EFFECT OF CHAOTROPIC REAGENTS ON BOVINE SERUM ALBUMIN (BSA) - A FLUORESCENCE STUDY

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CERTIFICATE

This is to certify that the dissertation entitled **"Effect of Chaotropic Reagents on Bovine Serum Albumin (BSA) – A Fluorescence Study"** being submitted by Miss Smruti Snigdha Mishra to the Department of Chemistry, National Institute of Technology, Rourkela, Orissa, 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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Smruti Snigdha Mishra

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INTRODUCTION

1.1 CONCEPT OF PROTEIN:

Proteins are biomolecules of one or more polypeptides folded into a globular or fibrous form in a biologically functional way required for the growth and maintenance of life systems [1]. A polypeptide is a single linear polymer chain of amino acids bonded together by peptide bonds between the carboxyl and amino groups of adjacent amino acid residues. In nature only 20 amino acids are found and the primary structure of different proteins results from just a permutation and combination of these amino acids providing them a unique function.

There are four distinct levels of protein structure.

1. Primary structure refers to the sequence of the different amino acids of the polypeptide or protein. The primary structure is held together by covalent or peptide bonds, which are made during the process of protein biosynthesis or translation.
2. Secondary structure refers to the regular repeating pattern of local structures stabilized by hydrogen bonds. The most common examples are the alpha helix, beta sheet and turns.
3. Tertiary structure defines the overall shape of a single protein molecule; the spatial relationship of the secondary structures to one another. Tertiary structure is generally stabilized by nonlocal interactions, most commonly the formation of a hydrophobic core, but also through salt bridges, hydrogen bonds, disulphide bonds, and even post-translational modifications. The tertiary structure is what controls the basic function of the protein.

4. Quaternary structure is formed by several protein molecules (polypeptide chains), usually called protein subunits in this context, which function as a single protein complex.

1.2 SERUM ALBUMIN:

Serum albumin is a highly soluble multi-domain protein, without prosthetic groups or bulky appending carbohydrates that is very stable and available at high purity andlow cost. It is highly soluble and of elliptical shape with a low intrinsic viscosity. Albumin is a very stable protein although more than 50 slight variants of the 585 amino acid sequences that comprises human albumin exists. Human serum albumin (HSA) structure has been revealed by high-resolution X-ray image of the protein[2, 3], which is directed towards the determination of the tertiary structureof other mammalian albumins as they resemble closely to it. Thus serum albumin molecule can be described as a very flexible protein that changes shape with variations in environmental conditions and with binding of ligands. Despite this albumin has a resilient structure and regains shape easily owing to the disulphide bridges, which provides strength especially in physiological conditions. After their rupture the molecule can re-establish these bridges and regain its structure [4]. Denaturation occurs only with dramatic and non-physiological changes in temperature, pH and the ionic or chemical environment.

Albumin, the most abundant extracellular protein accounts for total 60 % of the total serum content in human. It is manufactured in the liver and is a single polypeptide with 585 amino acids and a molecular weight of 66200 D.The primary structure of serum albumin differs from other extracellular proteins. Serum albumin has one cysteine group (Cys-34) and low tryptophan content. The secondary structure consists of approximately 67 % of α-helix as well as there are 9 loops and 17 disulphide bridges giving a heart shaped 3D structure

confirmed by X-ray crystallography studies. [2, 5]. The tertiary structure is composed of three domains I, II, and III, and each domain is constituted of two subdomains A and B.

1.2.1 Function of serum albumin:

Serum albumin has been one of the most extensively studied being the most abundant protein in blood plasma with typical concentration of 50 g/L. Some of the albumins most commonly studied are human serum albumin (HSA), bovine serum albumin (BSA), equine serum albumin (ESA) and rat serum albumin (RSA).

Physiological Roles of albumin:

1. Maintenance of the colloid osmotic pressure (COP)

   Albumin is responsible for the 75-80 % of osmotic pressure. It constitutes the main protein in the blood plasma and in the interstitial. So it is the COP gradient rather than absolute plasma value which is important. It defines the flow of fluid in and out of the capillaries [4].

2. Binding and transport, particularly of drugs

   Albumin helps in transport of drugs and ligands by binding to it and so reduces the serum concentration of these compounds. There are particularly four binding sites on albumin with varying specificity for different substances. Competitive binding of drugs may occur at same site or different sites leading to conformational changes, for example: warfarin and diazepam. In other words they can be considered as carriers for numerous exogenous and endogenous compounds in the blood. [2, 5]

3. Free radical scavenging

Albumin is a major source of sulfhydryl group, these "thiols" scavenge free radicals (nitrogen and oxygen species). It may be an important free radical scavenger in sepsis also [4].

4. Acid base balance

Albumin is a negatively charged protein in high concentration in the plasma. It contributes heavily to what called the “anion gap”: Classically the anion gap is calculated as (Na + K) − (Cl) = AG (mEq/l). The concentration of anions and cations in plasma should be equal so the remaining anions come predominantly from albumin, inorganic phosphate and haemoglobin. Thus, in hypoalbuminemic states meaning high albumin concentration in blood plasma, the anion gap should be narrowed [4].

5. Effects on vascular permeability

Albumin has a role in limiting the leakage from capillary beds during stress induced increase inthe capillary permeability. This is related to the ability of endothelial cells to control the permeability of their walls, and the spaces between them. Albumin may plug this gap or may have a deflecting effect, owing to its negative charge. This has led to the hypothesis that colloids are effective at maintaining vascular architecture [4].

1.2.2 Structure of Bovine Serum Albumin (BSA):

The primary structure of BSA was presented in the same year as HSA. The proposed structure was composed of 582 amino acid residues. The sequence has 17 disulphide bonds resulting in nine loops formed by the bridges. BSA contains one cysteine and 8 pairs of disulphide bonds similar to HSA [2]. BSA also contains a high content of Asp, Glu, Ala, Leu

and Lys as well as the four amino acid residues in the sequence determined later as Gly–Phe–Gln–Asn[6]

According to the amino acid sequence proposed by Brown, the structural featuresof BSA show that it is composed of three homologous domains [6, 7]. Circular dichroism measurements suggest that BSA secondary structure contentfor α- helix, β- sheet, turn and random coil are 48.7 %, 0 %, 10.9 % and 30.7 %, respectively [8,9]. In the secondary structure of BSA, it has been suggested thatthe α-helices are uniformly placed in the subdomains and in the connections between thedomains. Most of the residues in the long loops (except at the end) and the sections linking the domains possibly form α-helices, whereas the intra-domain hinge regions aremainly non-helical structure. The three long helices in the subdomain are considered as principle elements of the structure. These run parallel with each other, and a trough is formed owing to the middle helix (Y) being slightly lower in position. The helices are mainly linked together by disulphide bridges [5].

