Discussion
Fatty acid free fraction V albumin containing 0.05 to 0.1 mole of fatty acid per mole of protein is a costly protein. In contrast, commercial fraction V albumin is relatively cheaper but it contains 0.1 - 2.4 moles of fatty acid per mole. Removal of fatty acids was accomplished by incubating albumin with activated charcoal for 1 hour at pH 3.0 in an ice bath. Lowering of pH unfolds the protein and removes the negative charge from the carboxyl group of fatty acids. These changes facilitate the removal of fatty acids from albumin. Denaturation of albumin does not take place by this treatment and capacity of albumin to bind fatty acids remains intact when pH is returned to 7.0 (Chen 1967).

Commercial albumin contains significant amount of dimeric and oligomeric forms of albumin in addition to BSA monomer. BSA monomer and dimer were isolated by size exclusion chromatography and their purity was checked by gel chromatography and polyacrylamide gel electrophoresis at pH 8.2. The presence of a single symmetrical peak on Sephacryl S-200 HR column (100.5 x 2.24cm) and a single band on polyacrylamide gel electrophoresis indicate that the preparation was pure with respect to size and charge. Fatted BSA was prepared by mixing the requisite amount of fatty acid in ethanol to 50 parts of albumin (by volume) at
pH 9.0 for 1 hour. Recently heat denaturation of HSA containing subsaturating levels of bound fatty acid revealed the existence of different albumin populations containing different amounts of fatty acids (Shrake and Ross, 1988; 1990; 1992). To circumvent this problem, BSA preparation containing 6 moles of palmitic acid per mole of protein was used throughout this study. The fatty acid content of the two albumin preparations was determined by the method of Chen (1967) and was found to be 0.05 mole/mole of protein and 5.81 mole/mole of protein, respectively, in defatted and fatted BSA. The elution profile of the two albumin preparations and their relative mobilities were identical, indicating no measurable change in protein conformation and its molecular mass (addition of 6 moles of palmitic acid per mole of BSA would increase its molecular mass by 2%) takes place as a result of fatty acid binding to protein. However, the presence of small peaks at 295 nm and 288 nm in the uv difference spectrum and a blue shift of 3 nm in the emission spectrum of fatted BSA suggested a change in the environment of tyrosine and tryptophan residues. These findings are in agreement with the studies of Spector and John (1968) and Honore and Pederson (1989). Tabayashi et al. (1983) observed increase in the oxidation of Cys-34 as a function of fatty acid binding suggesting clearly a change in the conformation of the protein that could not be
detected by gel chromatography and polyacrylamide gel electrophoresis. Dielectric properties and viscosity measurements have also indicated that albumin becomes more compact and rounded upon fatty acid binding (Soeteway et al., 1972).

In order to follow the denaturation of the two preparations the first and foremost task is to determine the wavelength at which folded and unfolded states differ maximally. For solving this problem, UV absorption spectrum and emission spectra in the presence and absence of 9 M urea were measured. There was a clear blue shift in the UV absorption spectrum indicating a change in the environment of tyrosines and tryptophans from nonpolar to polar. A prominent trough at 288 nm and a shoulder around 281 nm are characteristics of tyrosyl exposure. Absence of any peak around 292 nm, which is usually obtained due to the exposure of the tryptophan, is presumably due to the fact that both tryptophans of BSA are exposed to the solvent in its native state (Sogami and Ogura, 1973). Secondly, the number of tryptophan (two) residues are outnumbered by tyrosine (twenty) residues. The maximum difference in magnitude was obtained at 288 nm, hence this wavelength was used to follow denaturation by UV difference spectroscopy.
The emission spectrum of BSA in the absence of urea gave a single peak at 340 nm confirming the class B nature of albumin and the hydrophillic environment of tryptophans. Addition of 9 M urea caused a decrease in the fluorescence intensity and a red shift in the emission spectrum. Red shift was found to be very small and therefore, fluorescence intensity at 340 nm was used to monitor the unfolding of the protein.

