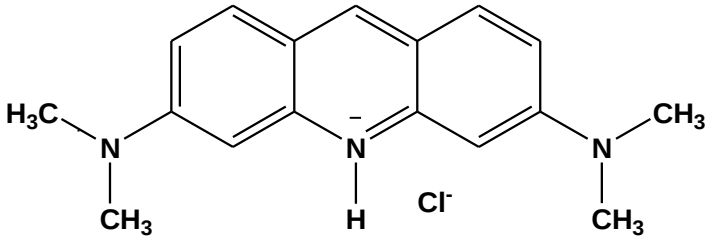



Fig.1.1:Molecular structure of Acridine orange

The photophysical property of AO is extremely sensitive to its environment and surrounding pH conditions. AO shows its absorption maximum at ~ 490 nm along with a weak shoulder peak at ~ 470 nm in aqueous media. This is because of the monomer absorption of AO and the presence of dimeric sites of AO in the solution respectively [5]. Its emission maximum appears at ~527nm [6]. It remains in its cationic form at pH below 10 and undergoes deprotonation at pH greater than 10 as its $pK_a$ value is around ~ 10.4 [5]. It was synthesized in 1889 and its first biological applications were reported in 1940. It has got numerous applications particularly in biological fields.

### 1.2.1 Biological applications of AO

AO is a metachromic dye, often used as a probe in the study of microheterogenous and biological systems such as micelles, vesicles, microemulsion, DNA, and nucleosides. The use of AO in clinical applications has become widely accepted; used in highlighting bacteria in blood cultures and is recommended for the use of fluorescent microscopic detection of microorganisms in direct smears prepared from clinical and non-clinical materials. The staining are performed at an acid pH in order to obtain the differential staining effect with bacteria showing orange stain and tissue components exhibiting yellow to green. It is also used for visualization of biological compartments and measurements of pH gradient across the membrane due to its photophysical and photochemical properties, which strongly depend on the nature of the surrounding environment [5 - 8]. AO can be used as a stain for the characterization of biopolymers. It is very much useful as a mitochondria labeling probe in living cells [9]. Recently it has been used to investigate the unusual DNA structure with parallel-stranded orientation and also useful for studying structural organization of RNA-DNA triplex and in the study of DNA thermal denaturation. The investigation of the chromatin functional state *i.e.* the ability of its DNA to serve as a primer in RNA synthesis can be assayed using AO which is based on two different approaches such as estimation of direct AO binding to cellular chromatin and study of spectral properties of cells strained with AO after different pretreatments [10]. The changes in its spectroscopic characteristics upon binding to proteins have been utilized for analytical determination of proteins in solutions. One of the most important applications of AO is its photosensitizing ability and thus extensively employed in photodynamic therapy [12]. Considering the excellent potential, AO based photodynamic therapy has already reached to clinical trial stage [13-15]. AO being a basic dye accumulates densely in lysosomes, the strongly acidic organelles. Cancer cells produce acidity in the form of protons in a hypoxic environment therefore containing many

strongly acidic lysosomes of large sizes and it is generally observed that the extracellular pH is more acidic in tumors than in normal tissues. Consequently, AO shows marked and prolonged accumulation in the acidic lysosomes of cancer cells as compared to normal cells. Photon energy when excites AO results in the production of active oxygen species, which oxidize the fatty acids of the lysosomal membrane, resulting in the leakage of lysosomal enzymes and protons, followed by apoptosis of the cancer cells. Therefore AO exhibits excellent potential towards selective anti-cancer cell activity and has been considered as a "Magic Bullet" for cancer treatment [11,12].

The photosensitizer when administered intravenously reaches to the target tissue by circulating through blood, which emphasizes its interaction with various biomolecules such as proteins, DNAs, biosurfactants *etc.* Although the interaction of AO with DNA, proteins and other synthetic surfactants has been widely studied [5-9,14,15], its interaction with biosurfactants such as bile salts is yet to be explored, which is the focus of the present work. Moreover, the interactions between dyes and surfactants, in general, are interesting for their complex nature governed and influenced by the chemical structure of dye and physicochemical properties of the surfactants [2-6]. AO is reported to exhibit induced dimerization, dye-surfactant complex formation and dye-surfactant mixed micelle formation in the presence of aqueous submicellar anionic surfactants which breaks down upon solubilization in the monomeric form in nonpolar region of micelles above CMC [5, 8].