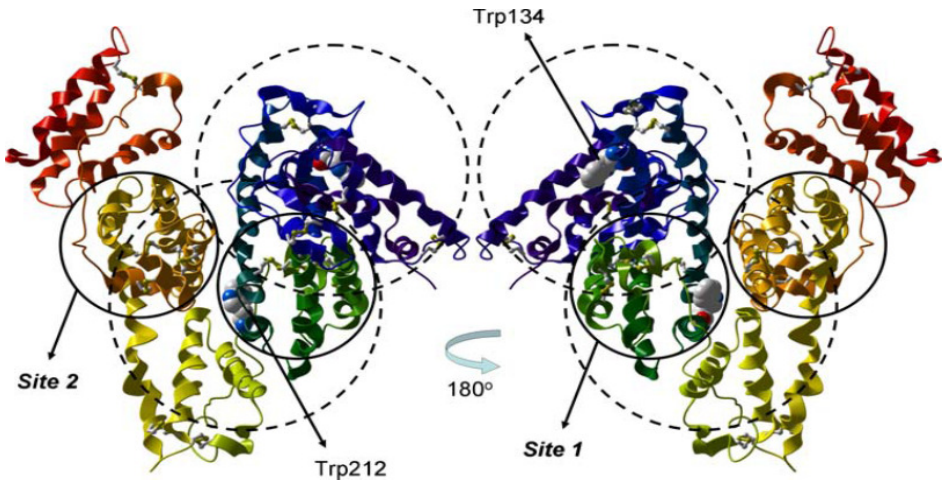


Fig1:Two side-on 3D graphic representation of a BSA model structure based on HSA X-raycrystal structure obtained from the Protein Data Bank (PDB ID:1UOR) [2,3].

This 3D graphic structure is in accordance with the proposed domains and sub- domains present in the BSA. It shows clearly the presence of two tryptophan residues that is basically responsible for the intrinsic fluorescence of BSA.

1.2.3 Difference between HSA and BSA:

HSA and BSA are the most studied serum albumin proteins. There occurs almost 76% homology and a repeating pattern of disulphides which is conserved. The major difference between the two occurs with respect to the number and positioning of tryptophan residues in them. HSA has only one tryptophan, located at position 214 which is equivalent to Trp-212 for BSA present buried in a hydrophobic pocket at sub domain IIA. BSA has one more additional tryptophan Trp-134, which is more exposed to solvent and found at sub domain IB [3, 4]. Thus BSA which is a homologous protein of HSA is selected as the protein model due to its medical importance, low cost, ready availability, and unusual ligand-binding properties [2, 9-10].

1.3 DENATURATION:

Protein denaturation is associated with any modification in conformation not accompanied by rupture of peptide bonds and ultimately resulting in a totally unfolded polypeptide structure which can be reversible or irreversible. It most often results in loss of bioactivity due to the alteration in the tertiary structure of the proteins. Other effects include, exposure of hydrophobic groups upon denaturation often leading to adsorption on the surfaces, aggregation, and precipitation. Denaturation sometimes also triggers the chemical degradation pathways often not seen with the native or natural tertiary (and/or quaternary) structure. The effects of denaturation are[11]

- Decreased solubility
- Altered water binding capacity

- Destruction of toxins
- Improved digestibility
- Increased intrinsic viscosity
- Inability to crystallize

1.3.1 Causes of protein Denaturation:

1. Temperature fluctuation

   (a) Effect of increased temperature:

   - Affect interactions of tertiary structure
   - Increased flexibility → reversible
   - H-bonds begin to break → water interaction
   - Increased water binding
   - Increased viscosity of solution
   - Structures different from native protein

   (b) Effect of decreased temperature:

   - Can result in Denaturation (*e.g.* Gliadins, egg and milk proteins)
   - Remain active (Some lipases and oxidases and Release from sub-cellularcompartments)
   - Proteins with high hydrophobic/polar amino residues and structures dependent on hydrophobic interactions do lose their activity.

2. Water content affects denaturation by the process of heat or thermal treatment.
3. Every protein has an optimal pH for its bioactivity. Slight changes in pH can affect itsactivity. Strong acidic or basic conditions can denature the protein. Physiological pH for maximum proteins is in the range 7.2 to 7.4.
4. Mechanical treatments induce denaturation.

5. Hydrostatic Pressure also induces denaturation
6. Irradiation assists for denaturation to occur.
7. Heavy metal salts act to denature proteins in much the same manner as acids and bases. Heavy metal salts usually contain Hg^{+2}, Pb^{+2}, Ag^{+1} Tl^{+1}, Cd^{+2} and other metals with high atomic weights act on by disrupting salt bridges in proteins being ionic in nature. The reaction of a heavy metal salt with a protein usually leads to an insoluble metal protein salt.
8. Heavy metals may also disrupt disulphide bonds because of their high affinity and attraction for sulphur and will also lead to the denaturation of proteins.
9. Alcohol Disrupts Hydrogen Bonding: Hydrogen bonding occurs between the amide groups in the secondary protein structure. Hydrogen bonding between "side chains" occurs in tertiary protein structure in a variety of amino acid combinations. All of these are disrupted by the addition of another alcohol. Thus alcohol denatures proteins by disrupting the side chain intramolecular hydrogen bonding. New hydrogen bonds are formed instead between the new alcohol molecule and the protein side chains.

1.3.2 Why denaturation study is important?

Proteins and peptides exhibit the following challenges to the formulation scientists

a. They exhibit maximal chemical instability.
b. They tend to self-associate.
c. They adopt multiple conformers.
d. They can also exhibit complex physical instabilities, such as gel formation.

Proteins are no doubt an important constituent of medication. Since the medicines move through the blood by binding with the serum albumins only, so it is necessary to account for their interaction to obtain the needed best results.

