STUDIES ON THE PHYSICO-CHEMICAL PROPERTIES OF SESAME (*Sesamum Indicum*) SEED PROTEINS

A THESIS SUBMITTED TO THE UNIVERSITY OF MYSORE
FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

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D_E_C_L_A_R_A_T_I_O_N

I hereby declare that this thesis on "Studies on the Physico-Chemical Properties of Sesame (*Sesamum indicum*) Seed Proteins" which is submitted herewith for the degree of DOCTOR OF PHILOSOPHY of the UNIVERSITY OF MYSORE is the result of the work done by me in the Protein Technology Discipline, Central Food Technological Research Institute, Mysore under the guidance of Dr. P.K. Nandi during the period 1973-1976.

I further declare that the results of this work have not been previously submitted for any degree or fellowship.

V. Prakash

(V. PRAKASH)

C_E_R_T_I_F_I_C_A_T_E

I hereby certify that the thesis on "Studies on the Physico-Chemical Properties of Sesame (*Sesamum indicum*) Seed Proteins" submitted by Sri V. Prakash for the degree of DOCTOR OF PHILOSOPHY of the UNIVERSITY OF MYSORE is the result of research work carried out by him in the Protein Technology Discipline, Central Food Technological Research Institute, Mysore under my guidance during the period 1973-1976.

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A_C_K_N_O_W_L_E_D_G_E_M_E_N_T_S

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ABBREVIATIONS

CM:	Carboxymethyl
DEAE:	Diethylaminoethyl
et al.	and co-workers
Fig.	Figure
GuHCl	Guanidine hydrochloride
NBS:	N-Bromosuccinimide
QAE:	Quarternaryaminoethyl
SDS:	Sodium dodecyl sulfate
SP:	Sulphopropyl
TEA:	Triethanolamine
TMEED:	N,N,N^{1}, N^{1}-Tetramethylethylene-diamine
Tris:	2-amino-2 hydroxymethyl propane-1, 3-diol.

S_Y_M_B_O_L_S

V_o:	Void volume of the gel column used in gel filtration.
V_t:	Total volume of the gel column used in gel filtration.
V_e:	Elution volume of the peak in gel filtration.
K:	Apparent association constant
k:	Binding constant
$[\eta]$:	Intrinsic viscosity
η_{sp}:	Specific viscosity
η_{red}:	Reduced viscosity
$[\alpha]$:	Specific rotation
ε:	Molar extinction coefficient
E:	Absorption coefficient
$\bar{\nu}$:	Moles of ligand bound per mole of protein
$\backsim$:	Approximately
Δ:	Difference
α:	Alpha
β:	Beta
γ:	Gamma
δ:	Delta
t^o:	t^oC
f_u:	Fraction of the total change observed in various measurements.

S Y N O P S I S

S_Y N_O_P_S_I_S

Proteins from unconventional sources e.g. from oilseeds have drawn considerable attention in recent years from the view point of their high nutritional quality, acceptable for human consumption. Among the oilseed proteins, the proteins from sesame seed has a special significance as a rich source of sulphur containing amino acids. This potential source of the protein in the sesame seed cake remaining after extraction of oil is mostly used as a cattle fodder. At present, very few studies are available regarding the proteins of sesame seed with reference to its isolation, characterisation and different chemical and physico-chemical properties. The study of these proteins with respect to their chemical and physico-chemical properties and association-dissociation and denaturation phenomena under different solution conditions can be expected to contribute to a better understanding of the molecular characteristics of the protein which may ultimately lead to better food formulations.

The present investigation on "Studies on the Physico-Chemical Properties of Sesame (Sesamum indicum) Seed Proteins" has been carried out as follows. We have

concentrated our study on the major protein fraction, α-globulin of sesame seed, its isolation, determination of some of its chemical and physico-chemical properties and studies on the association-dissociation and denaturation phenomena under various solution conditions, e.g. acid, alkali, electrolytes, urea, guanidine hydrochloride (GuHCl) and sodium dodecyl sulfate (SDS) which have been presented here. These studies on the association-dissociation and denaturation phenomena under the above solution conditions have been helpful in understanding the structural aspects of the protein.

(1) The protein α-globulin isolated by the procedures of Nath and Giri (1957b) and Ventura and Lima (1962) contains contaminations (both low, 2S and high molecular weight, 16S components) of ∽15% and ∽25% respectively as revealed by sedimentation velocity experiments. The α-globulin isolated in the present investigation is homogeneous, ∽95% by the techniques of gel filtration, polyacrylamide gel electrophoresis, DEAE-cellulose chromatography and sedimentation velocity experiments. The yield of α-globulin is increased by the procedure adopted here.

(2) The protein isolated is practically free from nucleic acid and carbohydrate contaminations as revealed by the low values of phosphorus and carbohydrate contents respectively.

(3) Amino acid analysis of the protein indicates that it is rich in acidic, sulphur containing and aromatic amino acid residues.

(4) The protein has a molecular weight of 2,50,000 ± 15,000 daltons as determined from Archibald's approach to equilibrium method.

(5) It is an oligomeric protein containing at least 11 subunits.

(6) The value of intrinsic viscosity is 0.03 dl/gm indicating the protein to be globular.

(7) In acid solution, in the pH range of 4.2-1.5, the results of gel filtration, fluorescence and viscosity measurements indicate that α-globulin dissociates and denatures upto pH ~3.

(8) The difference spectrum in this range of pH is characterised by blue shift with troughs at 280, 287 and 292 nm and arises from a combination of dissociation, denaturation and charge effect on the chromophore.

(9) In still stronger acid solution (pH < 3) reassociation of the dissociated fraction takes place as indicated by difference spectra, fluorescence spectra and sedimentation velocity measurements.

(10) The temperature effect on the association-dissociation phenomena of α-globulin at pH 1.5 indicates that the reassociation of the dissociated components takes place probably by hydrophobic interaction.

(11) In alkaline solution, in the range of pH 7-12, d-globulin dissociates around pH 8, and above pH 10, dissociation and denaturation proceed simultaneously as have been evidenced by sedimentation velocity, fluorescence spectra, ultraviolet spectral changes, optical rotation and viscosity measurements.

(12) The change in the shape of the ultraviolet spectrum with pH in alkaline solution indicates that both tyrosyl ionisation and conformational changes are taking place simultaneously in the protein. The constancy of absorbance at 242 mμ after tyrosyl ionisation is complete, can be taken as an evidence that no other reaction which might result from alkaline hydrolysis of disulfide groups is taking place.

(13) The phenolic group in the protein is abnormal (pK_{Int}=10.6). In 6M GuHCl the pK_{Int} of tyrosine groups is 9.6 which is the expected value for the pK_{Int} of tyrosyl groups. The denaturation in alkaline solution is irreversible.

(14). The characteristic pH values of transition from 10.6-10.8 indicates that the transition of the protein involves a single step in alkaline solution.

(15) Effects of various sodium salts on the association-dissociation phenomena of d-globulin show the following effectiveness towards dissociation of the

protein:

$SO_4^{--} < Cl^- < Br^- < ClO_4^- < SCN^- \leq I^- < CCl_3COO^-$

the first two members reduce the extent of dissociation. CCl_3COONa has been found to be the most effective amongst the series in inducing dissociation in the protein.

(16) The cations Li^+, Na^+, K^+ and Cs^+ induce association, the effectiveness being:

$$Cs^+ \backsimeq K^+ \gg Na^+ \gg Li^+$$

(17) Minor discrepancy from the Hofmeister series has been observed in the present investigation.

(18) The low concentration of salts (anions) necessary to induce dissociation does not involve any detectable change in the protein conformation.

(19) The temperature effect on the association-reaction 7S → 11S, indicates that the subunits of α-globulin are associated predominantly by hydrophobic interaction.

(20) The dissociating effect of the electrolytes may be due to a combination of favourable energetics of the chaotropic ions with the amide dipole against a low positive unfavourable free energy of interaction of the nonpolar groups with the same ions.

(21) d-globulin undergoes dissociation, aggregation and denaturation in urea and GuHCl solutions. The protein precipitates at low concentration of these reagents.

(22) The aggregation phenomenon is maximum at a critical concentration of either of the denaturants.

(23) The protein precipitated at low concentrations of these reagents goes back into solution at higher reagent concentration.

(24) The soluble aggregate having a sedimentation coefficient of ~12S is the precursor of this insoluble aggregate.

(25) Viscosity results indicate that the denaturation of the protein is complete at 8M urea and 6M GuHCl.

(26) The difference spectrum of the protein is characterised by blue shift with troughs at 280, 287 and 293 nm indicating that both tryptophan and tyrosine groups have been effected by the denaturants. A red shift at 300-305 nm is also observed in both the reagents and is attributed to the denaturation of the protein and anomalous tryptophan absorption change.

(27) Fluorescence measurements indicate a red shift of the spectrum from 325 to 345 nm with increasing

concentration of the denaturant. This indicates that the tryptophan fluorophor is experiencing increasing polar environment with increasing concentration of urea and/or GuHCl. The fluorescence transition of the protein is complete at a lesser concentration of both urea and GuHCl as compared to viscosity and difference spectral results.

(28) The simultaneous dissociation and aggregation reactions have been explained by considering two types of subunits present in the protein molecule, one leading to smaller sedimenting component (4S) and the other producing the aggregate (~ 12S component).

(29) The amino acid analysis of the aggregated fraction indicates that it is rich in aliphatic amino acid residues.

(30) The endothermic nature of the aggregation process has been explained as probably arising from hydrophobic interaction of aliphatic side chains of the relevant subunits i.e. which are rich in aliphatic amino acid residues.

(31) α-globulin exists in a more denatured state in GuHCl than in urea solution. The extent of denaturation in these solutions are reduced in the presence of .05M KCl.

(32) In SDS solution α-globulin undergoes stepwise dissociation initially and then denaturation.

(33) Gel filtration, polyacrylamide gel electrophoresis and sedimentation velocity measurements indicate the dissociation of the protein in SDS solutions. The dissociation is complete at $3x10^{-3}$M SDS and four components are observed in the sedimentation velocity pattern with $s_{20,w}$ values of 2S, 4S, 7S and 11S.

(34) The specific rotation measurements indicate a cooperative transition between $3x10^{-3}$M to $8x10^{-3}$M detergent suggesting conformational change.

(35) The difference spectra of the protein is characterised by blue shift with troughs at 280, 287 and 292 nm indicating that both tryptophan and tyrosine groups have been effected by the detergent.

(36) The appearance of a blue shift in the difference spectra of protein below $3x10^{-3}$M arises from the binding of the detergent near the tyrosine and tryptophan chromophores. Above $5x10^{-3}$M SDS, the conformational change contributes to the observed blue shift in the difference spectra.

(37) In the presence of detergent, the fluorescence intensity decreases and a red shift in the fluorescence maximum occurs resulting in a 'isoemissive point' at 355 nm. The red shift of the fluorescence maximum at

the highest concentration of the detergent used is 330-335 nm. This red shift is less, as compared to Urea and GuHCl solutions where it is 345 nm.

(38) The binding studies of SDS to protein indicates a steep binding curve above 2.5×10^{-3}M SDS. Analysis of the binding data in the range 1×10^{-5} to 1×10^{-3}M SDS indicates the presence of ~50 binding sites in the protein and a binding constant of 3×10^{3}.

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INTRODUCTION

I N T R O D U C T I O N

The importance of utilizing oilseed meals as supplementary protein sources for human consumption has received considerable attention in recent years. The different oilseed meals that are utilized for this purpose are from peanut, soyabean, rapeseed, mustard, sesame, cottonseed and sunflower seed. India is one of the major oilseed producing countries in the world and has the largest production of sesame seeds (0.5 million tonnes) representing nearly 40% of the world's production of sesame seed (CSIR, 1972; FAO, 1973). A major portion of this production ~80% is utilised for the extraction of oil and the rest for edible purposes in traditional foods. Among the vegetable proteins, the proteins from sesame seed has a special significance as a rich source of sulphur containing amino acids, acceptable for human consumption (Block and Bolling, 1951; Gastry et al., 1974). The nutritive value of sesame flour is significantly enhanced by supplementation with lysine which is the limiting amino acid in this protein (Almquist and Grau, 1944; Joseph et al., 1962). Also sesame proteins

constitutes a valuable supplement to pulse, soyabean, groundnut and Bengalgram proteins (Kuppuswamy et al., 1958). This potential source of protein in the sesame seed cake remaining after extraction of oil is mostly used as a cattle fodder. In the literature, very little work is available on the chemical and physico-chemical properties of sesame seed proteins. In what follows an account of information available on sesame seed proteins is presented.

Present state of knowledge regarding sesame seed proteins:

The work on sesame seed proteins has mostly centred round the isolation of the total protein from fat-free meal, the solubility of the protein as a function of pH and the fractionation of different protein components present in it. A few of its physico-chemical properties including electrophoresis, amino acid composition and isoelectric point have been reported. Limited studies on the association-dissociation of the major protein of the sesame seed at different pH and ionic strength, the molecular weight of this protein from ultracentrifugation studies and its diffusion coefficient have also been reported.

Extractability studies of sesame seed proteins:

Ritthausen (1880) obtained several preparations of proteins from sesame seed by extracting the protein from oil free cake under variable conditions of alkali,

sodium chloride concentration and temperature. The different preparations were analysed for elementary composition, e.g. carbon, nitrogen, etc.

Adolph and Lin (1936) carried out solubility of sesame seed proteins from the fat free meal in sodium chloride, sodium hydroxide and sodium carbonate and showed that prior treatment of the meal with methanol or a temp of 110° decreases the solubility considerably.

Rao and Sen Gupta (1947) carried out similiar solubility studies with defatted sesame seed proteins in various pH's, sodium bisulfite and sodium chloride solutions and showed that sodium chloride (6%) at pH 9.0 extracted nearly 87% of the protein.

Nath and Giri (1957a) carried out peptisation studies of sesame protein with 10% sodium chloride solution. The effect of particle size of the meal, ratio of sample weight to solvent volume, time of stirring, temperature, salt concentration and pH on the extractability of total protein from the meal were studied.

Deschamps et al., (1966) reported the dispersibility of sesame seed proteins with pH and showed that the protein was soluble below pH 3.0 and above pH 7.0. They also reported an isoelectric point of 4.5 from precipitation studies.

Guerra and Park (1975) carried out studies on the solubility of defatted sesame seed meal in aqueous solution over various pH's and salt solutions. Maximum solubility was found in alkaline solution and the proteins were almost insoluble in acidic solutions. The solubility of the protein in sodium chloride or calcium chloride solution was increased upon increasing the salt concentration upto 1M whereas in sodium sulfite or disodium monohydrogen phosphate solution, the solubility of the protein was higher at lower salt concentrations but decreased at higher salt concentrations at pH 8.

Isolation of different fractions and studies pertaining to them:

The available information in the literature indicates the presence of four different protein fractions constituting the total proteins from the sesame seed. There has been some ambiguity in nomenclature of the different fractions (Jones and Gersdorff, 1927; Nath and Giri, 1957b; Salem and Beikheit, 1964). For the present work we will adopt the nomenclature of Nath and Giri (1957b). The major protein which constitutes nearly 65-70% of the total protein present in the sesame seed, has been designated as α-globulin (Jones and Gersdorff, 1927). The other fractions have been named as β, γ and δ globulins (see subsequent discussion).

Jones and Gersdorff (1927) were the first to make serious attempt on fractionation of different protein components of sesame seed proteins. They utilised a variety of procedures e.g. heat coagulation, fractional precipitation with varying concentration of ammonium sulfate, $(NH_4)_2SO_4$, dilution of the total protein extract from high salt molarity and acid coagulation. These authors by their methods observed three different protein fractions in the sesame seed proteins.

Nath and Giri (1957b) following and modifying the approaches of Jones and Gersdorff (1927) have isolated different protein fractions as follows. The clarified 10% sodium chloride extract of the meal brought to pH 7.0 (in buffer) was diluted 1:10 times with distilled water and centrifuged. The precipitate obtained was redissolved in the same volume and concentration of sodium chloride and the process of precipitation and centrifugation repeated. The precipitate was dispersed in water and dialysed free of salt and dried. Nath and Giri (1957b) observed that this fraction was the major component α-globulin, of Jones and Gersdorff (1927). The fraction had an iso-electric point of 4.65 and was homogeneous between pH 3-12.

The supernatant left after centrifugation of the diluted 10% sodium chloride extract (see above) was saturated to 40% $(NH_4)_2SO_4$ and centrifuged. The precipitate so obtained was redissolved in 10% NaCl and was

precipitated again by 40% $(NH_4)_2SO_4$ and this fraction was identified as β-globulin of Jones and Gersdorff (1927). Nath and Giri (1957b) observed a slight contamination of α-globulin in β-globulin fraction from electrophoretic measurements.

The above supernatant remaining after β-globulin isolation was brought to 60% $(NH_4)_2SO_4$ saturation. The precipitate was subjected to similar treatment of dissolution and precipitation as in β-globulin isolation. The electrophoretic pattern of this fraction indicated two more protein components along with β-globulin and were designated as γ and δ globulins.

The major protein fraction α-globulin isolated by dilution method was termed amorphous α-globulin by Jones and Gersdorff (1927). The precipitate, obtained by adding 20-30% $(NH_4)_2SO_4$, when dissolved in 2% sodium chloride at 60° yielded crystalline α-globulin on cooling (Nath and Giri, 1957b).

Salem and Rakhsit (1964) also studied different procedures for fractionation of protein components from sesame seed proteins and reported their electrophoretic measurements.

Physico-chemical properties of α-globulin:

Serious attempts on the study of the physico-chemical properties of sesame seed proteins appeared in the early sixties. Sinha and Sen (1962) from the sedimen-

tation velocity studies observed the presence of four protein components in the total protein in 10% sodium chloride with sedimentation coefficients of 2, 7, 13 and 19S. Those authors obtained a sedimentation coefficient of 13S for the major protein, α-globulin, in 10% sodium chloride. The effect of pH below pH 4.5 and above pH 9.0 indicated dissociation of the protein, which was independent of the variety of the seeds. Between pH 4.5 and pH 9.0 α-globulin preparation appeared to be stable and indicated the presence of low concentrations of 19S and 23S components depending upon the pH of the solution. The moving boundary electrophoretic results of α-globulin indicated a single peak at pH's 4.1 and 3.2 although two partially resolved peaks were observed at pH 2.45. In addition, these authors have reported the absorption spectrum of α-globulin with an absorption maximum between 278-280 mμ. The value of $E^{1\%}_{1cm}$ at 280 mμ was found to be 10.8.

Ventura and Lima (1963) studied the sedimentation and diffusion characteristics of α-globulin. Ultracentrifugal analysis of this protein revealed a major component with a $S_{20,w}$ value of 13.0. The values of frictional ratio was 1.5 and partial specific volume 0.735 for the protein. The calculation of molecular weight by a combination of diffusion, sedimentation and partial specific volume data yielded a value of 450,000±30,000 daltons.

Guerra and Park (1975) recently investigated the subunit composition of total sesame seed protein by SDS-polyacrylamide gel electrophoresis in the presence of β-mercaptoethanol by the method of Weber and Osborn (1969). Seven fractions of molecular weights of 51,000; 31,000; 28,500; 25,500; 21,800; 20,500 and 17,900 have been identified by them.

SCOPE AND OBJECTIVES OF THE PRESENT INVESTIGATION

SCOPE AND OBJECTIVES OF THE PRESENT INVESTIGATION

From the foregoing discussion it is apparent that very little systematic study has been carried out to characterise the proteins of oilseed sesame from the view point of its physical and chemical properties. In addition association-dissociation and denaturation phenomena has not been studied in detail for the proteins present in sesame seed which could be helpful in understanding the molecular characteristics of the protein molecule.

From this consideration, we have undertaken a systematic study on the sesame seed protein. It has already been mentioned that four different protein components are present in the protein extract of sesame seed. The understanding of the molecular nature of the protein becomes more meaningful from a study of a single protein rather than from a mixture of components in which it is present.

To achieve the above objectives, the following investigation has been undertaken in the present report. The major protein fraction α-globulin which constitutes ~65-70% of total sesame seed protein has been isolated,

its homogeneity tested by a variety of analytical physico-chemical techniques and a few of the chemical and physico-chemical properties of the protein have been studied. These studies have been followed by the association-dissociation and denaturation behaviour of α-globulin under various solution conditions e.g. acid, alkali, electrolytes, temperature, urea, GuHCl and SDS solutions. These studies it is believed would help to understand the physico-chemical properties of α-globulin, the major component of sesame seed protein.

PRESENT STATE OF KNOWLEDGE REGARDING DISSOCIATION AND [illegible]RATION OF PROTEINS IN DIF[illegible] SYSTEMS

KNOWL[illegible]ING DISSO-CIATI[illegible]INS IN [illegible]

The protein molecules are made up of linear polymer chains containing a number of different types of peptide residues arranged in genetically predetermined way and linked end to end constituting the primary structure of the molecule. Noncovalent interactions e.g. hydrogen bonds, hydrophobic bonds, etc. which operate within the limit imposed by the primary structure establishes the conformation, i.e., secondary and tertiary structure of the protein molecule. The equilibrium conformation attained by a protein molecule will be a sensitive function of the nature of the peptide residues, their sequence and solvent environment. In solution the native protein molecules, which are isolated from their sources by mild processes, the chain-chain contact of the peptide residues are energetically more favourable than chain-solvent contacts (von Hippel and Schleich, 1969). The phenomenon of protein denaturation describes a major conformational change in the native structure of the protein molecule without any alteration in the amino acid sequence (Tanford, 1968). In this denatured state of the protein, chain-solvent contact is energetically more

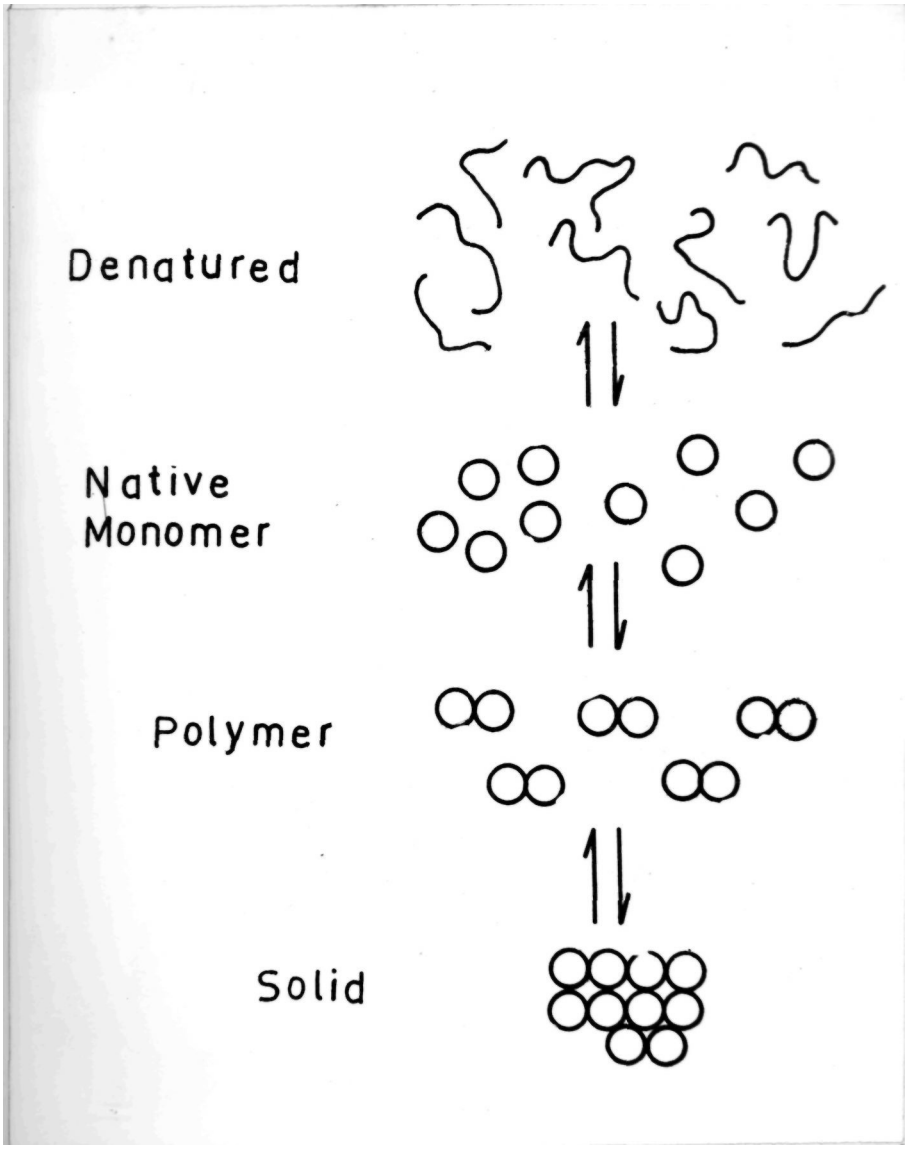
favourable than chain-chain contact in the molecule. Studies in the area of conformational change in the protein molecule have constituted a major area of physicochemical studies of the proteins. Reports on the predenaturation transition and cis-trans isomerisation of the protein molecule before the onset of major structural change in it, are also available (Lumry, 1974; Brandts et al., 1975).

The information from studies on protein denaturation has been helpful in understanding the nature of native structure of proteins and the mechanism of the conformational change. There are many proteins which in their native state are made up of several polypeptide chains or subunits (quaternary structure). The governing principle of the dissociation of these proteins into their subunits and conformational change in them, in general, are identical (Tanford, 1968). Several seed proteins have been observed to undergo stepwise dissociation in solution (Schwenke, 1975).

Protein molecules under various solution conditions, e.g. change in the pH, in presence of different electrolytes, urea and GuHCl, detergents, other organic additives and temperature undergoes conformational change. In case of oligomeric proteins dissociation into subunits may or may not involve denaturation. In Fig. 1 a schematic diagram of the dissolution of an oligomeric

Fig. 1 A schematic diagram of dissolution of an oligomeric protein, its dissociation and denaturation.

Fig. 1



protein and its dissociation and denaturation have been shown (Tobinson and Jencks, 1965). Each of the processes have their own equilibrium constants. The denaturation of protein molecule involves either a single step or multiple steps and may or may not be reversible. The denatured states of the protein molecule which are obtained under different solution conditions may not be the same. The foregoing conclusions have been found to be applicable to the dissociation process also (Tanford, 1968).

The dissociation and denaturation of the proteins in acid and alkaline solutions have been considerably studied (Joly, 1965; Tanford, 1968; Donovan, 1973a; Haschemeyer and Haschemeyer, 1973). Aggregation and precipitation of a few of the dissociated and denatured proteins in strong acid solution have been reported (Westhead, 1964; LeJohn et al., 1969; Flanagan and Hesketh, 1974; Warren et al., 1974; Vallee and Williams, 1975). In alkaline solution the aggregation and precipitation may result from polymerisation, chemical reaction of the S-S bonds and irreversible denaturation of the protein (Tanford, 1968; Donovan, 1973a; Aoki et al., 1973).

Different electrolytes induce dissociation and denaturation in proteins to different extents (Jencks, 1969; von Hippel and Schelich, 1969). Salts which contain sulfate, phosphate, citrate, acetate or fluoride tend

to precipitate proteins and protect it against dissociation and denaturation. Thiocyanate, iodide and perchlorate salts induce dissolution, dissociation and denaturation of the protein. The salts e.g. chloride, bromide and nitrite fall in the border line and lithium (Li^+) in general has been observed to dissociate and denature proteins (von Hippel and Schleich, 1969; Jencks, 1969; Sawyer and Puskridge, 1973; Harrington and Herskovits, 1975; Nakashima et al., 1975).

Dissociation and denaturation of the protein in high concentrations of urea and GuHCl solutions have been extensively studied (Tanford, 1968; Jencks, 1969; McKenzie and Ralston, 1973; Wallevik, 1973; Knapp and Pace, 1974; Teuwissen et al., 1974). A few instances are also available where the proteins are found to be resistant to urea or GuHCl denaturation even in concentrated solutions of these reagents (Leonis, 1956; Buckley et al., 1963; Stauffer and Sullivan, 1971; Asuma et al., 1974). GuHCl is more effective in dissociating and denaturing proteins than urea with minor exceptions (Tanford, 1968; Jencks, 1969; Raymond and Pace, 1974; Tsong, 1975). The results upto date indicate that the conformational transition of the proteins containing S-S bonds is often incomplete even in highest attainable urea or GuHCl concentration (Imai et al., 1963; Takagi and Isemura, 1966; Tanford, 1968; Sekok and Rawitch, 1969; Aoki et al., 1974). There are a few evidences available in literature

where the denatured protein in urea and GuHCl undergoes aggregation reaction (Fronsdorff et al., 1953; McKenzie et al., 1955; Cutter et al., 1957; McKenzie et al., 1963). Reports are also available, regarding the aggregation of a few proteins, at low urea or GuHCl concentration (Neurath et al., 1942; Clark, 1945; Mihyali, 1950; Jirgensons, 1952; Lytvynenko, 1960; Riddiford, 1966).

Detergents, particularly ionic, at very low concentrations are effective, in dissociating and denaturing proteins (Becker and Foster, 1966; Tanford, 1968). The measurement of different physico-chemical properties of certain proteins indicate that their α-helical structure is not completely destroyed in detergent solutions and in fact, instances are known, where detergents have been found to induce new type of helix formation in the protein molecule (Meyer and Kaumann, 1962; Jirgensons, 1967; Rosenberg et al., 1968). Urease is an instance, where high concentration of the detergent is unable to induce any conformational change in the protein (Pitts et al., 1974).

Considerable amount of information is available for the explanation of the mechanism of dissociation and denaturation of proteins in the above reagents based on either singly or in combination of (i) direct interaction between the reagents and protein and (ii) indirect action mediated through change in the solvent structure induced

by added reagents (Robinson and Jencks, 1965; von Hippel and Schleich, 1969; Jencks, 1969; Tanford, 1970; Nandi and Robinson, 1972a; Hamabata and von Hippel, 1973; von Hippel and Hamabata, 1973).

PRINCIPLES OF THE EXPERIMENTAL APPROACHES EMPLOYED IN THE PRESENT INVESTIGATION

PRINCIPLES OF THE EXPERIMENTAL APPROACHES EMPLOYED IN THE PRESENT INVESTIGATION

A brief outline of the principles involved in various chemical and physico-chemical methods, for characterisation and study of the association-dissociation and denaturation phenomena of sesame α-globulin in the present investigation, is presented below.

Gel filtration:

In gel filtration, separation of the molecules takes place on the basis of the difference in their molecular weight, the larger molecule elutes faster than the smaller ones because the former is retained relatively for less time in the gel. This separation does not involve any chemical interaction between the separating molecules and the gel matrix. For separation of the protein molecules of different molecular weights, commercially available gels like Sephadex (bead form of anhydroglucose polymer), Sepharose (bead form of alternating residues of D-galactose and 3,6 anhydro-l-galactose units), Bio-Gel P (granulated polyacrylamide gels) and Ultrogel (combination of agarose and polyacrylamide gels) are used. For homogeneous protein, the elution pattern in gel filtration consists of a single symmetrical peak. In the

case of oligomeric protein, dissociation into subunits can be monitored from their increased retention in the gel as compared to the parent protein molecule (Determann, 1968; Ackers, 1970).

Polyacrylamide gel electrophoresis:

Protein molecules migrate in an electrical field towards either anode or cathode to different extents depending upon the amount and nature of the charge present in them. The three widely used procedures are (i) moving boundary electrophoresis (ii) zone electrophoresis and (iii) continuous flow electrophoresis. In polyacrylamide gel electrophoresis, which is zone electrophoresis in principle, the protein in solution is loaded on top of of the acrylamide gel polymerised in tubes and will mitigate through the gel under the influence of applied electrical field. The extent of migration of the protein can be identified by precipitating and staining the protein in the gel, the mobility of which depends upon its overall charge and molecular size (Gordon, 1969).

Determination of subunits and their molecular weights

The method is based on the principle that in the presence of anionic detergent SDS of ~1% concentration and β-mercaptoethanol the oligomeric proteins will completely dissociate into its constituent subunits (Weber and Osborn, 1969). In this high concentration of the detergent solution,

all the polypeptide chains have the same charge density i.e. bind the same amount of detergent on a weight basis. Further, Stokes radius in this solution depends only on the molecular weight (Tanford et al., 1974). In polyacrylamide gels where significant molecular exclusion occurs, the mobility of the dissociated SDS-protein complex is a linear function of the molecular weight of the protein. Proteins with known molecular weights are used under identical conditions as that of the sample and a standard graph of the relative mobility against logarithm of the molecular weight is obtained. Depending upon the mobility of the subunits of the oligomeric protein, their molecular weights are calculated from the standard graph. In proteins containing, inter subunit S-S linkages β-mercaptoethanol (1%) in detergent solution is used for complete dissociation and the molecular weight of the subunits determined.

Ion-exchange chromatography:

The protein molecules can efficiently be separated on the basis of the net charge present in them by adsorbing them in gel matrix carrying opposite charge to that of protein molecules. There are two types of ion-exchangers (i) anion exchanger - e.g. DEAE-cellulose, QAE-sephadex, etc. and (ii) cation exchanger - e.g. CM-cellulose, SP-Sephadex, etc. The nature of the charged groups of the gel determine the type and strength of the ion exchanger. Depending upon

the relative strength of the binding of different protein molecules to the gel, the proteins can be separated with either a salt or a pH gradient. This gradient may be linear, concave or convex (Peterson, 1970).

Ultracentrifugation:

(i) Sedimentation velocity: In a sedimentation velocity run the solute molecules in solution are separated from the solvent due to the high centrifugal force. Due to this, two regions in the cell are set up (i) the solvent and (ii) the plateau region where the concentration of the solute is uniform. Between these two regions is a transition region known as the 'boundary' in which concentration varies with distance from the axis of rotation. Photographs taken at regular intervals of time measure the refractive index gradient (schlieren optical system) in the cell as a function of the distance 'x' from the center of rotation. Sedimentation coefficient (S_{app}) is calculated from the equation (Schachman, 1959),

$$S_{app} = \frac{\Delta x / \Delta t}{\left(\frac{x_1 + x_2}{2}\right)\left(\frac{2\pi \cdot r}{60}\right)^2} \quad \ldots\ (1)$$

where $\frac{\Delta x}{\Delta t}$ is the slope of the plot of the distance of the peak from the center of the rotor against time, $\frac{x_1+x_2}{2}$ is the mean distance of the peak from the center of rotor during the entire run, r is the revolutions per minute of the rotor and $\frac{2\pi \cdot r}{60}$ is the angular velocity. The S_{app}

value is corrected for temperature and viscosity effects of solvents and reduced to viscosity of water at 20°, when $S_{20,w}$ value is obtained. The homogeneity of a purified protein fraction is characterised by a single symmetrical peak of the protein in the schlieren pattern. In case of oligomeric proteins, the association-dissociation and denaturation phenomena can be studied under various solution conditions by determining the $S_{20,w}$ values of thedifferent schlieren peaks obtained. The phenomena of dissociation and denaturation can also be studied by molecular weight determinations from various sedimentation equilibriummethods.

(ii) Molecular weight determination from $S_{20,w}$ values: The molecular weight M can be determined from $S_{20,w}$ values by using the equation (Schachman, 1959).

$$M = \frac{4690\ (S_{20,w})^{3/2}\ [\eta]^{1/2}}{(1-\bar{v}\rho)^{3/2}} \quad \ldots\ldots \quad (2)$$

where $[\eta]$ is the intrinsic viscosity (dl/gm), $\bar{v}$ is the partial specific volume and ρ the density of the solution. However, the molecular weight determined by this method is only approximate.

(iii) Molecular weight determination by Archibald Method: This is basically a transient state sedimentation method and is based on the principle that a condition of equilibrium always exists in the centrifuge cell at the meniscus and at the bottom. During the run, no material transfer takes place across these boundaries, and so the equilibrium relationship always holds even though the con-

ditions in the immediate vicinity may be far from equilibrium. Thus for the meniscus in the cell, the molecular weight, M_m is given by Schachman, 1959).

$$M_m = \frac{RTF\left(\frac{dc}{dx}\right)_{x_0}}{(1-\bar{v}\rho)\,\omega^2 x_0\,\Delta x\left[\Sigma\frac{dc}{dx} - \frac{1}{x_0^2}\Sigma x^2\frac{dc}{dx}\right]} \quad \ldots\ldots (3)$$

where R is the gas constant, T is the absolute temperature, F is the $\overset{lens}{\wedge}$magnification factor, $\frac{dc}{dx}$ the concentration gradient, $\bar{v}$ is the partial specific volume, ρ is the density of the solution, ω is the angular velocity, x_0 is the radius corresponding to the air-liquid meniscus and x is the radius corresponding to a position in the plateau region. The values of dc/dx, x_0 and x are determined experimentally (See Page No. 58).

Viscosity:

Measurement of viscosity has been extensively used in the study of the size and shape of the protein molecules in solution. The experimental technique is simple although interpretation is often complex. Intrinsic viscosity $[\eta]$ which is defined as

$$[\eta] = \lim_{c \to 0}\left(\frac{\eta_{sp}}{c}\right) \quad \ldots\ldots (4)$$

is a complex function of the size and shape of the protein molecule, η_{sp} is the specific viscosity and c is the con-

centration of the protein in gm/dl. η_{sp} is defined as

$$\eta_{sp} = \frac{\eta - \eta_o}{\eta_o} \quad \ldots\ldots \quad (5)$$

where η and η_o are the viscosities of the protein solution and solvent respectively.

Globular proteins in general, have low values of intrinsic viscosity $\backsim$ 0.03 dl/gm (Yang, 1961). On denaturation due to increase in the asymmetry of the protein molecule the value of viscosity increases. The change in the viscosity upon denaturation, in general, will depend upon the nature of the denaturant. The viscosity change can be monitored either by plotting $[\eta]$ or η_{sp} or η_{red} against denaturant concentrations. In contrast to the globular proteins, the viscosity of rigid rodlike protein molecules e.g. myosin and soluble collagen decreases due to the decrease in the asymmetry of the molecule on denaturation (Bradbury, 1970).

Optical rotation:

The native protein molecules are levo rotatory although a few exceptions have been documented. The property of optical rotation in a molecule arises through the interaction of electrons in different groups in the asymmetric molecule. Any structural change, which alters the relative positions of the groups in an asymmetric molecule like protein, produces a marked change in its optical rotation, eventhough the chemical nature of the molecule remains unaltered (Knussmann, 1959). Upon denaturation the levo rotation of the

protein molecule increases. The changes in the spatial relationships between different parts of protein molecules result in this observed change. The amount of change in the optical rotation varies depending on the protein and on the denaturing conditions. However, the interpretation of the changes in optical rotation solely in terms of ordering and disordering of the conformation of the polypeptide chain involves an element of risk. In recent years the structure of proteins and other macromolecules have been studied by means of optical rotation over a broad range of wavelength, from 180-600 nm and the resulting spectrum is known as optical rotatory dispersion. In addition, circular dichroism which evaluates the molecule's unequal absorption of right and left-handed circularly polarised light is used extensively for the study of configuration of proteins and other macromolecules (Kauzmann, 1959; Adler et al., 1973).

Ultraviolet difference spectra:

The absorption spectra of proteins from 260-300 nm region arises mostly from the contribution of tyrosine and tryptophan residues present in theprotein molecules. The chromophores in general are perturbed by the environment, in which they are present. This is reflected in the small absorption shift in the spectrum of protein when its structure is appreciably altered. A quantitative expression of the shifts can be obtained by difference spectrophotometry,

where the spectrum of the protein in any given state is measured against that of the protein in a reference state at the same concentration. Under dissociating and denaturing conditions, the chromophoric groups which are present in the subunit interface and in the interior of the protein molecule respectively, would experience a change in the environment which will be reflected in the difference spectra, when measured against reference protein solution. Depending upon the conformational change, a blue or red shift is observed in the difference spectra. The difference spectral peaks at 292-293 and 278-280 nm are attributed to the perturbation of tryptophan and tyrosine moieties respectively. The difference spectral peak at 287-288 nm results from the perturbation of both tyrosine and tryptophan groups in the protein molecule (Wetlaufer, 1962; Donovan, 1969; Kronman and Robbins, 1970).

Fluorescence:

The fluorescence of non-conjugated proteins generally originates from the aromatic side chains of tyrosine, tryptophan and phenylalanine. In tryptophan containing proteins, the fluorescence spectra is dominated from the tryptophan moieties. As in absorption spectrum the fluorescence property e.g. intensity and fluorescence maximum are dependent upon the environment of the fluorophors. Change in the intensity of fluorescence and red shift in its maximum are

generally observed for dissociation and denaturation processes of the protein molecule. Fluorescence measurement has the advantage of sensitivity and specificity over other methods although interpretation is often complex (Chen et al., 1969).

Equilibrium dialysis:

The binding of small ligands to the macromolecules can be studied by equilibrium dialysis method. The method generally consists of equilibrating a fixed volume of a protein solution of known concentration in a dialysis bag dipped in a solution of ligand of known concentration at a particular temperature over a period of time. After the equilibrium is attained, the amount of ligand present in the outer solution is determined, from which the amount of ligand bound to the protein in the dialysis bag can be calculated (Steinhardt and Reynolds, 1969).

Turbidity measurements:

In protein solutions the measurement of turbidity gives a semi-quantitative idea of the kinetics of precipitation reactions (i) in presence of the added reagents (ii) with variation in temperature of the system. The appearance of cloudiness of small particles originates in the lateral scattering of light from the direction of the incident beam. The intensity of the beam is attenuated as

it penetrates the suspension, as if it were traversing in an absorbing medium. The turbidity of the solution plays a role in weakening of the primary beam by scattering, as does the absorption coefficient in absorption measurements. The measurement of turbidity is governed by the equation (West, 1949).

$$I = I_o e^{-\tau x} \qquad \ldots\ldots \quad (6)$$

where I_o is the intensity of the incident light, I the intensity of the emitted light in the direction of the incident light, x is the distance travelled by the light beam in the medium and the coefficient τ is called the turbidity of the medium. The turbidity τ, in general, is a function of wavelength of the primary light, the concentration of the protein, the size, shape and the relative refractive index of the scattering particles and the mutual solute-solute orientations and solvent-solute interactions.