**1.3 Bio-surfactants**

Bio-surfactants are the amphiphilic surface active compounds which are biologically synthesized. These are grouped as glycolipids, lipopeptides, phospholipids, fatty acids, neutral lipids, and particulate compounds [16]. Most of these compounds are either anionic or neutral amphiphilic molecules. Its hydrophilic part can be a carboxylic acid, carbohydrate, amino acid, cyclic peptide, phosphate or alcohol and the hydrophobic part is based on long-

chain fatty acids, hydroxy fatty acids or α-alkyl-β-hydroxy fatty acids. One of the important characteristic of these surfactants is, when dispersed in water; they are spontaneously organized in a definite fashion such as micelles, bi-layers, vesicles, emulsions *etc.* depending on their molecular structure, concentrations and experimental conditions. Some of the examples of bio-surfactants are phospholipids, glycosides, fatty acids, neutral lipids, bile salts *etc.* [17]. Among these, the phospholipids and bile salts have got special attention due to their applicability as delivery systems for drugs, vitamins, cosmetic materials *etc.* and both the biocompatible and biodegradable nature of these makes them a very safe and efficacious vehicle for medical applications [18, 19].

### 1.3.1 Bile salts as Bio-surfactants

Bile salts are the salts of bile acid which are synthesized from cholesterol in liver and stored in gall bladder. The major biosynthesized bile acids are classified into cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), and ursodeoxycholic acid (UDCA). The quantitative ratio is 12 (CA):7 (DCA):13 (CDCA):1 (UDCA) in human gallbladder [9]. Bile acid is the protonated (-COOH) form and bile salt is the deprotonated (-$COO^-$) form of compound [20]. Bile salts act as solubilizers for apolar materials like cholesterol, lipids and fat-soluble vitamins in the intestine [17]. Therefore, they play an important role in digestion. So, the function of bile salts is very different from another class of biologically relevant amphiphiles, lipids, which are the building blocks of biological membranes [18]. Apart from these biological applications, bile salts also play an important role as delivery systems for medicines, cosmetics and several other chemicals because of their unusual solubilizing and emulsifying capacity [21-23]. In the field of protein biochemistry bile salts are used to remove membrane bound proteins and other structures [19]. In the field of pharmaceutical sciences, bile salt micelles have been a very important role because of their ability in the drug carrier systems [17-21].

### 1.3.2 Structure of Bile Salts

All most all bile acids are consisting of two connecting units such as, a rigid steroid nucleus and a short aliphatic side chain. The steroid nucleus of bile acids has the saturated tetra-cyclic hydrocarbon containing three six-member rings (A, B and C) and a five member ring (D)[Fig. 1.2]. The hydroxyl groups are present in C3, C7 and C12 position in either alpha (α) or beta (β) orientation [Fig. 1.3]. All bile acids have a hydroxyl group at position 3, which was derived from the parent molecule, cholesterol. In cholesterol, the 4 steroid rings are flat and the position of the 3-hydroxyl is beta (β). In many species, the initial step in the formation of a bile acid is the addition of a 7-alpha (α) hydroxyl group. Subsequently, in the conversion from cholesterol to a bile acid, the junction between the first two steroid rings (A and B) is altered, making the molecule bent, as a result of which, the 3-hydroxyl is converted to the alpha(α) orientation. Thus, the simplest bile acid (of 24 carbons) has two hydroxyl groups at positions 3α and 7α. Human bile salts differ on the basis of number, position and stereochemistry of hydroxyl group in 3, 7 and 12 position of the steroid ring. The presence of taurine or glycine conjugation in the bile salt also contributes to the variety in human bile salts [17, 23].