Moreover in order to overcome the challenges the investigation of the mechanism of protein folding/ unfolding/ refolding is necessary [12]. Small changes occurring in the local environment also result in structural changes leading to the formation of alternate conformations resulting in loss of original function of the protein. The area of main concern is that the defects in native structure of protein folding lead to a wide range of human genetic disorders. In particular the rare neurodegenerative illnesses in mammals such as Alzheimer's and Parkinson's diseases [13,14] occurs due to competition of misfolded or partially unfolded protein with the normal protein leading to adverse effects. Hence a considerable attention is required to characterize partially unfolded protein states and to gain more insight into the information about the sequence and steps involved in protein folding mechanisms. This will help to solve the problem by revealing the causes of unfolding and the process involved in it to avoid the same for medication and other process requiring the effective storage of proteins.

1.3.3 Types of denaturing reagents:

The denaturation process can be achieved by any one of the following methods:

- Increasing temperature
- Changing pH
- Using denaturants (urea, guanidine hydrochloride, beta-mercaptoethanol, dithiothreitol)
- Inorganic salts (lithium bromide, potassium thiocyanate, sodium iodide)
- Organic solvents and (formamide, dimethylformamide, dichloro- and trichloroacetic acids and their salts)
- Detergents (sodium dodecyl sulphate)
- High pressure
- Ultrasonic homogenisation.

Hence the process of denaturation can be basically divided into physical and chemical agents accounting for the change. The driving force for denaturation for physical process is the increase in entropy that accompanies the transition of a single conformation into an ensemble of random ones. With increasing temperature the contribution of this entropy increases and becomes more significant, and at some temperature it overcomes the energy effect (the protein is heat denatured). The possible intermediate structures accounts to a great extend for understanding the process of denaturation or unfolding. The early unlocking of the tertiary structure deletes a large number of the bonds holding the structure together but increases the randomness only insignificantly. The later stages of denaturation lead to larger increases in entropy. The unfolding of the protein exposes the buried non-polar amino acid residues. Their intermolecular clustering leads to aggregation of the denatured protein. Consequently, heat denaturation is essentially irreversible [15].

In chemical denaturation the secondary bonds holding the protein segments together are disrupted by some chemicals capable of forming equally strong or stronger bonds with the groups holding the conformation together. For disrupting the hydrogen bonds, urea or guanidine hydrochloride is used. At high concentrations of these substances (e.g., 8M urea or 4M guanidine hydrochloride) many proteins adopt a highly unfolded conformation in solution. Proteins of multiple subunits are likely to be separated into their constituent polypeptide chains. Other proteins aggregate upon denaturation in urea or guanidine hydrochloride which is frequently due to the formation of disulphide bridges between sulfhydryl groups made accessible by the unfolding of the polypeptide chains. Such reactions may be inhibited by the addition of iodoacetate[16].Under these conditions, the denatured molecules remain in solution and may revert into native molecules if the denaturing agent is slowly dialysed away. Powerful detergents like SDS disrupt both hydrophobic and hydrogen bonds and effectively solvate the denatured molecule. Beta-Mercaptoethanol and

dithiothreitol (DTT) disrupt disulphide bonds and can be used in conjunction with urea or SDS to fully solubilise protein molecules.[17]

Thus the chemical denaturation can be basically described on the mode of breakage of the stabilising bonds in protein molecules resulting in their denaturation. There are basically two types of solvent structure modifying reagents which are of equal interest in describing the interaction with proteins, defined by Chaotropes and Kosmotropes. One of the cosolvents neutralises the dangerous solutes by decreasing their solubility and enhancing theformation of their aggregates. Such cosolvents are known as promoters of the water structure and are referred to as kosmotropes (order maker). Their stabilising function for proteins and aggregates of hydrophobic solute particles, and their importance for the osmotic balance in cells have triggered the interest in their study [18-19].

1.3.4 Concept of Chaotropic Reagents:

The other important cosolvent is the Chaotropic ones which increase the solubility of non - polar solute particles in aqueous medium [20-23]. For certain systems the solubility may be enhanced by several factors leading to a complete destabilisation of solute aggregates. In the case of protein solutions it leads to the complete denaturation and loss of function. [24-26].Some examples of substances displaying this property are urea, guanidine hydrochloride, thiourea and lithium perchlorate. These act as protein denaturant at a relatively higher concentration. Chaotropic cosolvents (order breaking) are less polar than water and so break hydrogen bonds between water molecules suppressing water structure formation. [19,27-29]. Chaotropic agents interfere with stabilizing inter-molecular interactions mediated by non-covalent forces such as hydrogen bonds, van der Waals forces and hydrophobic effects leading to the unfolding of protein structure. During the denaturation process the primary structure of protein remains almost unaffected but it disrupts both the secondary and

tertiary structures of proteins *i.e.* total or partial loss of three dimensional structures. Partially folded intermediates have been observed during the folding process for a number of proteins under in-vitro conditions [12]. The intermediate states of unfolding/refolding mechanisms can be studied through the denaturation of proteins. The complexity of the denaturation process is more evident when the protein contains multi-domain structures in which each domain can unfold dependently or independently and, the association of different domains in the whole protein through various short-range and long-range interactions may affect the overall process [30-31]. Thus chaotropic agents can be described as that they disrupt the inter-molecular forces between water molecules, allowing proteins and other macromolecules to dissolve more easily. But the exact physical mechanism for the changes in water structure and the destabilising effect of chaotropic agents is not fully understood.

1.3.5 Different techniques used for study of denaturation process:

The denaturation process can be followed using a lot of different experimental approaches [12, 32-33]. Fluorescence spectroscopy is a reliable tool in the study of proteins due to its great sensitivity and selectivity. In many cases, the protein of interest will contain one or more amino acids that are intrinsically fluorescent (phenylalanine, tyrosine, or tryptophan). The characteristics of these fluorescent amino acids have been clearly described in results and discussion part to account for the changes during the unfolding or denaturation process. The emissions of these intrinsic fluorophores are environmentally sensitive (generally via polarity) which thus allows effectively to follow the denaturation process. Extrinsic fluorophores attached to the protein can also be used to extend the possible range of protein denaturation studies in particular where an intrinsic fluorescent amino acid is absent [34, 35]. Geometrical effects can be successfully quantified using Forster resonance energy transfer (FRET) where the changes in distance between donor and acceptor centres can be monitored

[36-38]. The other techniques used are circular dichroism, dynamic light scattering, UV-Visible spectroscopy and many others to study the denaturation process of proteins depending on the agent which causes the unfolding to occur.