Precipitation reactions:

When a reagent is added to a solution of another compound a precipitate may result from (i) decrease in the solubility of the component in the presence of the reagent (ii) formation of an insoluble complex(es) between the reagent and the compound (iii) modification of structural properties in case of macromolecule in the presence of the reagent which alters its solubility property. The

amount of precipitate may vary depending upon the concentration of the reagents. From the supernatant of the precipitated solution, one can estimate the percent precipitation (Skoog and West, 1969).

Amino acid analysis:

In this method the protein is hydrolysed in presence of concentrated hydrochloric acid in vacuum when the peptide bond is hydrolysed and the amino acids are released. These are adsorbed on an ion exchanger and the different amino acids eluted with changes in pH. The eluted amino acids are mixed with ninhydrin, colour developed and quantitative analysis done by comparison with standard amino acid mixture loaded and analysed under similar conditions (Spackman et al., 1953).

Estimation of tryptophan:

Tryptophan is destroyed during acid hydrolysis of the protein and cannot be determined in an Amino Acid Analyser. It can be determined by using (i) NBS method (ii) Edelhoch's method and (iii) microbiological method.

(i) NBS method: In this method the indole chromophore oftryptophan absorbing strongly at 280 nm is converted to oxindole by oxidation with NBS. This oxindole has a much weaker absorbance at this wavelength. From the decrease in the absorbance at 280 nm with the addition of NBS, the trypa-

tophan content of the protein can be calculated using the equation (Spande and Witkop, 1967).

$$\% \text{ Try} = \frac{\triangle \text{Abs} \times 1.31 \times V \times 186}{W \times 5500} \times 100 \quad \ldots\ldots (7)$$

where $\triangle$Abs is the optical density decrease at 280 nm, 1.31 is empirical factor obtained from the ratio of extinction coefficient of free tryptophan at 280 nm to that of bound tryptophan, V is the initial volume of titrated solution (ml), 186 is the molecular weight of each bound tryptophan residue, W is the weight of the protein titrated (mg) and 5500 is the molar extinction coefficient at 280 nm for tryptophan. The dilution of the protein solution by the added aqueous NBS is taken into consideration while calculating $\triangle$Abs.

(ii) Edelhoch's method: In this method the absorption spectrum of the protein is measured in 6M GuHCl solution at neutral pH which will bring all the absorbing chromophores in contact with the aqueous surroundings. The extinction of the protein is calculated at 288 and 280 nm and the moles of tryptophan, M_{Try} determined from the equation (Edelhoch, 1967).

$$M_{Try} = 10^{-3} (0.322\ \varepsilon_{288} - 0.0969\ \varepsilon_{280}) \quad \ldots\ldots (8)$$

where ε_{288} is the extinction coefficient of the protein at 288 nm and ε_{280} is the extinction coefficient of the protein at 280 nm.

(iii) Microbiological method: In this method the amino acids are estimated using lactic acid bacteria. To different amounts of the hydrolysed protein sample is added known amount of basal medium containing all the nutrients except the amino acid to be assayed. The growth of the organism is directly proportional to the concentration of amino acid to be assayed over a certain range of concentration, which in turn is proportional to the amount of lactic acid produced. This is estimated by titration against standard alkali. A standard curve is obtained by assaying graded amounts of standard L-amino acids. Percentage amino acid in the sample is calculated by comparison with standard amino acids (Barton-Wright, 1952).

Spectrophotometric titration of tyrosine:

The main spectral change observed with tyrosine-containing proteins in the alkaline pH region is due to the ionisation of phenolic groups of tyrosyl residues. The ionisation of tyrosine is accompanied by both an intensification of the spectrum and a shift of the maximum from 275 towards longer wavelengths as compared to the neutral molecule. The difference spectra of the ionised tyrosine has an absorption maxima in the 293-295 nm region. The pH of the experimental solution is varied and the increase in absorbance at 293-295 nm determined, against a reference solution of identical concentration at neutral pH. The

change in the molar absorptivity is calculated and the number of tyrosyl groups estimated on the basis of 2300 change in the extinction value at 295 nm as a result of ionisation of 1 mole of tyrosyl residue. In addition to the determination of number of tyrosyl groups present in the protein molecule, the dissociation constant of the tyrosyl groups present in it can also be determined from spectrophotometric titration (Donovan, 1973b; Mihyali, 1968).

Carbohydrate estimation:

In this method, the carbohydrate is released by the action of hot concentrated sulphuric acid from the protein molecule. The released carbohydrate reacts with phenol in presence ofsulphuric acid to give a stable yellow-orange colour which has an absorption maxima at 490 nm. A standard curve for the colour developed at 490 nm is obtained using glucose as standard and the carbohydrate content of the material expressed in glucose equivalents (Montgomerry, 1961).

Phosphorus estimation:

In this method, the inorganic phosphorus released from the hydrolysis of proteins by sulphuric acid is converted to a blue coloured complex in presence of ferrous sulphate-ammonium molybdate reagent in a weakly acidic medium and the colour measured in a colorimeter. A stan-

dard curve is obtained using potassium hydrogen phosphate and the phosphorus content of the material expressed in terms of inorganic phosphate (Taussky and Shorr, 1953).

Proteolytic activity:

For the determination of proteolytic activity, the protein sample in which the proteolytic activity is to be determined is incubated at various levels with protein substrate (e.g. hemoglobin). After incubation, the protein is precipitated by trichloroacetic acid, cooled and centrifuged. The supernatant solution which contains free amino acids and peptides released due to hydrolysis of the substrate by the enzyme is estimated spectrophotometrically and the extent of proteolytic activity is determined (Greenberg, 1955).

MATERIALS AND METHODS

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Materials:

White variety of sesame seeds were obtained from the local market. They were identified as *Sesamum indicum* L., during the flowering stage of the plant, by the Botany Department of the University of Mysore, Mysore.

The chemicals and reagents used are the following: The source of these materials are shown in the parenthesis. Sepharose 6B-100, Sephadex G-50, Sephadex G-75, DEAE-cellulose, bovine serum albumin, egg albumin, pepsin, α-chymotrypsin, ribonuclease-A and CuHCl (Sigma Chemicals); hemoglobin substrate (Worthington Biochemicals); sodium trichloroacetate and sodium perchlorate (E.Merck); NBS (L.Light and Co. Ltd.); bisacrylamide (Koch Light Laboratories); TEMED and β-mercaptoethanol (Fluka); Coomassie brillant blue (Schwarz Mann); bromophenol blue, ammonium persulfate; methylene blue, sodium sulfate, sodium chloride, glucose and potassium hydrogen phosphate (BDH Chemicals); sodium bromide, sodium iodide, urea, trichloroacetic acid and TEA (Sarabhai M.Chemicals); tris (V.P.Chest Institute); sodium thiocyanate (Ajax Chemicals); perchloric acid (Riedel-

DC Haen, AG) and SDS (Hindustan Levers). SDS was re-crystallised twice from ethanol. The other reagents used were all of analytical grade.

Methods:

The sesame seeds were soaked in water for 6 hr after which they were scrubbed to remove the hull. The dehulled and dried seeds were flaked followed by defatting by extraction with solvent n-hexane, with a solvent to flaked seed ratio as 1:1. The process of extraction was repeated at least six times and a meal containing less than 3% fat was obtained. This defatted meal was air dried in a cabinet drier at 45° for 6 hr after which it was powdered in a microatomiser and then passed through a 60 mesh sieve. The flour so obtained was further washed with n-hexane and a flour containing less than 1% fat was obtained. The defatted flour obtained was dried and used for the extraction of total protein.

Extraction of total protein:

The protein in the defatted flour was extracted in solvent, 0.02M phosphate buffer, pH 7.5 containing 1M sodium chloride. The flour to solvent ratio was to 1:10. The slurry was stirred, not too vigorously, for ~1 hr and centrifuged at 4,000 rpm for 20 min. The supernatant obtained was dialysed against buffer system for ~ 24 hr in which subsequent studies were carried out.

Isolation of α-globulin:

The protein α-globulin was isolated by a minor modification of Nath and Giri's (1957b) procedure.

The supernatant obtained after centrifugation of the slurry was diluted with water 1:5.5 times instead of 1:10 times dilution done by Nath and Giri (1957b). The solution was centrifuged at 4000 rpm for 30 min. The precipitate was redissolved in the extraction solvent and the process of precipitation and centrifugation were repeated. α-globulin thus obtained was dissolved in the extraction solvent and dialysed against the buffer system for ∽ 24 hr in which subsequent studies were carried out.

From a batch of 100 gm of the flour nearly 20-22 gm of α-globulin was obtained, giving a percentage yield of ∽ 20% α-globulin on the basis of flour weight.

Protein concentration:

The concentration of α-globulin was determined by macro Kjeldhal procedure (Official Methods of Analysis of AOAC, 1975). Nitrogen was estimated in a weighed amount of lyophilised α-globulin and the nitrogen to protein conversion factor was determined as 6.25. A calibration curve relating the mg of nitrogen present in the protein sample with ultraviolet absorbance of the protein at 280 nm measured in a Carl Zeiss spectrophotometer was

was obtained for routine determination of protein concentration. The absorption coefficient, E of 1% protein solution at 280 nm i.e. $E^{1\%}_{1\ cm}$ gave a value of 10.8 for α-globulin. The corresponding value obtained for the total protein was 13.0.

Gel filtration:

Gel filtration was carried out mostly in Sepharose 6B-100 for homogeneity test of α-globulin and its dissociation behaviour in SDS solutions. The gel column used was 2x87 cms (V_t=265 ml) in all these experiments. Sephadex G-50 having the above column dimension and bed volume was used for the study of dissociation of α-globulin in acid solution. Sephadex G-75 column of the same dimension as above was used for the separation of protein fractions in urea solution.

The gel was equilibrated with approximately three times the bed volume of the column with buffer in which further experiment had to be carried out. Nearly 50 mg of the protein in buffer was loaded on the column. The flow rate was adjusted at 25-30 ml/hr and 2.5 ml fractions were collected in an Enonvee automatic fraction collector at room temperature ($\sim$ 25°). The protein concentration in the fractions were determined by measuring the absorbance at 280 nm.

Polyacrylamide gel electrophoresis:

Polyacrylamide gel electrophoresis was carried out in a Metrex Gel electrophoresis unit identical with Acrylophor apparatus in 0.02M phosphate buffer pH 7.5. A 7.5% gel was used for determining the homogeneity of α-globulin. For a typical run, 0.83 gm of acrylamide and 23 mg of methylene bisacrylamide were dissolved in 8 ml of the above buffer, filtered and the volume made upto 10 ml and kept in cold. To 9.5 ml of this cooled solution, 0.5 ml of freshly prepared ammonium persulfate solution (15 mg/ml) and 20 µl of TEMED solution were added. The solution was polymerised in glass tubes (eight in number) of 7.5x0.5 cm dimension. Protein samples (10 µgm/1 ul) containing ~40% sucrose and 0.05% bromophenol blue (indicator/dye) were loaded on the gel and electrophoresis was performed at a constant current of 3 mA per gel for 70 min. The gels were stained for 1 hr in 0.5% amido black in 7.5% (v/v) acetic acid solution. Destaining was carried out in 7.5% acetic acid solution, till the gels were colourless and stored in 7.5% acetic acid solution.

In presence of SDS, the electrophoresis was carried out using 10% polyacrylamide gel because of relatively poor resolution of the bands in 7.5% gel. For a typical run in this solution, the protein, gel and the

running buffer contained same concentration of the detergent. A 10% gel was used for electrophoresis in 8M urea solution. In this experiment the protein and the gel contained 8M urea.

Determination of subunits and their molecular weight:

The determination of the number of subunits and their molecular weight was carried out by the method of Weber and Osborn (1969). The protein α-globulin (0.6 mg/ml) and other standard proteins (1 mg/ml) viz., bovine serum albumin, egg albumin, pepsin, α-chymotrypsin and ribonuclease-A were incubated at 37° for 2 hr in 0.01M sodium phosphate buffer pH 7.0, containing 1% SDS and 1% β-mercaptoethanol. After incubation, the protein solutions were dialysed against large volumes of 0.01M sodium phosphate buffer pH 7.0 containing 0.1% SDS and 0.1% β-mercaptoethanol and subjected to gel electrophoresis. Gel buffer contained 7.8 gm of $NaH_2PO_4H_2O$, 38.6 gm of $Na_2HPO_4.7H_2O$ and 2 gm of SDS per litre. The gel buffer diluted 1:1 with water was used as running buffer in presence of 10% gels. For a typical run, 22 gm of acrylamide and 0.6 gm of methylene bisacrylamide were dissolved in water filtered and the volume made upto 100 ml and kept in cold. To 13.5 ml of this solution, 15 ml of buffer, 1.5 ml of freshly prepared ammonium persulfate (15 mg/ml) and 0.045 ml of TEMED

solution were added. The solution was polymerised in glass tubes of 10 cm x 0.6 cm dimension. About 60 µl of protein sample containing ~40% sucrose and 0.05% bromophenol blue (indicator dye) were loaded on the gel and electrophoresis performed at a constant current of 6 mA per gel for 6 hr. The gels were stained in coomassie brilliant blue (1.25 gm of the dye in 454 ml of 50% methanol and 46 ml of glacial acetic acid) for 6 hr. Destaining was carried out in acetic acid:methanol:water (75:50:875) solution. The relative mobility of the protein band was calculated using equation (Weber and Osborn, 1969).

$$\text{Relative mobility} = \frac{\text{distance moved by the protein band from the top}}{\text{length of the gel after destaining}} \times \frac{\text{length of the gel before staining}}{\text{distance moved by the dye from the top}} \quad .. (9)$$

The relative mobilities were plotted against the logarithm of the molecular weight of the standard proteins used and the molecular weight of the subunits of the protein was determined.

Ion-exchange chromatography:

DEAE-cellulose used in the present study was regenerated using 1N hydrochloric acid for the acid wash. The material was washed free of acid and then treated

with 1N sodium hydroxide for the alkali wash. The regenerated DEAE-cellulose was washed free of alkali with distilled water and was stored at 5° for further use. The material so obtained was equilibrated with 0.01M glycine sodium hydroxide buffer pH 9.0 and packed into a column (2.5x30 cm) by pressure. About 140 mg of the protein in 3 ml of the buffer was applied to the column and allowed to be absorbed. Protein was eluted with a linear gradient of 0-0.4M sodium chloride. 2.5 ml fractions were collected and the protein concentration determined by measuring the absorbance of the fractions at 280 nm. Sodium chloride was estimated in the fractions by Vohlards method (Vogel, 1961).

Ultracentrifugation:

(1) Sedimentation velocity: Sedimentation velocity experiments were carried out at 25° (unless otherwise stated) in a Spinco Model E Analytical Ultracentrifuge equipped with a phase plate schlieren optics and a rotor temperature indicator and control (RTIC) unit. For a typical run a standard 12 mm duraluminium cell centerpiece and 1% protein solution were used at a speed of 59,780 rpm (unless otherwise stated). Plates were read on a Gaertner microcomparator and $S_{20,w}$ values calculated by the standard procedure (Schachman, 1959) using equation 1 (see page No.36).

(ii) Molecular weight determination from $S_{20,w}$ values: The molecular weight of α-globulin from $S_{20,w}$ value was calculated using equation 2 (see page No.37).

(iii) Molecular weight determination by Archibald method: In thefirst part of the experiment the synthetic boundary cell described by Klainer and Kegeles (1955) was used for the determination of C_o. The two side holes of the cell were filled with 0.07 ml of 0.02M phosphate buffer pH 7.5 containing 1M sodium chloride and the centre of the cell with 0.16 ml of α-globulin in the above buffer. The cell was centrifuged at 3848 rpm and the resulting boundary photographed immediately after the formation of peak (Fig. 2a).

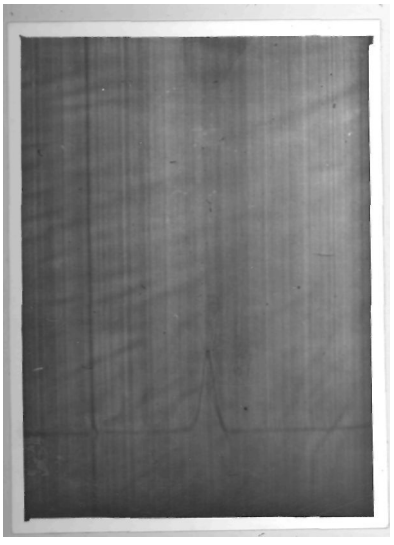
In the second part of the experiment a standard 4° sector duraluminium cell was used. 0.7 ml of protein solution was filled into the cell, and was centrifuged at 8766 rpm and the schlieren patterns were photographed at intervals of 15 min (Fig. 2b). The temperature of the cell was measured both at the beginning and at the end of the run. From these photographic plates dc/dx values were read on a two dimensional microcomparator and the numerical value proportional to C_o was computed. With the other pattern (Fig. 2a) dc/dx values were read starting from the top meniscus to the 'plateau' region.

Fig. 2 Molecular weight determination by Archibald method in 0.02M phosphate buffer pH 7.5 containing 1M sodium chloride.

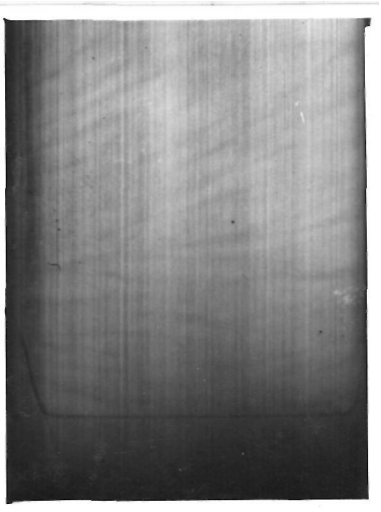
(a) Synthetic boundary pattern and

(b) Archibald run pattern.

Fig. 2



a



b

dc/dx at X_o was calculated by extrapolation of the above dc/dx values. Using these data the molecular weight M from equation 3 (see page No. 38) was calculated.

Viscosity:

Viscosity measurements were made at 28±0.1° with an Ostwald viscometer having a flow time of 345 sec with distilled water. Viscosity was calculated from the equation

$$\eta = \rho \left(At - \frac{B}{t} \right) \qquad \ldots\ldots \quad (10)$$

where η is the viscosity, ρ the density, t the flow time in sec, A and B the constants of the viscometer. A and B were determined by measuring the viscosity of water at different temperatures and a plot of $(\eta/\rho)t$ against t^2 was obtained. The slope of the graph gives A and the intercept on Y - axis gives the value of B. The value of B was found to be zero. Protein concentration (from 1%-6%) was used for the determination of intrinsic viscosity using equation 4 (see page No. 38). Reduced viscosities were determined using 1% protein solution.

Optical rotation:

Optical rotation was measured at 578 nm in a Carl-Zeiss spectropolarimeter using a 1 dm tube at 28°.

Protein solution (0.75%) was used. Specific rotation $[\alpha]$, was calculated from equation (Adler et al., 1973).

$$[\alpha] = \frac{\alpha}{\lambda c} \quad \ldots\ldots \quad (11)$$

where α is the observed rotation (degrees), λ is the length of the sample (dm) and C is the concentration of protein (gm/l).

Ultraviolet spectra and difference spectra:

The spectrum and the difference spectrum of the protein were recorded in a Perkin-Elmer 124 double beam spectrophotometer. Protein solution of 0.86 OD/ml (0.08%) was used for the determination of spectrum. For the difference spectrum 2 OD/ml (0.18%) protein solution in presence of SDS, 1.8 OD/ml (0.166%) in presence of urea and GuHCl, 0.76 OD/ml (0.07%) in acidic solutions and 0.73 OD/ml (0.067%) for the spectrum in alkaline solutions were used. Instead of using tandem cells, matched 1 cm cells were used for difference spectra measurements (Donovan, 1969; Donovan et al., 1969). By subtracting the absorbance of the perturbant, in the range of measurements, from the recorded difference spectrum, actual difference spectrum of the protein was determined. The change in the molar extinction coefficient, $\Delta\varepsilon$ of the protein was calculated taking 2.5×10^5 daltons as the molecular weight of α-globulin.

Fluorescence:

Fluorescence measurements were made in a Perkin-Elmer Hitachi Fluorescence Spectrophotometer at 28°. Protein solution having an absorbance of 0.04 (0.004%) at 280 nm was used. The excitation wavelength was 280 nm. The fluorescence emission was measured after 10 sec. when the fluorescence intensity attained constancy. The fluorescence of blanks were measured and used for obtaining fluorescence values due to the protein molecules.

Equilibrium dialysis:

The binding study of the detergent SDS with the protein α-globulin was carried out by equilibrium dialysis. 2.5 ml of 1% protein solution in the dialysis bag was equilibrated at 30° in a Brunswick incubator shaker with 10 ml of the detergent solution, having different concentrations. Attainment of equilibration was checked by taking out aliquots of the external solution at regular intervals of time and measuring the detergent concentration. The concentration of the detergent in the external solution attained a constant value within 36 hr and all the experimental solutions were equilibrated for this period of time. From the estimation of the amount of detergent bound to the protein molecule, (mole to mole basis) a binding isotherm was constructed.

Estimation of detergent:

The amount of detergent in the solution was estimated by mixing an aliquot of the external solution with $1x10^{-4}$M methylene blue in 0.01M hydrochloric acid and extracting the dye-detergent complex in chloroform and measuring the colour intensity at 650 nm in a Carl-Zeiss Spectrophotometer. A standard curve was obtained by using known concentration of SDS, from which SDS concentration of the external solution in the experimental solutions were determined.

Turbidity measurement:

The turbid protein solution in presence of different concentrations of urea indicated minimum transmittance at 340 nm. The percent transmittance of 0.1% protein solution was determined at this wavelength in presence of different concentrations of urea and at various temperatures viz. 15°, 25° and 45° (Jamieson et al., 1972).

Precipitation reactions:

Precipitation experiments in the presence of low concentration of urea and GuHCl were carried out by equilibrating, 0.1% protein solution, in varying concentration of these reagents at 30° in a Brunswick incubator shaker for 24 hr. The incubated solutions were

centrifuged at 20,000 x g. The supernatant of protein solutions in different concentrations of urea and GuHCl were measured in 6M urea and 4M GuHCl solution respectively against appropriate blanks. This was done in order to avoid any other effect which might contribute to the absorbance of protein due to the presence of different concentrations of the reagents present in the supernatants. Corrections for the absorbance by blanks were also made. The percentage of protein precipitated was determined by calculating the amount of protein present in the supernatant at each concentration of the denaturants as compared to the initial concentration of the protein solution.

Amino acid analysis:

The amino acid analysis was carried out in a Hitachi KLA-3B amino acid analyser. 2.5 mg of the protein in 1 ml of hydrochloric acid (6N) was frozen in a bath of liquid N_2, and evacuated with an oil pump and sealed. The hydrolysis was carried out in an oven at 110° for 24 hr, and after hydrolysis the hydrochloric acid was removed rapidly at 40° under reduced pressure. The residue was dissolved in 2.25 ml of 0.2N sodium citrate buffer pH 2.2. 0.5 ml of the buffer solution containing amino acid mixture was loaded on the column. Micromoles of each amino acid in the sample was calculated by the

height-width method using similarly eluted standard amino acids (Spackman et al., 1958).

Determination of tryptophan:

(i) NBS method: In this method, 2 ml of protein solution having an absorbance of 0.5 (0.046%) at 280 nm in 0.05M acetate buffer pH 4.0 containing 8M urea was pipetted out into a 2.5 ml cuvette. After noting the initial absorbance at 280 nm against buffer containing 8M urea, a 9mM aqueous solution of NBS was added in stepwise of 10 µl with a micro-syringe and stirred well. The decrease in absorbance was noted 5 min after each addition of NBS. Stepwise addition of NBS continued till no further decrease in absorbance at 280 nm is observed. The minimum absorbance was noted and a correction for the increase in volume was applied. The tryptophan content was calculated using equation 7 (see page No. 45).

(ii) Edelhoch's method: In this method, 0.8 OD/ml (0.074%) protein solution in 6M GuHCl in 0.02M phosphate buffer pH 6.5, was used for the determination of the spectra. The spectra was obtained in a Perkin-Elmer 124 double beam spectrophotometer with 6M GuHCl as blank. The extinction coefficients at 280 and 288 nm were calculated on the basis of 2.5×10^5 daltons for the molecular

weight of the protein and molar concentration of tryptophan M_{try} was calculated from equation 8 (see page No.45).

(iii) Microbiological methods Tryptophan in the sample was estimated by microbiological method using Lactobacillus arabinosus. 50 mg of the protein was hydrolysed in vacuum in presence of 50 ml of sodium hydroxide (6N) at 110° in an oven for 18 hr. The pH of the hydrolysate was adjusted to 6.8 and was taken in graded amounts along with the inoculated organism and the medium. The organisms were allowed to grow for 72 hr at 37° and the acid produced titrated against 0.1N sodium hydroxide using bromothymol blue as the indicator. The percentage of each of the amino acid was calculated by comparison with standard curve (Barton-Wright, 1952).

Spectrophotometric titration of tyrosine:

Spectrophotometric titration of phenolic groups in the protein was carried out by increasing the pH of the protein solution and measuring the absorbance change at 293 mμ resulting from tyrosyl ionisation (Donovan 1973b; Mihyali, 1968). 0.55 OD/ml (0.051%) protein solution in 0.5M KCl was used. The protein solution was adjusted to different pH's with 1M Annlar sodium hydroxide. The adjustment of pH was also carried out by

various buffers e.g. sodium phosphate, tris, glycine, carbonate-bicarbonate in their respective buffering ranges. Extinction changes were calculated from the change in absorbance at 295 nm. A Radiometer Titrator TTT 2 (Copenhagen) was used for the measurement of pH. The Radiometer Titrator was calibrated with 0.05M potassium acid phthalate (pH 4.01 at 25°), standard phosphate buffer (pH 6.5 at 25°) and 0.05M borax solution (pH 9.13 at 25°), prepared in deionised water.

Carbohydrate estimation:

The carbohydrate content of total protein and α-globulin was estimated by the method of Montogomerry (1961). 0.2 ml of 1% protein solution in 1M sodium chloride solution was mixed with 0.1 ml of 80% phenol and 5 ml of concentrated sulphuric acid. The solution was shaken well and was allowed to stand for 30 min at 27°. The absorbance of the developed colour was measured at 490 nm. A standard curve was obtained using glucose instead of the protein sample. The carbohydrate content of the protein was expressed as glucose equivalents.

Phosphorus estimation:

The phosphorus content of total protein and α-globulin was estimated by the method of Dawsky and Sherr (1953). To 0.5 ml of 0.5% protein solution was

added 0.5 ml of 10N sulphuric acid and was digested on a sand bath till the solution became dark brown in colour. The solution was cooled and a few drops of 60% perchloric acid ($HClO_4$) added to it and the digestion was continued till the solution became colourless. The volume was made upto 2 ml with glass distilled water. To this was added 2 ml of ammonium molybdate reagent and the solution allowed to stand for 30 min at room temperature. The developed colour was read at 660 nm in a Klett-Summerson colorimeter. A standard curve was obtained using 0-16 µg/ml of potassium hydrogen phosphate (KH_2PO_4).

Proteolytic activity:

The proteolytic activity of α-globulin was determined using denatured haemoglobin as substrate. The rate of hydrolysis was carried out in 0.1M borate buffer, pH 7.8. A 2% solution of haemoglobin and a 1% solution of α-globulin in the above buffer were used. The substrate to α-globulin ratio was 1:1. 2 ml aliquots of the solution were pipetted out at regular intervals of time till a period of 24 hr and the enzymatic activity (if any) inhibited by adding 2 ml of 10% trichloroacetic acid. The solutions were kept at 4° for 2 hr and then centrifuged at 4000 rpm for 20 min.

The absorbance of the supernatants at 280 nm was measured. A blank of both the substrate and α-globulin was carried out (Greenberg, 1955).

Preparation of protein solutions in different reagents:

For the measurements in acid solution, the protein was dialysed against 0.3M citrate-phosphate buffer, pH 3.0 and 0.1M acetate buffer pH 4.1 for 24 hr. The pH of the solution below pH 4.1 was adjusted by the addition of 1M hydrochloric acid to pH 4.1 solution. For the measurements in alkaline solutions upto pH 10, the extracted protein was dialysed against 0.05M Tris-HCl buffer of different pH's. The pH of the solution, above pH 10 was adjusted by the addition of 1M sodium hydroxide to pH 10 solution.

Due to the limitation of protein solubility and appearance of turbidity near neutral pH, the experiments in presence of electrolytes, urea, GuHCl and SDS were carried out at pH 9.0. The protein solution was dialysed against 0.05M Tris-HCl buffer, pH 9.0, for studying the effect of electrolytes. The protein in 0.05M Tris-HCl buffer pH 9.0, was used for the measurements in urea, GuHCl and SDS solutions. In all these experiments equal volumes of protein and reagent solutions were added to get the desired concentrations of both the components.

RESULTS AND DISCUSSION

ISOLATION AND CHARACTERISATION OF α_2-GLOBULIN

ISOLATION AND CHARACTERISATION OF α-GLOBULIN

α-globulin from total sesame seed protein was isolated by the methods of (a) Nath and Giri (1957b) (b) Ventura and Lima (1963) and (c) modification of the procedure of Nath and Giri (1957b) by us which has been adopted in the present investigation, and their homogeneity was checked by sedimentation velocity measurement.

From the sedimentation velocity patterns shown in Fig. 3a, 3b it can be seen that the protein obtained by the method of Nath and Giri (1957b) was more homogeneous as compared to that by the method of Ventura and Lima (1963). The protein obtained by the method of the latter authors consisted of a major 11S component along with considerable amount of a 2S component and small amounts of 7S and 16S components. Further the yield of α-globulin was only 5-6% on the basis of flour weight. The protein obtained by the method of Nath and Giri (1957b) also contained small amounts of 2S and 16S components. However, the yield of α-globulin was 15%.

Fig. 3 Sedimentation velocity patterns of α-globulin isolated by various methods in 0.02M phosphate buffer, pH 7.5 containing 1M sodium chloride.

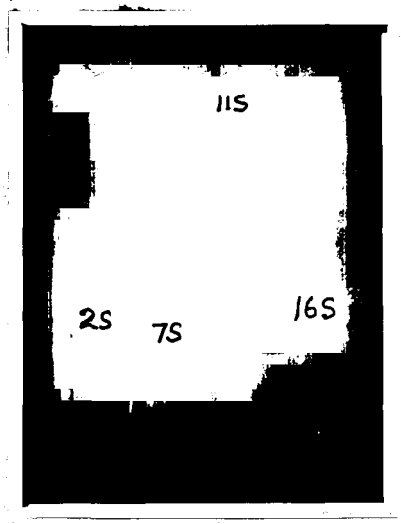
(a) α-globulin of Nath and Giri (1957b)

(b) α-globulin of Ventura and Lima (1963) and

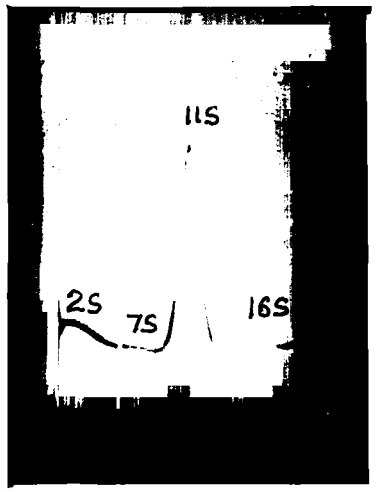
(c) α-globulin isolated in the present investigation.

Fig.3

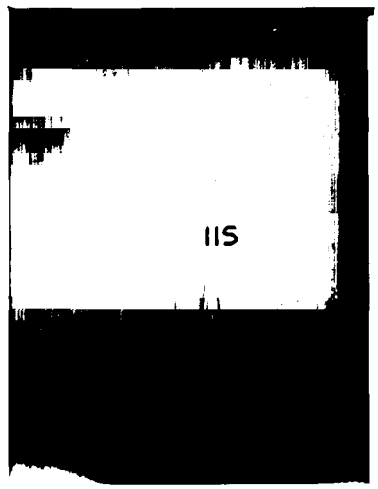
a



b



c



Hence with a view to get a homogeneous preparation of α-globulin a slight modification of the procedure of Nath and Giri (1957b) (see page No. 52) was adopted. The sedimentation velocity pattern (Fig. 3C) indicated a major peak with a $S_{20,w}$ value of 11.0. About 5% of the 16S component was also present. But no 2S component was detectable. In addition, the yield of α-globulin was 20-22%.

The homogeneity of α-globulin isolated by the method described in the present study was determined by using the techniques of gel filtration, DEAE-cellulose chromatography, polyacrylamide gel electrophoresis and sedimentation velocity experiments. For comparison, the total proteins were also analysed by these techniques.

Gel filtration:

Gel filtration of the sesame seed total protein in 0.02M phosphate buffer pH 7.5 containing 1M sodium chloride in Sepharose 6B-100 indicates three peaks (Fig. 4a). The first peak elutes at ∽95 ml (near the void volume) and constitutes about 10%, the second peak eluting at ∽160 ml constitutes about 70% (major fraction) and the third peak eluting at ∽230 ml constitutes about 20% of the protein recovered from the column (Fig. 4a).

The gel filtration pattern of α-globulin isolated by the method described in the present study indicates a single symmetrical peak eluting at ~ 160 ml (Fig. 4b). The peak portion when examined by polyacrylamide gel electrophoresis indicated a sharp single band (Fig. 4b). A fraction eluting near the void volume constitutes about 2-3% of the protein.

DEAE-cellulose chromatography:

DEAE-cellulose chromatography of the total protein in 0.01M glycine-sodium hydroxide buffer pH 9.0, indicated a sharp peak eluting at 0.04M sodium chloride concentration along with trailing portions, eluting upto 0.3M sodium chloride concentration (Fig. 5a).

α-globulin indicated a single peak with the peak portion eluting at 0.04M sodium chloride concentration (Fig. 5b). The different portions of the peak when examined by polyacrylamide gel electrophoresis show a sharp single band (Fig. 5b).

Polyacrylamide gel electrophoresis:

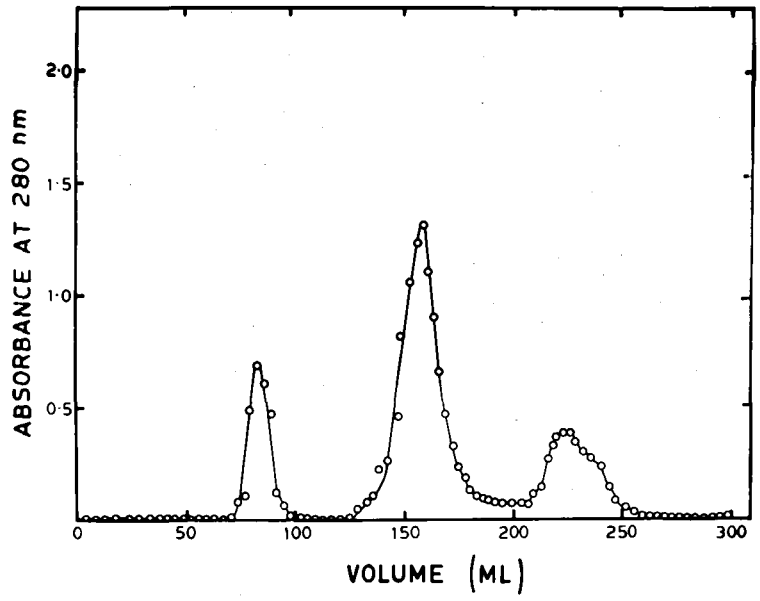
Gel electrophoresis of the total protein in 0.02M phosphate buffer pH 7.5 shows a major band along with several slow and fast moving components (Fig. 6a).

Fig. 4 Gel filtration patterns

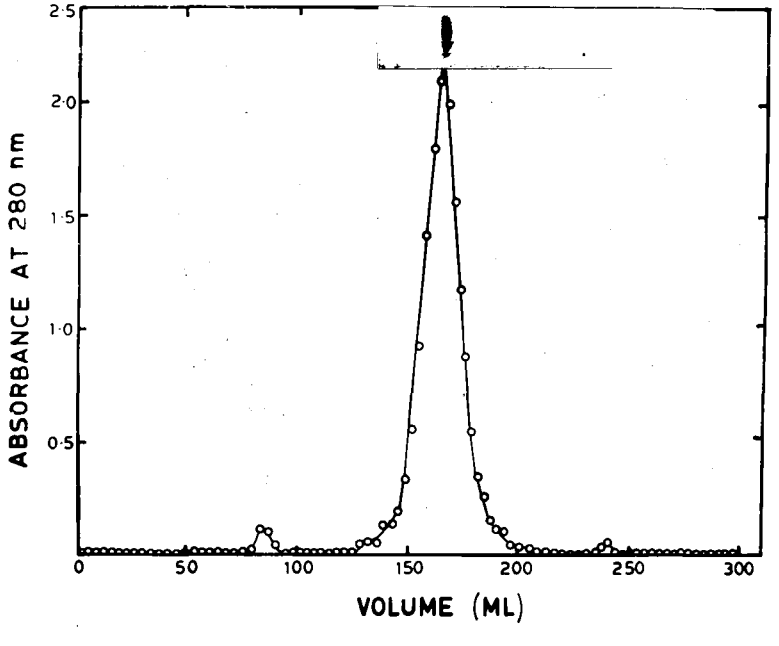
(a) total protein and

(b) α-globulin in 0.02M phosphate buffer, pH 7.5 containing 1M sodium chloride in Sepharose 6B-100 gel.

Fig.4


ABSORBANCE AT 280 nm
2·0
1·5
1·0
0·5
0
50
100
150
200
250
300
VOLUME (ML)

a


ABSORBANCE AT 280 nm
2·5
2·0
1·5
1·0
0·5
0
50
100
150
200
250
300
VOLUME (ML)

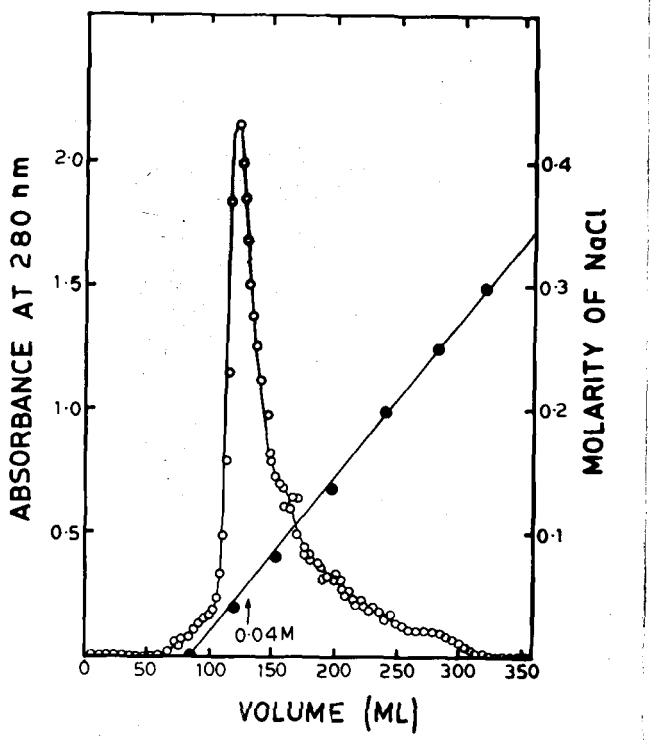
b

Fig. 5 DEAE-cellulose chromatographic patterns of

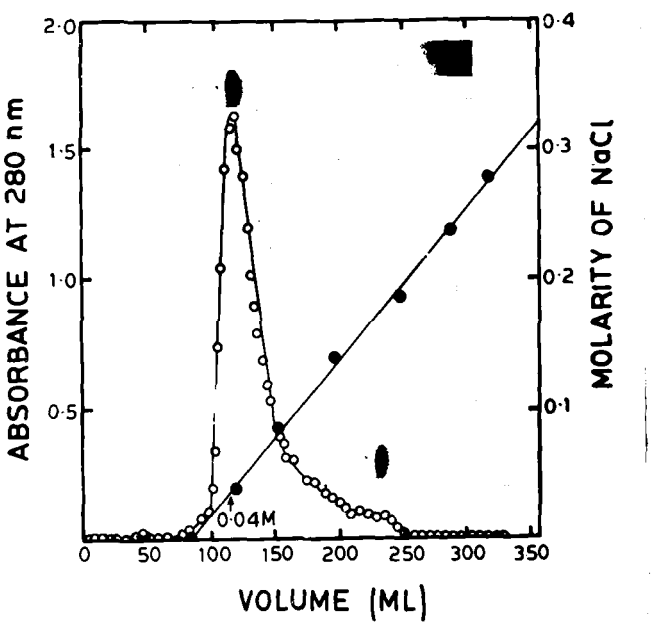
(a) total protein and

(b) α_1-globulin in 0.01M glycine sodium hydroxide buffer pH 9.0.

Fig. 5


ABSORBANCE AT 280 nm
2·0
1·5
1·0
0·5
0·4
0·3
0·2
0·1
MOLARITY OF NaCl
0·04M
0
50
100
150
200
250
300
350
VOLUME (ML)

a


ABSORBANCE AT 280 nm
2·0
1·5
1·0
0·5
0·4
0·3
0·2
0·1
MOLARITY OF NaCl
0·04M
0
50
100
150
200
250
300
350
VOLUME (ML)

b

α-globulin indicates a single sharp band corresponding to the major band of the total protein (Fig.6b).

Sedimentation velocity:

Sedimentation velocity experiments of the total protein in 0.02M phosphate buffer pH 7.5 containing 1M sodium chloride shows four components with $S_{20,w}$ values of 2, 7, 11 and 16 constituting nearly 20, ~5, 65 and 10% respectively (Fig. 7a).