The most noticable aspect of the denaturation transitions was the occurrence of a stable denaturation intermediate in defatted BSA when the transition was followed by uv difference spectroscopy. In contrast, the denaturation of fatted BSA by both the techniques gave a simple two state transition with a shift towards higher urea concentration indicating significant protein stabilization towards denaturation by bound fatty acids. A native globular protein in solution undergoes conformational fluctuations primarily from the ease of rotation about the single bonds of the backbone and side chains of the protein molecule. Thus it can be said that flexibility arises due to the equilibrium between the native and partially unfolded forms. Binding of ligands like fatty acids in BSA, Ca$^2+$ in lactalbumin (Okazaki et al., 1994) and subtilisin (Pantoliano et al., 1992) etc. shifts the equilibrium infavour of single form. Thus flexibility is reduced causing
a decrease in conformational entropy. Despite this decrease the protein molecule is stabilized in one conformation because of the binding energy between the ligand and the protein. Probably this is the reason that $\Delta G_D(H_2O)$ values of fatted BSA were much higher than that of the defatted BSA. The most popular and widely used method of linear extrapolation (Santoro and Bolen, 1988; 1992) was used for calculations of $\Delta G_D(H_2O)$. The D1/2 values i.e., the mid point of denaturation also showed an increase towards higher urea concentration for fatted BSA. The slope ($m$) values were also more in case of fatted BSA indicating that denaturation of fatted BSA is cooperative.

The characterization of denaturation intermediate by uv difference spectroscopy at 4.5 M, a concentration at which stable intermediate occurs in defatted BSA, further substantiated the occurrence of denaturation intermediate. The presence of a prominent peak at 287-289 nm in defatted BSA and its absence in fatted BSA was a clear indication that conformational changes occur only in defatted BSA. The difference molar absorption change in defatted BSA at 4.5 M urea was 1890 M$^{-1}$ L$^{-1}$ which is approximately 40% of the change observed in 9 M urea. The difference in the emission spectra of fatted and defatted BSA was insignificant strongly indicating that environment of tryptophans is not
perturbed up to this urea concentration. This also explains why denaturation intermediate was not seen in denaturation transition of defatted BSA by fluorescence technique.

Serum albumin is a multidomain protein and each domain has a specific/particular function. The independent folding and unfolding of domains in such proteins has been much documented (Privalov, 1982). Domain III of the albumin molecule has a very loose structure and is reported to be very unstable. It undergoes conformational changes before the other two domains (Carter and Ho, 1994; Khan, 1986). Domain III also contains very strong fatty acid binding sites (Reed, 1986). The results of uv difference spectroscopy suggest that part of the albumin molecule, probably domain III, undergoes denaturation earlier than rest of the molecule. Binding of fatty acids stabilizes this domain and hence it denatures at the same urea concentration at which the other two domains denature. Frontal analysis of defatted and fatted BSA in the absence and presence of 9 M urea was identical indicating that the folded and unfolded states of the two preparations have similar conformations under both conditions. However, the results of defatted BSA in the presence of 4.5 M urea were different from that of fatted BSA. The fast movement of defatted BSA in the presence of 4.5 M urea can be attributed to the loss in the substructure of defatted BSA. The elution volumes of the
two preparations obtained by peak analysis were identical in the presence and absence of 9 M urea whereas a totally different behaviour was observed for defatted BSA in the presence of 4.5 M urea concentration. Significant aggregation was observed in defatted BSA whereas fatted BSA gave a single symmetrical peak with a decrease in elution volume as compared to the native conditions, suggesting that conformation of fatted BSA is not altered by the addition of 4.5 M urea. Decrease in elution volume can be attributed to the changes in the properties of the eluant and not to conformational changes produced in the protein molecule. The presence of the two fast eluting peaks in the elution profile of defatted BSA suggested that there is an association between the partially unfolded protein molecules. Such type of aggregation has been reported for several other proteins like rhodonase (Horowitz and Butler, 1993), bovine growth hormone (Brems et al., 1988) and oligomers of lupin seed (Guerrieri and Cerletti, 1990). The Ve/Vo ratio of the 1st fast eluting peak was similar to that of BSA dimer suggesting that dimerization of albumin molecules (defatted) takes place in the presence of 4.5 M urea. The PAGE pattern of defatted BSA, fatted BSA, BSA dimer and defatted BSA mixed with 6 moles of fatty acid in the presence of 4.5 M urea were in close agreement with results of gel chromatography. In addition to the 2 major
bands, 2 clear distinct bands were observed in defatted BSA whereas fatted BSA moved as a single band. One of the major bands of defatted BSA corresponded to fatted BSA while the other one had a $R_m$ value equal to that of defatted BSA dimer strengthening the fact that dimerization has taken place in defatted BSA. The behaviour of defatted BSA mixed with 6 moles of palmitic acid was not different from that of defatted BSA indicating that addition of fatty acids does not affect PAGE pattern of defatted BSA. The Ve/Vo ratio and $R_m$ values in the presence of 9 M urea were very much smaller indicating that shape of the molecule plays an important role both in gel chromatography as well as in PAGE. The effect of time on aggregation clearly indicated that process of aggregation is kinetically a slow process and takes at least 12 hours for completion. The effect of fatty acids on aggregation showed that aggregation can be considerably reduced even at 1:2 molar ratio of protein to fatty acid and completely eliminated at 1:6 ratio. In order to understand the nature of aggregation, the aggregated products were isolated on Sephacryl S-200 HR column (66x2.02cm) in the presence of 4.5 M urea. The isolated peaks showed considerable heterogeneity particularly peak I, hence rechromatography was performed to get the pure preparations. The Ve/Vo ratio and $R_m$ values in the presence and absence of 4.5 M urea suggested apparently that peak I is a dimer,
peak II is a mixture of peak I and peak III and peak III is identical to that of defatted BSA monomer. The results of gel filtration and PAGE indicate that aggregation is an irreversible process there by proving some sort of covalent linkage is involved. Since in proteins disulfide bond is the only other covalent linkage besides the peptide bond, the possibility of the formation of an intermolecular disulfide bond cannot be ruled out. The uv difference spectral features and the emission spectra clearly indicated that isolated peaks had a different conformation. The emission peak and uv difference spectral features of dimer were completely different from that of peak I suggesting that tertiary structure of peak I is different from BSA dimer although size and charge parameters are similar to that of the BSA dimer.