Recently Deniso *et al.* have studied the thermodynamics to account for the changes induced by chaotropic agent guanidine hydrochloride resulting in the denaturation of BSA using the fluorescence decay kinetics at different stages [39]. Sulkowska *et al.* by using fluorescence measurements have shown that the destabilisation of its tertiary structure BSA occurs by guanidine hydrochloride [40]. The denaturant guanidine hydrochloride destroys the native protein structure by the interaction between its amino group and the carbonyl group in the peptide bond, bythe destabilization of water structure or by hydrophobic interactions between the aliphatic side chains of the denaturant and the hydrophobic amino acid residues.

The unfolding of BSA seems to be a complex process in view of the independent unfolding behaviour of all the three domains. The effectiveness of urea as a protein denaturant is known. It acts by increasing the aqueous stability around the hydrophobic domains of protein and/or weakening the hydrogen bonding in the protein structure [41]. Amit Das *et al.* have investigated and revealed the presence of any folding intermediates during urea denaturation of BSA and have reported about the changes occurring in the shape and size during the unfolding of BSA by using small angle neutron scattering technique for thermal denaturation [42]. The kinetics of BSA denaturation in the absence and the presence of urea was also studied by the isoconversional method and the master plots using differential scanning calorimetry by Xiaomin Cao *et al.* This helps in exploring the kinetics process of protein denaturation which shows reduced thermal and kinetic stability of BSA [43].

The mechanism of the denaturing activity of urea differsfrom that of guanidine hydrochloride. Urea breaks the hydrogen bonds or makes them weaker. Urea molecules form intra-molecular complexes with parts of the polypeptide chain. Guanidine hydrochloride

probably stimulates the formation of new hydrogen bonds between the denaturant molecule and the proteinafter the destruction of intra-molecular hydrogen bonds [44]. This has been extensively studied by Aschi Adel *et al*. from the study of thermally and chemically unfolded conformations of bovine serum albumin by means of dynamic light scattering. Sułkowska *et al.* have also studied extensively the denaturation of BSA and HSA in presence of urea and also the effect of urea on the serum albumin complex and antithyroid drugs by fluorescence spectroscopy using the tryptophan emission [41].

The interests of our study are the chaotropic reagents urea and guanidine hydrochloride these being less polar than water act upon by leading to an energetically unfavourable disruption ofwater structure. The addition of these denaturing agents to aqueous solutions therefore resultsin an increase in solvent-accessible surface area which destabilises hydrophobic aggregates, micelles and native protein structures ultimately leading to the process of unfolding or denaturation.

1.4 AIM OF THE PRESENT WORK:

- To study the effect of two chaotropic reagents Urea and Guanidine hydrochloride on BSA structure
- To use the intrinsic fluorescence of protein meaning all the three fluorescent active amino acids present phenylalanine, tyrosine and tryptophan to account for the denaturation process by exciting at 280 nm.
- To use selectively the intrinsic fluorescence of tryptophan to follow the denaturation process by exciting at 295 nm.
- To use different fluorescent parameters such as fluorescence intensity, emission maximum, emission energy and fluorescence anisotropy to follow the denaturation process of BSA.

CHAPTER-2

MATERIALS AND METHODS

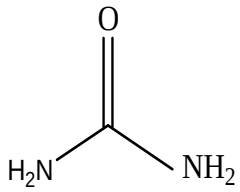
2.1 MATERIALS:

2.1.1: Protein used:

Bovine serum albumin fraction V (BSA- protease free) was purchased from SRL India. The intrinsic fluorescence of the amino acids present *i.e.* tryptophan, tyrosine and phenylalanine is used for the study of denaturation process.

2.1.2: Chaotropic agents:

Urea and guanidine hydrochloride of AR grade were purchased from SRL India.



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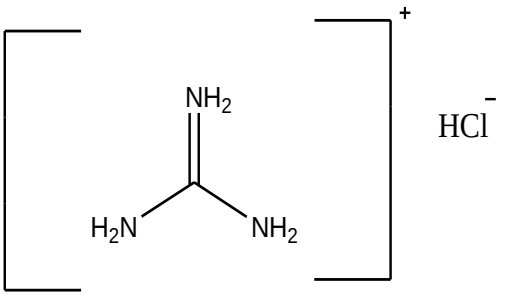


GUANIDINE HYDROCHLORIDE

2.1.3 Solvents :

Deionised water was used for all the experiments.

2.1.4 Instrumentation:

The absorption spectra were recorded using ***Shimadzu Spectrophotometer (UV-2450)*** and the emission spectra and steady-state fluorescence anisotropy values were recorded using ***Horiba Jobin Yvon Spectrofluorimeter (Fluoromax-4P).***

2.2 METHODS:

2.2.1 Preparation of BSA Solution:

The BSA stock of 10^{-4} M was prepared in deionised water fresh every time. The experimental solutions were made by dilution of stock such that 2 x 10^{-5} M and 1 x 10^{-5} M was maintained for absorption study and fluorescence study, respectively. The studies werecarried out after giving an equilibration time of about 30 minutes everytime.

2.2.2 Preparation of Chaotropic Agents Solutions:

The stock solutions of urea (14 M) and guanidine hydrochloride (10 M) were prepared taking care of the fact that they drastically increase volume, so the volume make up was done carefully.Then the dilution was done to obtain the required concentration of experimental solution.

2.3 TECHNIQUES USED:

2.3.1 Measurement of Absorption Spectrum:

The main elements of UV-VIS spectrophotometer are a light source, a monochromator and a detector. The monochromator works as a diffraction grating to dispense the beam of light into various wavelengths. The detectors role is to record the intensity of the light which has been transmitted.

Before the samples are run, a reference must first be taken. This calibrates the spectra to screen out an spectral interference. In this case milipore water was used as solvent as reference.

2.3.2 Measurement of Steady-State Fluorescence Spectrum:

The spectrofluorimeter is an instrument which takes advantage of intrinsic or extrinsic fluorescent properties of the compounds in order to provide information regarding their concentration and chemical environment in a sample. A certain excitation wavelength is

selected, and the emission is observed either at a single wavelength or a scan is performed to record the intensity versus wavelength also called an emission spectra. The slit widths are also fixed for a particular experiment at which the best output is obtained. In the present experiment the excitation wavelengths selected was 295 nm and the emission spectra were recorded in the range of 305 nm-570 nm, respectively with slit widths of 3/3 nm.