α-globulin shows a single symmetrical peak with a $S_{20,w}$ value of 11 along with about < 5% of a 16S component (Fig. 7b).

The above results indicate that α-globulin isolated by the method described in the present investigation had better than 95% homogeneity.

Storage:

Lyophilised α-globulin (< 1% moisture) was stored at room temperature for a period of ~1 year to see if there was any degradation of the protein. The results of polyacrylamide gel electrophoresis of the stored sample does not show any degraded products (Fig. 8 a and b). This indicates that the protein is quite stable during storage (~1 year).

Proteolytic activity:

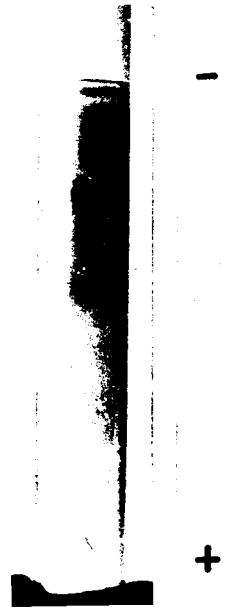
With denatured hemoglobin as substrate, α-globulin indicated no proteolytic activity over a period

Fig. 6 Polyacrylamide gel electrophoretic patterns of

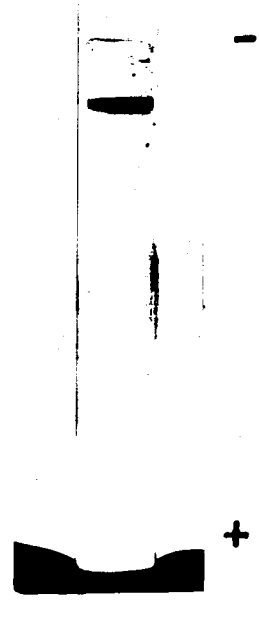
(a) total protein (120 min running time) and

(b) α-globulin (70 min. running time) in 0.02M phosphate buffer pH 7.3.

Fig. 6



a



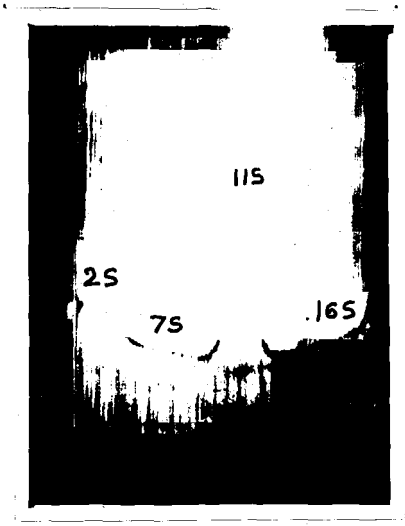
b

Fig. 7 Sedimentation velocity patterns of

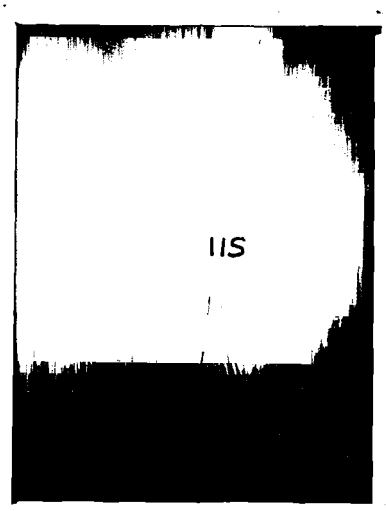
(a) total protein and

(b) α-globulin in 0.02M phosphate buffer pH 7.5 containing 1M sodium chloride.

Fig. 7



a



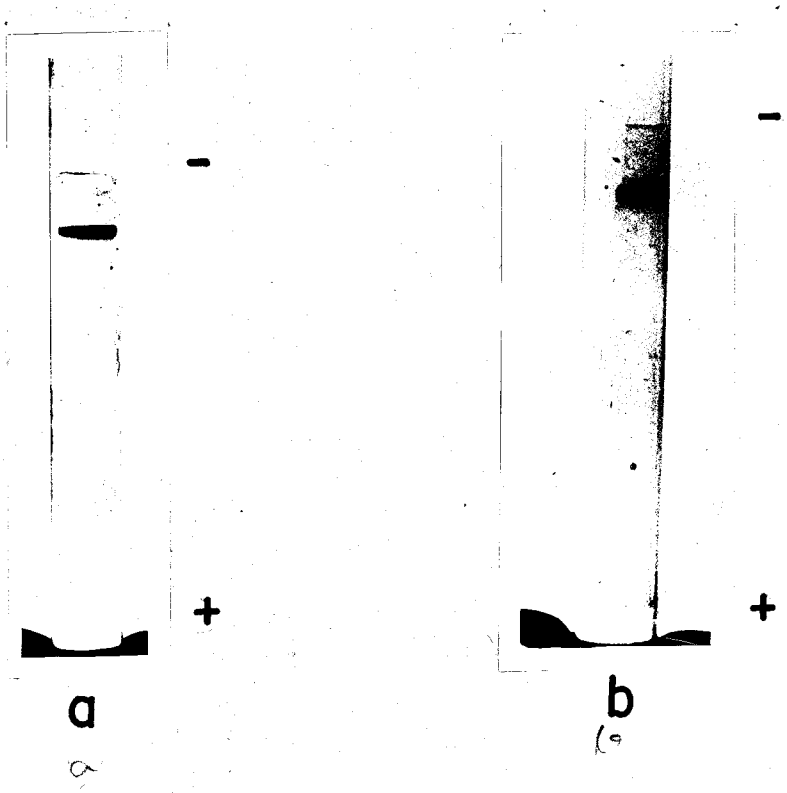
b

Fig. 8 Effect of storage on the polyacrylamide gel electrophoretic pattern of α-globulin in 0.02M phosphate buffer, pH 7.3

(a) freshly isolated

(b) lyophilised and stored.

Fig. 8



of 24 hr as determined by the absorbance at 280 nm of the trichloroacetic acid precipitated supernatant.

Ultraviolet spectrum

The ultraviolet spectrum of the protein in 0.02M phosphate buffer pH 7.5 containing 1M sodium chloride in the range 230-350 nm indicated an absorption maximum at 278-280 nm (Fig.9) and a minimum at 250 nm.

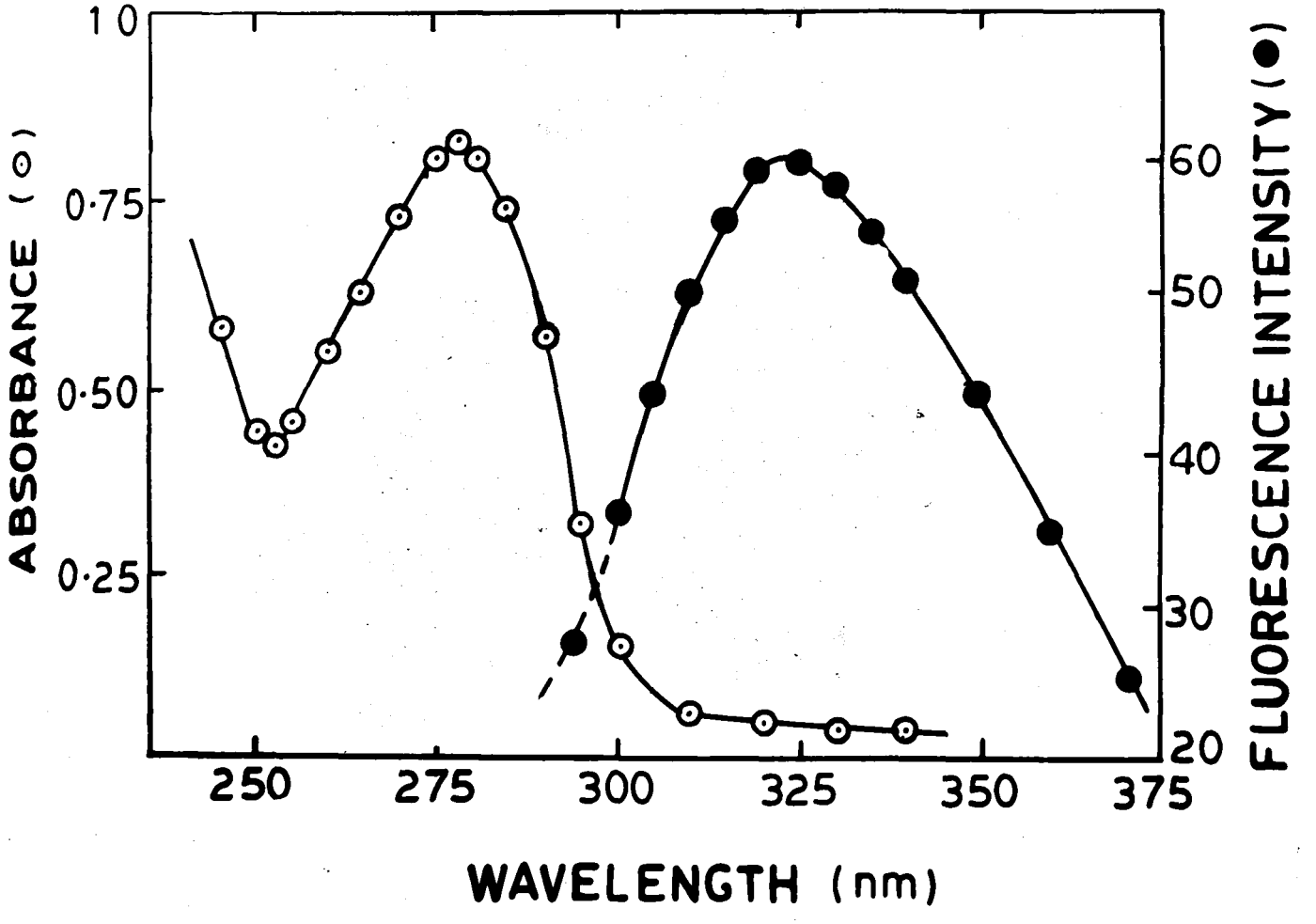
Fluorescence spectrum

The fluorescence spectrum of the protein in the range 300-375 nm in 0.02M phosphate buffer pH 7.5 containing 1M sodium chloride indicated an emission maximum at 328 nm (Fig.9).

The different physical and chemical properties of α-globulin are given in Table 1. The intrinsic viscosity of α-globulin, 0.03 dl/gm, indicates the globular nature of the protein (Yang, 1961; Bradbury, 1970) (Fig. 10). The molecular weight of α-globulin in 0.02M phosphate buffer pH 7.5, containing 1M sodium chloride, determined by Archibald method and sedimentation velocity experiments yielded a value of $2.5 \times 10^5 \pm 15{,}000$ daltons. This value is considerably lower than the value of $4.5 \times 10^5 \pm$ 30,000 daltons reported by Ventura and Lima (1963). The sedimentation coefficient of α-globulin was observed to be 11. This is also lower than the value of 13S reported by Sinha and Sen (1962) and Ventura and Lima (1963).

Fig. 9 Ultraviolet and fluorescence spectra of α-globulin in 0.02M phosphate buffer pH 7.5 containing 1M sodium chloride.

Fig. 9


1·0
0·75
0·50
0·25
ABSORBANCE (○)
60
50
40
30
20
FLUORESCENCE INTENSITY (●)
250
275
300
325
350
375
WAVELENGTH (nm)

The sedimentation coefficient of α-globulin isolated by the methods of Sinha and Sen (1962) and Ventura and Lima (1963) using the present sesame flour was also observed to be 11S. The fluorescence emission maximum of the protein at 328 mμ indicates that the fluorescence is dominated by tryptophan groups which are embeded in a non polar environment of α-globulin (Lakshmi and Nandi, unpublished results). The amide content of α-globulin was 2%. The low content of phosphorus and carbohydrate indicates that α-globulin is a non-conjugated protein. The oligomeric nature of the protein is indicated by the number of subunits present in it (Fig. 11). From the standard graph (Fig. 12) of logarithm of molecular weight of standard proteins against their relative mobility, the molecular weight of the subunits of α-globulin was calculated. The molecular weight ranged from $\sim 1 \times 10^4$ to $\sim 1 \times 10^5$ daltons.

Amino acid composition:

The amino acid composition of α-globulin is shown in Table 2. The amino acid analysis of α-globulin by Nath and Giri (1957c) is given for comparison. The values obtained by Nath and Giri (1957c) for lysine, threonine, half-cystine and leucine are significantly higher than the present values. Also their values for arginine, aspartic acid, serine, glutamic acid, proline

and phenylalanine are considerably lower than the present values. The protein contains high amounts of aspartic acid, glutamic acid and arginine.

TABLE 1

CHEMICAL AND PHYSICO CHEMICAL PROPERTIES OF α-GLOBULIN OF SESAME SEED

(A few values of total protein have been included for comparison).

Property	Total protein	α-globulin
Intrinsic viscosity[a] $[\eta]$	–	0.03 dl/gm
Isolectric point, PI	4.50	4.90
Sedimentation velocities[a] $S_{20,w}$	2,7,11 & 16	11
Molecular weight[a] M	–	2,50,000±15,000
Specific rotation $[\alpha]_{578\ nm}^{28°}$	–	-40°
Absorption coefficient[a] $E^{1\%}_{1\ cm}$ at 280 nm	13.0	10.8
Absorption maximum[a] nm	278-280	278-280
Fluorescence emission maximum[a] nm	–	328
Nitrogen content[a] %	–	15.9
Amide nitrogen %		2.0
Phosphorus content %	0.09	0.04
Carbohydrate content %	4.3	0.8
Subunit number	–	11
Proteolytic activity	–	Nil

[a] Values are in 0.02M phosphate buffer pH 7.5 containing 1M sodium chloride.

Fig. 10 Determination of intrinsic viscosity of α-globulin in 0.02M phosphate buffer pH 7.5 containing 1M sodium chloride at 28°

Fig.10

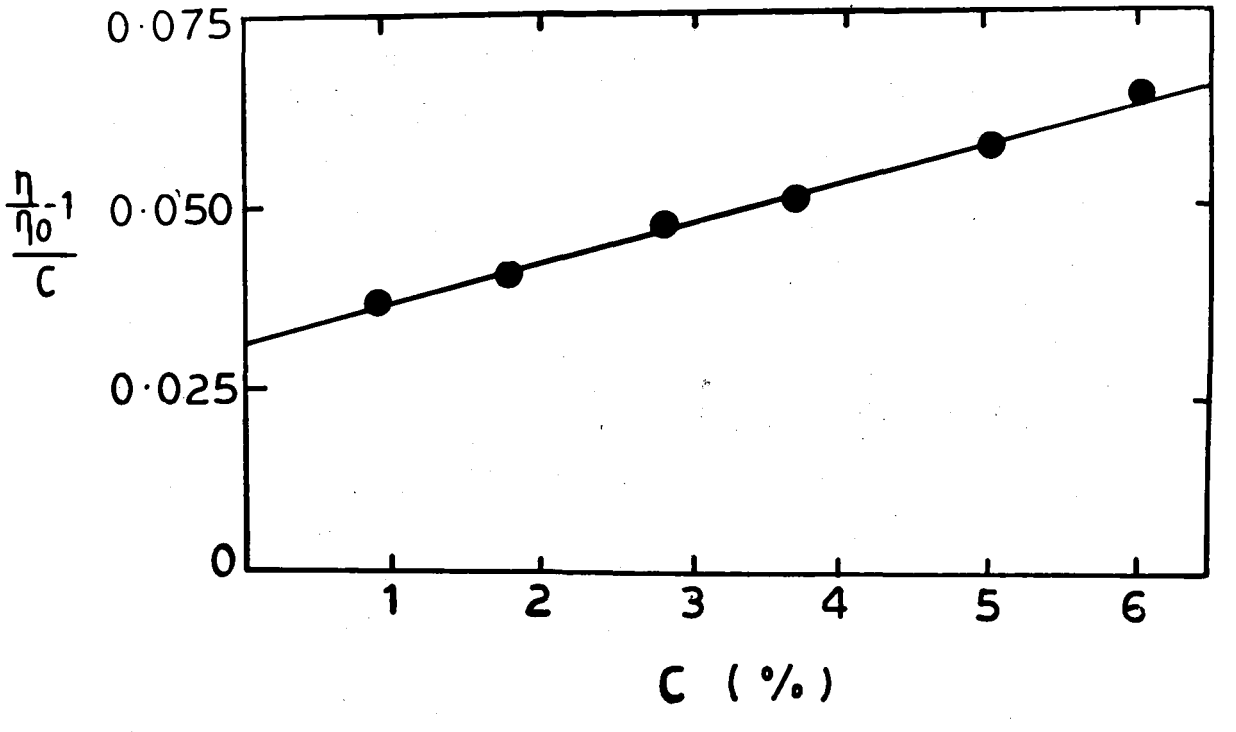


Fig. 11 SDS-polyacrylamide gel electrophoretic pattern of α-globulin.

Fig. 11

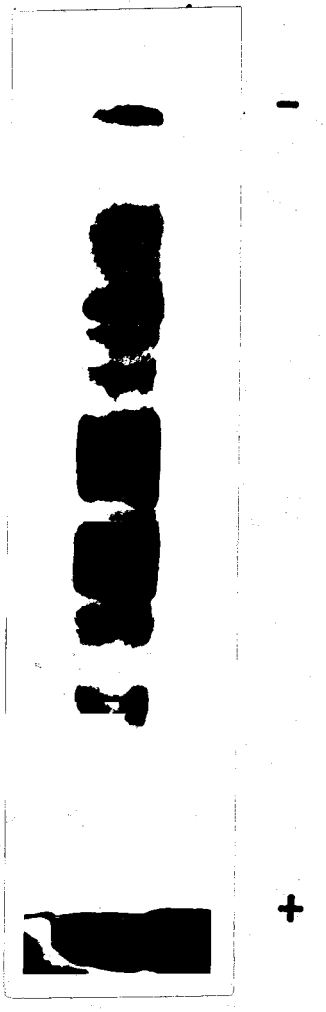


Fig. 12 Plot of logarithm of molecular weight as a function of relative mobility of various standard proteins used in SDS-polyacrylamide gel electrophoresis.

Fig.12

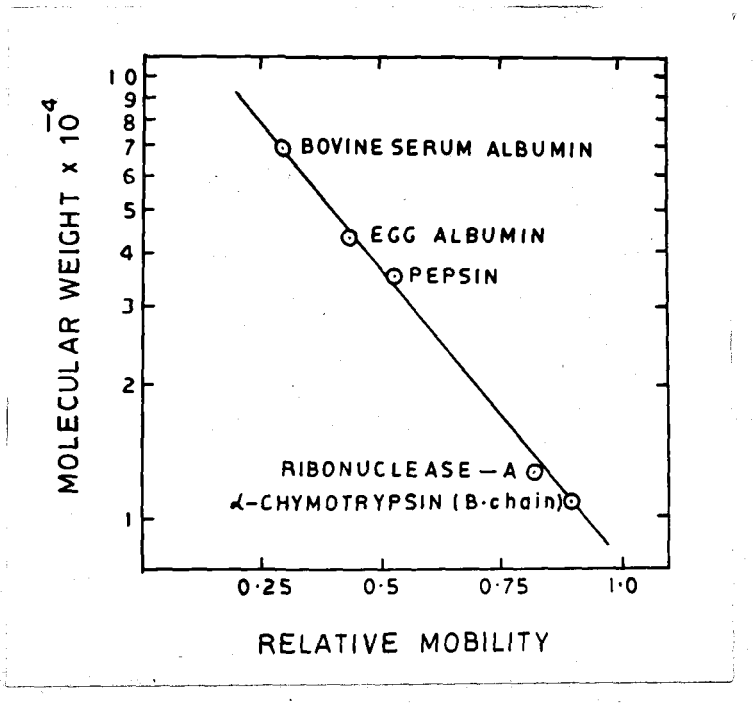

MOLECULAR WEIGHT x 10^{-4}
10
9
8
7
6
5
4
3
2
1
BOVINE SERUM ALBUMIN
EGG ALBUMIN
PEPSIN
RIBONUCLEASE — A
α-CHYMOTRYPSIN (B-chain)
0·25
0·5
0·75
1·0
RELATIVE MOBILITY

TABLE 2

AMINO ACID COMPOSITION OF α-GLOBULIN

Amino acid	By Nath and Giri (1957c) gm/100 gm protein	gm/100 gm protein[a]	Residues per 2,50,000 gm protein[b]
Lysine ..	2.90±0.24	2.01	34
Histidine ..	3.60±0.20	2.78	45
Ammonia ..	-	2.00	294
Arginine ..	8.40±0.26	14.18	204
Aspartic acid	4.30±0.22	9.63	181
Threonine ..	6.13±0.2	4.14	87
Serine ..	3.17±0.1	5.11	122
Glutamic acid	7.83±0.1	19.98	340
Proline ..	1.05±0.07	2.00	43
Glycine ..	3.45±0.1	5.16	172
Alanine ..	3.30±0.1	5.02	141
Half cystine	3.50±0.1	0.71	15
Valine ..	4.65±0.2	4.54	97
Methionine ..	2.65±0.2	2.59	43
Isoleucine ..	3.92±0.34	3.37	68
Leucine ..	9.00±0.14	7.09	135
Tyrosine ..	4.10±0.1	3.95	55
Phenylalanine	2.65±0.17	4.94	75
Tryptophan[c] ..	1.72±0.17	2.00±0.1	25

[a] Average of three analysis.

[b] To the nearest integer values

[c] Average of NBS method (Spande and Witkop, 1967), Edelhoch's method (Edelhoch, 1967) and microbiological method (Barton-Wright, 1952).

STUDIES IN ACID SOLUTIONS

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The isoelectric point of α-globulin is ∽ 4.9 (see page No. 94). The low solubility and turbidity of the protein solutions prevented any measurements in the range of pH 4-6. The effect of decreasing pH in the range, 4.1-1.5 on the protein α-globulin has been studied by the techniques of gel filtration, sedimentation velocity, ultraviolet difference spectra, fluorescence spectra and viscosity measurements.

Gel filtration:

The protein elutes as a single peak near the void volume (∽ 90 ml) in Sephadex G-50 column in 0.02M phosphate buffer of pH 7.5 (Fig. 13a). In 0.1M acetate buffer pH 3.8, the major protein fraction elutes at the void volume of the gel. This is followed by elution of low molecular weight fractions (Fig.13b). The appearance of these fractions indicates that α-globulin has dissociated partially in acidic solution. Results in 0.3M citrate phosphate buffer pH 3.0 also indicate the dissociation of the protein as shown in Fig.13c.

Fig. 13 Gel filtration pattern of α-globulin in

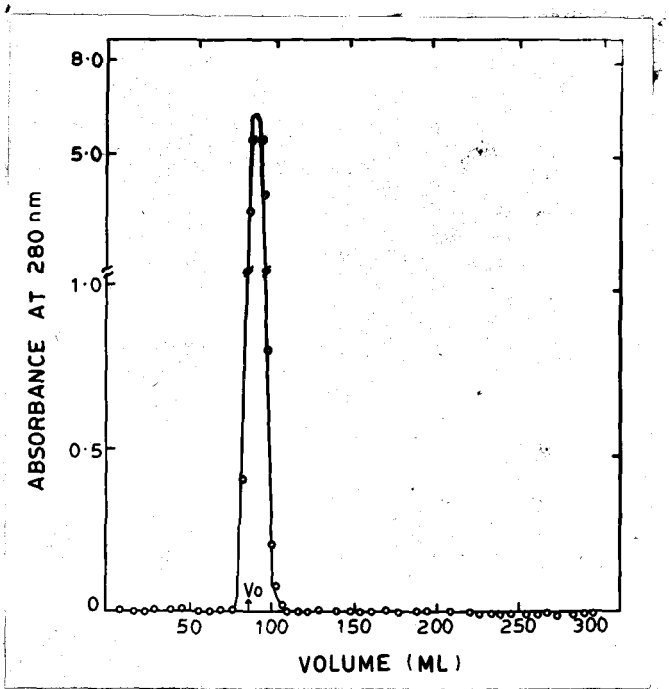
(a) 0.02M phosphate buffer, pH 7.5

(b) 0.1M acetate buffer, pH 3.8 and

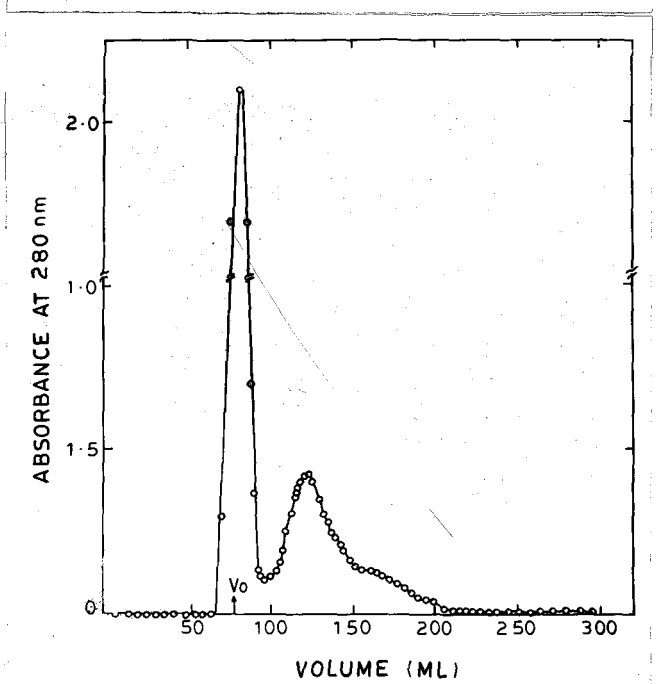
(c) 0.3M Citrate-Phosphate buffer pH 3.0 in Sephadex G-50 gel.

Fig.13

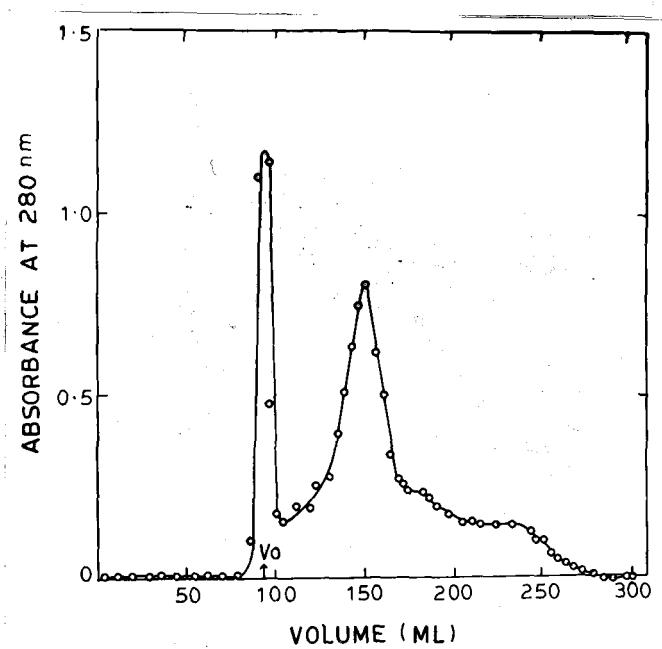
a


8·0
5·0
1·0
0·5
0
ABSORBANCE AT 280 nm
Vo
50
100
150
200
250
300
VOLUME (ML)

b


2·0
1·0
1·5
0
ABSORBANCE AT 280 nm
Vo
50
100
150
200
250
300
VOLUME (ML)

c


1·5
1·0
0·5
0
ABSORBANCE AT 280 nm
Vo
50
100
150
200
250
300
VOLUME (ML)

Sedimentation velocity:

The sedimentation velocity patterns of α-globulin in 0.1M acetate buffer at pH 3.8, 2.5 and 1.5 show that in acid solution α-globulin (11S) dissociates to a 2S component (Fig. 14). The concentration of 2S component increases at the cost of 11S component till pH 2.5 (Fig. 14b). With further decrease in pH to 1.5, the concentration of the 2S component decreases with a concomittant increase in the concentration of the 11S component (Fig. 14c). The relative percentages of 11S and 2S components at pH 1.5 is similar to the results at pH 3.8. This indicates that at pH 1.5, reassociation of the dissociated 2S component to the 11S component occurs. This 11S component may or may not be the same 11S component present initially at neutral pH.

Difference spectra:

The difference spectra of the protein were measured at nine different pH values in the range of pH 4.1-1.5. A few representative spectra are shown in Fig. 15a. All the difference spectra show blue shift and are characterised by peaks at 280, 287 and 292 nm, which indicates that both tyrosine and tryptophan groups in the protein are perturbed in acid solution (Wetlaufer, 1962; Donovan, 1969). The difference in the extinction coefficient values $\Delta\varepsilon$ at various wavelengths

Fig. 14 Effect of acidic pH on the sedimentation velocity patterns of α-globulin. pH of the solutions are

(a) 3.8

(b) 2.5 and

(c) 1.5

Fig. 11

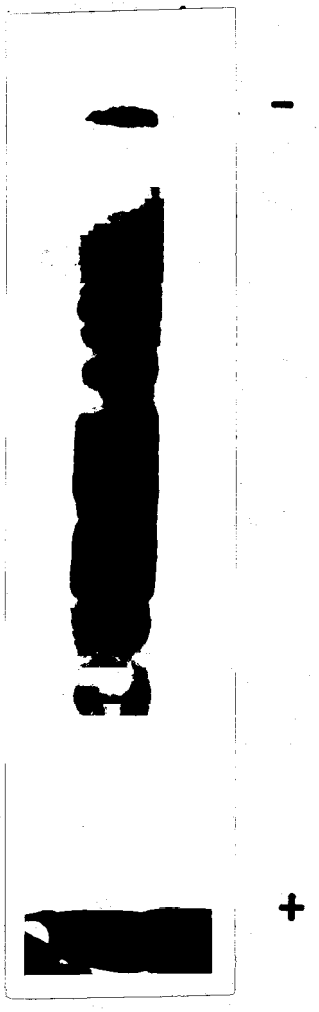


Fig. 12 Plot of logarithm of molecular weight as a function of relative mobility of various standard proteins used in SDS-polyacrylamide gel electrophoresis.

Fig.12

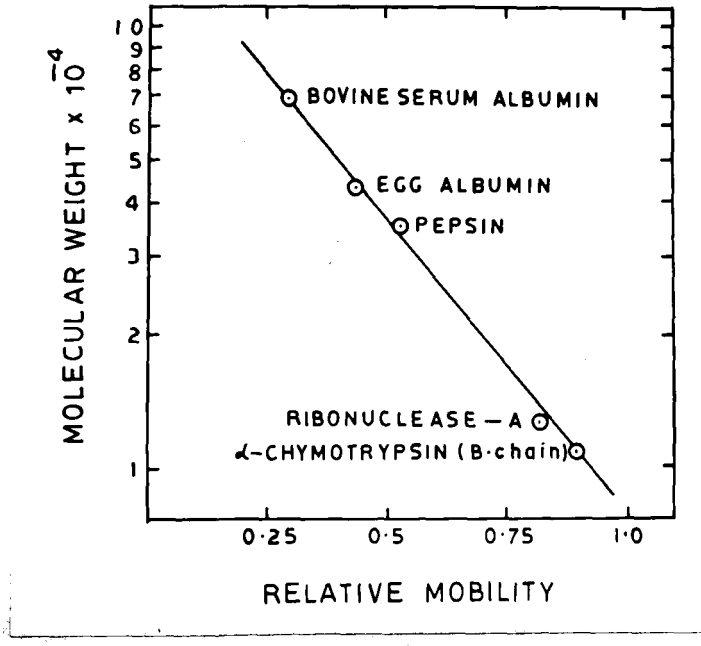

MOLECULAR WEIGHT x 10^{-4}
10
9
8
7
6
5
4
3
2
1
BOVINE SERUM ALBUMIN
EGG ALBUMIN
PEPSIN
RIBONUCLEASE — A
α-CHYMOTRYPSIN (B-chain)
0·25
0·5
0·75
1·0
RELATIVE MOBILITY

TABLE 2

AMINO ACID COMPOSITION OF α-GLOBULIN

Amino acid	By Nath and Giri (1957c) gm/100 gm protein	gm/100 gm protein[a]	Residues per 2,50,000 gm protein[b]
Lysine ..	2.90±0.24	2.01	34
Histidine ..	3.60±0.20	2.78	45
Ammonia ..	-	2.00	294
Arginine ..	8.40±0.26	14.18	204
Aspartic acid	4.30±0.22	9.63	181
Threonine ..	6.13±0.2	4.14	87
Serine ..	3.17±0.1	5.11	122
Glutamic acid	7.83±0.1	19.98	340
Proline ..	1.05±0.07	2.00	43
Glycine ..	3.45±0.1	5.16	172
Alanine ..	3.50±0.1	5.02	141
Half cystine	3.50±0.1	0.71	15
Valine ..	4.65±0.2	4.34	97
Methionine ..	2.65±0.2	2.59	43
Isoleucine ..	3.92±0.34	3.57	68
Leucine ..	9.00±0.14	7.09	135
Tyrosine ..	4.10±0.1	3.95	55
Phenylalanine	2.65±0.17	4.94	75
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[a] Average of three analysis.

[b] To the nearest integer values

[c] Average of NBS method (Spande and Witkop, 1967), Edelhoch's method (Edelhoch, 1967) and microbiological method (Barton-Wright, 1952).

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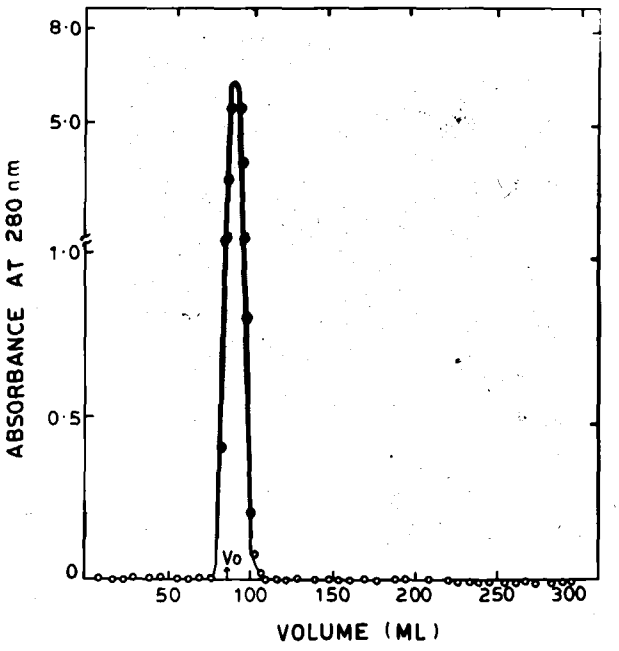
(b) 0.1M acetate buffer, pH 3.8 and

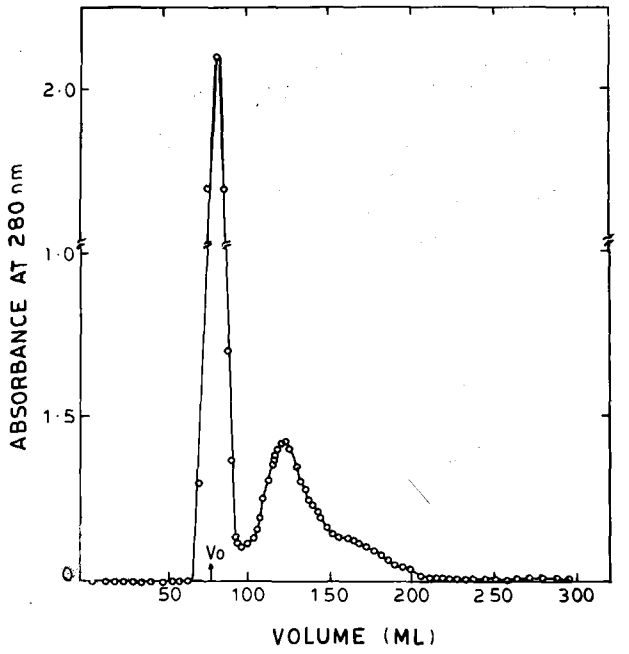
(c) 0.3M Citrate-Phosphate buffer pH 3.0 in Sephadex G-50 gel.

Fig.13

a


8·0
5·0
1·0
0·5
0
ABSORBANCE AT 280 nm
Vo
50
100
150
200
250
300
VOLUME (ML)

b


2·0
1·0
1·5
0
ABSORBANCE AT 280 nm
Vo
50
100
150
200
250
300
VOLUME (ML)

c

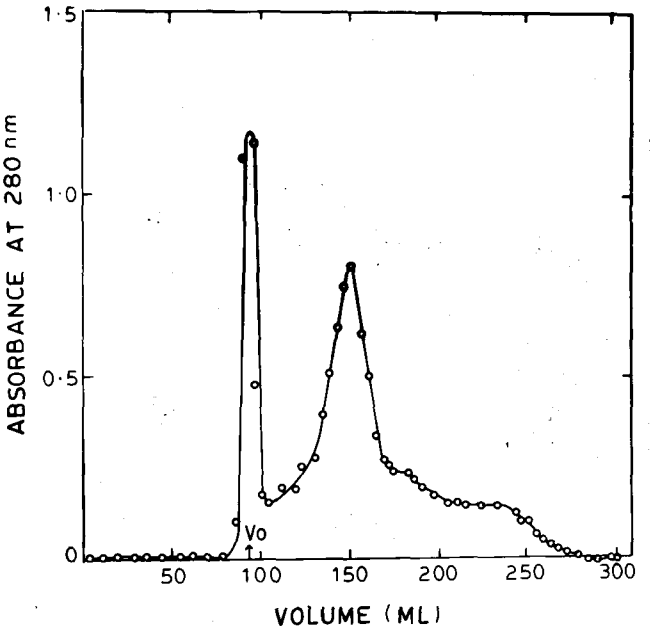

1·5
1·0
0·5
0
ABSORBANCE AT 280 nm
Vo
50
100
150
200
250
300
VOLUME (ML)

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Fig. 14 Effect of acidic pH on the sedimentation velocity patterns of α-globulin. pH of the solutions are

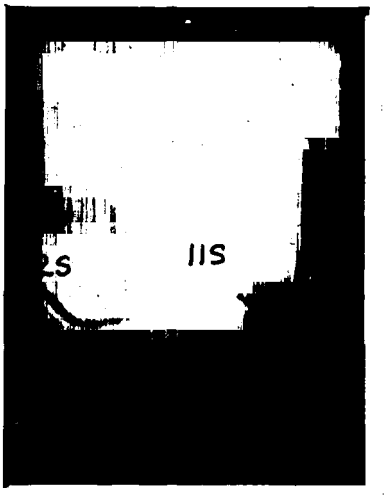
(a) 3.8

(b) 2.5 and

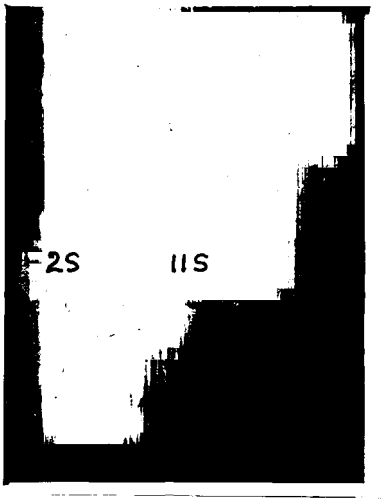
(c) 1.5

Fig.14

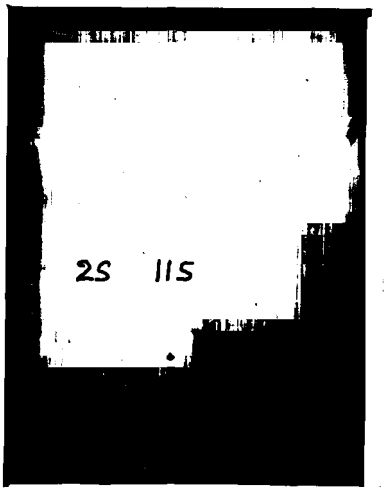
a



b



c



indicate that $\Delta\varepsilon$ at 287 nm > $\Delta\varepsilon$ at 292 nm > $\Delta\varepsilon$ at 280 nm, a trend observed in other proteins also (Donovan, 1973a). Further after the attainment of maximum blue shift at pH 3.1, a decrease in the blue shift is observed in higher acid solution (Fig. 15a). A plot of $\Delta\varepsilon$ against pH is shown in Fig. 15b. The above observation would suggest that at pH's < 3.1, the number of aromatic chromophores exposed or perturbed is less than the number at pH's > 3.1.