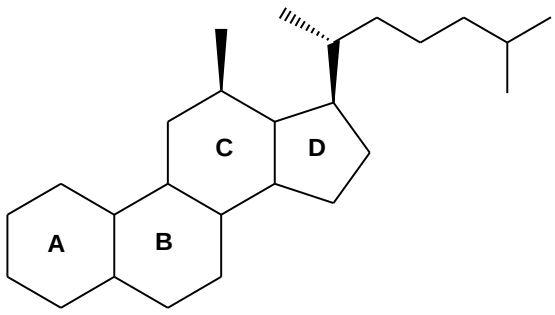



Fig.1.2:Sterol ring of bile salt

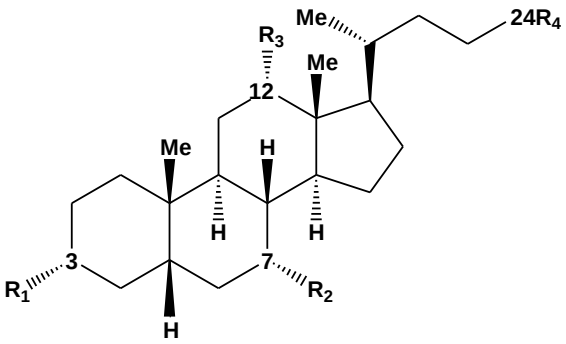



Fig. 1.3: Bile salt molecule with hydroxyl groups ($R_1$,$R_2$,$R_3$) and head group ($R_4$)

Table 1.1Position and orientation of hydroxyl groups in a typical bile salt [12].

| BILE ACID | CORRESPONDING BILE SALT | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|---|
| Cholic Acid | Cholate | αOH | αOH | αOH | -COOH |
| Deoxycholic Acid | Deoxycholate | αOH | H | αOH | -COOH |
| Glycocholic Acid | Glycocholate | αOH | αOH | αOH | $-CONHCH_2COOH$ |
| Glycodeoxycholic Acid | Glycodeoxycholate | αOH | H | αOH | $-CONHCH_2COOH$ |
| Taurocholic Acid | Taurocholate | αOH | αOH | αOH | $-CONHCH_2CH_2SO_3H$ |
| Taurodeoxycholic Acid | Taurodeoxycholate | αOH | H | αOH | $-CONHCH_2CH_2SO_3H$ |

### 1.3.3 Micellisation of Bile Salt

When the surfactant molecules in water-air interface become so packed in the monolayer that no more molecules can be accommodated with ease, they agglomerate in the bulk of the solution leading to the formation of aggregates, known as micelles [22]. Owing to the unique molecular structure, bile salt micelles behave differently from the conventional surfactants. Conventional surfactants show clear-cut polarity gradient between the hydrophilic and hydrophobic parts and both the domains are clearly separated where the hydrophilic head is smaller and the hydrophobic tail is larger [Fig. 1.4]. But bile salts have a facial polarity gradient with a convex hydrophobic side and a concave hydrophilic side [Fig. 1.4]. The hydrophilic and hydrophobic domains are hence not as clearly separated as in classical amphiphiles. The micelles formed by conventional surfactants are larger than bile salt micelles due to the planner structure of the bile salt [17]. The micelles of conventional surfactant have a spherical structure but the micelles of bile salt have mostly rod like structure [Fig.1.5].

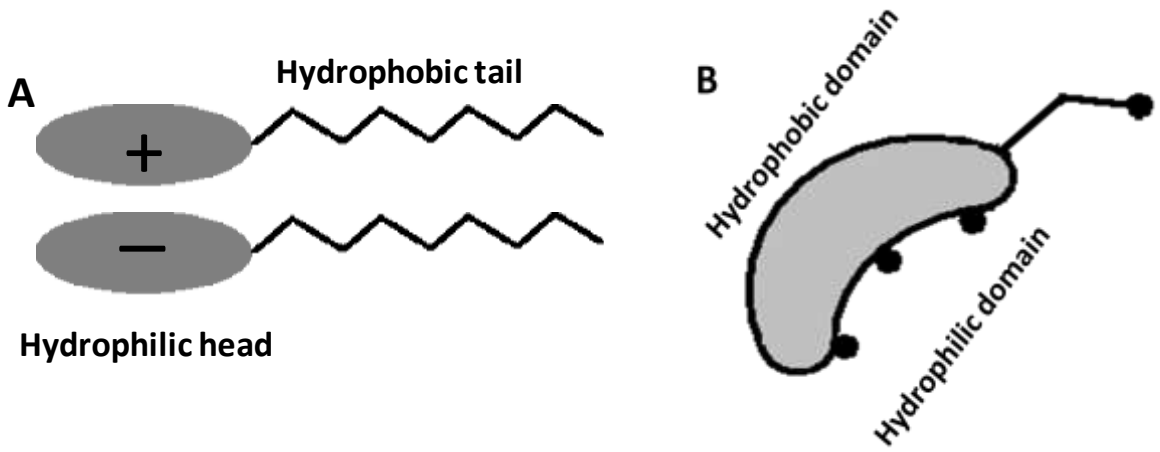



Fig.1.4: Cartoon representation showing difference in structure of a (A) conventional surfactant and (B) bile salt