Basic Components of fluorimeter:

1) Excitation energy is provided by a light source (Xenon lamp).

2) Light passes through a primary (excitation) filter before entering sample compartment.

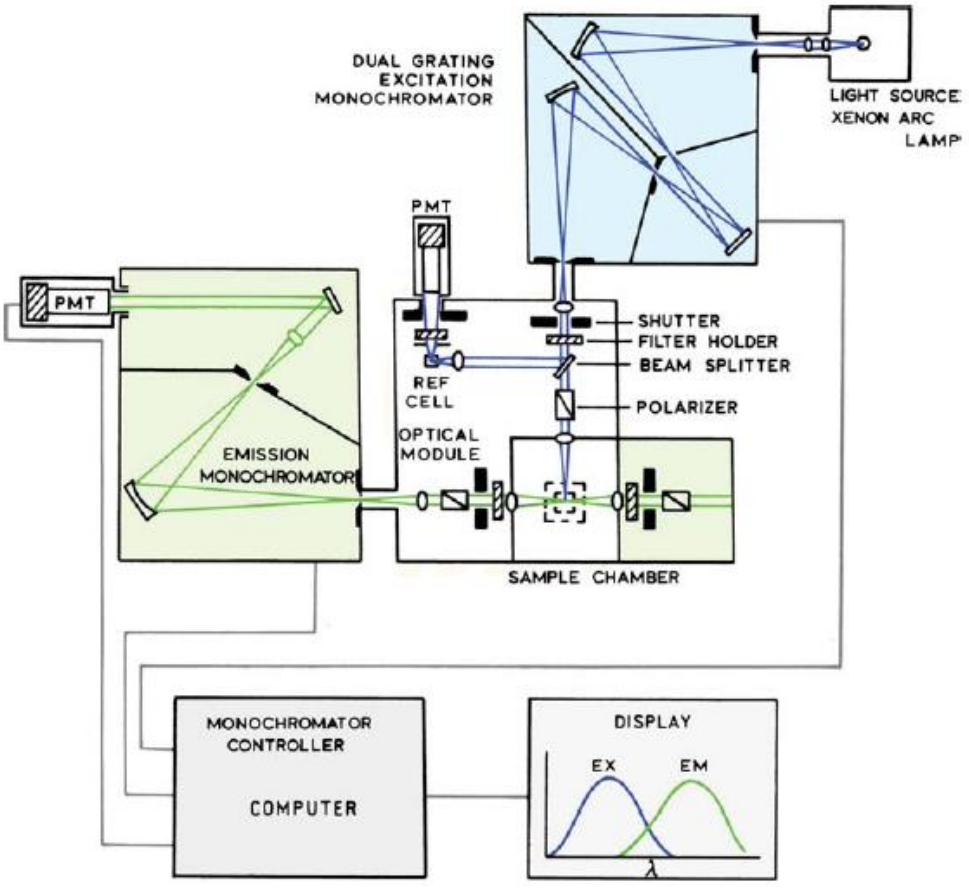
3) Light is absorbed by the fluorescent sample.

4) After excitation of the fluorescent substance, return to lower energy state occurs and light with a longer wavelength (fluorescence) is emitted.

5) Fluorescent light passes through a secondary filter (emission) which is opaque to light passing the primary filter and is at 90 degree angle to the primary light path.

6) The amount of light passing through the secondary filter ismeasured on a photomultiplier.

- Fluorophores preferentially absorb photons whose electric vectors are aligned parallel with transition moment of the fluorophore. In an isotropic solution, fluorophores are oriented randomly. Upon excitation with polarized light, one selectively excites those fluorophore molecules whose absorption transition dipole is parallel to the electric vector of the excitation. This selective excitation results in partially oriented population of fluorophores and in partially polarized fluorescence emission.



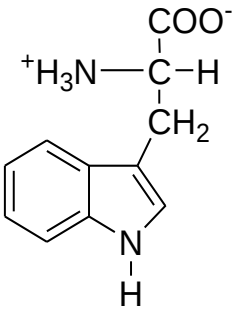
BLOCK DIAGRAM OF SPECTROFLUORIMETER

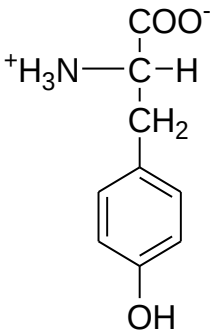
Reproduced from "Fluorescence Spectroscopy for biomolecularstudies":Exp. Biomol. Physics/ Appl. Phys. AlbaNova University Centre, KTH, Stockholm

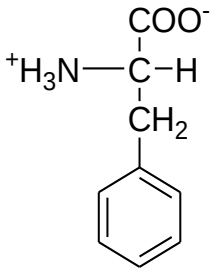
2.4 Parameters Studied:

Fluorescence intensity depends upon the polarity of the medium as well as the rigidity of the fluorophore.

Selection of Excitation Wavelength







TRYPTOPHAN TYROSINE PHENYLALANINE

These are the 3 amino acids responsible for inherent fluorescence of protein. Tyrosine and phenylalanine are weakly fluorescent. In other words basically the tryptophan emission is followed for the study of unfolding of BSA.

CHAPTER-3

RESULTS AND DISCUSSION

3.1: FLUORESCENT AMINO ACIDS PRESENT IN BOVINE SERUM ALBUMIN:

The three amino acid residues that are primarily responsible for the inherent fluorescence of proteins are tryptophan, tyrosine and phenylalanine. These residues have distinct absorption and emission wavelengths and differ in the quantum yields (Table 3.1). Tryptophan is much more fluorescent than either tyrosine or phenylalanine. However, the fluorescent properties of tryptophan are solvent dependent. As the polarity of the solvent decreases, the spectrum shifts to shorter wavelengths and increases in intensity. For this reason, tryptophan residues buried in hydrophobic domains of folded proteins exhibit a spectral shift. This phenomenon has been utilized to study protein denaturation [34].