Fluorescence:

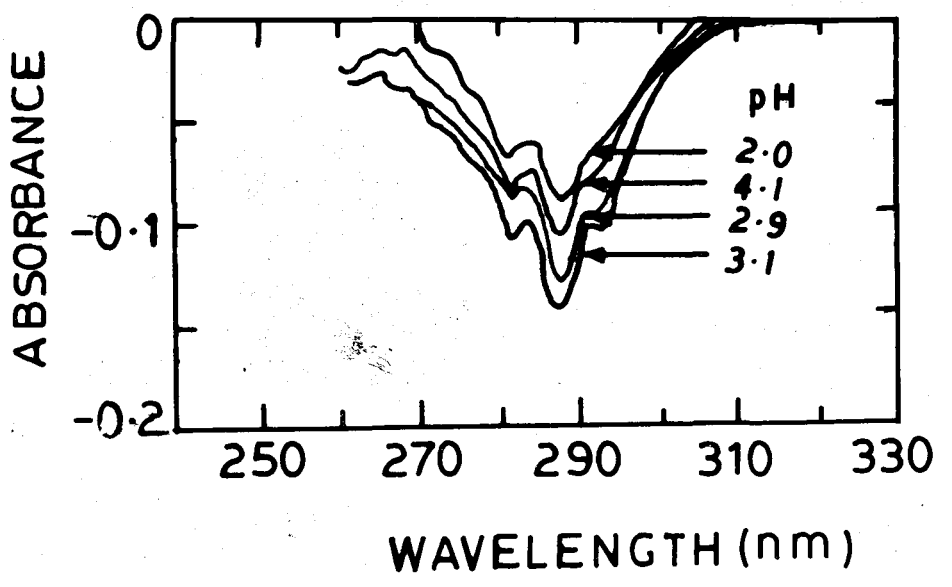
The fluorescence spectra were recorded at nine different acid concentrations in the pH range 4.1-1.3 a few of which are shown in Fig. 16a. Initially, in the pH range 4.1-2.6, a decrease in the fluorescence intensity is observed. This is accompanied by a progressive red shift of the emission maximum. The decrease in the fluorescence intensity and the red shift of the emission maximum suggest that the tryptophan groups are experiencing a high dielectric aqueous medium due to the exposure from the interior of the protein to the surrounding aqueous phase (Teale, 1960). This may be a consequence of dissociation and/or denaturation (Edelhoch et al., 1967). However, the pH dependency of protein fluorescence is complex. Therefore dissociation and denaturation causing a red shift may

Fig. 15 Effect of acidic pH on

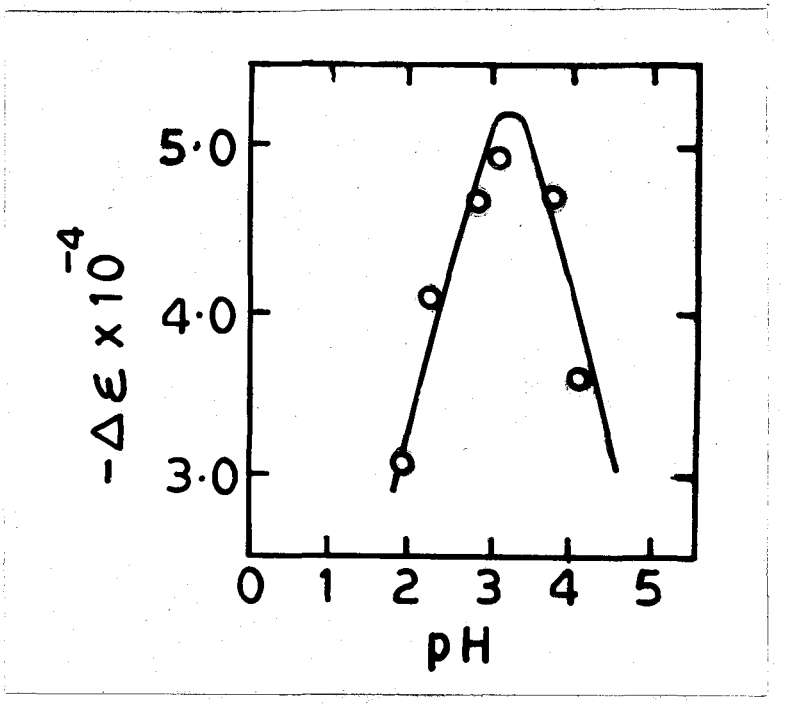
(a) Ultraviolet difference spectra of α-globulin and

(a) Change in the molar extinction coefficient values at 287 nm of the protein.

Fig. 15


ABSORBANCE
0
-0·1
-0·2
250
270
290
310
330
WAVELENGTH (nm)
pH
2·0
4·1
2·9
3·1

a


$-\Delta\epsilon \times 10^{-4}$
5·0
4·0
3·0
0
1
2
3
4
5
pH

b

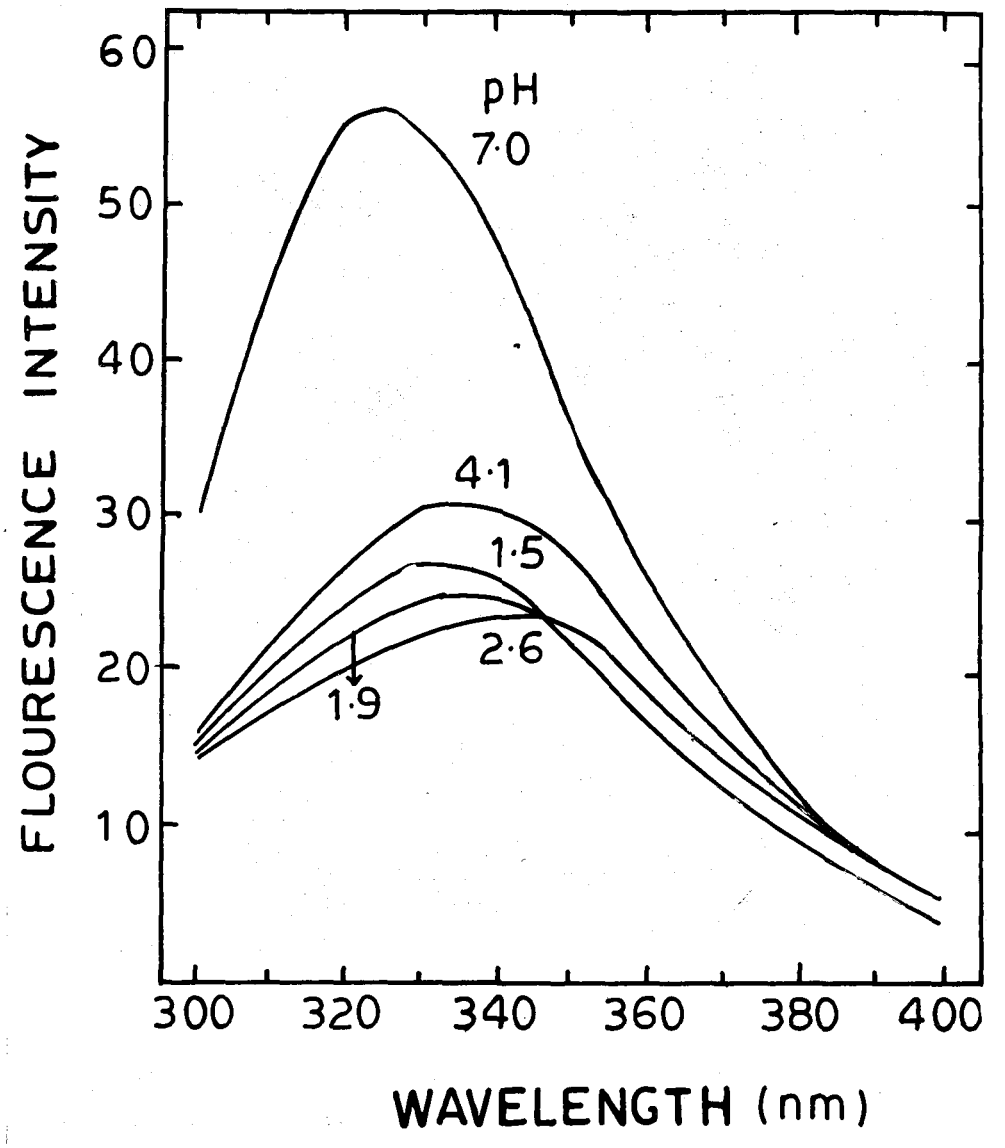
Fig. 16 Effect of acidic pH on

(a) fluorescence spectra of α-globulin and

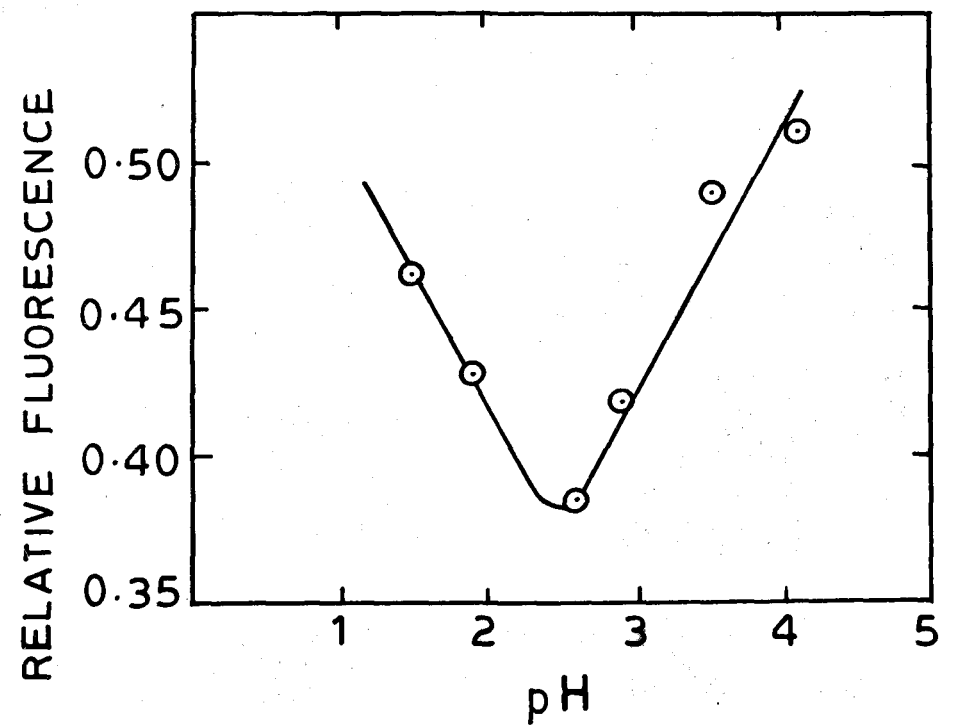
(b) relative fluorescence intensity of the protein at 325 nm.

Fig.16

a


FLOURESCENCE INTENSITY
60
50
40
30
20
10
pH
7·0
4·1
1·5
2·6
1·9
300
320
340
360
380
400
WAVELENGTH (nm)

b


RELATIVE FLUORESCENCE
0·50
0·45
0·40
0·35
1
2
3
4
5
pH

not be the only reason for the quenching observed in acid solutions (Longworth, 1971; Flanagan and Hesketh, 1974). With further decrease in pH below 2.6, an increase in the fluorescence intensity accompanied by a progressive blue shift of the emission maximum was observed (Fig. 16a). A plot of relative fluorescence against pH is shown in Fig. 16b.

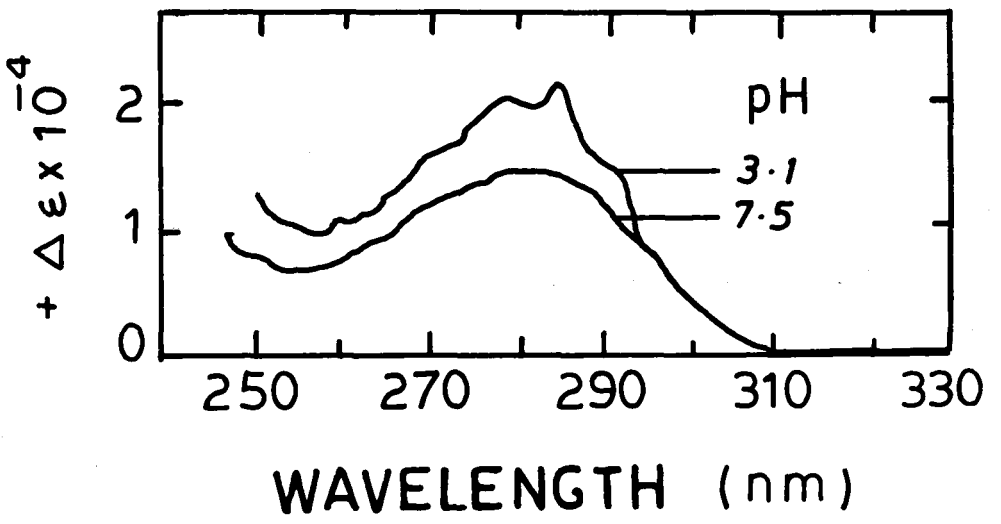
The results of gel filtration and sedimentation velocity experiments indicate that in acidic solution α-globulin dissociates. This dissociation probably accompanies unfolding of protein molecule as suggested from fluorescence measurements. The blue shift in the difference spectra may arise from (i) the exposure of the chromophores present at the interfaces upon dissociation and from the interior of the protein on denaturation or both (Herskovits, 1967; Donovan, 1973a; Imoto et al., 1975); (ii) the changes in the distribution of charges in the vicinity of the chromophores and disruption and formation of side chain hydrogen bonds involving tyrosyl and tryptophanyl residues (Herskovits, 1967; Donovan, 1973a). The availability of different number of chromopheric groups in the protein to the aqueous solution can be ascertained by the solvent perturbation technique developed by Herskovits and Laskowski (1960). Upon addition of reagents which

do not induce any structural change in the protein molecule, the absorption intensity of the protein increases as a consequence of red shift in its spectra. This red shift arises mostly from the change in the refractive index in the solvent surrounding the exposed chromophores of the protein molecule (Kronmann and Robbins, 1970; Laskowski, 1966). From the extent of intensity change in the perturbation spectra the number of tyrosyl and tryptophanyl groups exposed to the aqueous phase can be determined by using sucrose, glucose, ethylene glycol, etc. as perturbants. In acid solution, ethylene glycol was preferred over sucrose as perturbant of protein difference spectra. For meaningful comparison of the number of aromatic groups exposed in acid and neutral solutions, the protein difference spectra at pH 7.5 was also perturbed with ethylene glycol.

The protein difference spectra at pH 7.5 and 3.1 were perturbed by 20% ethylene glycol and measured against pH 7.5 protein solution. Comparison of the perturbation difference spectra at these two pH's show that more number of tyrosine and tryptophan groups are accessible to ethylene glycol at pH 3.1 (Fig.17). This indicates increased exposure of the chromophoric groups to the aqueous solvent at pH 3.1.

Fig. 17 Effect of 20% ethylene glycol on the difference spectra of α-globulin at pH's 7.5 and 3.1 measured against a protein solution of pH 7.5.

Fig. 17



The calculation of the number of tyrosyl and tryptophanyl groups from the difference spectrum at pH 3.1, based on the extinction change of -700 and -1600 per mole of tyrosone and tryptophan respectively (Donovan, 1973a), shows that approximately 65 tyrosine and 22 tryptophan groups are exposed at pH 3.1. From the total number of tyrosine and tryptophan groups in α-globulin (obtained from amino acid analysis of the protein) (see page No. 101), it appears that the majority of these groups are in contact with the solvent. This would indicate that a major conformational change in α-globulin has occurred at pH 3.1. To ascertain the extent of conformational change, viscosity was measured at pH 3.0. The reduced viscosity, η_{red}, at this pH has a value of 0.17 dl/gm. The η_{red} value for the denatured protein in 6M GuHCl solution is 0.46 dl/gm (see page No. 207). The native protein in 0.05M TEA-HCl buffer pH 9.0, has a η_{red} value of 0.06 dl/gm (see page No. 199). This means that the protein is only partially denatured in acid pH. This partial denaturation of the protein molecule would indicate that not all the extinction coefficient changes observed in the difference spectrum are arising from conformational change of α-globulin in acid solution.

The decrease in blue shift below pH 3.1 can be considered to have arisen from association of the

protein molecule. Sedimentation velocity experiments suggest reassociation of the protein. From increase in fluorescence, decrease in blue shift and changes of Cotton effect Flanagan and Hesketh (1974) have concluded that in strong acid solution (pH ~1), bovine carbonic anhydrase B is partly refolded though not necessarily to the original native conformation. Catsimpoolas et al., (1971), observed a blue shift in the difference spectrum of the 11S soybean protein, glycinin, in acid solution. A decrease in the blue shift occurs at pH 2. These authors have explained the decrease in the blue shift as arising from the association of the protein by H-bonding between the uncharged carboxylic groups. The results from different laboratories during the last few years, however, have indicated that intermolecular mono and bifunctional hydrogen bonds have no appreciable stability in water (Klotz and Franzen, 1962; Susi et al., 1964; Gill and Noll, 1972).

The association of the subunits of α-globulin which occurs in acid solution at pH ≤ 3 results in the removal of aromatic groups from the aqueous phase as has been evidenced from spectral measurements. The effect of temperature on the association-dissociation process of α-globulin at pH 1.5 by sedimentation velocity measurements shown in Fig.19 indicates that at 18°

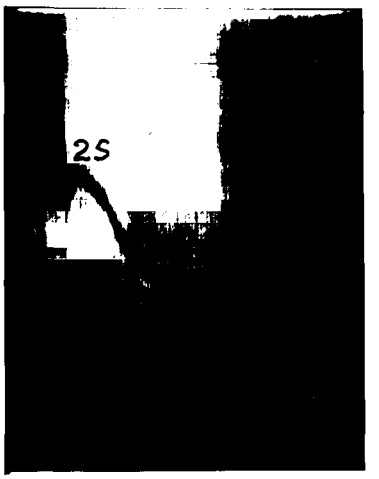
Fig. 13 Effect of temperature on the sedimentation velocity patterns of α-globulin at pH 1.5

(a) 19° and

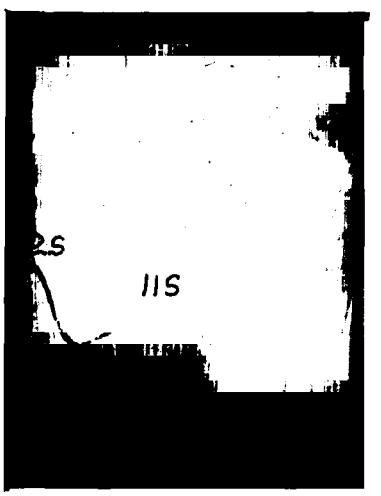
(b) 42°

Fig.18

a



b



no associated (11S) component is present in the system. The concentration of the 11S component increases with increase in temperature. This indicates that the nature of the association process is endothermic. Probably this results from entropically controlled hydrophobic interaction of the exposed nonpolar side chains of the dissociated subunits and not by H-bonding between the peptide chain(s) which is energetically exothermic in nature (Kauzmann, 1959; Jencks, 1969). From the results of the present investigation, it is not certain whether this reassociation process of the subunits of α-globulin is accompanied by refolding of the protein molecule also.

STUDIES IN ALKALINE SOLUTIONS

STUDIES IN ALKALINE SOLUTIONS

The effect of increasing the pH above 7 on the structure of α-globulin has been investigated by sedimentation velocity, fluorescence spectra, ultraviolet spectra, optical rotation, viscosity and tyrosyl ionisation measurements.

Sedimentation velocity:

In 0.05M Tris-HCl buffer pH 7.0, α-globulin exists as a single peak with $S_{20,w}$ value of 11S. With increase in pH, the 11S component dissociates to a 7S component (Fig. 19). The dissociation to 7S component is not rapid till pH 8.5. Above pH 8.5, the concentration of the 7S component increases rapidly with increase in pH. At pH 9.0, nearly 50% of 11S component has dissociated to the 7S component. At pH 11, the 7S component represents 100% of the protein present in solution (Fig. 19g). The variation of the percentage of 7S and 11S components with pH is shown in Fig. 20. At pH 12, a third component with 2S value represents 100% of the protein present in the solution (Fig. 19h). This has perhaps resulted from the dissociation of the 7S component. These results would suggest that α-globulin

Fig. 19 Effect of alkaline pH on the sedimentation velocity patterns of α-globulin. pHs of the solutions are

(a) 7.0
(b) 7.5
(c) 8.0
(d) 8.5
(e) 9.0
(f) 10.0
(g) 11.0 and
(h) 12.0.

Fig. 19

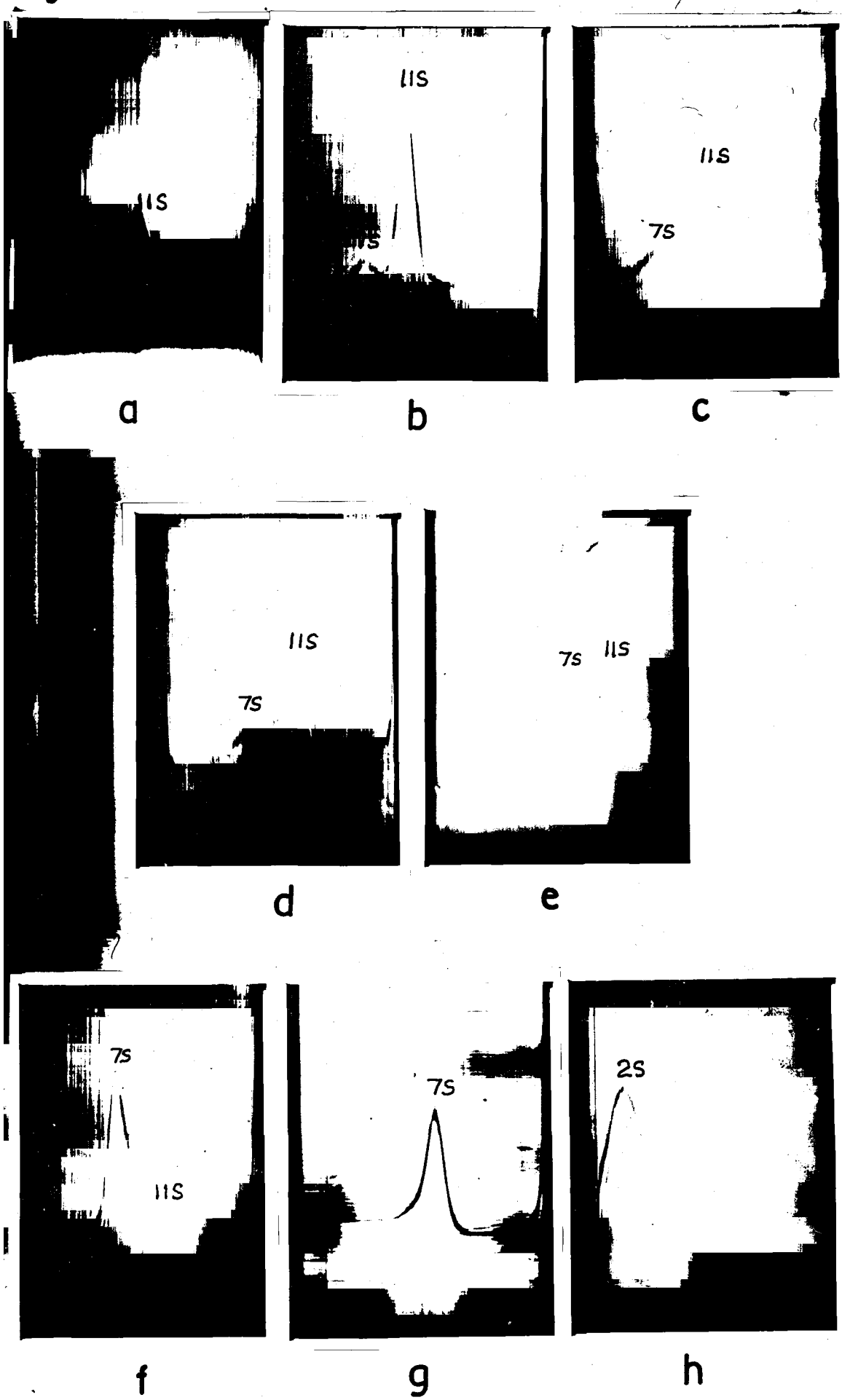
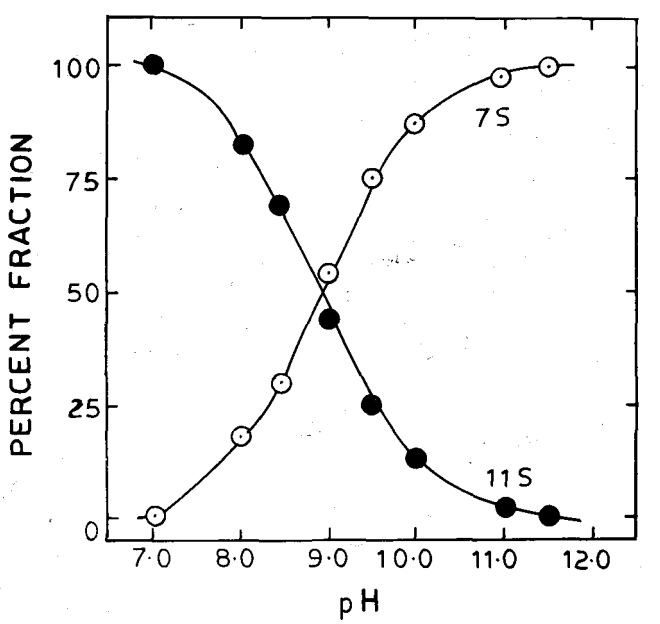


Fig. 20 Variation of percent fraction of 11S and 7S components as a function of pH (> 7.0).

Fig. 20



in alkaline solution dissociates according to the following scheme:

11S ⟶ 7S ⟶ 2S

Fluorescence:

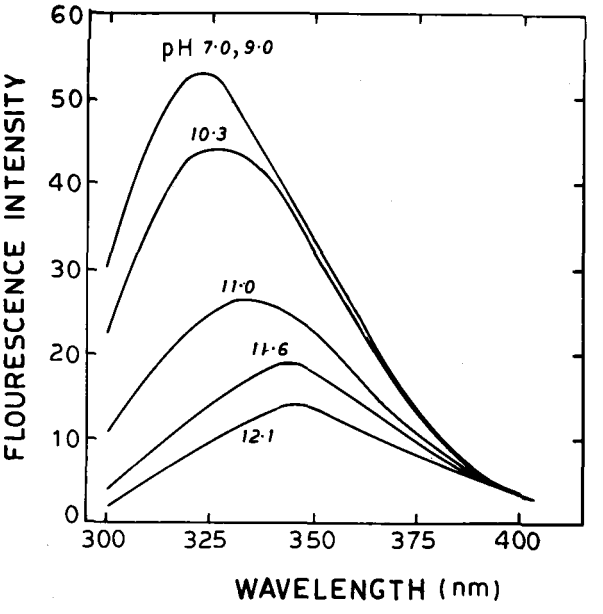
The fluorescence spectra of α-globulin were measured at various pH's from 7.0 upto pH 12.1 (Fig. 21a). Till pH 9.3, there is no change in the fluorescence intensity at 325 nm nor is there any shift in the fluorescence maximum. Above pH 9.5 a sharp decrease in the fluorescence intensity accompanied by a shift in the emission maximum towards red is observed (Fig.21a). Above pH 12 there is no change in either the fluorescence intensity or emission maximum. A plot of relative fluorescence as a function of pH is shown in Fig. 21b. The decrease in the fluorescence intensity may result from quenching of tryptophanyl fluorescence by resonance energy transfer from indole to phenolate ion (Brand and Witholt, 1967). The red shift of the emission maximum would indicate the gradual exposure of the tryptophan groups to the aqueous phase (Teale, 1960). These results show that although ultracentrifugal measurements indicate dissociation of the protein (11S to 7S) at pH 9.3, the relative fluorescence intensity at 325 nm does not change, nor is there any shift in the emission maximum (Fig.21a and b). Hence

Fig. 21 Effect of alkaline pH on the

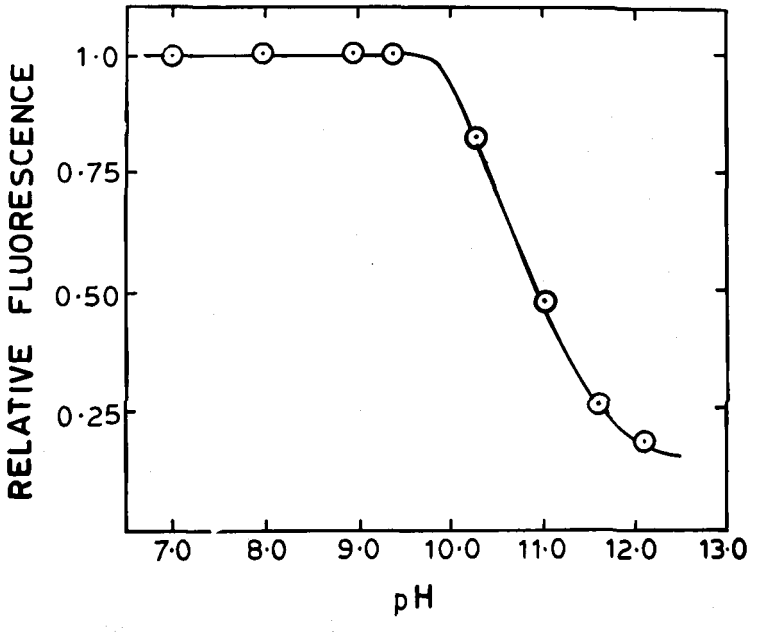
(a) fluorescence spectra of α-globulin and

(b) relative fluorescence intensity of the protein at 325 nm.

Fig. 21


FLOURESCENCE INTENSITY
60
50
40
30
20
10
0
pH 7·0, 9·0
10·3
11·0
11·6
12·1
300
325
350
375
400
WAVELENGTH (nm)

a


RELATIVE FLUORESCENCE
1·0
0·75
0·50
0·25
7·0
8·0
9·0
10·0
11·0
12·0
13·0
pH

b

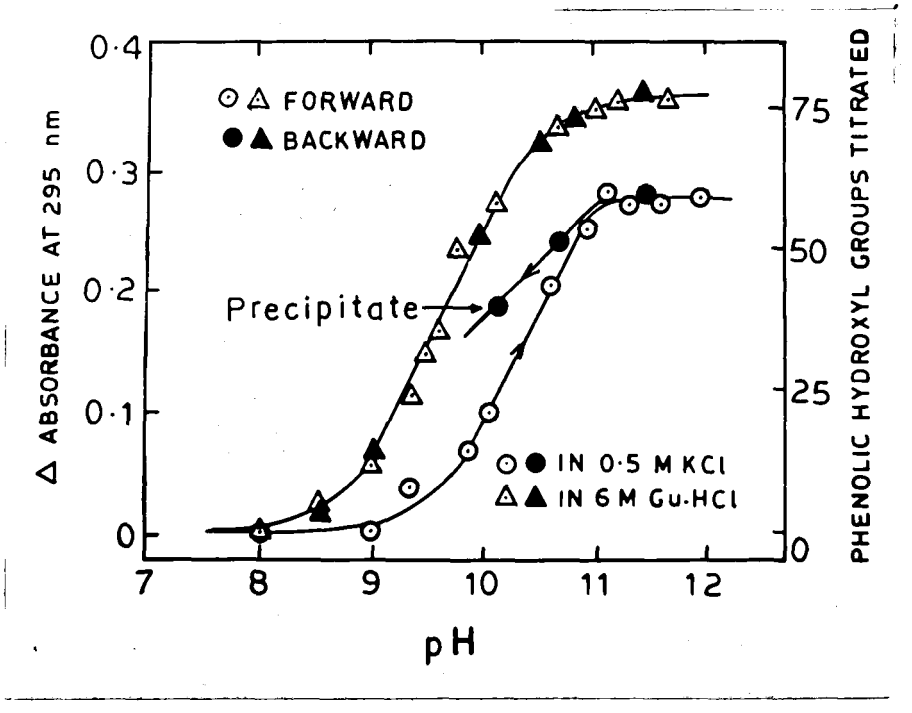
the dissociation of the 11S component to the 7S component does not involve any major change in the fluorophor environment. The mid point of the fluorescence transition occurs at pH 10.8 (Fig. 21b). Probably the majority of the fluorophors are in nonpolar environment.

Spectrophotometric titration of phenolic groups:

The titration of the phenolic groups was carried out from pH 7.0-12.0 by adjusting the pH of the protein solution with 1M NaOH. Measurements in buffered and unbuffered solutions (pH adjusted with 1M NaOH) gave the same results. In Fig. 22 increase in absorbance at 295 nm and the corresponding number of phenolic hydroxyl groups titrated are given as a function of pH. The gradual increase of absorbance (Fig. 22) below pH 9.5 indicates that a few of the tyrosyl groups are in the normal state i.e. exposed at the surface of the molecule. Above pH 9.5, the titration curve is steep. At pH $\backsim$12, nearly 60-62 phenolic groups have been titrated. The pK_{Int} of the tyrosyl groups derived from spectrophotometric titration yields a value of 10.6. This indicates that the tyrosyl groups in the protein are abnormal and are removed from the aqueous phase. The reversibility test of the titration could not be carried out over the entire range of pH since the lowering of the pH from pH 11.5 to 10, resulted in the

Fig. 22 Spectrophotometric titration of α-globulin, in 0.5M KCl and 6M GuHCl solutions.

Fig. 22


0·4
0·3
0·2
0·1
0
Δ ABSORBANCE AT 295 nm
FORWARD
BACKWARD
Precipitate
IN 0·5 M KCl
IN 6 M Gu·HCl
7
8
9
10
11
12
pH
75
50
25
0
PHENOLIC HYDROXYL GROUPS TITRATED

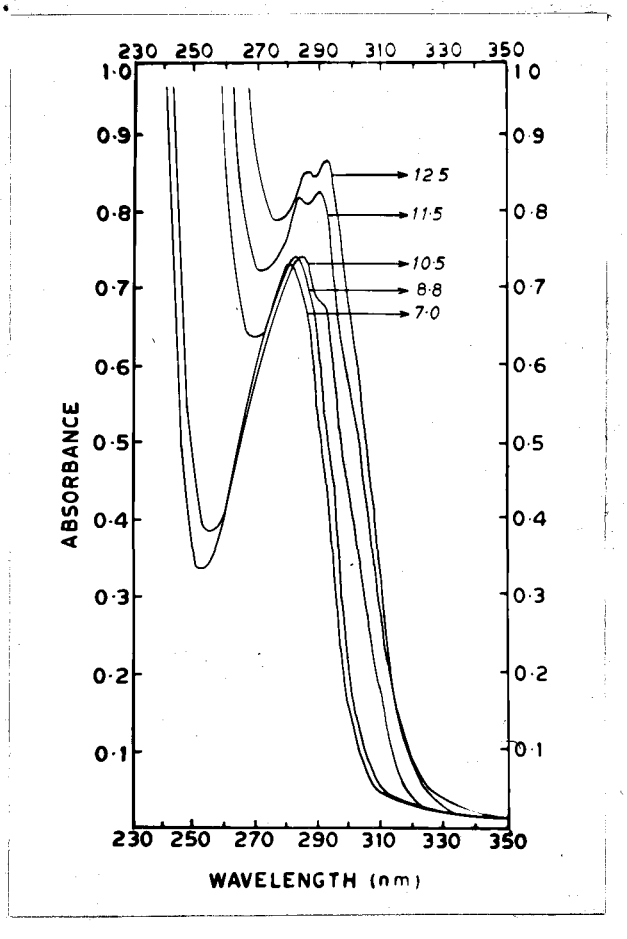
precipitation of the protein (Fig. 22). This precipitation of the protein at lower pH could be due to irreversible denaturation of the protein in alkaline solution which occurs possibly due to ionic forces (Tanford, 1961; Donovan, 1973a).

Spectra of the protein:

The spectrum of the protein was recorded in the 230-350 nm range at different pH's. The spectrum of the protein at pH 7.0, shows a well defined absorption maximum at 280 nm (Fig. 23). With increase in pH upto 10.0 the spectrum only becomes broader and the intensity increases. This indicates ionisation of the tyrosyl hydroxyl groups (Donovan, 1973a). At pH 10.5 (Fig. 23), a shoulder at 292 nm is observed which becomes more distinct at pH 10.7 accompanied by a red shift in the 280 nm peak to 284-285 nm. The intensity of the two peaks (i.e. 292 nm and 284-285 nm peaks) become identical at pH 11.0. With further increase in pH, the height of 292 nm peak increases compared to the 285 nm peak upto pH 12.5. The shape of the protein spectrum at alkaline pH and the gradual change in the intensity of the peaks at 285 nm and 292 nm indicate that both tyrosyl ionisation and conformational changes of the protein take place in

Fig. 23 Absorption spectra of α-globulin in the region 230-350 nm at various pH's.

Fig. 23

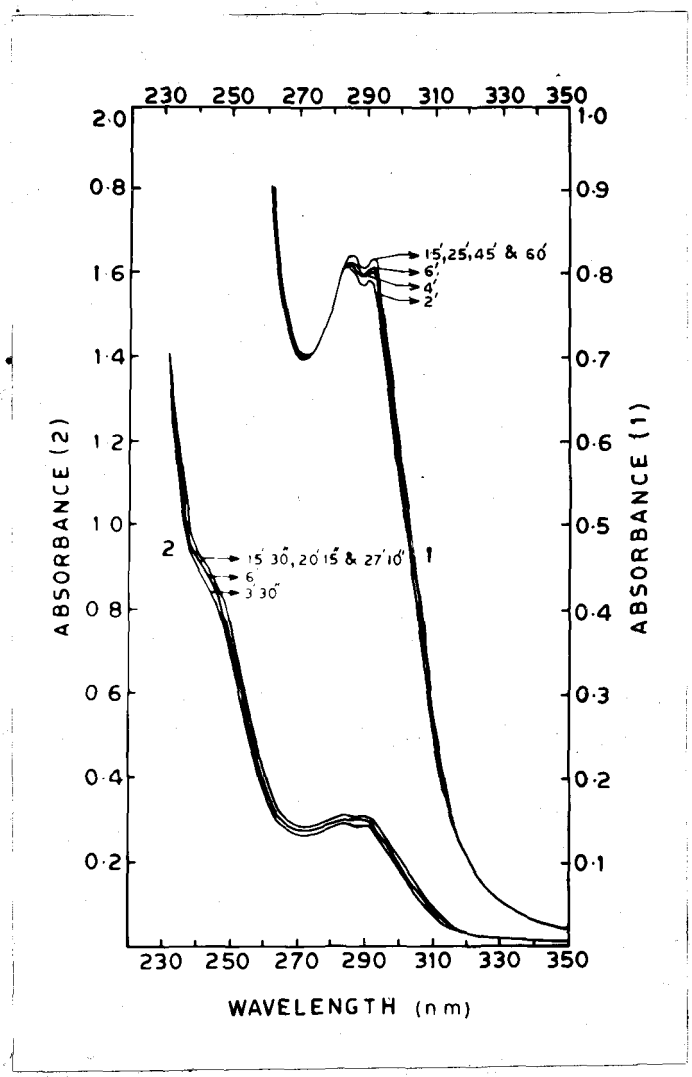

230 250 270 290 310 330 350
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
12·5
11·5
10·5
8·8
7·0
ABSORBANCE
WAVELENGTH (nm)

alkaline solution (Donovan, 1973a). However, this may not be the only reason for the observed results. At high alkaline pH, in addition to conformational changes, reactions catalysed by hydroxide ion, or in which hydroxide ion is a reactant, or hydrolysis of disulphide bonds are not uncommon (Donovan, 1973a). These reactions may perturb the absorption spectra of other chromophores. In ovomucoid the results of spectral measurements at different intervals of time in the region 220-340 nm showed that at pH 13, in addition to tyrosyl ionisation, hydrolysis of the disulfide bonds took place (Donovan, 1967).

The absorption spectrum of α-globulin at pH 11, was measured as a function of time both in the lower wavelength region (230-260 nm) and the longer wavelength region (260-350 nm). The spectra are shown in Fig. 24. In the longer wavelength region the large absorption change which take place in the initial ∽ 200 sec is due to tyrosyl ionisation which attains a constant value at ∽ 900 sec (Fig. 24). The successive scans after this period were superposable. The constancy of absorbance at 242 nm after tyrosine ionisation is complete, (Fig. 24) can be taken as an evidence that no other reaction which might result from alkaline hydrolysis of disulfide groups or other reactions are possibly taking place.

Fig. 24 Absorption spectra of α-globulin at pH 11.0 measured as a function of time.

Fig. 24


230 250 270 290 310 330 350
2·0
0·8
1 6
1·4
1 2
1 0
0 8
0 6
0·4
0·2
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
ABSORBANCE (2)
ABSORBANCE (1)
15', 25', 45' & 60'
6'
4'
2'
2
15' 30", 20' 15" & 27' 10"
6"
3' 30"
1
WAVELENGTH (n m)

Optical rotation:

The specific rotation of α-globulin at neutral pH at 578 mμ is - [40]°. This value of specific rotation does not change upto pH 9.5. Above this pH a sharp increase in the levo rotation is observed (Fig. 25) and attains a constant value at pH 12.0 which does not increase further even at pH 12.5 where the value of specific rotation is - [73]°. The transition point occurs at pH 10.8.

Viscosity:

The reduced viscosity, η_{red} of α-globulin is 0.06 dl/gm in 0.1M phosphate buffer of pH 7.5. With increase in pH there is no appreciable change in the value of η_{red} till pH 9.5 (Fig. 26). Above this pH a rapid increase is observed and it reaches a constant value of 0.16 dl/gm at pH 12.5; this value of η_{red} does not increase further. The transition point occurs at pH 10.8.

The results in alkaline solutions indicate that the 11S component dissociates completely to the 7S component at pH 11.5. Above this pH, the 7S component dissociates further to the 2S component. These results would indicate that α-globulin in alkaline solution dissociates according to the following scheme:

$$11S \xrightarrow{pH < 11.5} 7S \xrightarrow{pH > 11.5} 2S$$

Fig. 25 Effect of alkaline pH on the specific rotation of α-globulin at 578 mμ at 28°.