SDS-PAGE in the presence and absence of β-mercaptoethanol (βRSH) confirmed the involvement of disulfide exchange reactions. Rm values obtained in the presence of RSH were identical for all the preparations. The mobility of peak I and III was similar to that of BSA monomer and BSA dimer in the absence of βRSH whereas peak II was found to be a mixture of peaks I and III. These results clearly suggested that urea induced denaturation of defatted BSA is accompanied by disulfide exchange reactions.
The urea induced difference spectrum and the emission in the presence and absence of 9 M urea indicate that all the three preparations retain their respective native structures. The denaturation plots clearly indicate that peak I follows a different path of unfolding as compared to other protein preparations including defatted BSA dimer. Peak I gave a three state denaturation transition whereas all the other protein preparations followed a simple two state transition. $\Delta G_p(H_2O)$ values also suggested that peak I is a fairly stable protein.

Binding of BCG and bilirubin with isolated protein preparations showed similar characteristics whereas ANSA binding appeared to be more complex. The decreased binding of BCG, bilirubin and ANSA with peak I can be attributed to the loss of the binding sites and hydrophobicity due to the formation of wrong disulfides in the presence of urea.

The uv difference spectrum of alkylated BSA obtained in the presence of 4.5 M urea showed characteristics similar to that of defatted BSA. The presence of single peak in the elution profile and a single band on PAGE in the presence of 4.5 M and 9 M urea of alkylated BSA clearly proved the involvement of lone sulfhydryl (Cys - 34). The low $pK_{SH}$ (5) of Cys - 34 and its close proximity to His 39 makes it highly reactive. Since earlier studies have shown that
domain III of albumin molecule is less stable, therefore, it is probable that opening of this domain at 4.5 M urea concentration sets the stage for inter and intra molecular disulfide exchange reactions.

As mentioned earlier domain III of albumin molecule is substantially stabilized upon fatty acid binding. Recent X-ray crystallographic studies have shown that fatty acid binding produces a slight opening of the interface between the two valves of the molecule and rotation of domain I. Further the environment of Cys-34 becomes more exposed resulting in the change in the microenvironment of Cys-34 thereby making it less reactive. These three reasons explain absence of intermediate which is mixture of aggregated products in fatted BSA.

The present study finds a strong application in the field of biotechnology. Being a valued therapeutic agent serum albumin has been expressed as an intracellular protein in a number of organisms (Etchewerry et al., 1986; Latta et al., 1987). This protein has a tendency to aggregate within the cell and therefore extraction with a denaturant and subsequent renaturation is required. Renaturation is being mainly done by diluting the denaturant to a level which favours renaturation (Teale and Benjamin, 1976; Johanson et al., 1981). The addition of fatty acids and a disulphide.
correcting enzyme could prove fruitful for increasing the percent yield of purely correctly refolded serum albumin monomer.