Table 3.1: Fluorescent Characteristics of the Aromatic Amino Acids. [34]

	Absorption		Fluorescence	
Amino Acid	**Wavelength (nm)**	**Absorptivity**	**Wavelength (nm)**	**Quantum Yield**
Tryptophan	280	5,600	348	0.20
Tyrosine	274	1,400	303	0.14
Phenylalanine	257	200	282	0.04

Tyrosine can be excited at wavelength similar to that of tryptophan, but emits at a distinctly different wavelength. While tyrosine is less fluorescent than tryptophan, it can provide significant signal, as it is often present in large numbers in many proteins. Tyrosine fluorescence has been observed to be quenched by the presence of nearby tryptophan moieties via resonance energy transfer, as well as by ionization of its aromatic hydroxyl

group. Phenylalanine is very weakly fluorescent and can only be observed in the absence of both tryptophan and tyrosine [34].

3.2: TRYPTOPHAN EMISSION FOR THE DENATURATION STUDY:

Tryptophan has amino nitrogen in the indole group, which can form a hydrogen bond with solventand thus gives rise to the spectral shift [34].Due to tryptophan's greater absorptivity, higher quantum yield, and resonance energy transfer, the fluorescence spectrum of a protein containing the three amino acids usually resembles that of tryptophan. The absorption spectrum of BSA in aqueous mediumas shown by Fig 3.1 has characteristics of all the three amino acids with maximum around 280 nm.

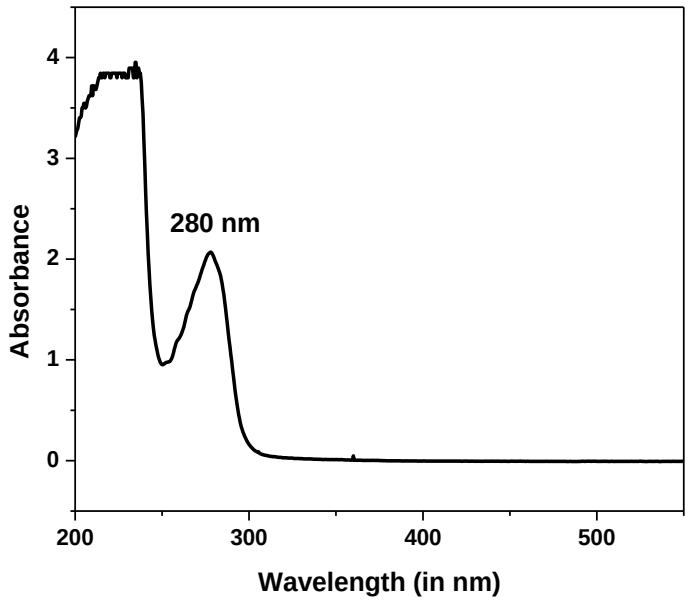


Fig 3.1: Absorption spectrum of BSA (2 x 10^{-5} M) in water

3.3:EFFECT OF UREA ON BOVINE SERUM ALBUMIN:

3.3.1:Absorption Studies:

The UV-Visible absorption technique was used for studying the denaturation or unfolding process of BSA.But from absorption studies on referring to the Fig 3.2, it does not clearly

give any indication about the denaturation studies with no significant changes in the absorbance with the increasing concentration of urea. This is so may be due to the reason that it is a combined effect on the three amino acids present not clearly signifying the result. But the slight increase in the absorption values which coincides with each other at higher urea concentration was observed reflecting that the denaturation is occurring.

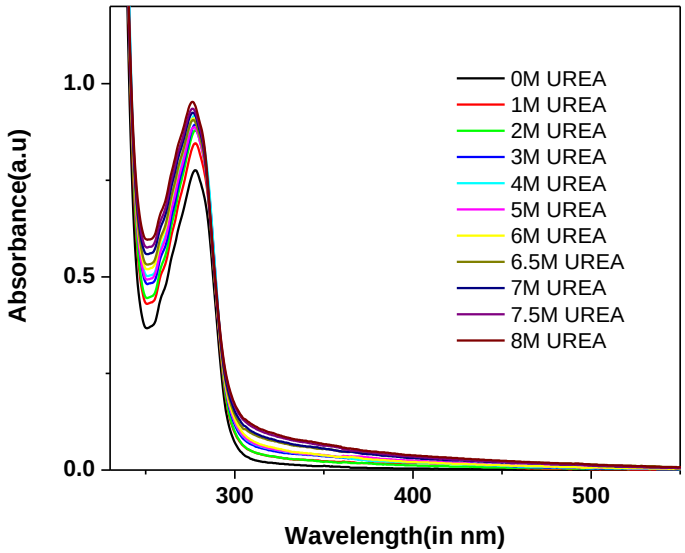


Fig 3.2: Absorption spectra BSA (2 x 10^{-5}M) with increasing concentrations of urea

3.3.2: Fluorescence Studies:

Initially at lower concentration of urea (< 4 M) there is a slight blue shift in the emission maximum observed, which is more clear in the plots of fluorescence maximum and emission energy with concentrations of urea (Fig 3.5A & 3.5B). The blue shift observed for less than 4 M urea can be due to the movement of Trp212 to a less polar inner core of hydrophobic cavity of sub domain IIA. This movement may occur due to the partial rearrangement of domain II or due to the alteration in the packing structure of domain II and III because of the change in the distance of separation between them.