Fig. 25

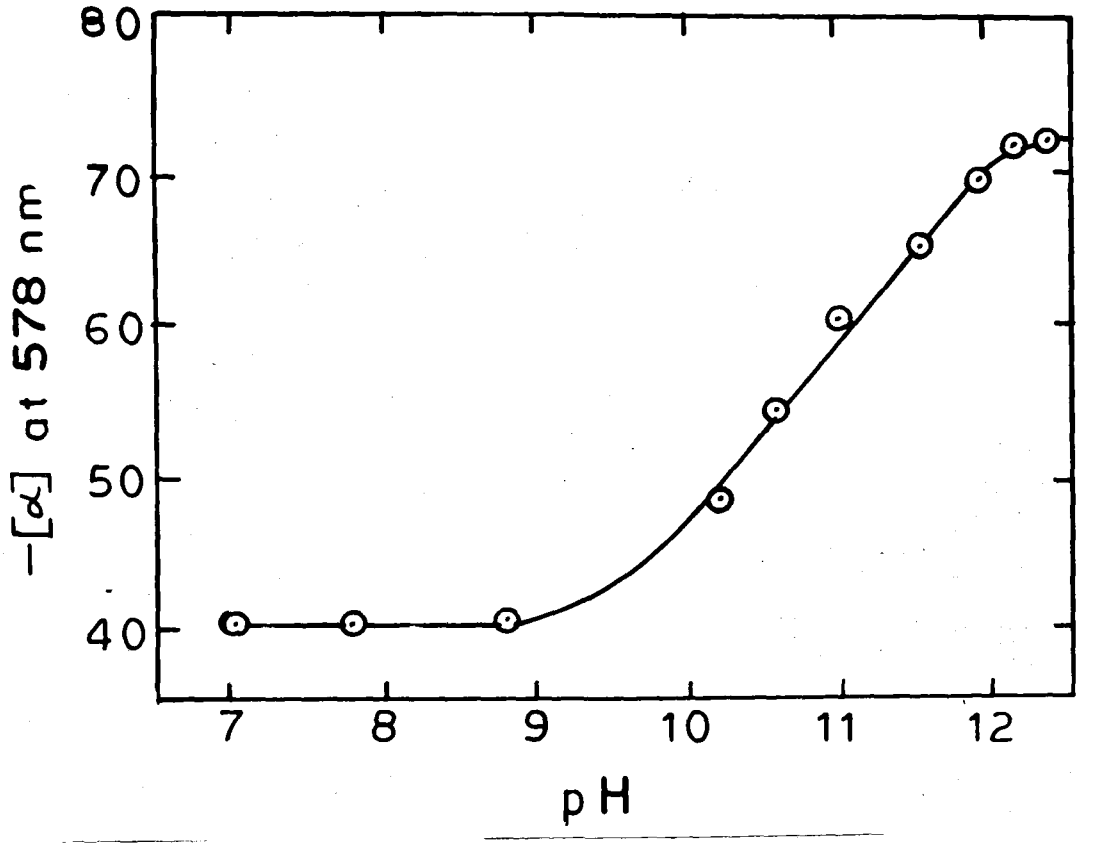
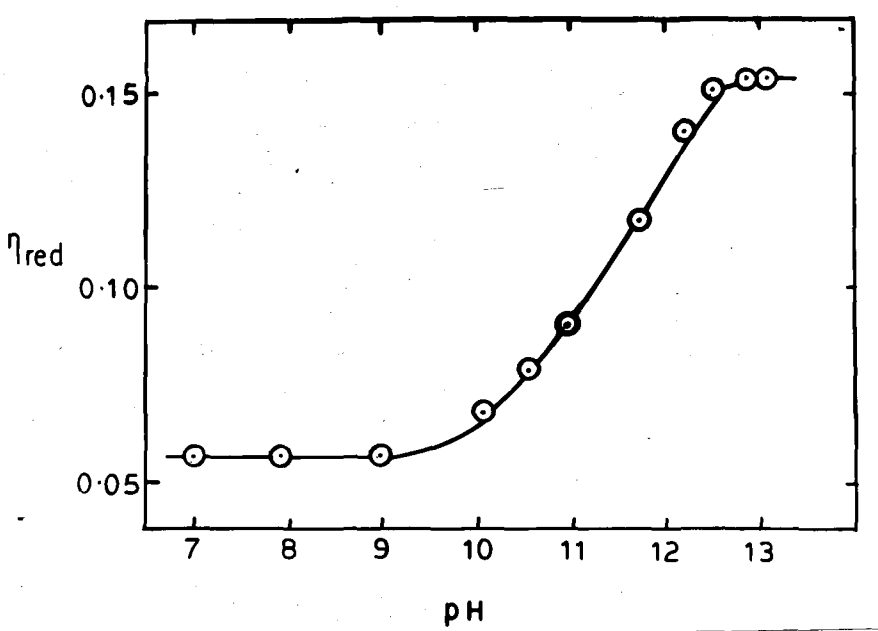


Fig. 26 Effect of alkaline pH on the reduced viscosity of α-globulin at 28°

Fig. 26



The spectral results show that both tyrosyl ionisation and conformational change take place simultaneously. The extent of this denaturation as determined from η_{red} values and specific rotation indicates that even at pH 12.0, the protein is not extensively denatured; these values are lower than the value obtained in 6M GuHCl solution (Table 3). Further the pK_{Int} of tyrosine groups of α-globulin measured in 6M GuHCl is 9.6 (Fig. 22), which is the expected value for the pK_{Int} of tyrosyl groups (Nozaki and Tanford, 1967; Donovan et al., 1969). Also in presence of GuHCl, 13-15 more tyrosyl groups are titrated (Fig. 22) showing that in the absence of the above denaturant not all the titratable groups are available to the solvent even at pH 12.

The amino acid analysis of α-globulin gives a value of 55 tyrosyl groups (see page No. 101). This number is considerably lower than the number of phenolic groups titrated in 0.5M KCl or in 6M GuHCl (Table 4). It has been reported that in ribonuclease A, the addition of methionine sulfoxide and the presence of high amounts of aspartic acid lead to a loss tyrosyl groups (Blackburn, 1968). α-globulin contains relatively high percentage of methionine (~2.6%) and

TABLE 3

VALUES OF REDUCED VISCOSITY (η_{red}, dl/gm) AND SPECIFIC ROTATION $[\alpha]_{578}$ AT pH 12 AND IN 6M CaHCl SOLUTIONS.

	pH 12.0	6M [illegible]
η_{red}	0.16	0.41
$[\alpha]_{578\ nm}$	-73°	-130°

TABLE 4

NUMBER OF PHENOLIC GROUPS TITRATED AND pK_{Int} OF THE PHENOLIC GROUPS OF THE PROTEIN IN 0.5M KCl and 6M GuHCl SOLUTIONS

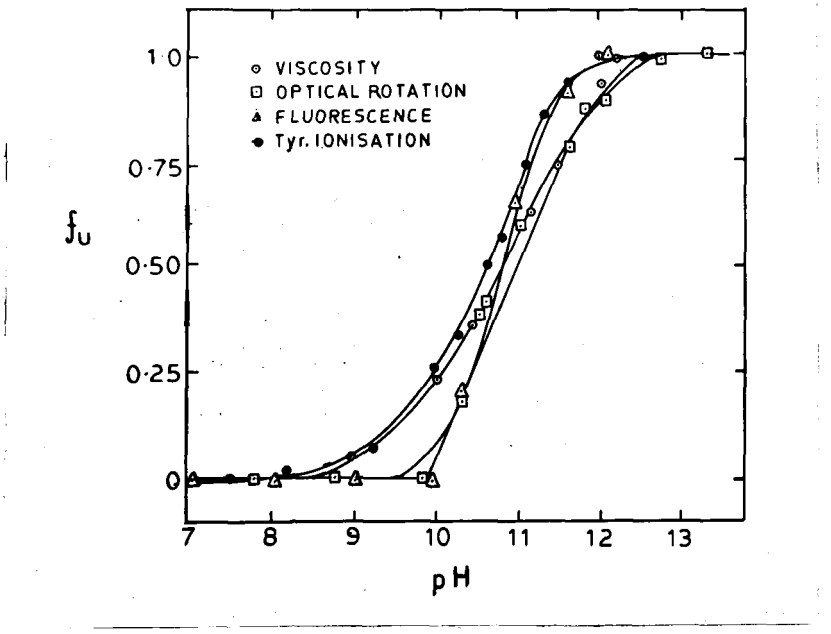
	0.5M KCl	6M GuHCl
No. of phenolic groups titrated at pH $\sim$ 12.	60-62	75
pK_{Int}	10.6	9.6

aspartic acid (9.6%) (see page No.104). This may be the reason for the observed low values of tyrosine. On the other hand the ratio of tyrosine and tryptophan determined by the procedure of Beneze and Schmid (1957) and the number of tryptophan groups determined by NBS method (Spande and Witkop, 1967) and Edelhoch's (1967) method indicated that ~70 tyrosyl groups are probably present in the protein molecule.

The results in alkaline solution have been analysed by plotting f_u as a function of pH (Fig. 27) where f_u is the fraction of the total change of viscosity, optical rotation, fluorescence and tyrosyl ionization of α-globulin at different pH's (Donovan et al, 1969; Wong and Tanford, 1973). The characteristic pH of the transition can be determined from the 50% change i.e. at f_u = 0.5 of the different changes observed in the above properties. The mid point of transition, (i.e. f_u = 0.5) lies in the pH range of 10.6-10.8 obtained for different measurements (Fig. 27). This sharp transition in the above properties indicate that conformational change in the protein involves a single step in alkaline solution (Donovan, 1967).

Fig. 27 Plot of f_u as a function of pH. f_u is the fraction of the total change that has taken place in viscosity, optical rotation, fluorescence and tyrosyl ionisation measurements.

Fig. 27


VISCOSITY
OPTICAL ROTATION
FLUORESCENCE
Tyr. IONISATION
f_U
1·0
0·75
0·50
0·25
0
7
8
9
10
11
12
13
pH

STUDIES IN ELECTROLYTE SOLUTIONS

STUDIES IN ELECTROLYTE SOLUTIONS

The effect of different electrolytes on the association-dissociation phenomena of α-globulin has been studied in 0.05M Tris-HCl buffer pH 9.0 by sedimentation velocity experiments. Mostly the effect of anions has been studied here. The conformational change, if any, has been followed by optical rotation measurement. The experiments were carried out at 25°.

The sedimentation velocity patterns of α-globulin in the presence of 0.025M concentration of sodium sulfate (Na_2SO_4) and 0.25M concentration of sodium chloride (NaCl), sodium bromide (NaBr), sodium iodide (NaI) sodium perchlorate ($NaClO_4$), sodium trichloroacetate (CCl_3COONa) and sodium thiocyanate (NaSCN) are shown in Fig. 28. The percentages of 11S and 7S components with variation of salt concentration are shown in Fig. 29 and 30 respectively. The sedimentation velocity patterns indicate that Na_2SO_4 and NaCl reduce dissociation in α-globulin. In 0.025M Na_2SO_4 nearly 30% of 11S component is present comparedto 60% in buffer (Fig. 28 a and b). Further increase in the concentration of Na_2SO_4 reduces dissociation and at 0.1M nearly 90% of the protein is

Fig. 28 Effect of various anions at 0.25M concentration on the sedimentation velocity pattern of d-globulin in 0.05M Tris-HCl buffer pH 9.0

(a) in buffer alone

(b) Na_2SO_4 (0.025M)

(c) NaCl

(d) NaBr

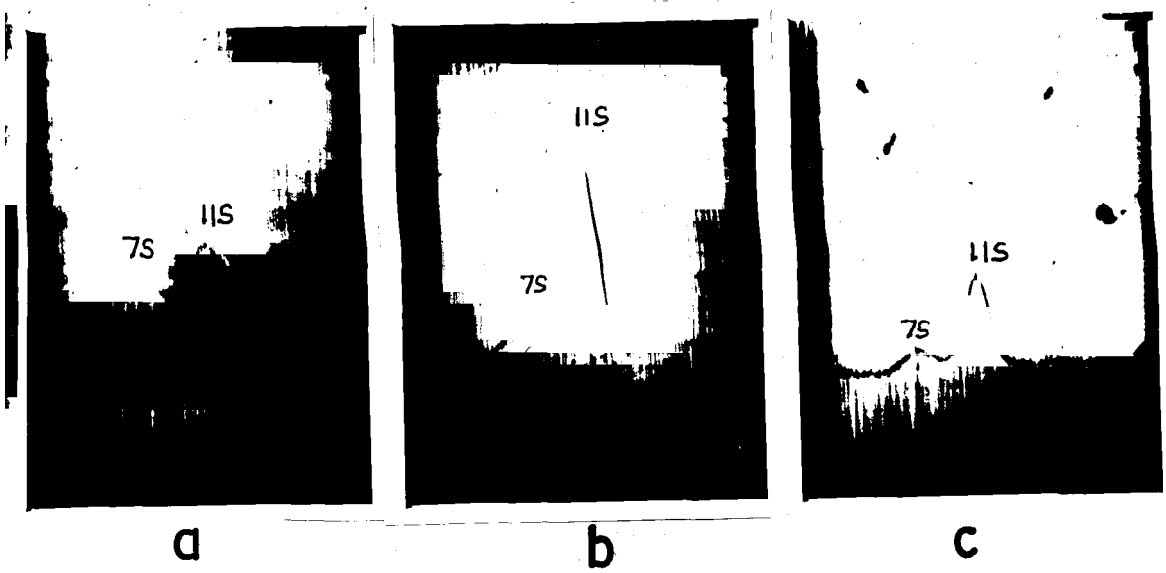
(e) NaI

(f) NaSCN

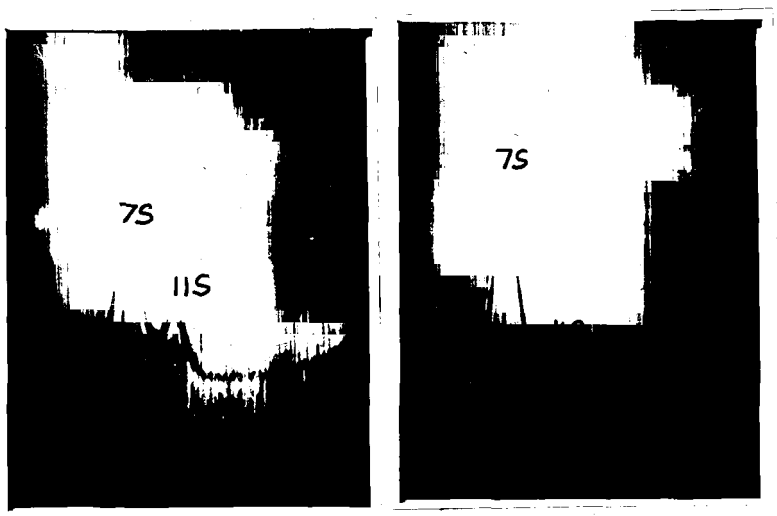
(g) $NaClO_4$ and

(h) CCl_3COONa.

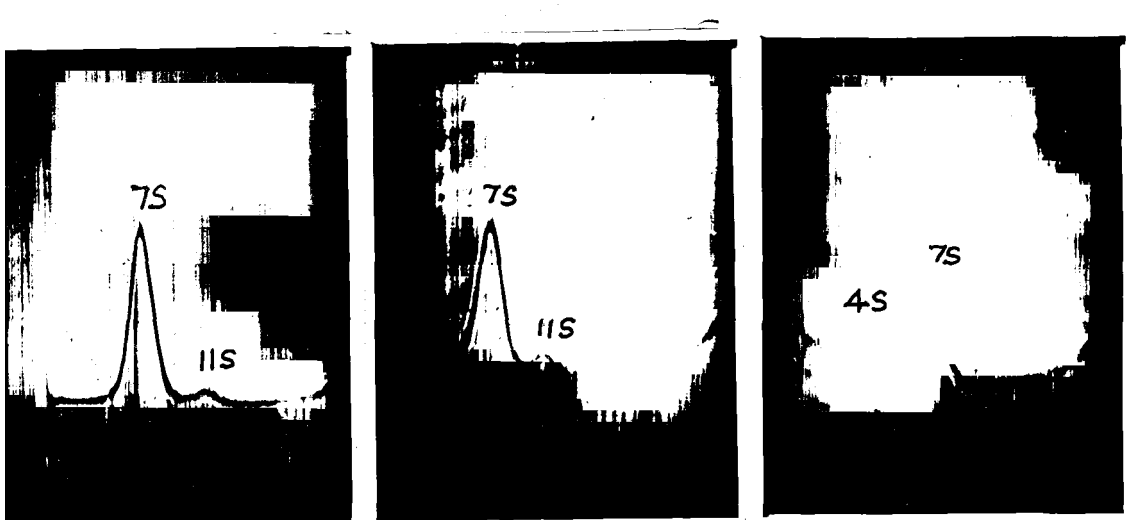
Fig. 28



a b c



d e



f g h

Fig. 29 Variation of percent fraction of 11S component with various concentrations of anions in 0.05M Tris-HCl buffer pH 9.0.

Fig. 29

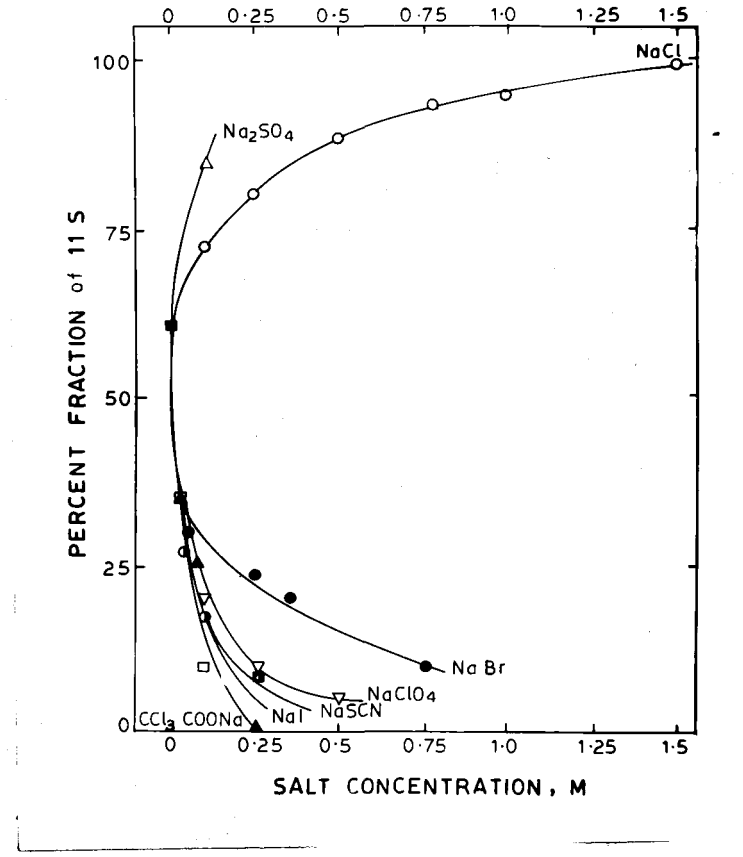
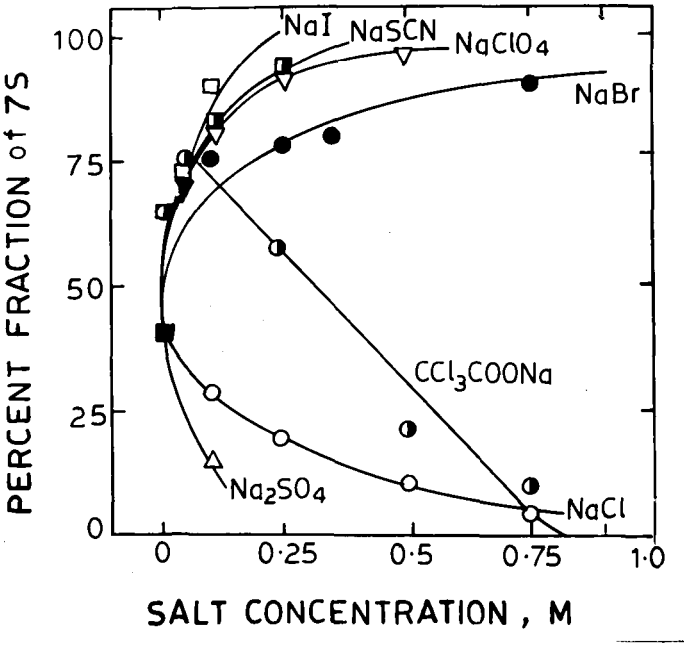

0
0·25
0·5
0·75
1·0
1·25
1·5
100
75
50
25
0
PERCENT FRACTION of 11 S
SALT CONCENTRATION, M
NaCl
Na2SO4
Na Br
NaClO4
NaSCN
NaI
CCl3 COONa

Fig. 30 Variation of percent fraction of 7S component with various concentrations of anions in 0.05M Tris-HCl buffer pH 9.0.

Fig.30


NaI
NaSCN
NaClO4
NaBr
CCl3COONa
Na2SO4
NaCl
100
75
50
25
0
0
0·25
0·5
0·75
1·0
PERCENT FRACTION of 7S
SALT CONCENTRATION , M

in the 11S form (Fig. 31b). Above this concentration of the salt, precipitation of the protein occurred. Similarly in 0.25M NaCl, nearly 80% of the protein is in 11S form (Fig. 28e). Above 1M NaCl, the sedimentation velocity pattern consists entirely of 11S component (Fig. 31c). These results indicate that at the same concentration of the salt, Na_2SO_4 is more effective in reducing the dissociation of α-globulin than NaCl.

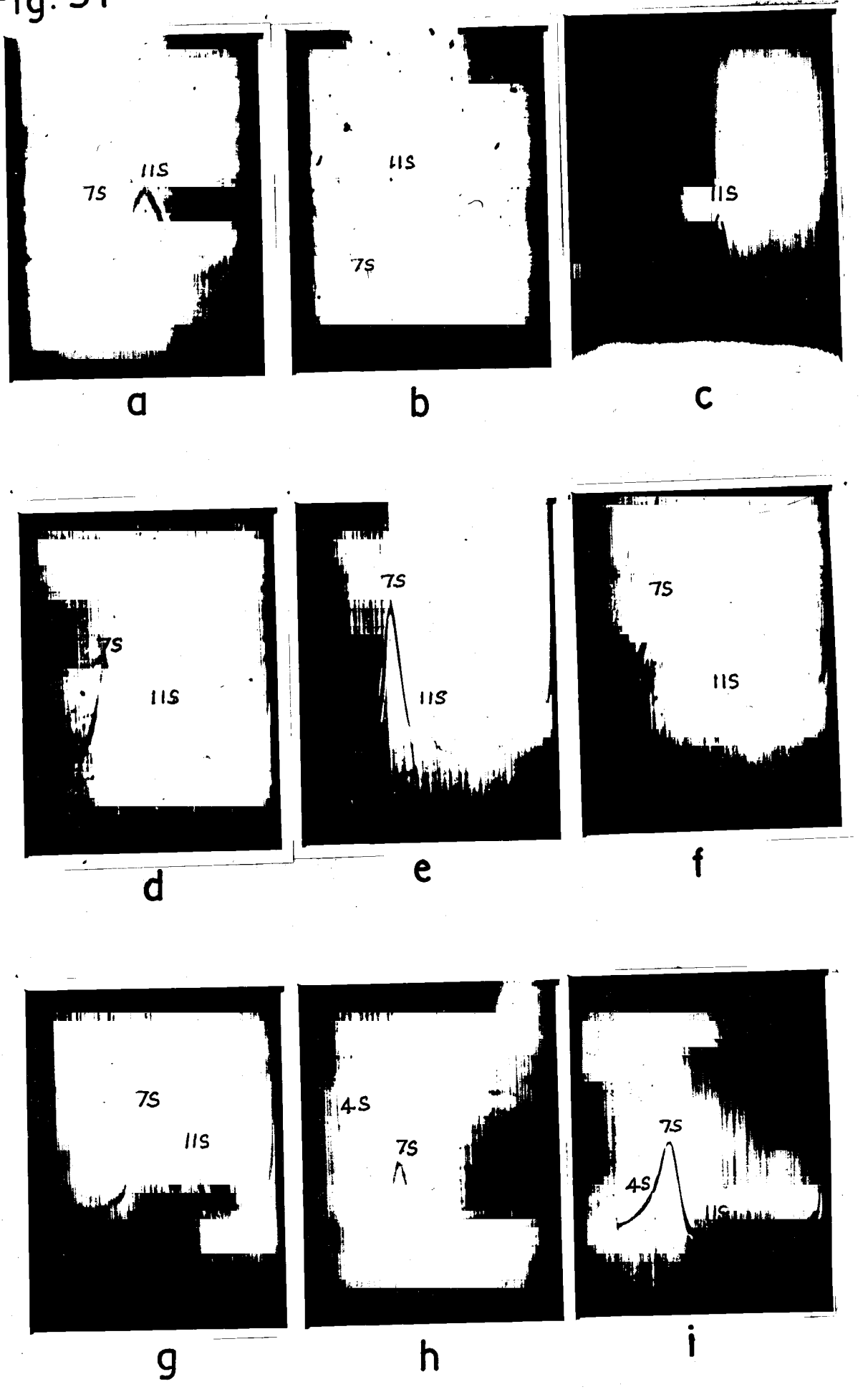
The other salts viz. NaBr, $NaClO_4$, CCl_3COONa and NaSCN enhanced dissociation; the effectiveness depending upon the nature of the anion. In 0.1M NaBr (Fig. 31d), the proportion of the 7S component is nearly twice as that found in buffer alone (Fig. 31a) and at 0.75M, nearly 90% of the protein exists as 7S component (Fig. 31c). NaI is more effective than NaBr in inducing dissociation (Fig. 28 c and d). At 0.25M NaI, the protein consists almost entirely of 7S component (Fig. 28e). $NaClO_4$ was less effective than NaI in dissociating the protein. At 0.5M $NaClO_4$ nearly 95% of the 7S component was present in the system (Fig. 31f).

In CCl_3COONa in addition to the 7S component, 4S component is also observed. At 0.1M salt concentration nearly 75% of the protein is in the 7S form

Fig. 31 Effect of various anions at different concentrations on the sedimentation velocity pattern of α-globulin in 0.05M Tris-HCl buffer pH 9.0

(a) in buffer alone
(b) 0.1M Na_2SO_4
(c) 1M NaCl
(d) 0.1M NaBr
(e) 0.75M NaBr
(f) 0.5M $NaClO_4$
(g) 0.1M CCl_3COONa
(h) 0.75M CCl_3COONa and
(i) 0.05M NaSCN.

Fig. 31


7S
11S
a
11S
7S
b
11S
c
7S
11S
d
7S
11S
e
7S
11S
f
7S
11S
g
4S
7S
h
7S
4S
11S
i

(Fig. 31g) and at 0.75M nearly 85% of the protein is in the 4S form (Fig. 31h). These results indicate that CCl_3COONa was more effective in dissociating α-globulin than either NaBr or NaI or $NaClO_4$. In 0.25M NaSCN solution nearly 95% of the 7S component was present in the system and at 0.5M about 15% of 4S component was observed (Fig. 28f and 31i). These results indicate that NaSCN was less effective in dissociating α-globulin than CCl_3COONa, and the effectiveness in dissociating was similar to that of NaI at the same concentration of salt.

The effectiveness of the anions in dissociating the protein follows the order:

$$SO_4^{--} < Cl^- < Br^- < ClO_4^- < SCN^- \leq I^- < CCl_3COO^-$$

the first two salts in the series reduce the extent of dissociation.

The effect of different cations viz. Li^+, Na^+, K^+ and Cs^+ on the association-dissociation phenomena of α-globulin has been studied at 0.1M salt concentration (Fig. 32). It can be seen from Fig. 32 that the cations are not as effective as anions in altering the association-dissociation equilibria in α-globulin. The examination of the sedimentation velocity pattern in presence of these salts indicate the following

Fig. 32 Effect of various cations at 0.1M concentration on the sedimentation velocity pattern of α-globulin in 0.05M Tris-HCl buffer pH 9.0

(a) in buffer alone

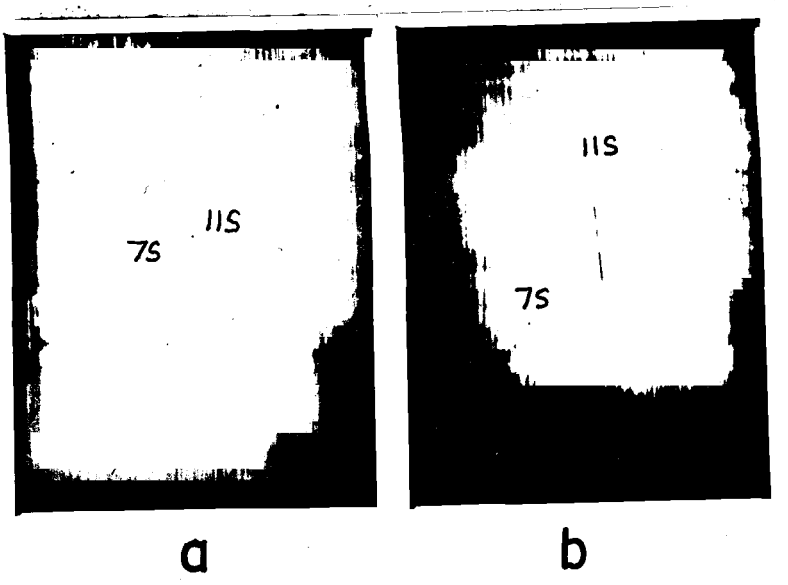
(b) CaCl

(c) KCl

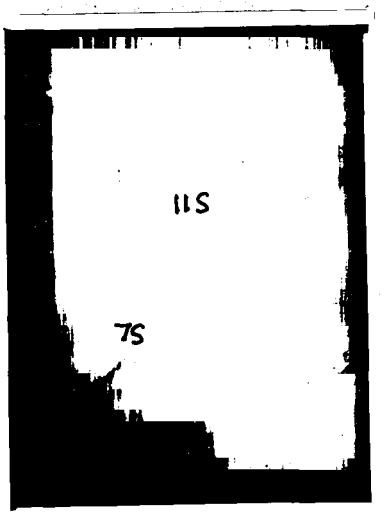
(d) NaCl and

(e) LiCl.

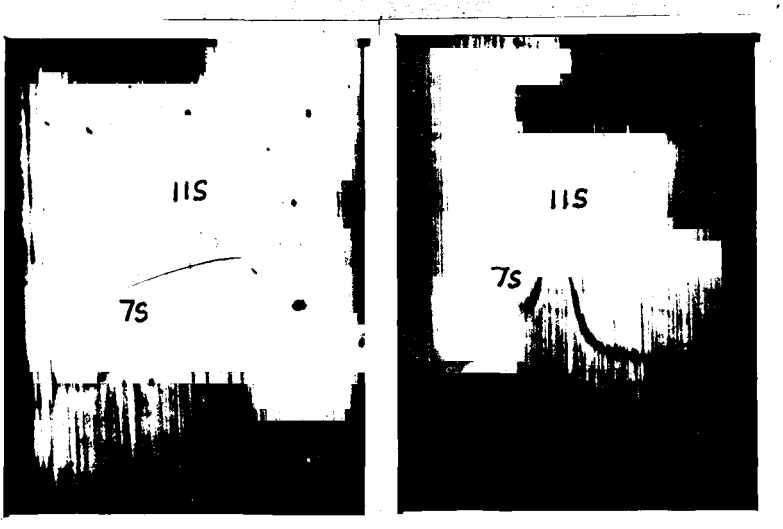
Fig.32



a b



c



d e

effectiveness in inducing association in the protein.

$$Cs^{+} \backsimeq K^{+} \geqslant Na^{+} \geqslant Li^{+}$$

The specific rotation of the protein did not change in the presence of the salts at all the concentrations studied. This indicates that no detectable conformational change in the protein had taken place in the concentration range of the salts studied.

Several mechanisms have been suggested to explain protein denaturation in concentrated salt solutions (Jencks, 1969; von Hippel and Schlieich, 1969). The neutral salts can cause change in the protein molecules (i) by direct electrostatic interaction of the charged groups in the protein molecule (ii) by weakening the ion pairs of salt bridges formed between charged carboxylate and charged lysine and/or arginine side chains (iii) by effecting the nonpolar groups (iv) by interacting with dipolar groups e.g. peptide, amino, carboxyl and hydroxyl groups, primary, secondary and tertiary amides, etc. and (v) indirect effect mediated via change in the solvent structure.

That the direct electrostatic interaction is responsible for the protein denaturation has been discounted from the consideration, that the physical state of these charged groups is nearly the same in both the

native and denatured state. In the present investigation the experimental pH is 9.0, where the existence of salt bridges can be ignored (Thomas and Edelstein, 1973). Matsfi and Hanstein (1969) and Hanstein et al. (1971) have observed that certain salts which denature protein increase the solubility of nonpolar groups. Work from different laboratories has indicated that peptide groups which are exposed from the interior of the protein on denaturation are energetically favourable in electrolyte solution as compared to in water and the stoichiometric interaction of the anion with the amide dipole has been suggested (Robinson and Jenks, 1965). A detailed understanding of water structure is still needed before it can be seriously contemplated to explain the conformational change in the macromolecules.

Recently Sawyer and Puckridge (1973) studied the effect of chaotropic salts i.e. salts whose anions favour the transfer of apolar groups to water, on the dissociation of several proteins. They observed that the salts $NaNO_3$, NaBr, $NaClO_4$, CF_3COONa, NaSCN and CCl_3COONa follow the Hofmeister series in dissociating β-lactoglobulin and hemoglobin whose subunits are associated by hydrogen bonds. On the other hand, in the dissociation of β-casein-A and

concanavalin-A, the subunits of which are hydrophobically associated, the salts did not follow the Hofmeister series. The order of effectiveness of the anions in causing dissociation was as follows: SCN^-, ClO_4^-; $CBr_2COO^- > CCl_2COO^- > CCl_3COO^- > NO_3^- > Cl^- > CF_3COO^-$. In comparing this order with the Hofmeister series, CCl_3COO^- was a notable misplacement in the series. The above conclusions were drawn by Sawyer and Puckridge (1973) from their ultracentrifugal studies. Their results indicated that $NaClO_4$ was more effective in polymerising β-casein A than NaBr; and $NaNO_3$ and CCl_3COONa were less effective than either NaSCN or $NaClO_4$ in dissociating concanavalin A.

In order to determine the nature of the association of the subunits of α-globulin, the effect of temperature (18°-39°) on the association-dissociation phenomena of 7S $\rightarrow$ 11S is carried out by sedimentation velocity experiments in 0.05M Tris-HCl buffer pH 9.0.

The sedimentation velocity patterns at five different temperatures are shown in Fig. 33. From the figure it can be seen that in buffer at 18°, the 7S and 11S components constitute nearly 80% and 20% respectively (Fig. 33a). With increase in temperature, the percentage of the 11S component increases at the

Fig. 33 Effect of temperature on the sedimentation velocity pattern of α-globulin in 0.05M Tris-HCl buffer pH 9.0

(a) 18°

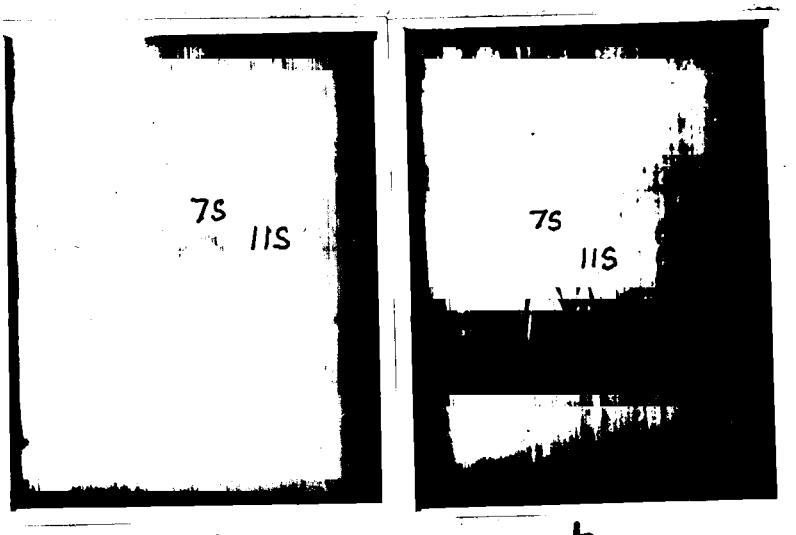
(b) 23°

(c) 27°

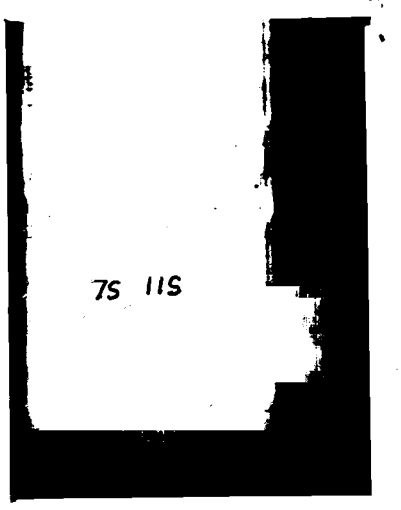
(d) 32° and

(e) 39°

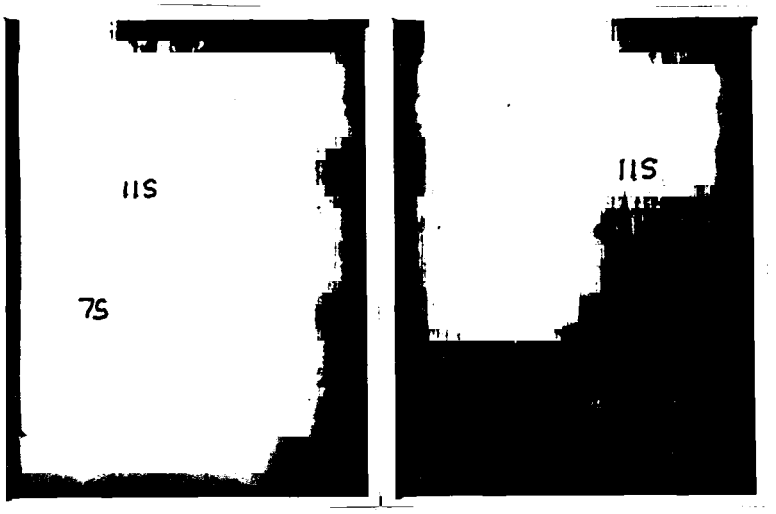
Fig.33



a b



c



d e

cost of the 7S component. At 27° (Fig. 33c) nearly 60% of the protein is in the 11S form, the concentration of which increases 75% and 90% at 32° and 39° respectively (Fig. 33d and e).

To characterise the nature of the association reaction in α-globulin the following procedure was adopted. From the ratio of the concentration of 11S to 7S component the apparent association constant K for the association reaction at three different temperatures were calculated. A plot of log K as a function of reciprocal of absolute temperature (T) is found to be linear as has been represented in Fig. 34. From the slope of this plot, a value of 13 Kcal/mole is obtained for the enthalpy of the association reaction. The free energy

$$\Delta F = - RT \ln K$$

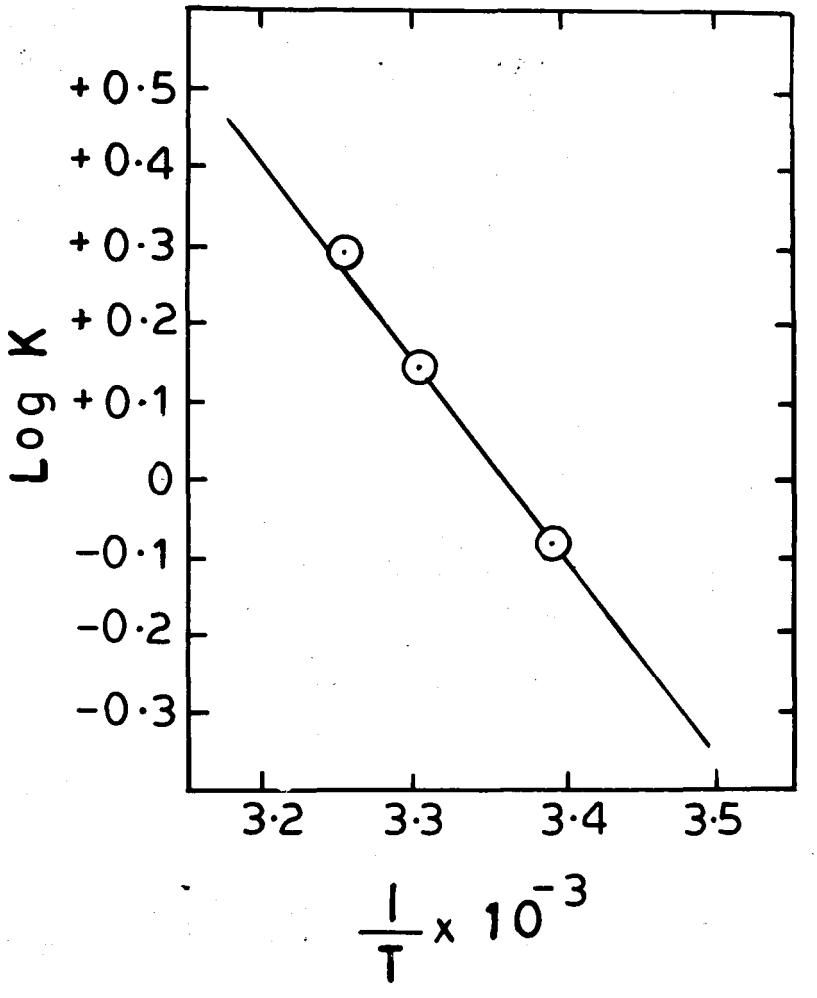
where R is the gas constant, T the absolute temperature and K the apparent association constant, gives a value of -0.19 Kcal/mole at 27°. These results yields a value of ∽ 42 e.u. for the entropy of the association reaction at 27° using the equation

$$\Delta F = \Delta H - T \cdot \Delta S$$

where ΔF is the free energy, ΔH is the enthalpy, ΔS is the entropy of the reaction and T is the

Fig. 34 Plot of logarithm of apparent association constant, K for α-globulin as a function of reciprocal of the absolute temperature.

Fig. 34



absolute temperature. This indicates that the association of the subunits of the protein α-globulin is predominantly governed by hydrophobic interaction (Kauzmann, 1959).

The effect of chaotropic salts on the dissociation of α-globulin does not strictly follow Hofmeister series. The salts that violate the series are $NaClO_4$, NaI and NaSCN. Also the concentration of these salts for inducing dissociation is much lower than that is normally needed for lyotropic effect. However, such low concentration of the salt has been found to effect actin (Nagy and Jencks, 1965), hemoglobin (Guidotti, 1967) and antigen-antibody complexes (Dandliker et al., 1967).

Aune et al. (1971) in their study of dimerisation of chymotrypsin observed that the Hofmeister series is followed in solubility measurements. This is a consequence of the interaction of the solvent with the entire protein surface and the interacting sites between the protein subunits need not be similar to those on the protein surface.