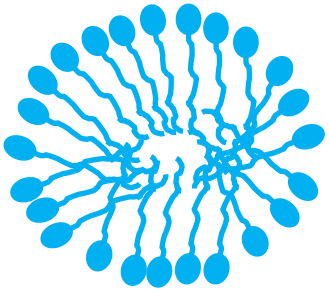


Fig. 1.5: Cartoon representation showing a micelle of conventional surfactant

Bile salts have a smaller aggregation number as compared to the conventional surfactants [23-25]. It was also suggested that bile salts micelles are smaller in size compared with sodium dodecyl sulfate micelles although their molecular length is almost similar [24]. Micellisation process of bile salts, in fact is still a topic of controversy. The two most debated models in this respect are: i) Small's model of primary and secondary micellisation and ii) Oakenfull and Fishers' model. Out of these two models Small's model is the widely accepted one.

### 1.3.3.1 Small's model

D. M. Small's model was based on his study using high-resolution $^{1}H$ nuclear magnetic resonance (NMR) technique. He has proposed that bile salts form two types of aggregates primary and secondary [26]. Primary micelles consist of two to nine monomers and they are

held together by hydrophobic interaction between the steroid nuclei. The primary micelles further aggregate and give rise to large aggregates, held together by hydrogen bonding between the hydroxyl groups of the primary micelles [Fig. 1.6]. The primary micelles are suggested to be globular in shape and the secondary micelles are roughly globular and have oblate ellipsoidal structure. Though there still remains ambiguity, this model however has gained more popularity among the researchers.

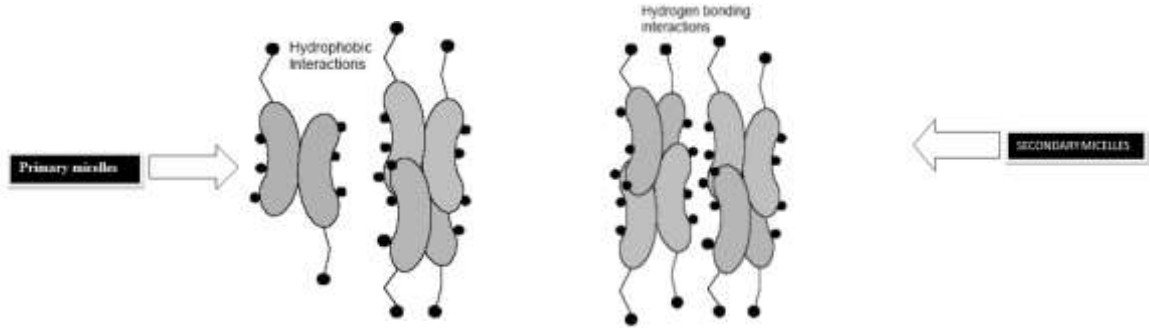



Fig.1.6: Pictorial representation of bile salts micelles as suggested by Small [26].

**1.3.3.2 Oakenfull and Fishers' Model*:***

Oakenfull and Fisher suggested that the first stage of aggregation is due to dimer formation which includes maximum number of hydroxyl and charged carboxylic groups. The back-to-back hydrophobic interaction between the dimers helps formation of layer aggregates of bile salts (Figure 1.5). This back-to-back hydrophobic interaction between the dimers is in synchronization with Small's model for formation of primary micelles. The stacked micelles give a rod like structure to the secondary aggregation [27].

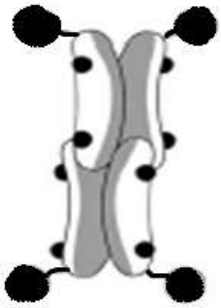


Fig. 1.7: Pictorial representation of bile salts micelles as suggested by Oakenfull and Fisher [27]

### 1.3.4 Factors affecting aggregation of bile salts

#### i. Temperature effect

Bile salt micelles are very sensitive to the temperature. As the temperature increases the kinetic energy of the molecules increases and the aggregation happens at relatively higher concentrations. The CMC of bile salts is known to increase with increase in temperature [28, 29]. With increase in temperature at high concentration of bile salt the concentration of anion increases with increasing temperature until it reaches the CMC. The narrow temperature range over which the solution changes from monomers to the bile salt micelles is called critical micellisation temperature [30].