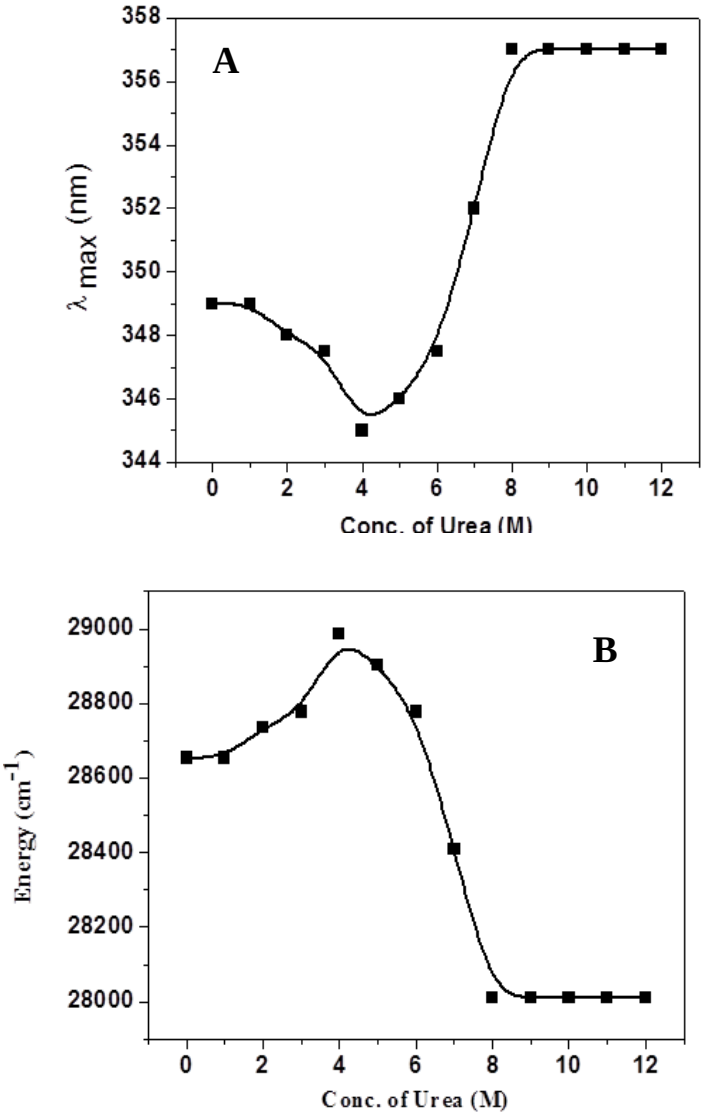


Fig 3.5: Variation of (A) emission maximum and (B) emission energy of BSA as a function of urea concentration.

After 4M urea there is a clear red shift in the emission maximum observed up to 8 M of urea beyond which the emission maximum remains constant. This can be due to the exposure of Trp212 to a more polar environment thus allowing water molecules to quench the fluorescence or quenching occurs by other amino acid residues which become close to the tryptophan. [41, 45-47]. In other words the denaturation or unfolding starts by 4M of urea and is almost complete by 8M of urea. The variation of emission energy maximum of BSA as a function of urea concentration also supports it as evident from the nature of Fig 3.5 B.

The above argument is further supported by the variation of fluorescence intensity with urea concentration shown by Fig 3.6.

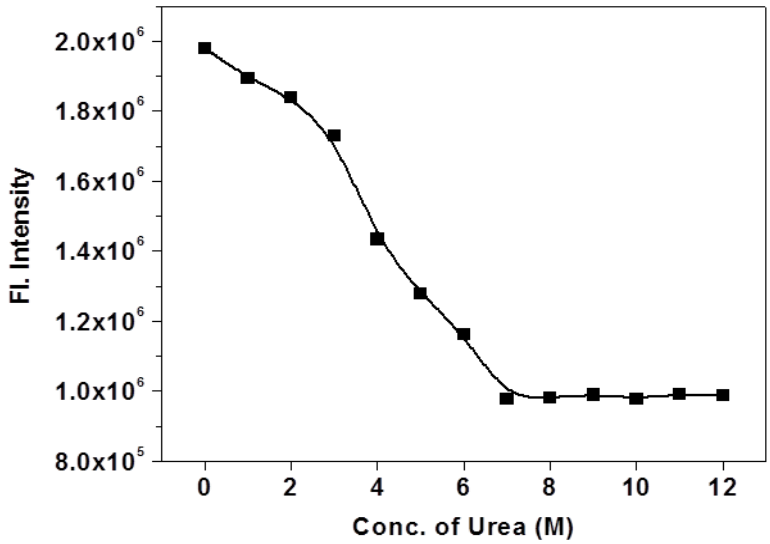


Fig 3.6: Variation of Fluorescence intensity of BSA as a function of urea concentration.

The intensity gradually decreases with the increasing urea concentration and beyond 8 M of urea it remains constant. From the plot it can be clearly seen that the maximum change occurs between 4M– 8M of urea, which means the denaturation or unfolding is almost complete by 8M of urea. The denaturation of BSA is complete by 8 M of urea, which exposes the tryptophan to the aqueous medium resulting in a bathochromic shift in the emission maximum and quenching of the tryptophan fluorescence. So with increasing concentration of urea the individual contribution of tyrosine and tryptophan towards the protein emission is clearly seen.

3.4: EFFECT OF GUANIDINE HYDROCHLORIDE ON BSA:

3.4.1:Absorption Studies:

Guanidine hydrochloride is the other chaotropic agent used to study the effect on BSA. The chaotropic property was first tried to be investigated by absorption studies but as mentioned

earlier for urea it does not give much information about the unfolding process being the average effect of all the three amino acids.