Sawyer and Buckridge (1973) have suggested from their work and from the results available in literature, that the dissociation by chaotropic salts possibly involves either singly or in combination the following mechanisms: (i) preferential binding

of salt to the dissociated species resulting possibly from the availability of extra binding sites; (ii) breakdown of water "structure" by the ions which may enhance the "solubility" of nonpolar areas of the protein and (iii) the salts may directly disrupt the intersubunit hydrogen bonds.

Robinson and Jencks (1965) from their studies on the model peptides have suggested that the chaotropic ions may interact with the amide dipole and have calculated the free energy of interaction between the amide dipole and different salts. Nandi and Robinson (1972a) have observed that formamide is salted in (favourable free energy of interaction as compared to in water) in most of the neutral salts. A possible mechanism suggested by them for the salting in of formamide and the peptide group may be a direct ion amide group interaction to form soluble complexes. Recently von Hippel and Wasmbata (1973) have concluded from extensive gel chromatographic and solubility studies that the binding of the ions is 'non-salt-specific' to the 'ideal' amide dipole and becomes 'salt specific' with the insertion of vicinal methyl groups. Kaussmann (1959) has suggested that hydrophobic interaction increases in salt solution. Similar increase in the hydrophobicity in electrolyte solutions has been observed by Prakash and Nandi (1975) from their study of the

interaction of model peptide esters with Sephadex LH-20 gel. Matsfi and Hanstein (1969) and Hanstein et al. (1971) have explained the increased solubility of nonpolar molecules in chaotropic salt solutions as resulting from the disruption of 'ordered' water structure making it more disordered or lipophilic. Nandi and Robinson (1972a) in their study of the solubility of model, N-acetyl peptide ester, $CH_3CONHCH(R)COOC_2H_5$ (R= side chain), in electrolyte solutions observed that the solubility of the ester did not increase as compared to the unsubstituted derivative. This observation contradicts the second suggestion of Sawyer and Puckridge (1973) i.e. the 'solubility' of nonpolar areas of the protein might increase in electrolyte solutions due to the breakdown of the water structure by the ions. Further, their suggestion that certain anions interact very specifically with the subunit contact areas (specially in the case of hydrophobically associating protein) may not be valid since these areas may not be accessible to the solvent. Similarly the accessibility of solvents to the inter-subunit hydrogen bonds is also not probable.

It has been suggested that a balance between salting in of the polar groups and salting out of the nonpolar groups would determine the stability of one

state or the other of a protein molecule (von Hippel and Schleich, 1969; Nandi, 1974). In α-globin, the decreased dissociation in Na_2SO_4 and NaCl would be predominantly due to the positive unfavourable free energy of interaction of the nonpolar groups with the solvent. The dissociation effect by other electrolytes may be due to a combination of favourable energetics of the chaotropic ions with the amide dipole (see page No. 167) against a low positive unfavourable free energy of interaction of the nonpolar groups with the same ions.

It is obvious from the above discussion that a complete mechanistic description of the effect of salts on the dissociation and denaturation of protein is extremely difficult. According to von Hippel and Hamabata (1973) a detailed attempt of Hofmeister behaviour of the ions can be made only when detailed information regarding the equilibrium structure of water around various groups is available.

STUDIES IN UREA AND GUANIDINE HYDROCHLORIDE SOLUTIONS

STUDIES IN UREA AND GUANIDINE HYDROCHLORIDE SOLUTIONS

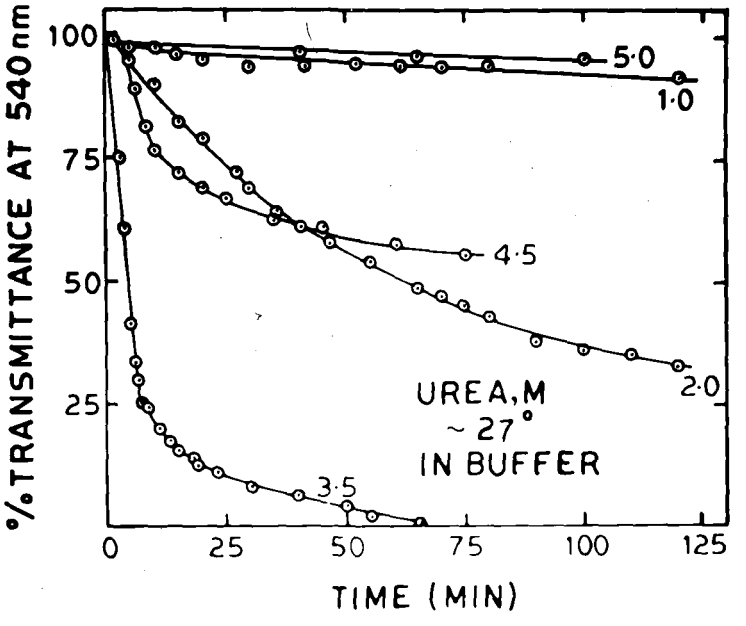
The effect of urea and GuHCl on the dissociation, aggregation and denaturation of α-globulin has been studied in 0.05M TRA-HCl buffer pH 9.0 and the buffer containing 0.5M potassium chloride (KCl) by sedimentation velocity, viscosity, difference spectra and fluorescence spectral measurements. The aggregation of the protein in presence of the above denaturants has been monitored by sedimentation velocity, turbidity and precipitation experiments.

Turbidity:

The addition of low concentration of urea (< 5.0M) and GuHCl (< 2.0M) induces turbidity in the protein solution. In GuHCl solutions instantaneous precipitation of protein prevented any measurements of turbidity. With 0.1% protein concentration precipitation of the protein occurred at 130,80 and 30 min after mixing in 2.0, 2.5 and 3.0M urea at 27°. In 1M urea at 27° the amount of turbidity was very small till a period of 4 hr after mixing (Fig. 35). The increase in urea concentration upto 3.5M induces

Fig. 35 Effect of urea concentration on the rate of appearance of turbidity of α-globulin in 0.05M TEA-HCl buffer pH 9.0.

Fig. 35


% TRANSMITTANCE AT 540 nm
100
75
50
25
0
25
50
75
100
125
TIME (MIN)
5·0
1·0
4·5
2·0
3·5
UREA, M
~ 27°
IN BUFFER

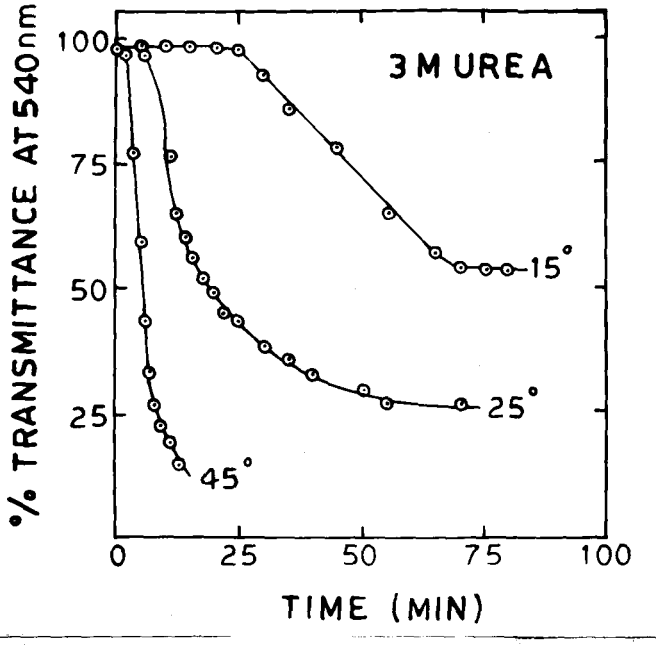
faster appearance of turbidity (Fig. 35). Above 3.5M urea the amount of turbidity decreases and at 5M urea no turbidity is observed (Fig.35). Increase in the protein concentration upto 0.5% causes increased turbidity at 3M urea.

Similar experiments in the presence of buffer containing 0.5M KCl showed that the appearance of turbidity is delayed in the presence of the salt. In 3M urea, with 0.1% protein concentration, turbidity appears after 24 hr in presence of salt as against 20 min in buffer alone. In 1.0M and 1.5M GuHCl solutions, the turbidity appears after 4 hr in presence of salt whereas instantaneous precipitation occurs in low ionic strength buffer.

The effect of temperature on the rate of appearance of turbidity at 3.0M urea and 0.1% protein concentration was studied in low ionic strength buffer. At low temperatures (15°) the appearance of turbidity is slow and there is a lag period before turbidity appears (Fig. 36). At 45° the appearance of turbidity is faster than at either 15° or 25° and within 20 min the percentage transmittance reaches a value of 10% (Fig. 36).

Fig. 36 Effect of temperature on the rate of appearance of turbidity of α-globulin in urea solution at 3M in 0.05M TEA-HCl buffer pH 9.0.

Fig.36


% TRANSMITTANCE AT 540nm
100
75
50
25
0
25
50
75
100
TIME (MIN)
3M UREA
15°
25°
45°

Precipitation:

The amounts of precipitate obtained at different concentrations of denaturants was determined in low ionic strength buffer by equilibrating the protein solution with the denaturants for 24 hr (see page No. 64). The amount of precipitate obtained with different concentration of urea is shown in Table 5. Maximum precipitation (∽ 30%) is observed at ∽ 3M urea. In GuHCl solution maximum precipitation (∽ 70%) is observed at ∽ 0.025M. Above these concentrations of the reagents the amount of precipitate decreased and above 4.5M urea no precipitate is observed.

Precipitation results of the protein in buffer containing 0.5M KCl indicates turbidity at < 0.5M urea and < 1.0M GuHCl solutions and no phase separation occurs even after equilibration at 30° for 24 hr in these solutions.

Sedimentation velocity:

Sedimentation velocity measurements were carried out both in the presence of urea and GuHCl in the range 0.5-8M and 1.5-6M respectively. In 0.05M TRA-HCl buffer pH 9.0 the protein exists as a mixture of 11S (40%) and 7S (60%) components (Fig. 37a). Upon addition of urea from 0.5-8M, five components sedimenting with 2, 4, 7, 11 and ∽ 12OS are observed (Fig. 37). A plot of percent fraction of all the five

TABLE 5

PERCENTAGE PRECIPITATION OF α-GLOBULIN AT DIFFERENT CONCENTRATIONS OF UREA

Urea, M	Absorption at 280 nm of supernatant of protein solution, after equilibration with varying concentrations of urea.	% Protein precipitated
-	1.00	0
1.0	1.00	0
1.5	0.83	17
2.0	0.76	24
2.5	0.54	46
3.0	0.59	41
3.5	0.68	32
4.0	0.88	12
4.5	1.00	0

Fig. 37 Effect of increasing concentrations of urea on the sedimentation velocity pattern of α-globulin in 0.05M TRA-HCl buffer pH 9.0

(a) in buffer alone

(b) 0.5M

(c) 1.0M

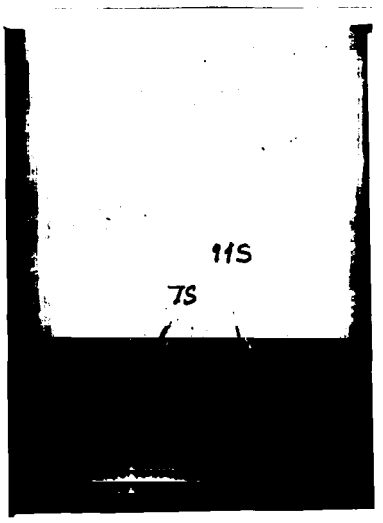
(d) 2M (17,280 rpm), ∽ 120S

(e) 1.5M

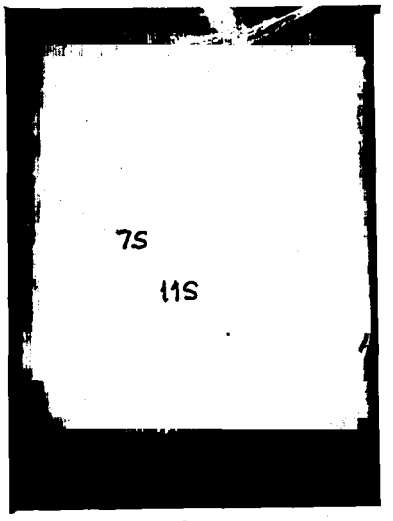
(f) 2.8M and

(g) 4.0M urea solution.

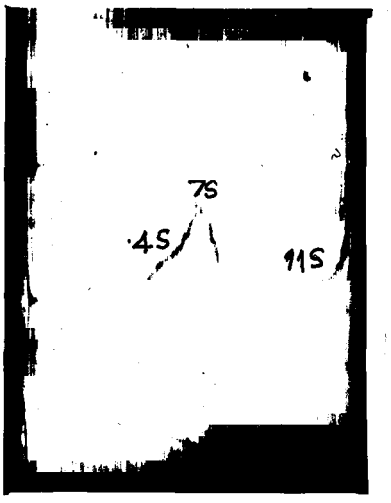
Fig. 37



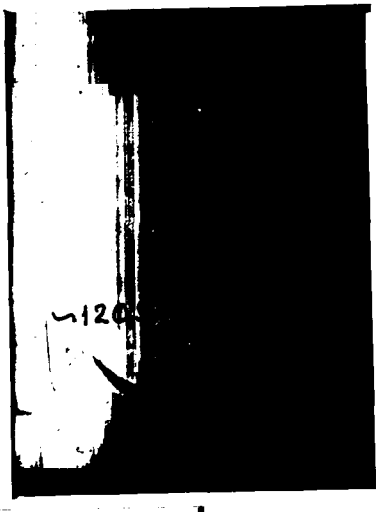
a



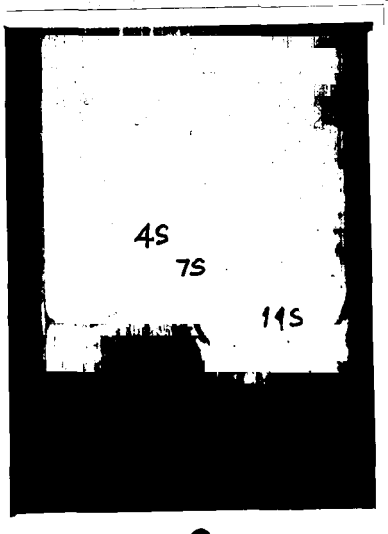
b



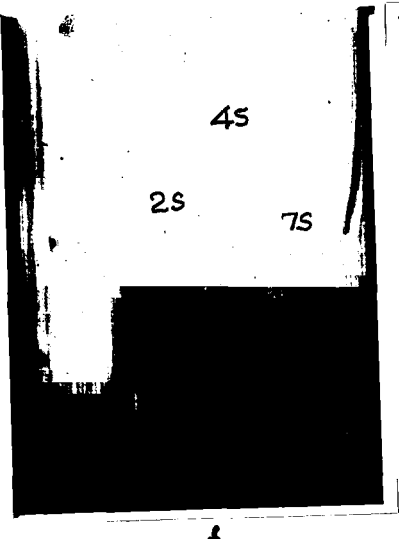
c



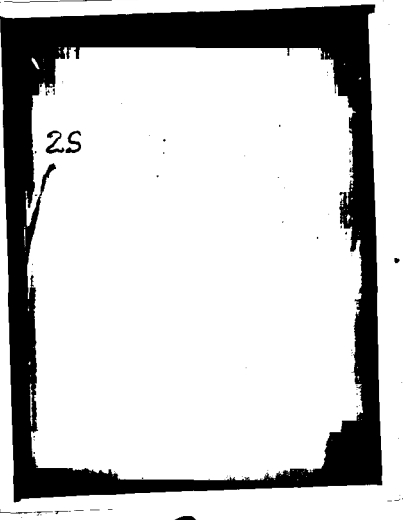
d



e



f



g

components against urea concentration is shown in Fig. 38. Below 1M urea, the concentration of 7S increases at the cost of 11S. The new 7S component may or may not be the same 7S component originally present. This possibly, new 7S component obtained due to the action of urea, is also designated as 7S. Around 1M urea concentration, two new components are observed, one, a very fast moving component (or a mixture of components) having ∽ 120S value and another sedimenting with 4S value (Fig. 37 c, d and Fig. 38). Around 2M urea, the concentration of 4S component was found to be maximum and ∽ 2.8M urea, the concentration of ∽120S component was found to be maximum (Fig. 38). With further increase in urea concentration, above 3M, the proportion of both the components i.e. 4S and ∽120S decreases with a concomittant increase in the proportion of the 2S component. Above 5M urea, the solution consists solely of the 2S component. These results suggest that α-globulin in urea solution undergoes aggregation and dissociation reactions. The soluble polymer (∽ 120S) is probably the precursor of the insoluble aggregate as could be seen from Fig. 39 where the amount of ∽120S component has been compared and found to be directly proportional to the amount of insoluble aggregate with varying urea concentration.

Fig. 38 Variation of percent fraction of 2^S, 4S, 7S, 11S and ~12S components with increase in urea concentration in 0.05M TEA-HCl buffer pH 9.0

Fig. 38

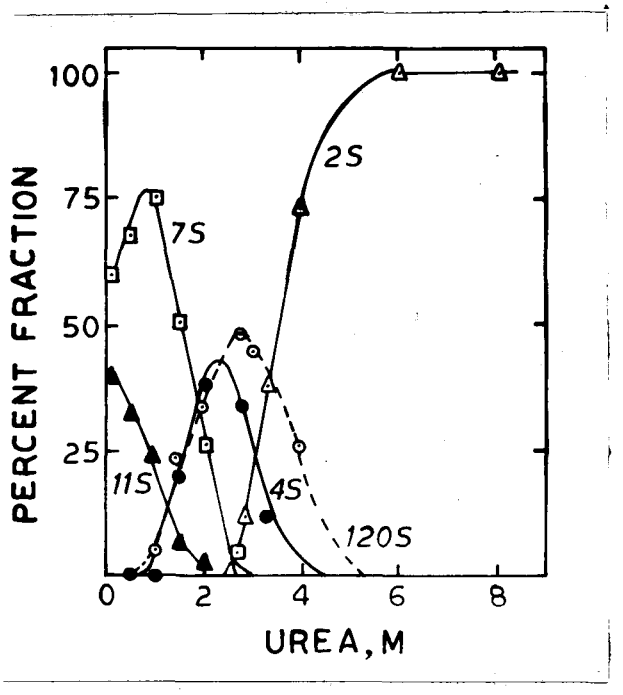
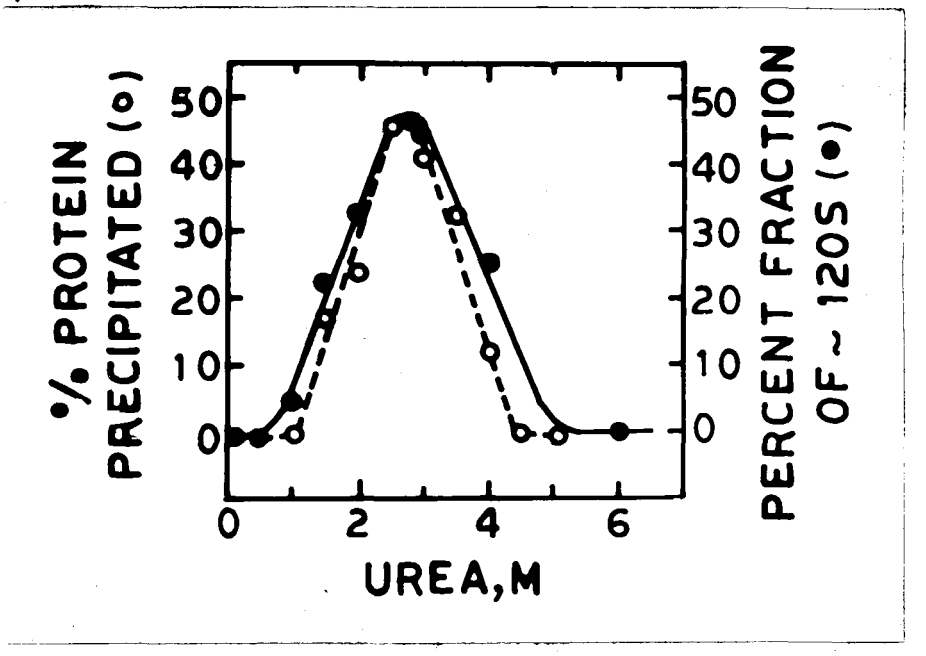


Fig. 39 Effect of increasing concentrations of urea on the percentage protein precipitated and ~120S component remaining in solution in 0.05M TCA-HCl buffer of 9.0.

Fig. 39


% PROTEIN PRECIPITATED (o)
PERCENT FRACTION OF ~ 120S (•)
50
40
30
20
10
0
0
2
4
6
UREA, M

Sedimentation velocity experiments could not be carried out in the presence of low concentrations of GuHCl, due to the precipitation of protein. The precipitation at these low reagent concentrations probably results from neutralisation of charges on the protein molecule due to the preferential binding of GuH^+ ions to it. Experiments could be carried out at $\geqslant$ 0.5M GuHCl as the amount of protein precipitated at these concentrations is less. At 0.5M GuHCl the protein consists of both 7S and 11S components in the proportion of $\backsim$95% and $\backsim$5% respectively and at 0.75M, $\backsim$95% of the protein is present as 4S component (Fig. 40b and c). Above 2M concentration of the denaturant the protein contained only the 2S component (Fig. 40d and e).

Similar experiments in the presence of buffer containing 0.5M KCl were carried out both in urea and GuHCl solutions. At 2M urea, in the presence of salt the percentage of 4S component was very little ($\backsim$ 5%) whereas in buffer alone nearly 30% of the 4S component was present in the solution (Fig.41b). Similarly at 0.5M GuHCl nearly 15% of the 11S component is dissociated to the 7S component (Fig. 41c). These results indicate that the extent of dissociation of the protein by urea and GuHCl is less in presence of 0.5M KCl.

Fig. 40 Effect of increasing concentrations of GuHCl on the sedimentation velocity pattern of α-globulin in 0.05M TEA-HCl buffer pH 9.0

(a) in buffer alone

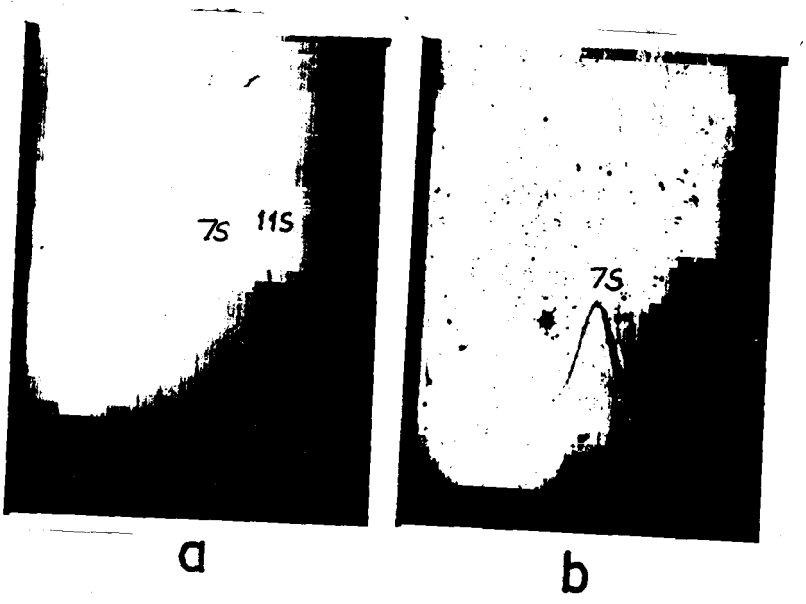
(b) 0.5M

(c) 0.75M

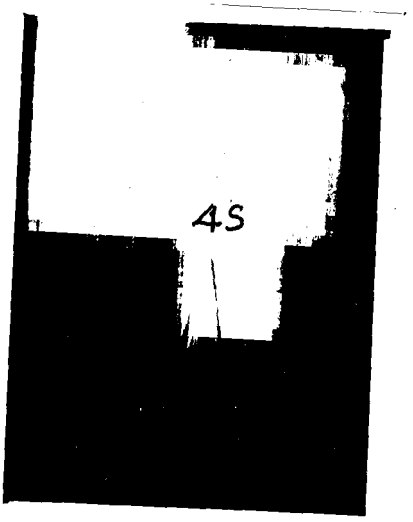
(d) 2M and

(e) upper, 4M and lower, 6M GuHCl.

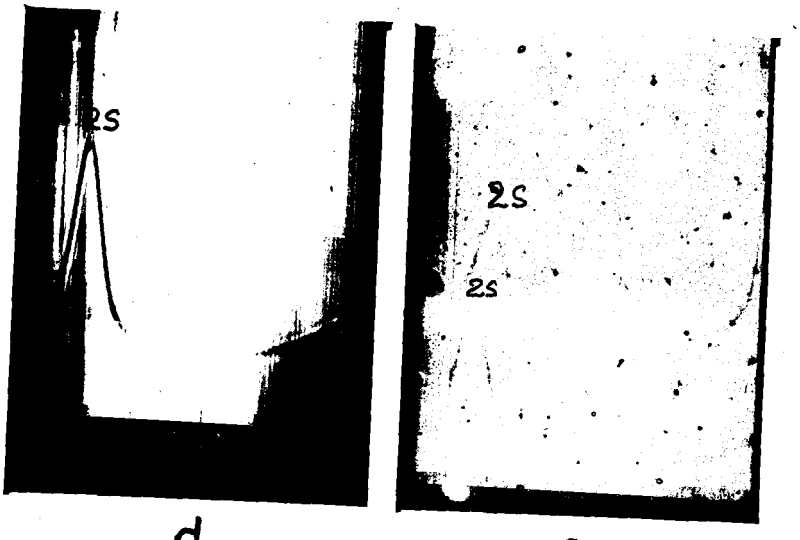
Fig.40



a b

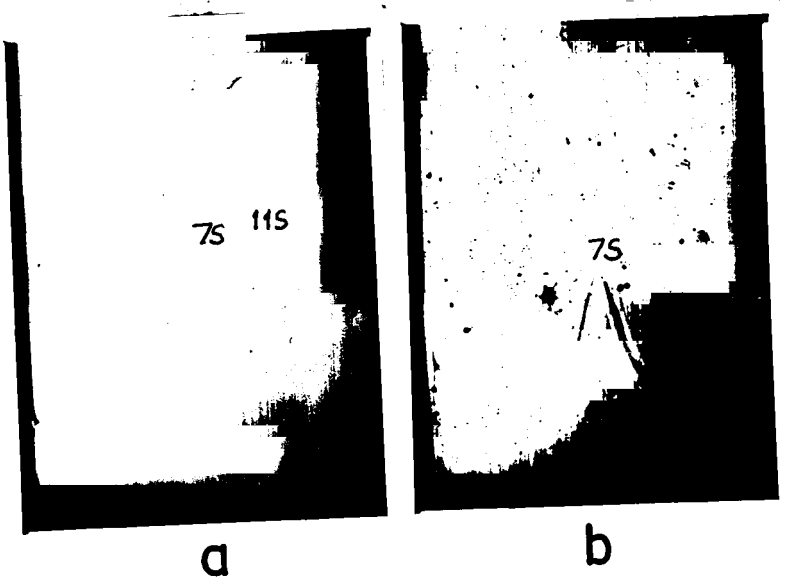


c

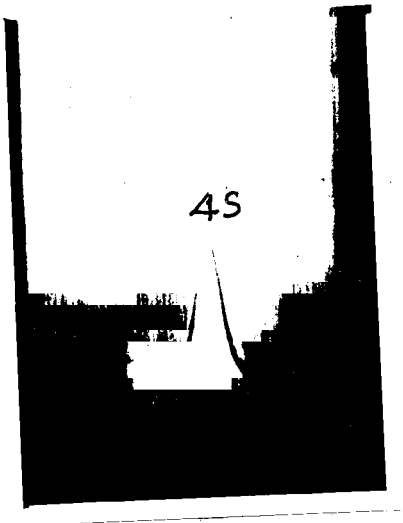


d e

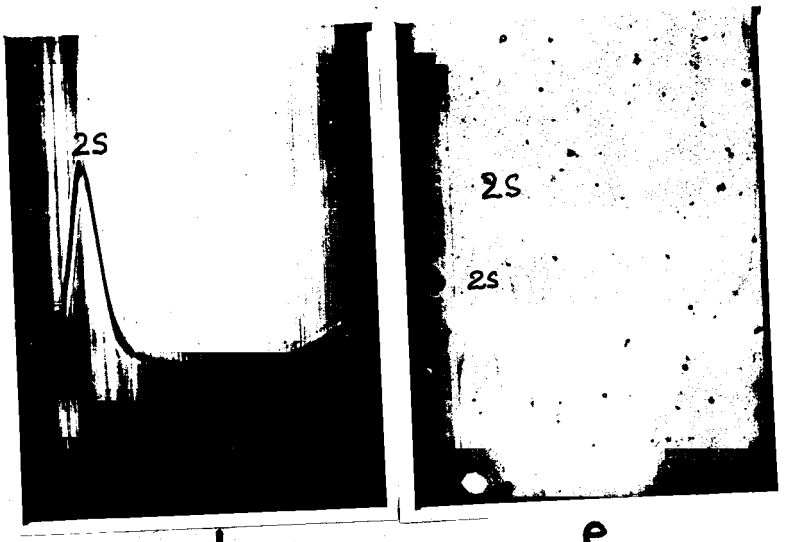
Fig. 40



a b



c



d e

Fig. 41 Effect of various concentrations of urea and GuHCl on the sedimentation velocity pattern of α-globulin in 0.5M KCl-TRA-HCl, pH 9.0 buffer (0.05M) system.

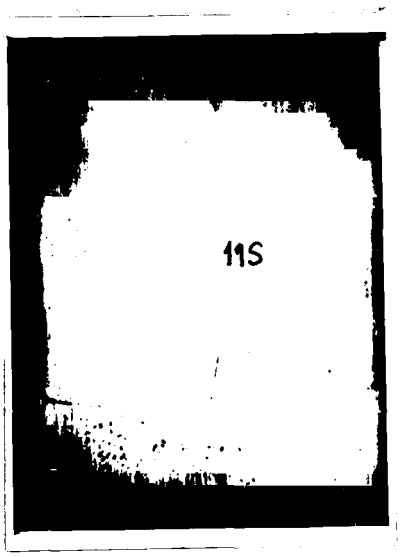
(a) in buffer alone

(b) 2.0M urea and

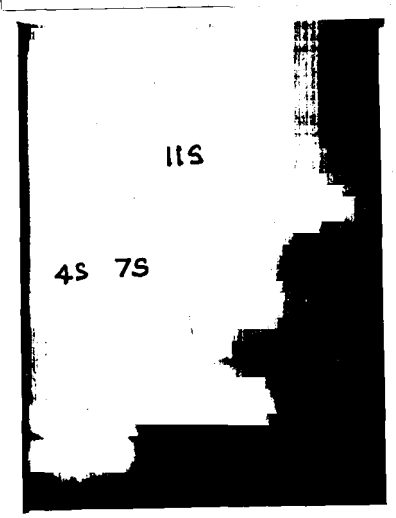
(c) 0.5M GuHCl.

Fig. 41

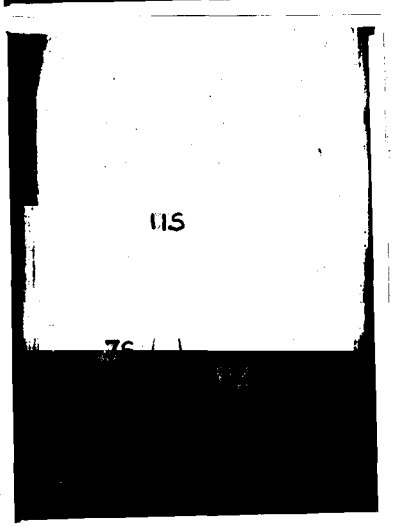
a



b



c



Polyacrylamide gel electrophoresis:

Polyacrylamide gel electrophoresis of α-globulin in presence of urea could not be carried out at pH 9.0, as the resolution of bands were not sharp. However at pH 7.5 in 0.02M phosphate buffer, electrophoresis of α-globulin in presence of 8M urea indicated five well resolved components (Fig. 42). This result also indicates that α-globulin dissociates in urea solution.

Viscosity:

Addition of urea upto 1M to 1% α-globulin solution causes opacity but no phase separation is observed on standing for 36 hr although at protein concentrations of 0.1% the protein precipitates (see page No.185). The values of η_{red} determined with 1% protein concentration at different concentration of urea (0-10M) is shown in Fig. 43. The value of η_{red} increases two-fold in 2M urea solution as compared to in buffer. The measurements taken at different intervals of times after mixing upto 36 hr did not show any change in the value compared to the initial value.

Further to know if any adsorption of protein to the viscometer occurs which may in turn effect the flow time, the following experiment was carried out. The viscosity of the protein solution in 1.5M urea was determined 4 hr after mixing the reagent and the protein

Fig. 42 Effect of urea on the polyacrylamide gel electrophoretic pattern of α-globulin in 0.02M phosphate buffer pH 7.5

(a) in buffer alone and

(b) in 8M urea.

Fig. 42

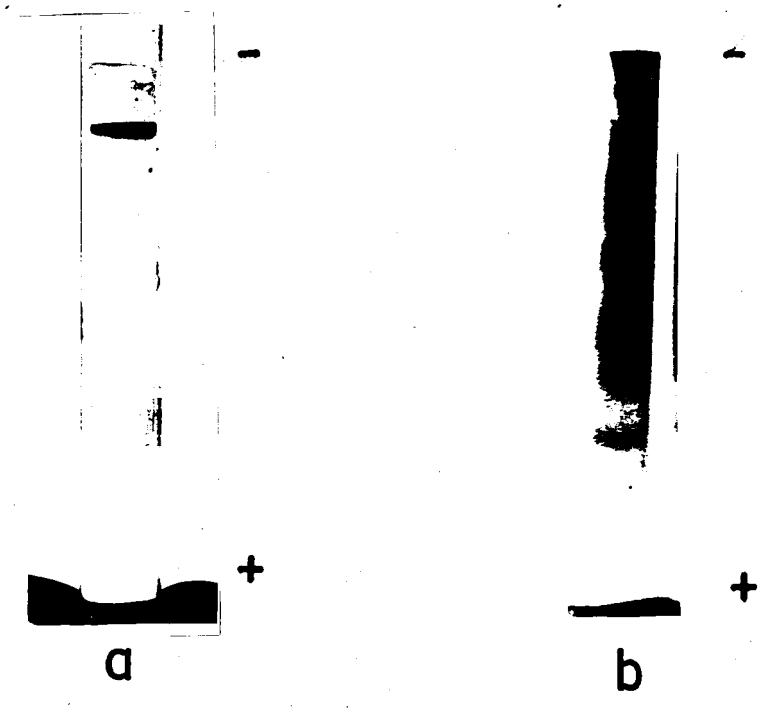
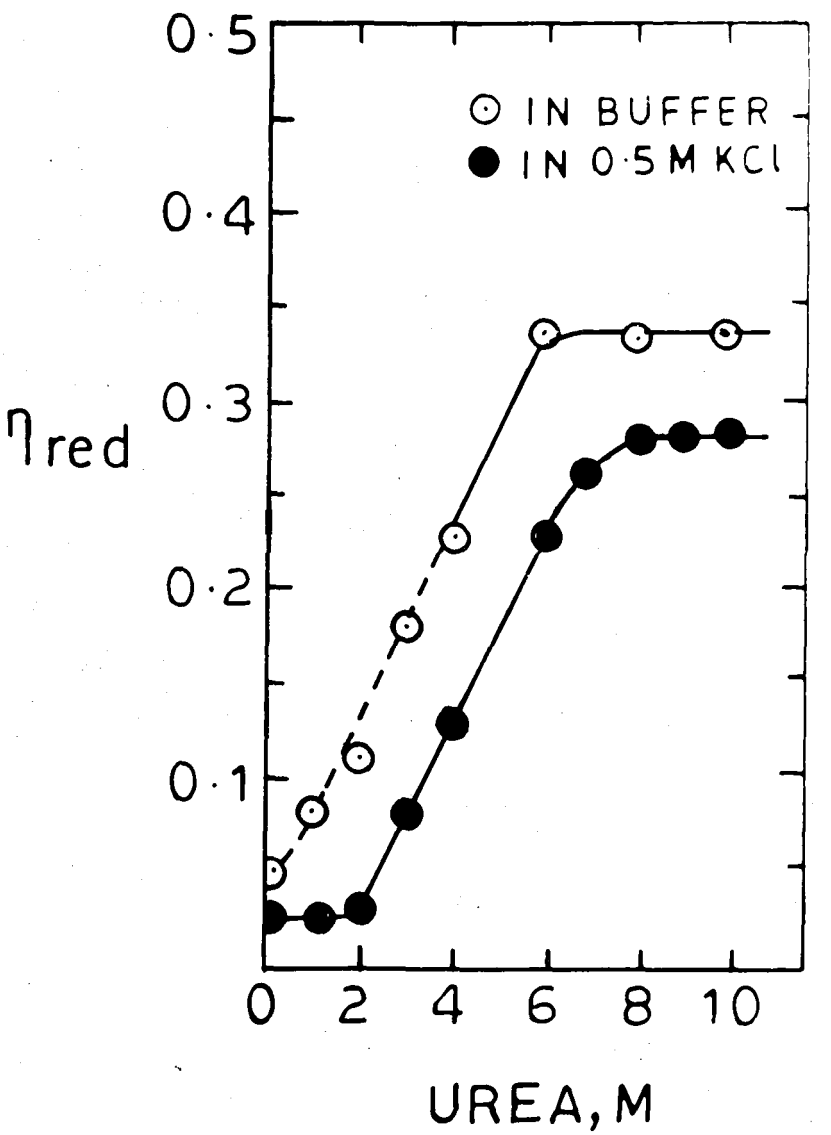


Fig. 43 Effect of increasing concentrations of urea on the reduced viscosity of α-globulin in 0.05M TEA-HCl buffer pH 9.0, and the buffer containing 0.5M KCl.

Fig. 43



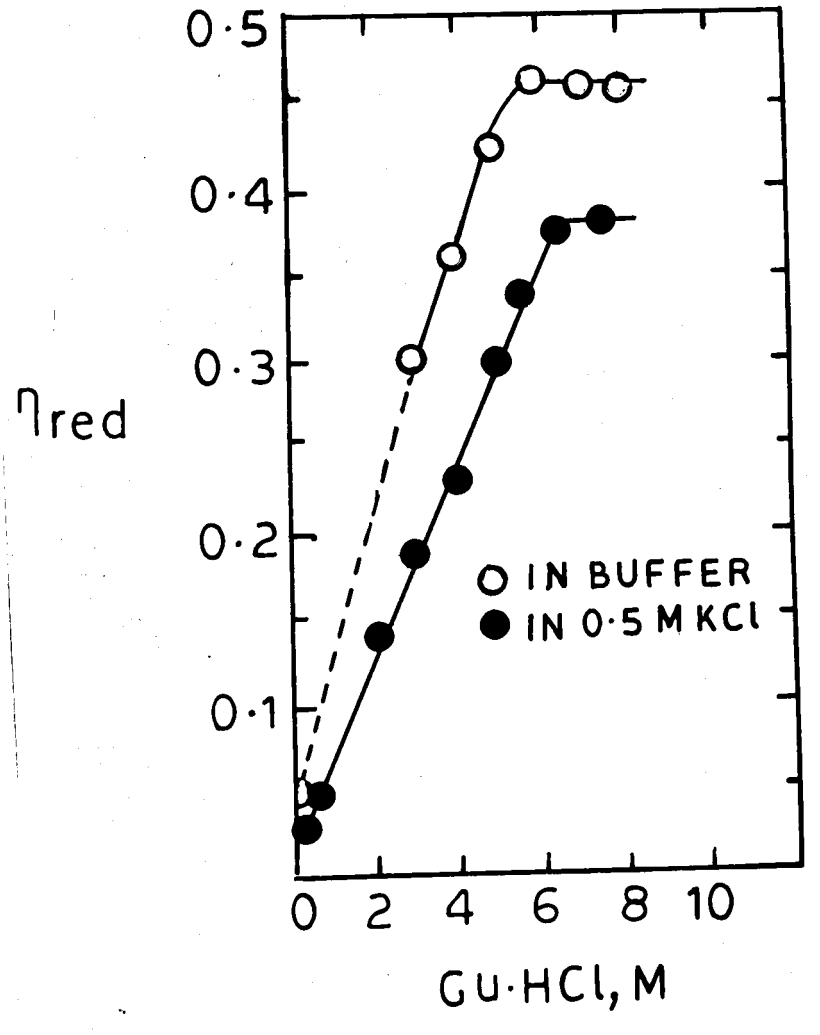
solution. The viscosity of the same solution kept at ∽25° for nearly 30 hr when measured in a cleansed and dried viscometer showed the same viscosity as that of the solution kept for ½ hr. This indicates that the observed result is not an artifact. Mihyali (1950) while studying the effect of urea on fibrinogen obtained an opaque solution the viscosity of which increased compared to the control protein. At higher urea concentration (∽5M) α-globulin solution is clear.

The value of η_{red} attains a value of 0.30 dl/gm in 6M urea which does not change with higher urea concentrations (Fig. 43). The protein in urea solution in 0.5M KCl does not show any opacity within 24 hr. The viscosity results in these solutions show that the mid-point of transition is shifted to 4.5M urea as compared to 3.5M in the absence of KCl. The value of η_{red} is also less (0.28 dl/gm) in KCl solution. The above results indicate that α-globulin in urea solutions is in a denatured state (Tanford, 1968) and the extent of denaturation (η_{red} = 0.28 dl/gm) is less in KCl solution.

Viscosity in GuHCl could not be measured in buffer below 2M concentration due to precipitation of protein. In 2M GuHCl, the value is considerably higher than that of the native protein (Fig.44). Viscosity increases further with increase in GuHCl concentration

Fig. 44 Effect of increasing concentrations of CuCl2 on the reduced viscosity of α-globulin in 0.05M TRA-HCl buffer pH 9.0, and the buffer containing 0.5M KCl.