#### ii. pH effect

The $pK_a$ of natural unconjugated bile acids is close to 5.1 and glycine conjugated is moderately stronger acids with a $pK_a$ of 3.7. The $pK_a$ value points towards the pH value at which the concentration in solution of protonated bile acid molecules is equal to that of the bile acid anion. The anion concentration (and total solubility) rises exponentially with increasing pH. The concentration of bile acid anions can be increased by increasing the pH when protonated bile acid is present in excess. When the concentration of bile acid anions is raised to the CMC, micelle formation occurs. The solubility of the bile salt increases very rapidly with further increase in pH, but the anion concentration remains practically constant

at the CMC. Thus, there occurs a critical micellisation pH or CMpH. The CMpH can be calculated by

$$CMpH = \log[CMC] + pK_a + pS_{BA}$$

$pS_{BA}$ is negative logarithm of the aqueous solubility of protonated acid. This equation indicates that the CMpH is directly correlated with CMC but inversely correlated with aqueous solubility of the protonated bile acid [30, 31].

**iii. Ionic strength effect**

As the ionic strength of an aqueous media increases, it helps in promoting the micellar aggregates of bile salts. Due to the counter ion effect, the ionic charges on the carboxylate group become neutralized which highly affect the CMC of bile salt micelles [22]. The literature survey has brought out various explanations about the effect of salt on the micellisation process. These counter ions reduce the CMC in case of ionic and non ionic detergents by a numerous mechanisms [32]. The major effect on the CMC due to added electrolyte is charge neutralisation but salting out effect concept proposed by Mukerjee [33] is quite considerable. The dependence of CMC and ion concentration can be proved thermodynamically. In order to prove it has been assumed that above CMC the bile salts exist as monodispersed micelles and monomers in equilibrium [34]. As the concentration of NaCl increases, the CMC of the bile salts decreases [22, 32].

**iv. Co-solvent effect**

In general, as the concentration of the cosolvents increases, the CMC increases and becomes less well-defined. [35]. The addition of cosolvents acts as water structure beakers resulting in decrease of hydrophobic effect leading to the decrease in the partition coefficients as well as the decreased dielectric constant plays an important role in deciding the extend of increase of CMC of the bile salts. Breaking of iceberg water structure stabilizing micelle facilitates the

interactions between hydrophobic part of the surfactant and the hydrophobic part of the cosolvent and consequently the local concentration of cosolvent increases around surfactant monomers than the bulk [36]. Thus solvation of the surfactant molecules by hydrophobic part of the organic solvents leads to delay in the aggregation of the surfactant monomers to form micelles and hence the increase in CMC.

## 1.4 Objectives of the study

The objectives of the present work are

1. To study the interaction of the cationic dye acridine orange (AO) with bile salts sodium deoxycholate (NaDC), sodium cholate (NaC), sodium taurodeoxycholate (NaTDC) and sodium taurocholate (NaTC).
2. To determine the binding constant, partition coefficient and thermodynamic parameters for the interaction of AO with all the bile salts.
3. To investigate the effect of ionic strength on the aggregation of AO and on the micellisation of bile salt.
4. To examine the effect of cosolvents (methanol, dioxane, acetonitrile) on the aggregation of AO and on the micellisation of bile salt.