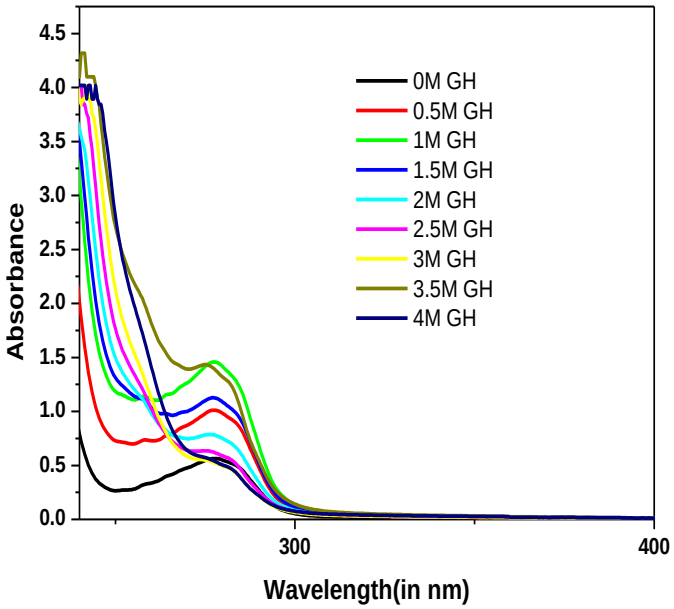


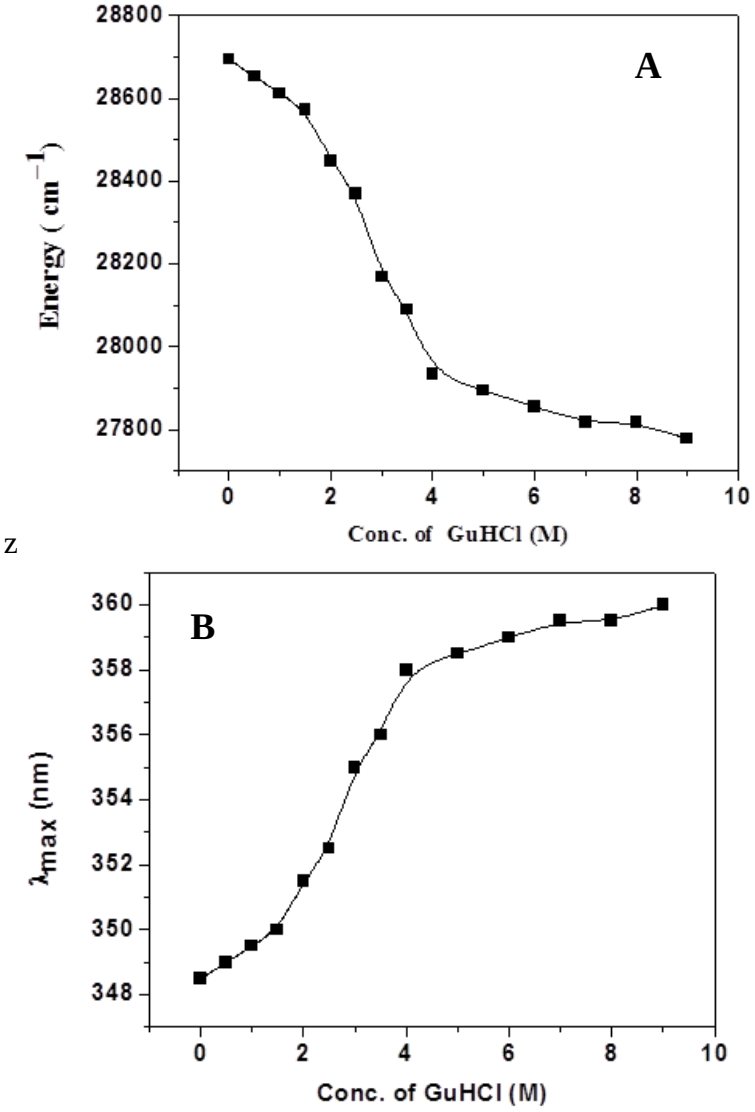
Fig 3.8: Absorption spectra of BSA ($2x10^{-5}$M) with increasing concentrations of guanidine hydrochloride with baseline correction done with guanidine hydrochloride solution

Since it was observed that guanidine hydrochloride in water has contribution close to the BSA 280 nm characteristic peak, the experiment was modified with each time doing the base line correction with the required concentration of guanidine hydrochloride solution in water as shown in Fig 3.8. This reflects the increase in absorption value with increasing guanidine hydrochloride with a pattern being followed. Thus unfolding of protein is no doubt occurring but these results are not much significant so fluorescence study needs to be done to account for the changes.

3.4.2:Fluorescence Studies:

The spectral shift is clearly described by the plot of variation of emission maximum as a function of concentration of guanidine hydrochloride as shown by Fig 3.11A. As the guanidine hydrochloride concentration increases there is red shift in the emission maximum observed with maximum change occurring between 2 M and 4 M guanidine hydrochloride

beyond which it almost remains constant. This may be attributed due to the exposure of Trp212 to a more polar environmentIn other words the unfolding of BSA is almost complete by 4 M of guanidine hydrochloride. This is again supported by energy emission maximum plot shown by Fig 3.11B. Thus the maximum change is occurring in the range of 2 M – 4 M guanidine hydrochloride.



z

Fig 3.11: (A) Variation of emission maximum,(B) Variation of emission energy of BSA as a function of Guanidine hydrochloride concentration.

The above data can be further accounted for by the variation of fluorescence intensity. It is known that intensity depends on the polarity of medium. With the denaturation or unfolding proceeding the intensity is quenched gradually leading to an equilibrium value after complete denaturation by 4 M of guanidine hydrochloride

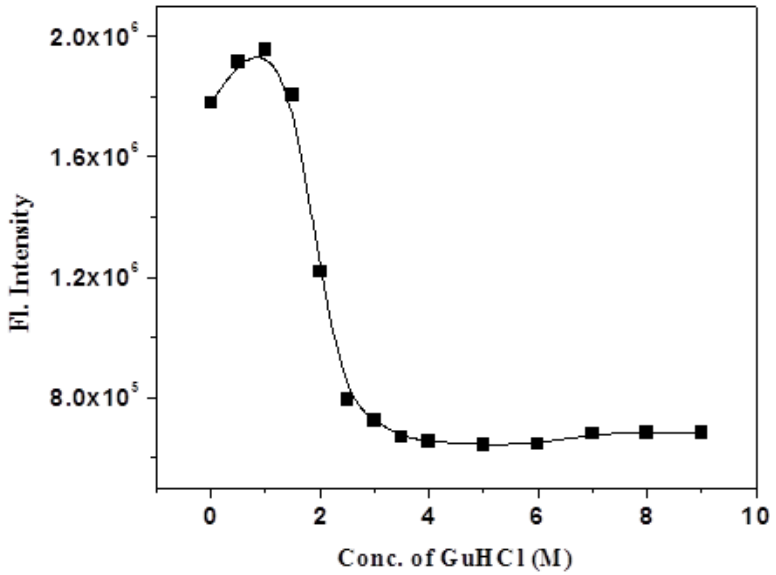


Fig 3.12: Variation of Fluorescence intensity of BSA as a function of Guanidine hydrochlorideconcentration.

CONCLUSIONS:

- Absorption studies were not effective in providing much information regarding the denaturation or unfolding of BSA by both the chaotropic agents urea and guanidine hydrochloride.

- Fluorescence studies provide a better insight into the denaturation or unfolding process due to the effect of chaotropic agents on BSA.

- A fluorescence study for excitation at 295 nm describes successfully the emission spectra of tryptophan accounting for the changes during the unfolding of BSA and the reasons behind it.

- The fluorescence intensity and anisotropy values well complemented each other for assigning the microenvironment and rotational mobility of tryptophan to account for the changes during the unfolding of BSA.

- The guanidine hydrochloride has stronger chaotropic property than urea. For the bovine serum albumin denaturation less amount of guanidine hydrochloride 4 M is required for complete denaturation where as it is 8 M for urea.

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