Fig. 44



upto 6M, above which the value remains constant at 0.46 dl/gm (Fig. 44). In presence of 0.5M KCl the mid-point of transition shifts to a higher value by 1M. The extent of denaturation (η_{red}= 0.38 dl/gm) is also less in KCl solution. These observations are similar to those made in urea solution, which indicate that the protein is resistant to denaturation in urea and GuHCl solutions in the presence of 0.5M KCl. Viscosity data show that α-globulin is in a more denatured state in GuHCl solution than in urea solution, a phenomenon which is common for most of the proteins (Tanford, 1968).

Difference spectra:

The difference spectrum of the protein in buffer was measured above 5M urea and above 2M GuHCl concentrations to avoid the opacity of the solution at low concentrations of the reagents. In buffer containing 0.5M KCl, the difference spectral measurements were made from 2-10M urea and 1-8M GuHCl concentrations. The difference spectra at various concentrations of urea and GuHCl in buffer and buffer containing 0.5M KCl are shown in Fig. 45 and 46 respectively. The changes in the extinction values, $\Delta\varepsilon$ at various concentrations of these reagents are shown in Fig. 47. The difference spectra are characterised by strong

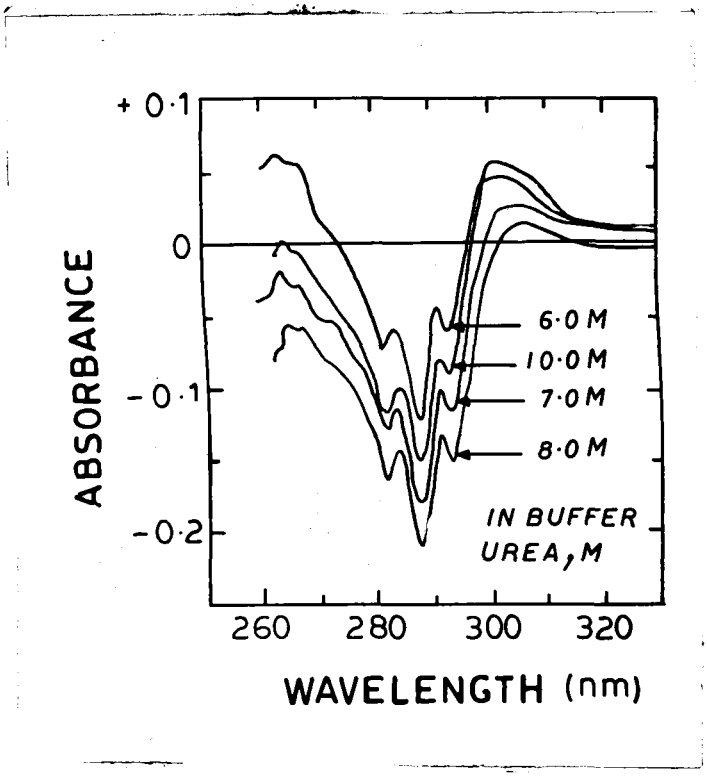
Fig. 45 Effect of increasing concentrations of urea on the ultraviolet difference spectra of α-globulin in

(a) 0.05M TEA-HCl buffer, pH 9.0 and

(b) the buffer containing 0.5M NCl.

Fig. 45

a


+0·1
0
-0·1
-0·2
ABSORBANCE
6·0 M
10·0 M
7·0 M
8·0 M
IN BUFFER
UREA, M
260
280
300
320
WAVELENGTH (nm)

b

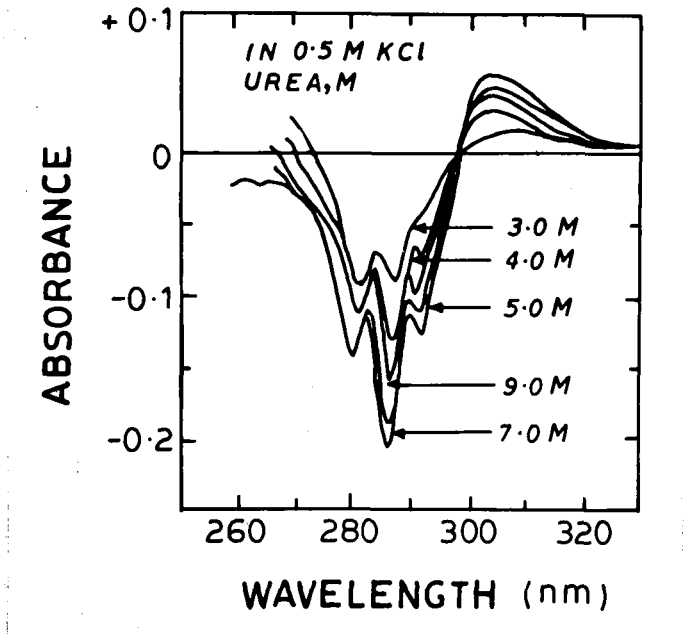

+0·1
IN 0·5 M KCl
UREA, M
0
-0·1
-0·2
ABSORBANCE
3·0 M
4·0 M
5·0 M
9·0 M
7·0 M
260
280
300
320
WAVELENGTH (nm)

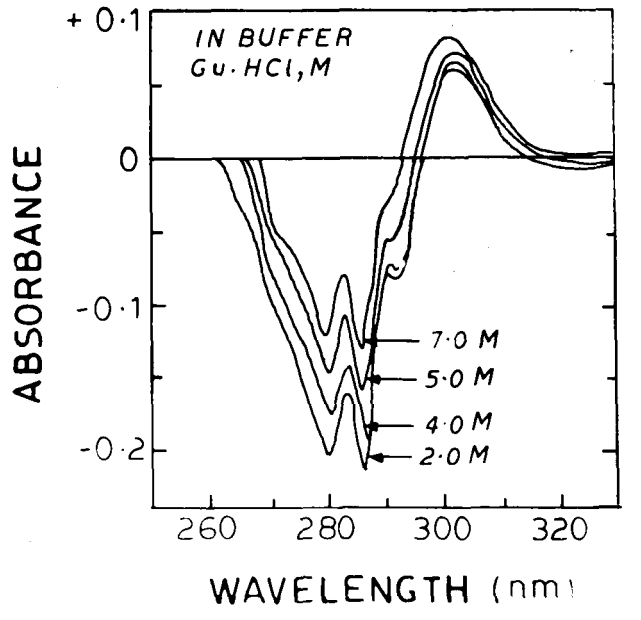
Fig. 46 Effect of increasing concentrations of GuHCl on the ultraviolet difference spectra of α-globulin in

(a) 0.05M TEA-HCl buffer pH 9.0 and

(b) the buffer containing 0.5M KCl.

Fig.46

a


+ 0·1
0
-0·1
-0·2
IN BUFFER
Gu·HCl,M
7·0 M
5·0 M
4·0 M
2·0 M
ABSORBANCE
260
280
300
320
WAVELENGTH (nm)

b

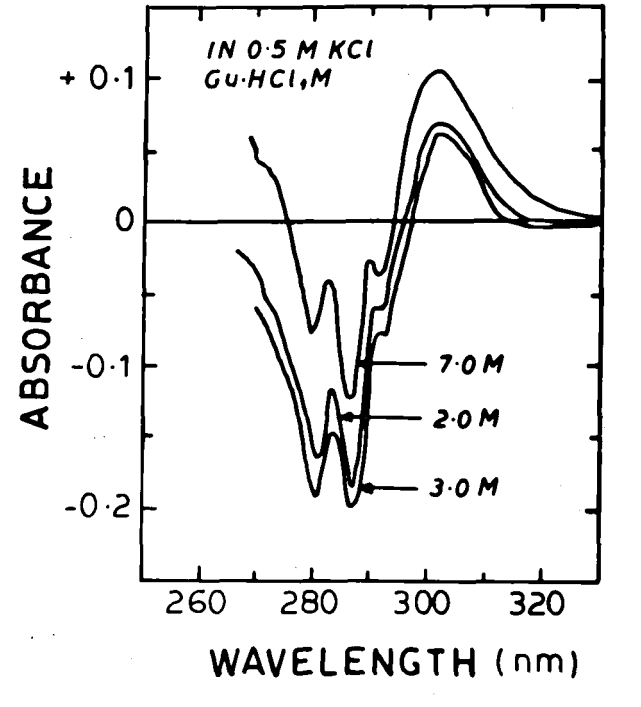

+ 0·1
0
-0·1
-0·2
IN 0·5 M KCl
Gu·HCl,M
7·0 M
2·0 M
3·0 M
ABSORBANCE
260
280
300
320
WAVELENGTH (nm)

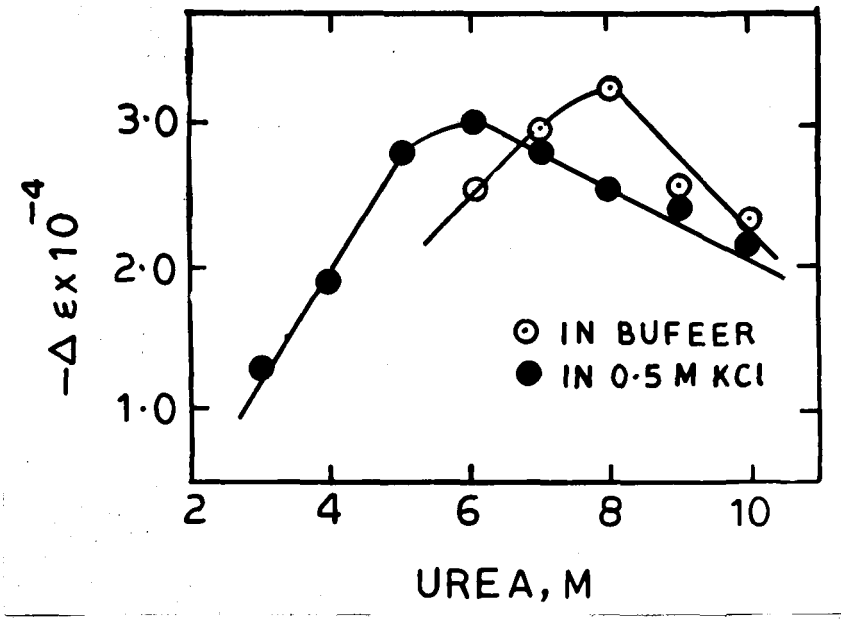
Fig. 47 Plot of change in the molar extinction coefficient value, $\Delta\epsilon$ at 287 nm, in 0.05M TEA-HCl buffer pH 9.0 and in the buffer containing 0.5M KCl as a function of increasing concentrations of

(a) urea and

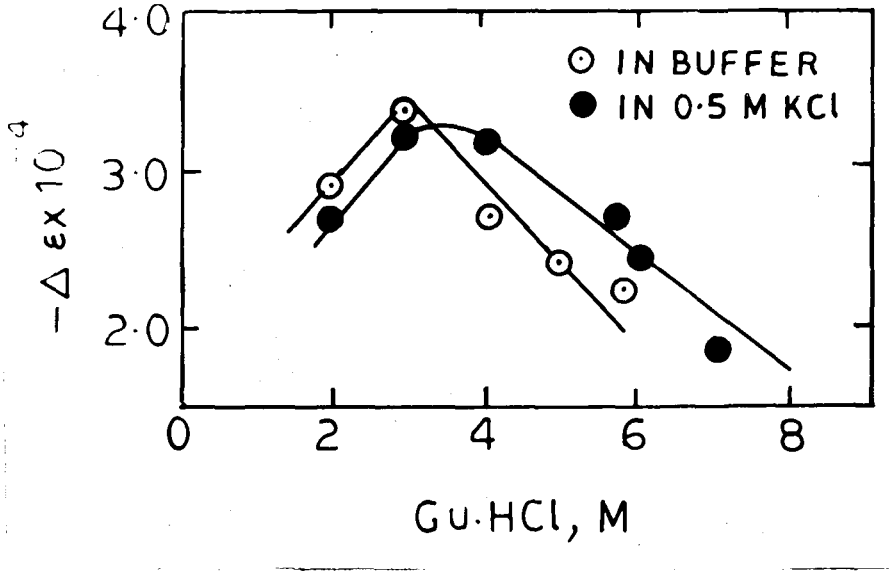
(b) GuHCl

Fig.47

a


3·0
2·0
1·0
$-\Delta\varepsilon \times 10^{-4}$
⊙ IN BUFEER
● IN 0·5 M KCl
2
4
6
8
10
UREA, M

b


4·0
3·0
2·0
$-\Delta\varepsilon \times 10^{-4}$
⊙ IN BUFFER
● IN 0·5 M KCl
0
2
4
6
8
Gu·HCl, M

'blue shift' with characteristic peaks at 280, 288 and 292 nm and a red shift at 300-305 nm (Fig. 45 and 46). The extent of red shift at 300-305 nm is more in GuHCl than in urea. The 292 nm peak in urea is more pronounced as compared to that in GuHCl solutions whereas the 280 nm peak is more pronounced in GuHCl. The appearance of the peaks at 280, 288 and 292 nm in the difference spectra suggests that tyrosine and tryptophan groups of the protein are in a polar environment in urea and GuHCl solutions (Wetlaufer, 1962; Donovan, 1969). From the results of viscosity it can be seen that α-globulin is in a denatured state in presence of the above denaturants. This blue shift may result from the exposure of the aromatic side chains of tyrosine and tryptophan from the non-polar interior of the protein to the more polar aqueous environment, as a result of conformational changes in the protein leading to denaturation.

The red shift at 300-305 nm is not generally found with most of the proteins in denaturant solutions. Hamaguchi and Kurono (1963) have observed similar red shifts in the 300 nm region while studying lysozyme in GuHCl solutions which they concluded as arising from denaturation of the protein. Similarly, the observed red shift at 300-305 nm in this investigation may arise from the denaturation of α-globulin in urea and GuHCl solutions. The increased red shift at 300-305 nm

in GuHCl solution compared to the results in urea solution probably arises from increased denaturation in the former solvent as has been indicated from the viscosity results (see page No. 199). However, Anantha-narayanan and Bigelow (1969) have explained that the red shift in 300-305 nm region may also arise from the anomalous tryptophan absorption.

A comparison of the $\Delta\varepsilon$ values in urea and GuHCl solutions in buffer and buffer containing 0.5M KCl shows that more number of groups are exposed in GuHCl than in urea (Fig. 47a and b). This is in conformity with the results of viscosity measurements which shows that the protein is in a more denatured state in GuHCl. Further the extent of blue shift in the presence of salt is considerably less than in its absence (Fig. 47a and b). This may be due to the protein being more resistant to denaturation in the presence of 0.5M KCl as can be seen from the viscosity data. In addition, to obtain the same extent of blue shift as in buffer, higher concentration of the denaturant is necessary. Above 8M urea and 3M GuHCl solution a decrease in the $\Delta\varepsilon$ values at 280, 288 and 292 nm is observed (Fig. 47a and b). This 'red shift' at higher denaturant concentration may be explained as arising from the change in the refractive index of the solvent at this high concentration of the reagents (Edsall, 1963).

Fluorescence:

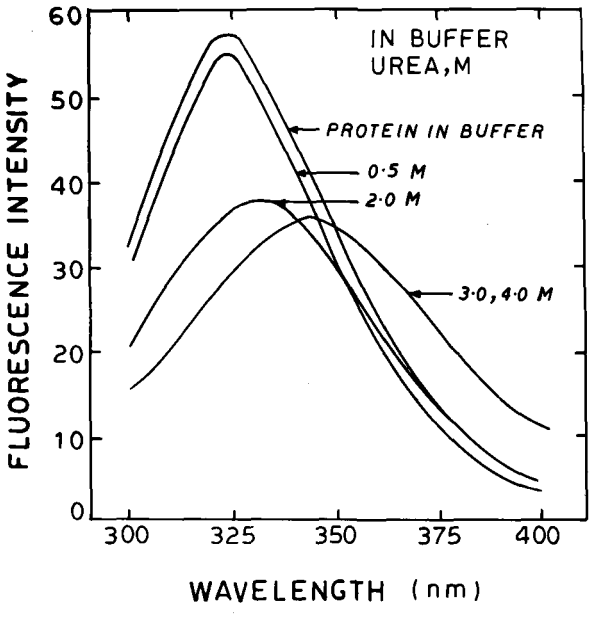
Fluorescence measurements were made in 0-10M urea and 0-6M GuHCl solutions both in buffer and buffer containing 0.5M KCl. The fluorescence emission maximum of the protein in buffer is at 325 nm (Fig. 48). With increasing amount of either urea or GuHCl the fluorescence intensity decreases accompanied by a shift in the emission maximum towards longer wavelengths (Fig. 48 and 49). The fluorescence intensity becomes constant at 3.0M urea and 1.5M GuHCl solution. The emission maximum at these concentration of the reagents is at 343 nm. Above these concentrations of the reagents the fluorescence intensity remains constant and there is no shift in the emission maximum. From a study of model compounds and their fluorescence property in different systems it has been found that tryptophan or its amide have their emission maximum around 345-350 nm in aqueous phase (Shifrin et al., 1971). The present results indicate that the tryptophan fluorophor is experiencing increasing polar environment with increasing concentrations of urea and/or GuHCl. Further it can be seen from Fig. 50 a and b that the relative fluorescence when it is constant, is less in presence of GuHCl than in urea which may be due to the fact that α-globulin is denatured to a greater extent in GuHCl than in urea.

Fig. 48 Effect of increasing concentrations of urea on the fluorescence spectra of α-globulin in

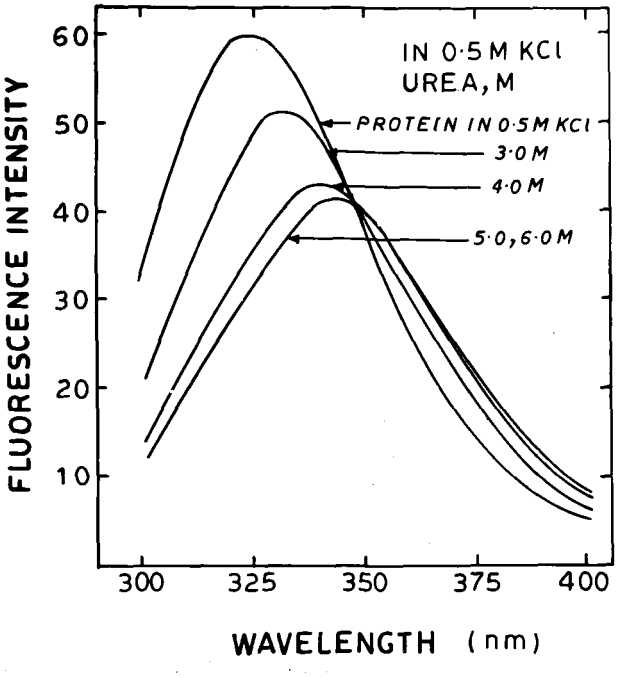
(a) 0.05M TEA-HCl buffer pH 9.0 and

(b) the buffer containing 0.5M KCl.

Fig.48


IN BUFFER
UREA,M
PROTEIN IN BUFFER
0·5 M
2·0 M
3·0,4·0 M
FLUORESCENCE INTENSITY
WAVELENGTH (nm)
0
10
20
30
40
50
60
300
325
350
375
400

a


IN 0·5 M KCl
UREA,M
PROTEIN IN 0·5M KCl
3·0 M
4·0 M
5·0,6·0 M
FLUORESCENCE INTENSITY
WAVELENGTH (nm)
10
20
30
40
50
60
300
325
350
375
400

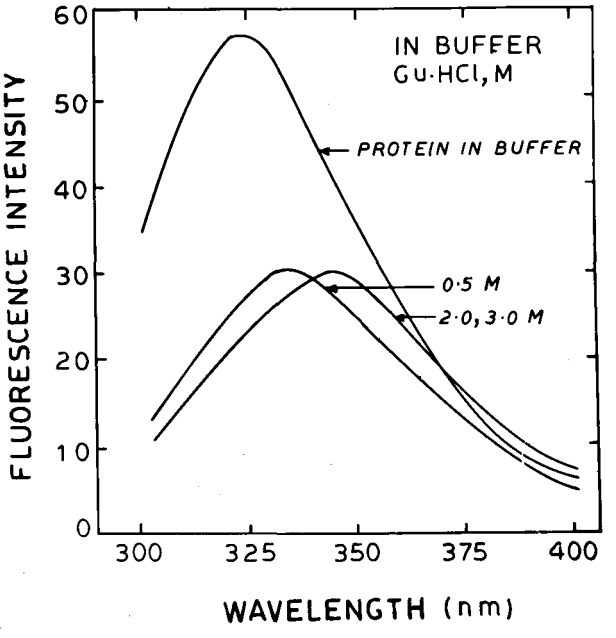
b

Fig. 49 Effect of increasing concentrations of GuHCl on the fluorescence spectra of α-globulin in,

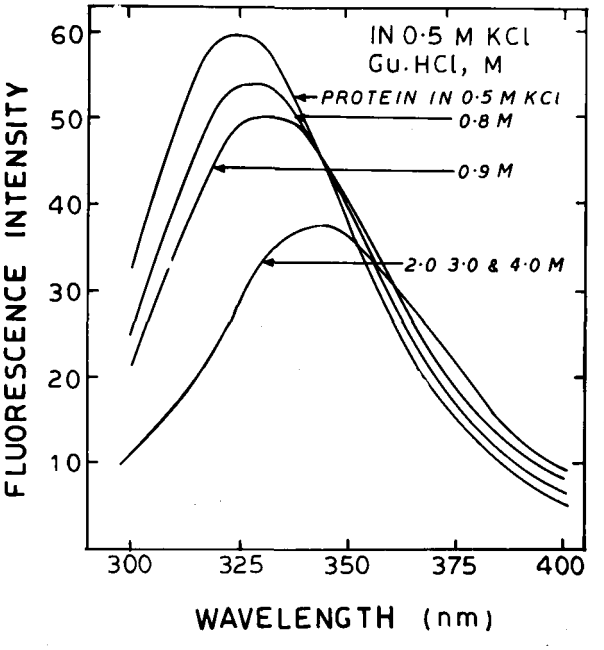
(a) 0.05M TEA-HCl buffer pH 9.0 and

(b) the buffer containing 0.5M KCl.

Fig. 49


IN BUFFER
Gu·HCl, M
PROTEIN IN BUFFER
0·5 M
2·0, 3·0 M
FLUORESCENCE INTENSITY
WAVELENGTH (nm)
0
10
20
30
40
50
60
300
325
350
375
400

a


IN 0·5 M KCl
Gu·HCl, M
PROTEIN IN 0·5 M KCl
0·8 M
0·9 M
2·0 3·0 & 4·0 M
FLUORESCENCE INTENSITY
WAVELENGTH (nm)
10
20
30
40
50
60
300
325
350
375
400

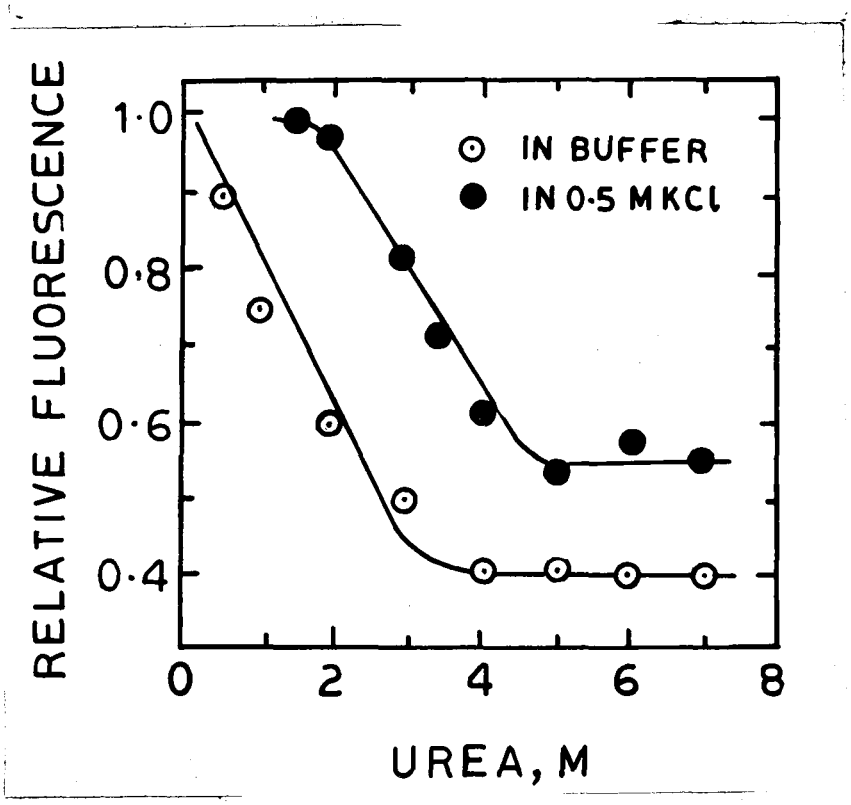
b

Fig. 50 Plot of relative fluorescence of α-globulin at 325 nm in 0.05M TEA-HCl buffer pH 9.0 and the buffer containing 0.5M KCl as a function of increasing concentration of

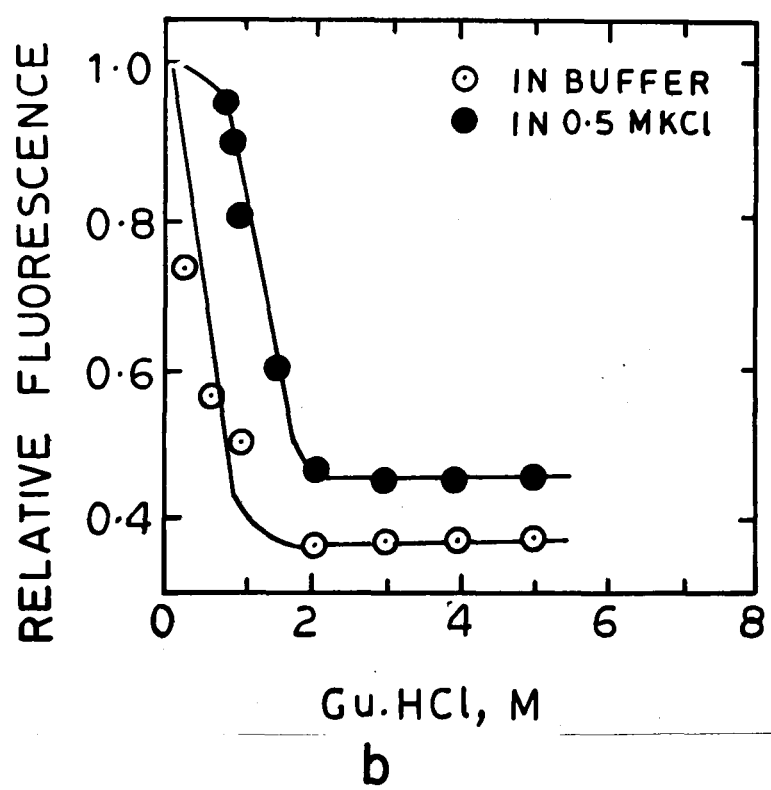
(a) Urea and

(b) GuHCl

Fig.50


RELATIVE FLUORESCENCE
1·0
0·8
0·6
0·4
0
2
4
6
8
IN BUFFER
IN 0·5 M KCl
UREA, M

a


RELATIVE FLUORESCENCE
1·0
0·8
0·6
0·4
0
2
4
6
8
IN BUFFER
IN 0·5 M KCl
Gu.HCl, M

b

Further no exposure of tryptophan from the interior of the protein molecule is taking place at $\geq$ 3.0M urea and $\geq$ 1.5M GuHCl. But the results of difference spectra indicates that the value of $\Delta\varepsilon$ reaches a constant value at 8M urea and 3M GuHCl concentration (Fig. 47). Similarly viscosity data indicate that conformational change is not complete at 3.0M urea and 1.5M GuHCl. The discrepancy between fluorescence results and other measurements as far as denaturation of α-globulin is considered, remains inexplicable. Whether this (completion of transition at a lesser concentration of both urea and GuHCl) is due to the high ratio of the denaturant to protein used in fluorescence measurements is not clear.

Measurement of fluorescence in the presence of 0.5M KCl shows that the relative fluorescence value attains a constant value at 4M urea and 2M GuHCl concentration and the steepness of the transition curve decreases considerably (Fig. 50). These results show that α-globulin in 0.5M KCl is resistant to denaturation (exposure of tryptophanyl residues) in urea and GuHCl as compared to buffer. Similar observation is made in viscosity and difference spectral measurements (see page Nos. 207 and 215).

The results obtained above indicate that α-globulin undergoes dissociation, aggregation and de-

naturation depending upon the concentration of urea and GuHCl solutions. Also the extent of dissociation, aggregation and denaturation is less at high ionic strength. Further the protein is in a more denatured state in GuHCl solution than in urea solution.

Aggregation in presence of urea has been observed with several proteins. Clark (1945), Foster and Samsa (1951) observed that the aggregation of egg albumin (ovalbumin) in low urea concentration is followed by the dissociation of the protein at higher reagent concentrations. Lytvynenko (1960) observed aggregation of myogen in urea solution between 1.6-4.1M above which dissociation of the protein into smaller components occurred. Mihyali (1950) reported the aggregation of fibrinogen below 3.3M urea above which no aggregate was observed. Aggregation of horse serum pseudoglobulin in GuHCl solution was observed by Neurath et al. (1942) below 2M concentration. Similar observation has been made with paramyosin in GuHCl solution by Riddiford (1966). Clark (1945) and Foster and Samsa (1951) concluded that the aggregation and dissociation reactions can proceed independently, a conclusion which was also arrived at by Connell (1960) who studied the effect of urea on fish myosin.

α-globulin aggregates at low concentrations of urea (< 3M) and GuHCl (< 0.025M) and above these concentration of the reagent the protein dissociates. This aggregation phenomenon may be due to the oxidation of -SH groups or complexation between the denaturants and amide or other polar groups in the protein or by some other process(es).

Under the experimental conditions employed namely pH 9 the possibility exists that -SH groups are oxidised as a consequence of structural changes leading to polymerisation of the protein by the formation of covalent S-S linkage (Fraenkel-Conrat et al., 1953; Cutter et al., 1957; McKenzie et al., 1963). At higher denaturant concentrations, where α-globulin would be in a more unfolded state, the amount of polymer resulting from -SH oxidation should be more, which is contrary to the observed results. Further the covalently linked aggregate formed by S-S bonds would not be soluble at higher urea or GuHCl concentration which is again contrary to the observed results. In addition, the presence of reagents such as β-mercaptoethanol which disrupt S-S linkages and N-ethylmaleimide which prevents the formation of S-S bonds, enhances the aggregation (precipitation) reaction of the protein at low urea

or GuHCl concentration. Similar increase in precipitation was observed by Connell (1961) in fish myoglobin in 0.5M urea in the presence of sulphydryl blocking agents. These results would suggest that aggregation of α-globulin at low urea or GuHCl concentration takes place by some process(es) other than disulfide linkages.

The complexation between the reagents and amide or other polar groups would involve H-bond formation between the reactants which would be exothermic in nature. But the relatively faster appearance of turbidity with increase in temperature (Fig.36). indicates that the aggregation does not result from H-bonded complexation. Also the appearance of soluble polymer ∽12S is noticed much earlier at 42° than at room temperature (∽25°) in the sedimentation velocity experiments. The ∽12S polymer is observed ∽80 min at 27° whereas at 42° it is observed at ∽30 min. These results could mean that the aggregation reaction is controlled by entropy driven hydrophobic interaction.

The effect of temperature on the sedimentation velocity pattern of α-globulin at pH 9 in the above buffer is shown in Fig.31. This indicates the hydrophobic nature of association of subunits in

Fig. 51 Effect of temperature on the sedimentation velocity pattern of α-globulin in 0.05M TEA-HCl buffer pH 9.0,

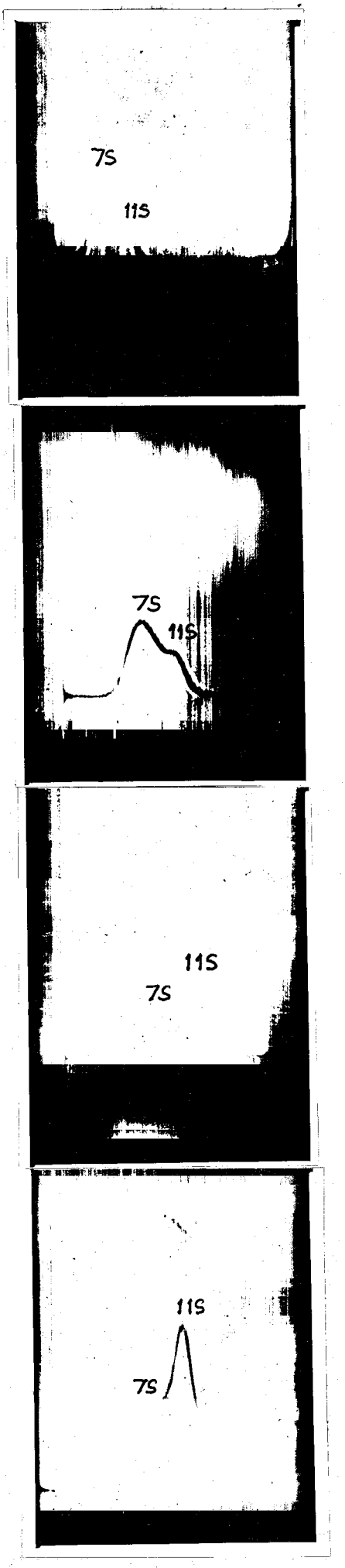
(a) 18°

(b) 22°

(c) 27° and

(d) 34°

Fig. 51



the protein (see page No.174). Further the results of fluorescence and difference spectra between pH 7 and 9 where nearly 60% of the 11S component is dissociated to the 7S component show (i) no change in the intensity of emission or shift in the emission maximum of fluorescence and (ii) the absence of any difference spectra which might result either from tyrosine or tryptophan residues. The above results indicate that predominantly aliphatic hydrocarbon chains are involved in the association-dissociation of the subunits of the protein.

The results of viscosity, fluorescence and sedimentation velocity measurements suggest that conformational changes in the protein has taken place at ∽ 2M urea concentration. At this concentration of urea, three components are present in the system viz. 4S, 7S and ∽ 12S and hence the interpretation is not easy. The increase in viscosity may be due to the polymer ∽ 12S or due to the partially unfolded 4S or 7S or by all the three components. Similarly the changes in fluorescence pattern may arise from either any or all ofthe above three components. Nandi and Robinson (1971) in their study of the effect ofurea and GuHCl on the model (blocked) peptides, $CH_3CONHCH(R)COOC_2H_5$, where R is aliphatic or

aromatic side chain, observed that aliphatic groups including leucine are energetically unfavourable upto 6M urea and 4M GuHCl, whereas aromatic groups are always favourable in these solutions as compared to water. In α-globulin, as a consequence of dissociation at low concentration of urea and GuHCl, the newly exposed aliphatic side chains, would try to decrease the positive free energy of the system by aggregating between themselves. This explanation, however, is not valid since if the groups are energetically unfavourable in urea or GuHCl solution they would not have been exposed in these solutions at all.

The simultaneous appearance of 4S and ∽12S at 1M urea concentration from the single 7S component would seem thermodynamically unlikely. It is possible that 7S component originally present and the new 7S component produced in urea (< 1M) may follow independent pathways to form different end products viz. 4S and ∽12S components. This would seem unlikely since (i) the maximum amount of 7S produced in urea (< 1M) from the dissociation of 11S is only 15% (Fig. 38) which cannotaccount for the formation of either 4S or ∽12S each of which attains 40-50% of the total protein present in solution and (ii) in buffer containing 0.5M KCl at 2M urea, where only 20% of the

7S fraction is present , the same course of reactions as in buffer only is observed (Fig. 41). Similarly even if it isassumed that the polymerisation results from 4S, then also simultaneous appearance of 2S and the polymer (at < 3M urea) from the same 4S component would be thermodynamically unlikely. The result could possibly be explained by two types of subunits present in the 7S component (originally present and produced in ureasolution). One type of subunits (A) produces the component sedimenting at 4S and the other type of subunits (B) produces the soluble polymer ($\backsim$ 12S) and ultimately the insoluble aggregate.

The subunit B leading to aggregation ($\backsim$ 12S) at low urea concentration may be expected to be richer in aliphatic amino acid residues than the subunit A from which the 4S component is produced (see page No.229).

To obtain evidence for this postulate the 4S and the $\backsim$ 12S components were isolated and the amino acid analysis of the fractions were carried out(see page No.65).

Isolation of 4S and ~12S component in presence of urea:

Urea (8M) solution in buffer was added to a known volume of α-globulin in 0.05M TRA-HCl buffer pH 9.0 such that the final concentration of urea was 2.8M. The solution was stirred well. The precipitate obtained was washed with 2.8M urea, then washed several times with water and dialysed against distilled water, lyophilized and used for amino acid analysis. The supernatant, containing both 7S and 4S components were separated on a Sephadex G-75 column equilibrated with 2.8M urea in the above buffer. The column was eluted with the buffer containing 2.8M urea and 2.5 ml fractions were collected. The fractions corresponding to the 7S component constituting 10-15% of the protein loaded on the column eluted in the void volume of the gel i.e. ~ 80-85 ml. The fractions corresponding to the 4S component eluting as a fairly broad peak between 120-150 ml were pooled and dialysed extensively against distilled water, lyophilized and used for amino acid analysis. The results of the amino acid analysis are shown in Table 6.

The amino acid analysis reveals differences between the two type of subunits. Further the minimum amount of 7S component at urea concentration where

TABLE 6

AMINO ACID ANALYSIS OF 4S AND ~12OS COMPONENTS

Amino acid	4S component (gm/100 gm protein)	12OS component (gm/100 gm protein)
Alanine ..	4.3	5.3
Valine ..	3.8	5.1
Leucine ..	3.2	4.0
Isoleucine ..	7.2	6.6
Phenylalanine ..	4.4	5.1
Tyrosine ..	4.0	4.1
Tryptophan ..	0.8	0.2

both 4S component and ∽ 12S component is maximum (Fig. 38) is indicative of this mechanism (Wolf and Tamura, 1969). Similar evidence for two different types of subunits, one leading to aggregation and the other sedimenting at 3-4S has been reported by Wolf and Tamura (1969) in their study of the thermal aggregation of glycinin, the 11S fraction of soybean proteins.

With further increase in urea or GuHCl concentration the 4S component dissociates to the 2S component (subunits) which from other measurements appears to be in a denatured state. The amount of aggregation after a critical urea or GuHCl concentration is reduced as the aliphatic side chains find the higher reagent concentration to be energetically favourable to them as compared to water. From Fig. 38 it can be seen that the precursor of aggregation (∽ 12S component) at high urea or GuHCl concentration dissociate to 2S component.

STUDIES IN SODIUM DODECYL SULFATE SOLUTIONS

STUDIES IN SODIUM DODECYL SULFATE SOLUTIONS

The effect of anionic detergent, sodium dodecyl sulfate, on α-globulin has been investigated by gel filtration, sedimentation velocity, viscosity, optical rotation, difference spectra and fluorescence spectral measurements in 0.05M TEA-HCl buffer, pH 9.0. The binding of the detergent to the protein has been determined by equilibrium dialysis.

Gel filtration

In the buffer, α-globulin elutes as a single peak near the void volume of Sepharose 6B-100 gel column. In the presence of SDS upto 5×10^{-3}M the protein elutes as a single peak, but the elution volume shifts to higher values (Fig. 52). Above this concentration of SDS, no further change in the elution volume is observed. However, the elution profiles are broad. The broad elution profiles at $\geq 5 \times 10^{-3}$M SDS may be due to the presence of dissociated fractions of varying molecular weight and size. A plot of V_e/V_o as a function of

Fig. 52 Effect of increasing concentrations of SDS on the gel filtration pattern of α-globulin in 0.05M TRA-HCl buffer pH 9.0 in Sepharose 6B-100.