# 2. Materials and Methods

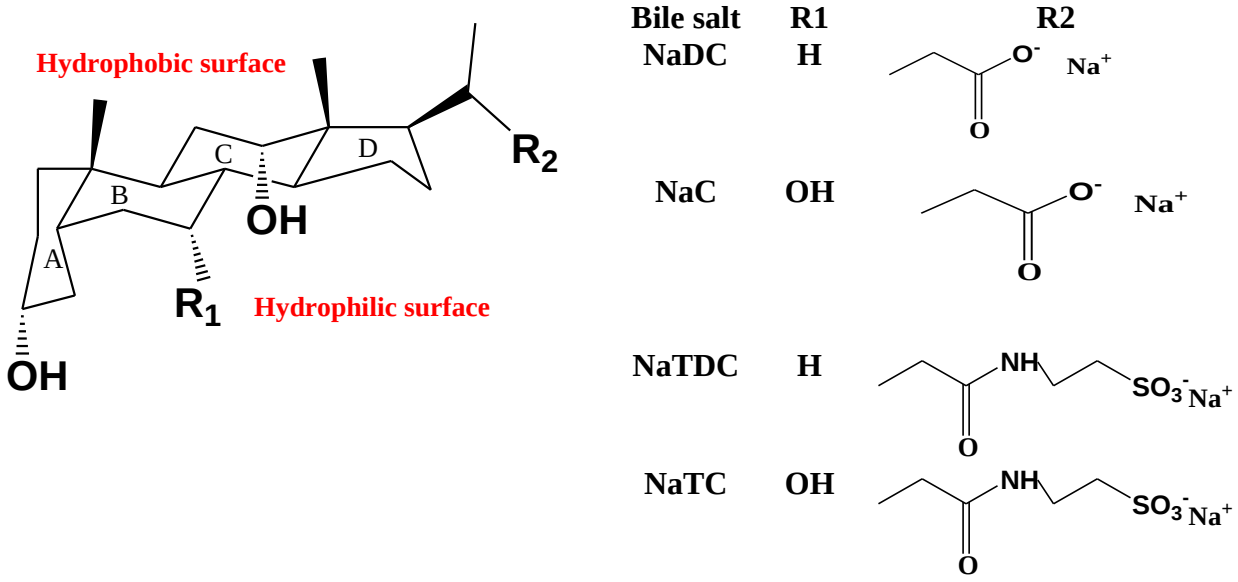
## 2.1 Materials

### 2.1.1 Chromophore Used

Acridine orange was purchased from SRL India and used as such.

### 2.1.2 Medium Components

The bile salts sodium cholate (NaC), sodium deoxycholate (NaDC) were obtained from SRL India and sodium taurocholate (NaTC), sodium taurodeoxycholate (NaTDC) were obtained from Sigma Chemical Company and used as such. Sodium chloride was obtained from SRL India and used as such.




Scheme 2.1: Molecular structure of different bile salts used.

### 2.1.3 Solvents

Deionised water was used for all the experiments. Methanol (SRL Chemical Co., India) of spectroscopic grade was used to prepare the stock solution of Acridine orange. The cosolvents methanol, 1,4-dioxane and acetonitrile were of spectroscopic grade and used as such to prepare 10% cosolvent – water mixture.

### 2.1.4 Instrumentation

The absorption spectra were recorded using Shimadzu Spectrophotometer (UV-1800).

## 2.2 Methods

### 2.2.1 Preparation of Bile Salts Solution

A desired stock solution of 40 mM for the bile salts was made in deionised water. Then on serial dilution the required concentrations of experimental solutions were obtained. The stock for the study of salt effect was made in the desired amount of salt solutions and the dilution was carried out using the salt solution instead of water. Every time fresh solutions of bile salt were made to avoid the problem of ageing.

### 2.2.2 Preparation of AO Solution

The stock solution (1mM) of acridine orange was made in methanol. The experimental solutions were made by adding a desired amount of AO to the bile salt solutions such that the final concentration in the experimental solution is maintained at $5 \times 10^{-6}$ M. The contamination of methanol in the experimental solution was maintained very low to avoid any perturbation to the micellisation process.

# 3. Important Findings

- Bile salts below the CMC induce aggregation in the cationic metachromic dye acridine orange.
- Thus it can be concluded that at very low concentrations (< 3 mM) of bile salts AO exist in its monomeric, dimeric as well as multimeric forms. When the concentrations of bile salts are close to their CMCs only the monomers and dimers exist and at concentrations above the CMCs, AO is solubilised in bile salt micelles in its monomeric form.
- The AO dimers formed are of H type.
- Dihyroxy bile salts induce the aggregation of AO more efficiently as compared to the corresponding trihydroxy bile salts.
- The plot of $A_M/A_D$ with the concentration of bile salt can be utilised for the estimation of CMC value.
- Binding and partitioning of AO to bile salt micelles is spontaneous and exothermic in nature.
- AO shows higher binding and partitioning efficiency towards the dihydroxy bile salts. The order of binding and partitioning efficiency of AO towards the four bile salts is NaDC > NaTDC > NaTC > NaC.
- With increasing NaCl concentration, the CMC of NaDC and NaC decreases with higher value of $\beta$ for NaDC.
- For both the bile salts the $\Delta G_m^0$(energy for micellization) decreases with increasing concentration of NaCl.
- The cosolvents affect the micellization of bile salts as well as the aggregation of AO and follows the order Dioxane> Methanol > Acetonitrile.

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