Fig. 52

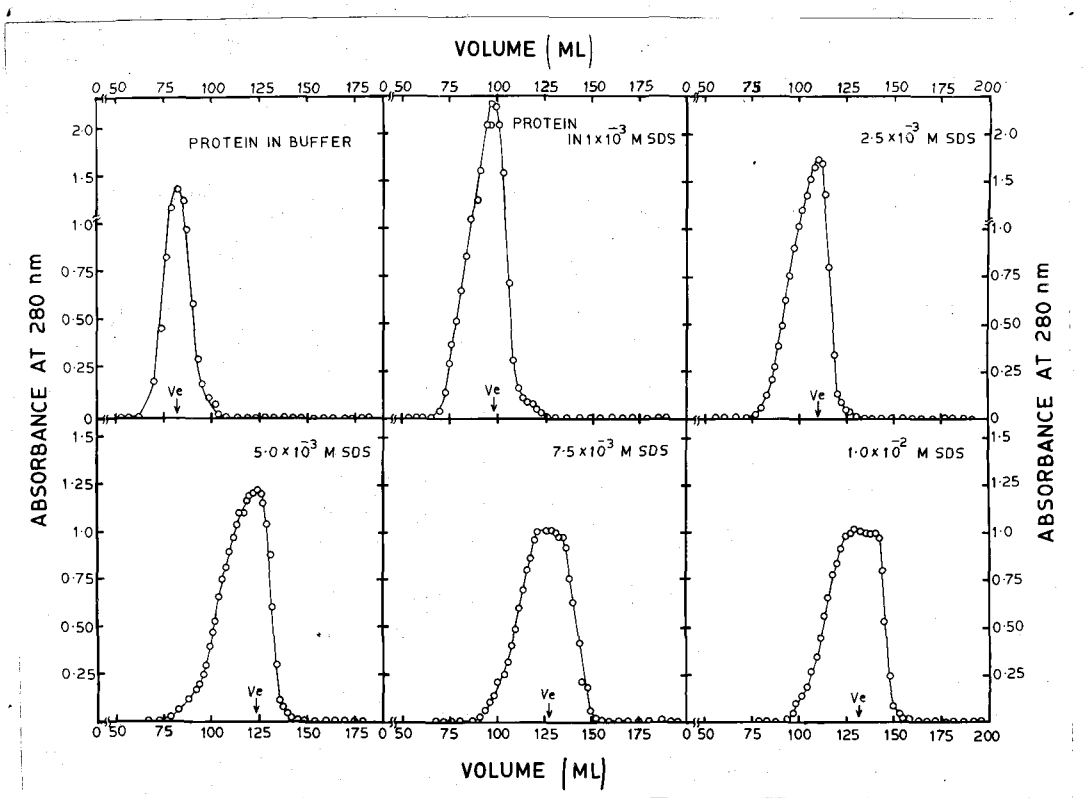
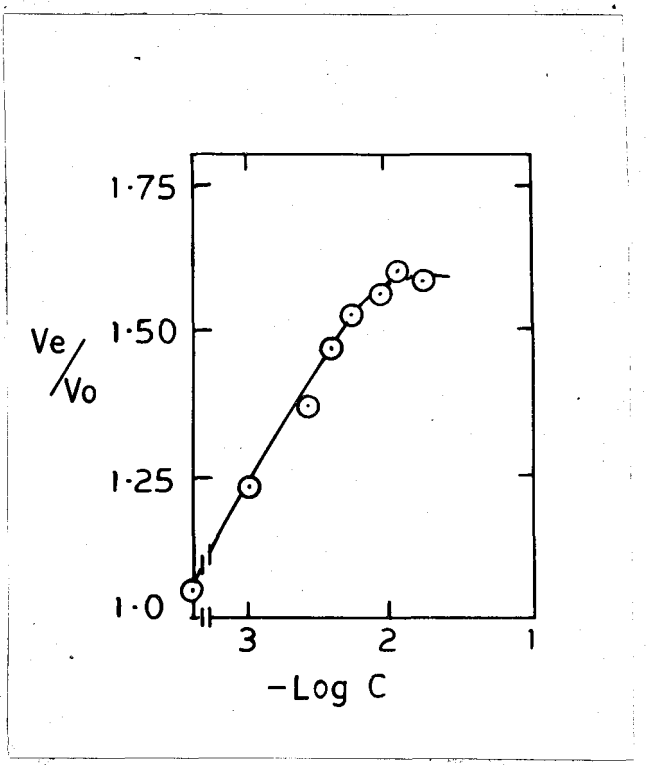

VOLUME (ML)
PROTEIN IN BUFFER
PROTEIN IN 1x10⁻³ M SDS
2·5x10⁻³ M SDS
5·0x10⁻³ M SDS
7·5x10⁻³ M SDS
1·0x10⁻² M SDS
Ve
ABSORBANCE AT 280 nm
VOLUME (ML)

Fig. 53 Variation of V_e/V_o with increase in detergent concentration in 0.05M TEA-HCl buffer pH 9.0.

Fig. 53



centration indicates that at $\geq 5x10^{-3}$M SDS concentration the elution volume does not change further (Fig. 53). These results indicate that α-globulin dissociates with increasing concentration of SDS.

Sedimentation analysis:

In the buffer, α-globulin exists as 11S and 7S components in the proportion of 40 and 60 respectively (Fig. 54a). At low concentrations of the detergent no change in the $S_{20,w}$ values are observed; however, the proportion of 7S component increases at the cost of 11S component upto $1x10^{-3}$M SDS (Fig. 54b and c). The new 7S component obtained by the dissociation of 11S component by SDS may or may not be the same 7S component obtained by the dissociating effect at alkaline pH on the 11S component (see page No.124). However, for the sake of convenience, this new 7S component has also been designated as 7S. With further increase in the SDS concentration, a 4S component and a 2S component are observed. In the concentration range of 1.75 to $4x10^{-3}$M SDS all the four components viz. 2S, 4S, 7S and 11S are present (Fig. 54 d, e and f). At still higher detergent concentration, $\geq 5x10^{-3}$M predominantly the 2S component is present. Above this concentration of SDS, only the 2S component represents the entire pattern (Fig. 54 g and h). In

Fig. 54 Effect of increasing concentrations of SDS on the sedimentation velocity pattern of α-globulin in 0.05M TEA-HCl buffer pH 9.0,

(a) in buffer alone

(b) lower, $1x10^{-4}$M and upper, $5x10^{-4}$M

(c) $1x10^{-3}$M

(d) $1.75x10^{-3}$M

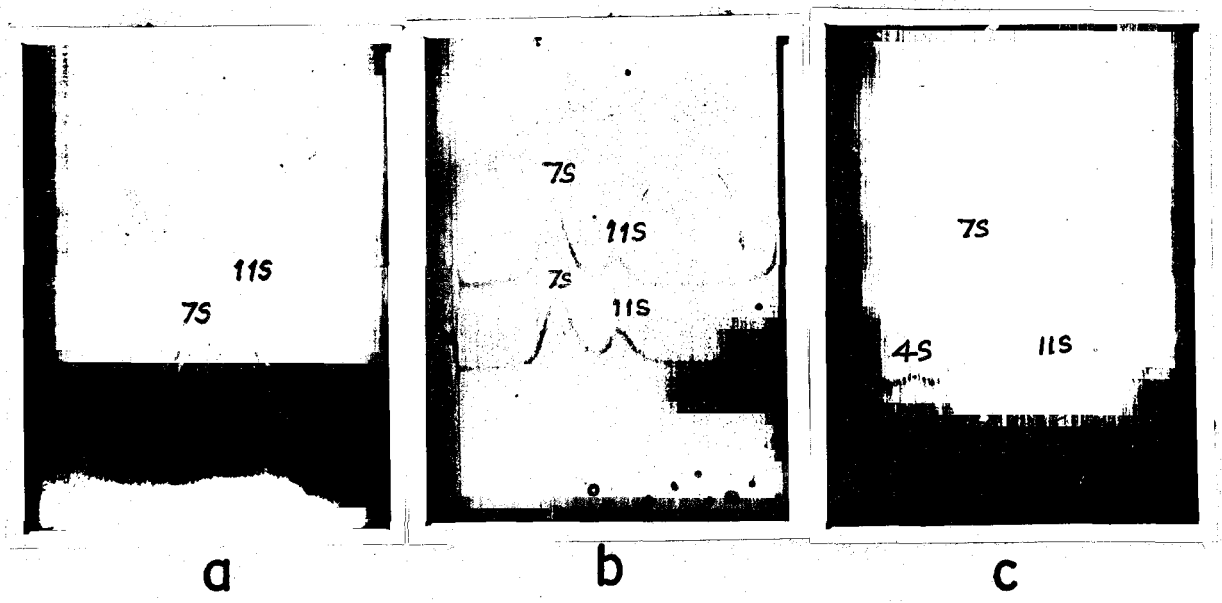
(e) $2.5x10^{-3}$M

(f) $3.5x10^{-3}$M

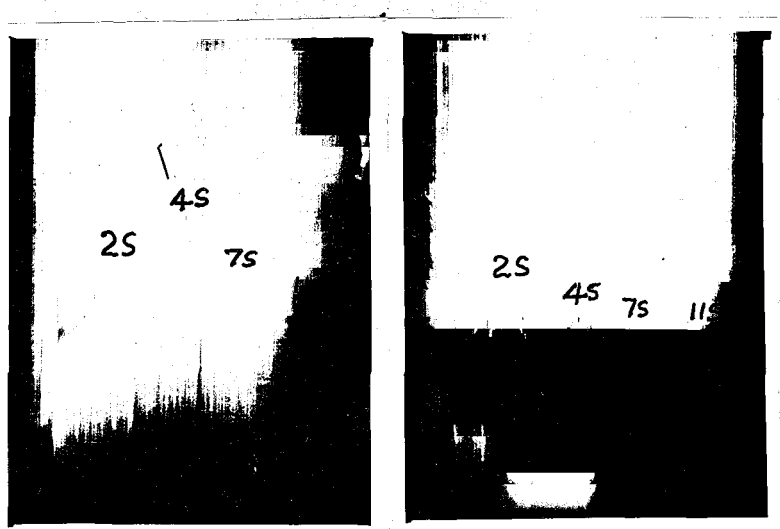
(g) $5x10^{-3}$M and

(h) $1x10^{-2}$M detergent solution.

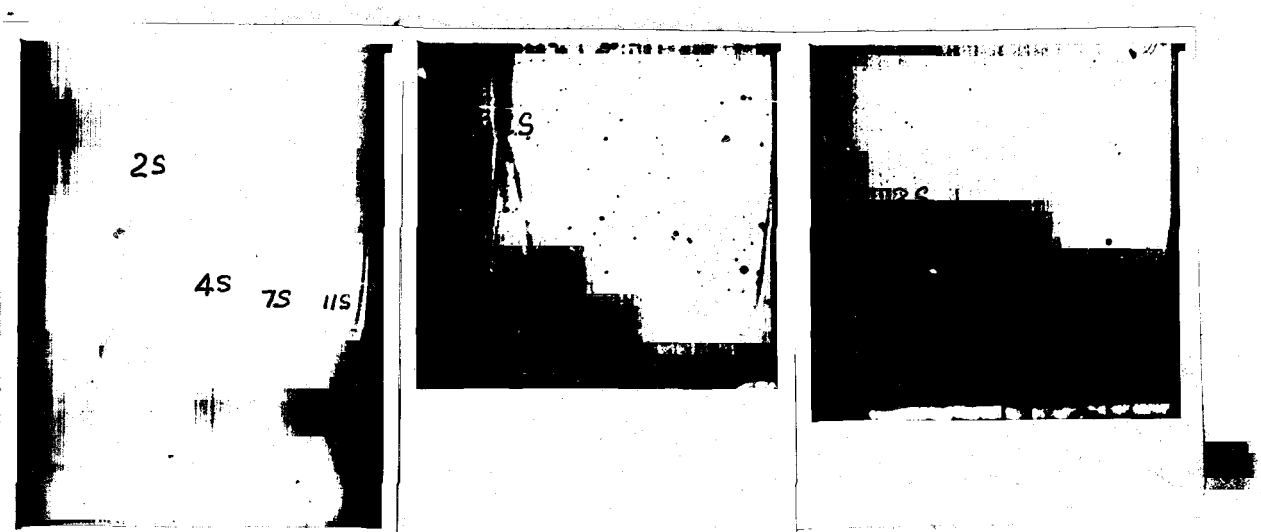
Fig.54



a b c



d e



f g h

Fig. 55 the percentage of all the four components is plotted as a function of SDS concentration. The results would indicate that α-globulin in SDS solution dissociates according to the following scheme:

$$11S \longrightarrow 7S \longrightarrow 4S \longrightarrow 2S$$

Polyacrylamide gel electrophoresis:

Polyacrylamide gel electrophoresis of α-globulin in different concentrations of SDS could not be carried out at pH 9.0 as the resolution of bands were not sharp. However at pH 7.5, in 0.02M phosphate buffer, electrophoresis of α-globulin in presence of varying concentrations of SDS indicated fast moving well resolved components (Fig. 56). The mobility of these bands increased, with increase in SDS concentration. The results indicate that α-globulin dissociates in SDS solutions.

Comparison of the sedimentation velocity and polyacrylamide gel electrophoresis results at the highest concentration of SDS used, i.e. 1×10^{-2}M, indicate that the protein is completely in 2S form (Fig. 54 h) whereas at this concentration of SDS several bands are observed in the gel electrophoretic pattern (Fig. 56 e). This may be due to the he-

Fig. 55 Variation of percent fraction of 2S, 4S, 7S and 11S components with increasing concentration of SDS in 0.05M TEA-HCl buffer pH 9.0.

Fig.55

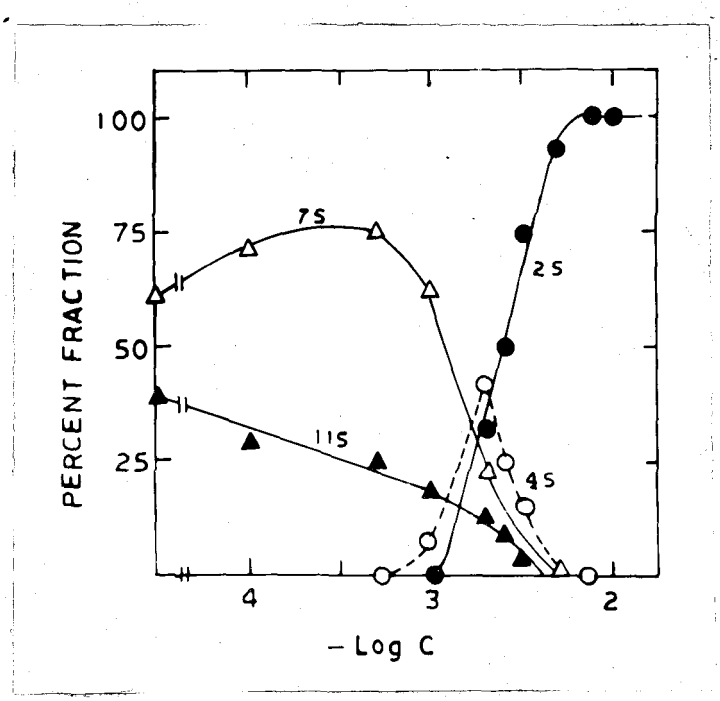


Fig. 56 Effect of various concentrations of SDS on the polyacrylamide gel electrophoretic pattern of α-globulin in 0.02M phosphate buffer pH 7.5,

(a) in buffer alone

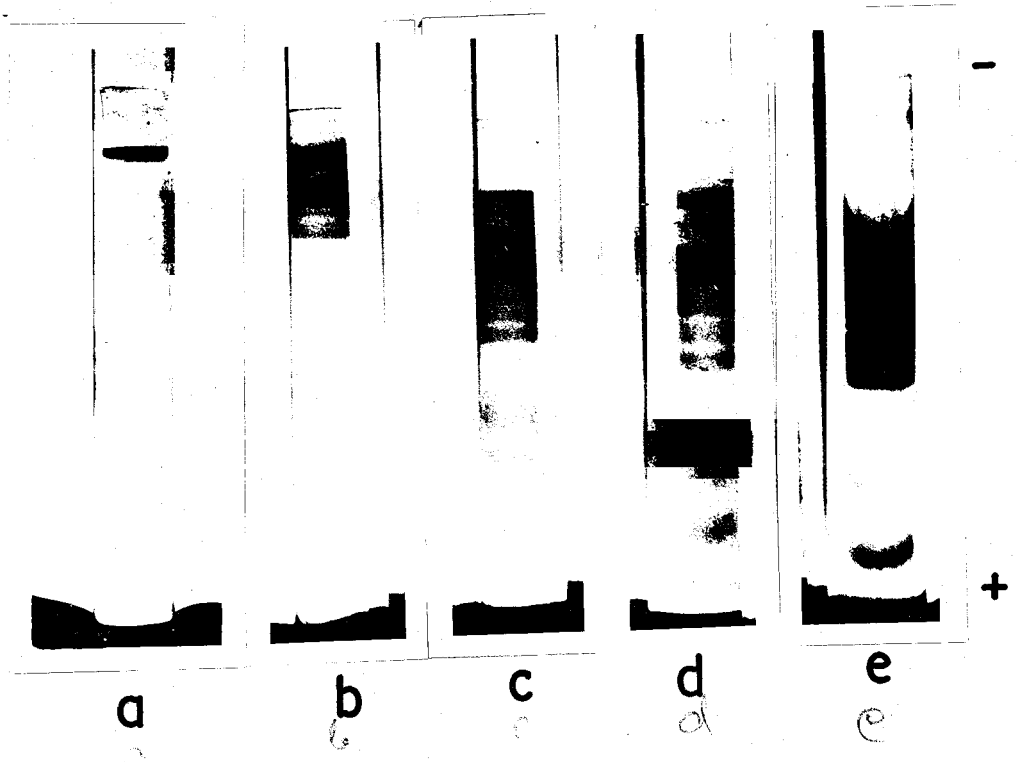
(b) 5×10^{-4}M

(c) 1×10^{-3}M

(d) 5×10^{-3}M and

(e) 1×10^{-2}M detergent solution.

Fig. 56



terogeneity of the 2S component itself containing several components of nearly the same sedimentation value. Similar anomalous mobility in presence of detergent has been observed by Williams and Gratzer (1971) and Ohno et al., (1975).

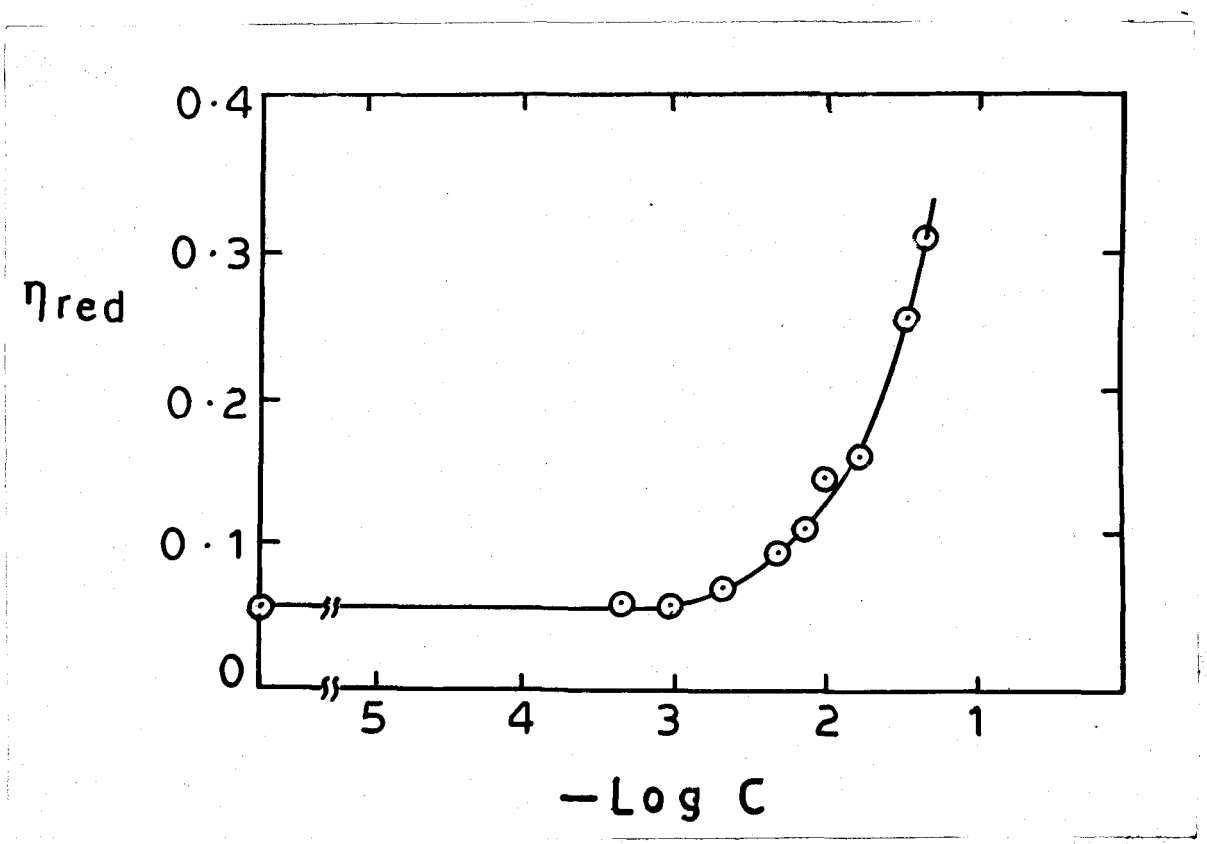
The results of gel filtration, polyacrylamide gel electrophoresis and sedimentation velocity experiments indicate that α-globulin, dissociates stepwise in SDS solutions. To monitor whether this dissociation process involves conformational changes or not, viscosity, optical rotation, difference spectrum and fluorescence spectrum of the protein in the detergent solution were measured.

Viscosity:

The reduced viscosity η_{red} of α-globulin in 0.05M TEA-HCl buffer pH 9.0 is 0.06 dl/gm. The η_{red} of the protein were determined at different concentrations of the detergent upto $3x10^{-2}$M SDS. The value of η_{red} remains unaltered upto $1x10^{-3}$M SDS. Above this concentration, a small but gradual change in the value of η_{red} is observed. With further increase in the detergent concentration, a sharp increase in the value of η_{red} takes place, which does not attain a constant value even at $3x10^{-2}$M SDS concentration (Fig. 37). The value of η_{red} at $3x10^{-2}$M

Fig. 57 Effect of increasing concentrations of SDS on the reduced viscosity of α-globulin in 0.05M TEA-HCl buffer pH 9.0 at 28°

Fig.57



SDS is 0.3 dl/gm. This change in the value of η_{red} of the protein from 0.06 dl/gm in buffer to 0.3 dl/gm in $5x10^{-2}$M SDS indicates unfolding of the protein molecule (Tanford, 1968). This takes place at $\geq 3x10^{-3}$M SDS concentration.

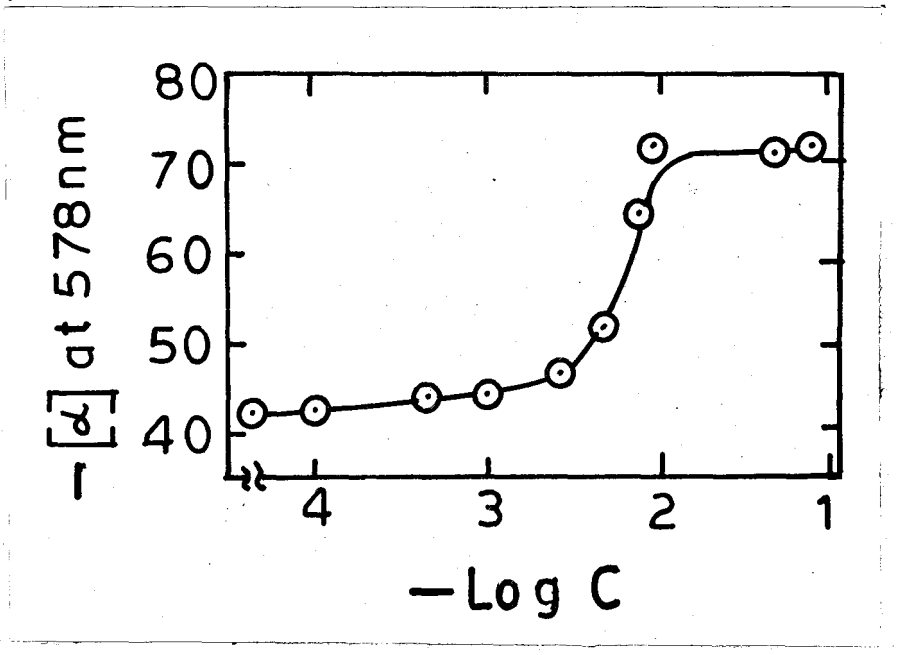
Optical rotation:

With increase in SDS concentration, the specific rotation value of $-[\alpha]_D^{20}$ of α-globulin does not change upto $2.5x10^{-3}$M SDS (Fig. 58). Above this concentration a sharp increase in levo-rotation is observed, which attains a constant value of $-[\alpha]_D^{20}$ at $1x10^{-2}$M SDS. This sharp increase in the levo rotation indicates conformational transition in the protein. The change in the specific rotation which takes place within this narrow range of detergent concentration ($\backsim 3x10^{-3}$M to $8x10^{-3}$M SDS) can be considered as an indication of a cooperative transition (Tanford, 1968).

The results of viscosity, optical rotation and sedimentation velocity experiments suggest that dissociation at $\geq 5x10^{-3}$M SDS is accompanied by the denaturation of the protein.

Fig. 58 Effect of increasing concentrations of SDS on the specific rotation of α-globulin at 578 nm in 0.05M TEA-HCl buffer pH 9.0 at 28°

Fig. 58



Difference and perturbation spectra:

The difference spectra of the protein were measured from $1x10^{-4}$M SDS to $2x10^{-2}$M SDS concentration (Fig. 39a). The difference spectra are characterised by 'blue shift' with troughs at 280 nm, 287 nm and 292 nm. The appearance of these troughs indicate that both tyrosine and tryptophan groups of the protein are perturbed in the presence of SDS (Wetlaufer, 1972; Donovan, 1969; Donovan, 1973a). With increase in detergent concentration a progressive decrease in the magnitude of the troughs is observed. However, the trough at 280 nm region which is due to tyrosine only (Wetlaufer, 1962) is not affected appreciably like the other two troughs which shows progressive decrease in absorbance till $7.5x10^{-3}$M SDS. With further increase in the concentration of SDS till $3x10^{-2}$M, no further change in the difference spectrum is observed. Fig. 39b shows the changes in the value of the extinction coefficient, $\Delta\varepsilon$ between the protein in buffer and in detergent solution against SDS concentration, at the three different wavelengths mentioned above.

Generally the blue shift observed in presence of detergents indicates denaturation of the protein (Wetlaufer, 1962; Donovan, 1973a). However, this

Fig. 39 Effect of increasing concentrations of SDS on the

(a) Ultraviolet difference spectra of α-globulin,

(1) 5×10^{-4}M

(2) 1×10^{-3}M

(3) 1.5×10^{-3}M

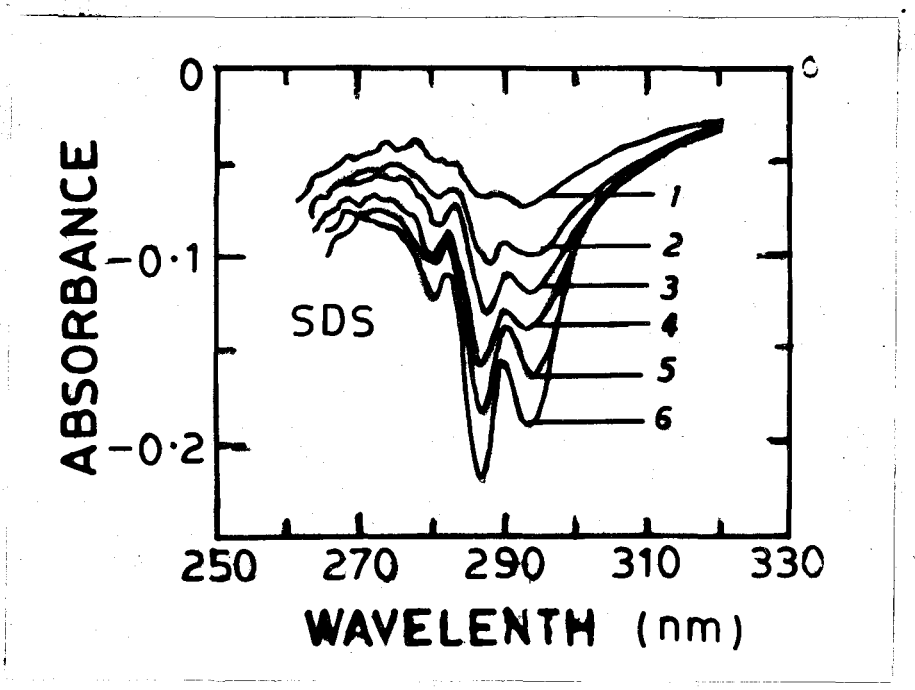
(4) 2.0×10^{-3}M

(5) 5.0×10^{-3}M

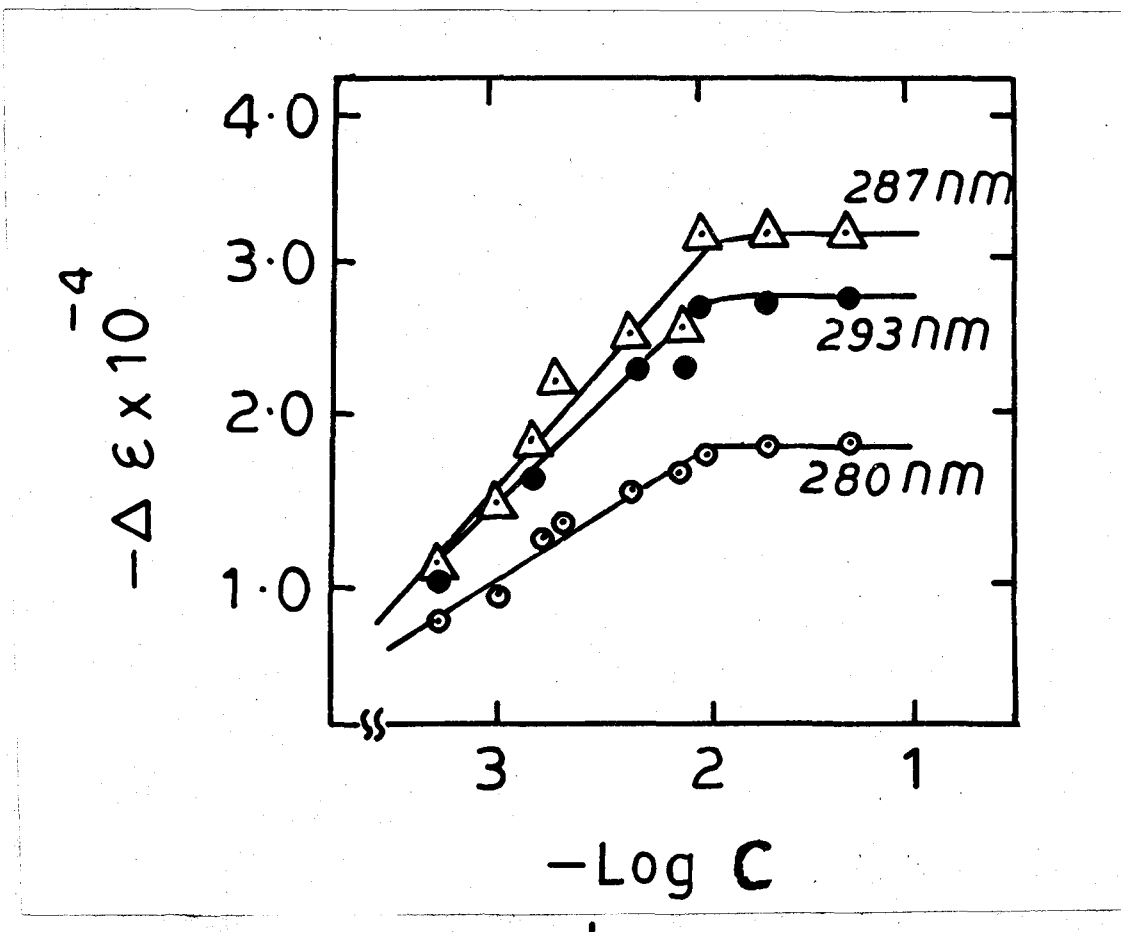
(6) 7.5×10^{-3}M, 1.0×10^{-2}M and 2.0×10^{-2}M detergent solution.

(b) changes in the molar extinction coefficient values of the protein at different wavelengths in 0.05M TEA-HCl buffer pH 9.0

Fig.59


0
-0·1
-0·2
ABSORBANCE
SDS
1
2
3
4
5
6
250
270
290
310
330
WAVELENTH (nm)

a


4·0
3·0
2·0
1·0
$-\Delta\varepsilon \times 10^{-4}$
287nm
293nm
280nm
3
2
1
−Log C

b

may not be true in all the cases; a blue shift may occur without any denaturation or conformational change in the protein. The results of viscosity and optical rotation discussed above indicate that the protein starts unfolding at $\geq 5 \times 10^{-3}$M SDS. Below this concentration of SDS considerable amount of blue shift has taken place as indicated from the difference spectral results.

In order to determine whether the aromatic chromophores are exposed to the surrounding solvents at lower concentration of the detergent ($\leq 5 \times 10^{-3}$M SDS) perturbation of the SDS induced difference spectrum by sucrose was studied (Laskowski, 1966). In Fig. 60 the dotted line indicates the perturbation of 20% sucrose on the difference spectrum of α-globulin in buffer. The solid line (1) indicates the perturbation by 20% sucrose when 5×10^{-3}M SDS is present in both the sample and reference cell, whereas the sample cell contains sucrose also. The identical difference spectrum obtained compared to that of control indicates that at this concentration of the detergent more tyrosyl and/or tryptophanyl groups are not accessible to sucrose. In contrast, similar perturbation experiment with 20% sucrose at 5×10^{-2}M SDS (Fig. 60) indicates the accessibility of sucrose to

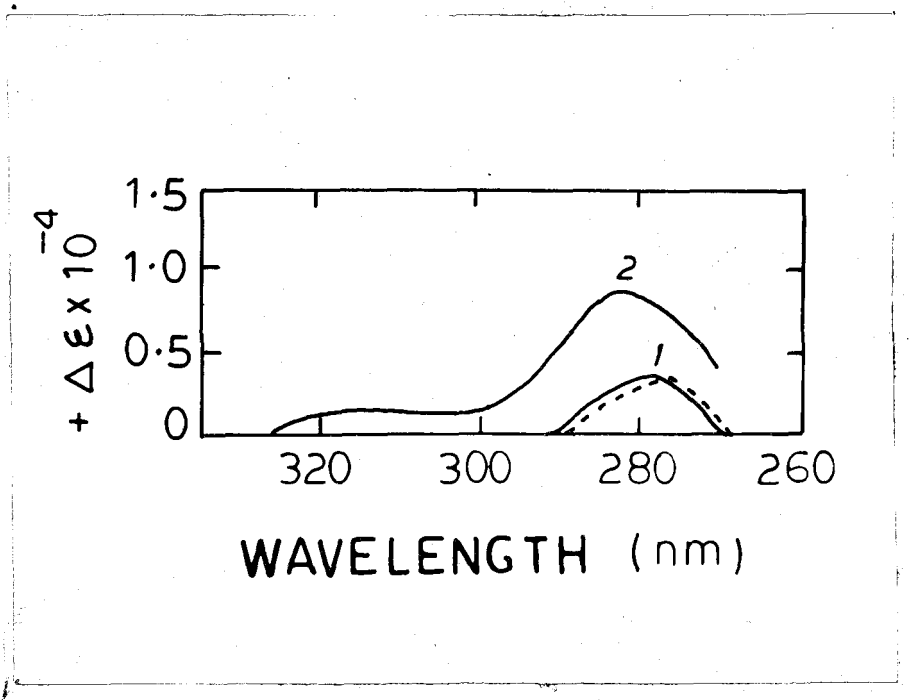
Fig. 60 Effect of sucrose on the difference spectra of α-globulin in 0.05M TEA-HCl buffer pH 9.0

(---) in buffer

(1) in 5×10^{-3}M and

(2) in 5×10^{-2}M SDS solution.

Fig. 60



the newly exposed tyrosyl and tryptophanyl groups. The viscosity and optical rotation measurements indicate that at this concentration i.e. at $5x10^{-2}$M SDS, the protein is in an unfolded state. The above results suggest that the difference spectrum observed below $5x10^{-3}$M SDS is not due to the unfolding of the protein molecule. It may be due to the perturbation of the chromophores by some effect other than denaturation. Perhaps it may be attributed to an alteration of ionic charges in the microenvironment of tyrosyl and tryptophanyl residues due to the binding of the detergent molecule(s) nearthese chromophoric groups which distort their spectra. However, the difference spectrum above $5x10^{-3}$M SDS concentration, results from both perturbation of the chromophores and also from the unfolding of the protein molecule.

Fluorescence measurements:

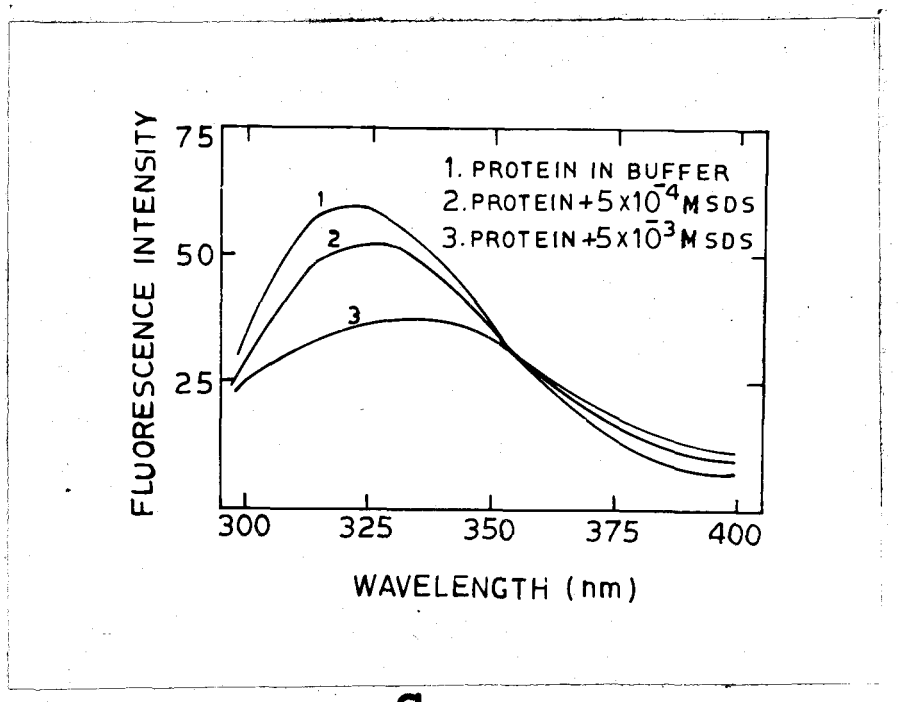
The flourescence measurements were carried out from $1x10^{-4}$M SDS to $1x10^{-2}$M SDS concentration. With increase in the SDS concentration from $1x10^{-4}$M onwards the fluorescence intensity of the protein at 325 nm decreases and the fluorescence emission maximum shifts to 330-335 nm at $\geq 5x10^{-3}$M SDS as shown in Fig. 61a. The spectra show the presence of an 'iso-emissive point' around 333 nm which may indicate the

Fig. 61 Effect of various concentrations of SDS on

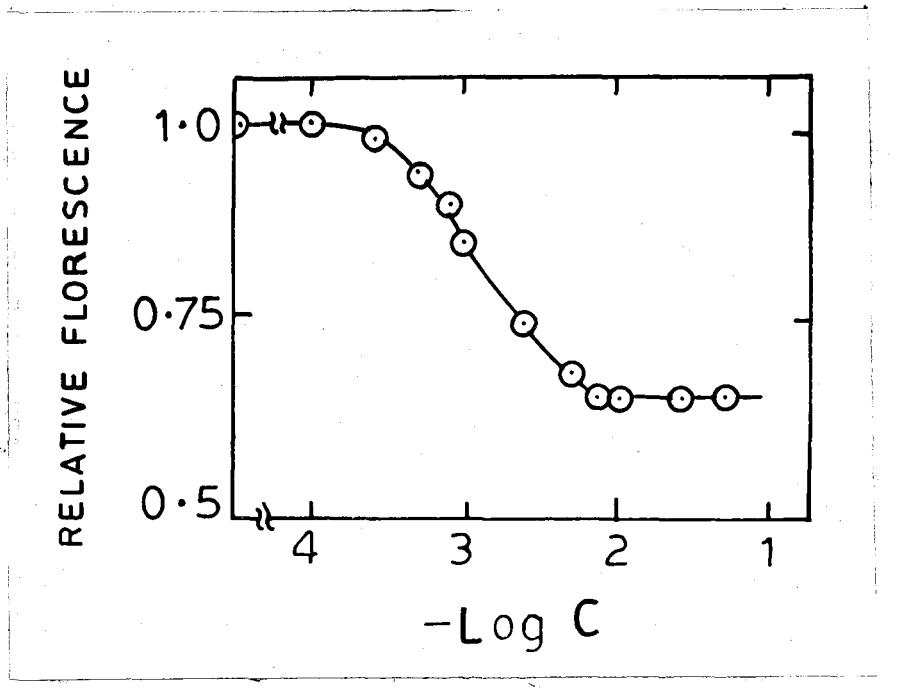
(a) the fluorescence spectra of α-globulin and

(b) relative fluorescence intensity of the protein at 325 nm in 0.05M TEA-HCl buffer pH 9.0

Fig.61


FLUORESCENCE INTENSITY
75
50
25
1. PROTEIN IN BUFFER
2. PROTEIN+5x10⁻⁴M SDS
3. PROTEIN+5x10⁻³M SDS
1
2
3
300
325
350
375
400
WAVELENGTH (nm)

a


RELATIVE FLORESCENCE
1·0
0·75
0·5
4
3
2
1
-Log C

b

presence of not more than two contributing components towards the fluorescence (Shifrin et al., 1971). But the ultracentrifugation results suggest that in the SDS concentration range at the isoemissive point, more than two types of protein components are present. Reynostad and Smith (1968) have pointed out that the presence of an isosbestic point cannot always be taken as an evidence for the presence of only two types of components contributing to either absorption or emission process. The above results also show that though the isoemissive point is present more than two types of components are responsible for it.

The plot of relative fluorescence intensity at 325 nm with increasing SDS concentration shows a sharp decrease after $5x10^{-4}$M SDS (Fig. 61b). The fluorescence intensity attains a constant value at $7.5x10^{-3}$M SDS. In this region of SDS concentration ($5x10^{-4}$M to $7.5x10^{-3}$M) no major conformational change has taken place in the protein as can be seen from viscosity and optical rotation measurements. Shifrin and Steers (1967) have observed that fluorescence change is complete before any conformational change takes place in β-galactosidase of E.coli in urea solution. In α-globulin, the decrease in fluorescence at $\leq 5x10^{-3}$M detergent solution where there is no

conformational change as evidenced by viscosity and optical rotation may arise from the binding of the detergent molecule(s) at site(s) near the tryptophan moieties. This binding of the detergent molecule would increase the electronegativity in the micro-environment of the fluorophor and quench the fluorescence as have been observed in the case of model peptides by Cowgill (1963).

The fluorescence maximum of the protein was 330-335 nm at 8×10^{-3}M SDS concentration when the specific rotation value is constant. This indicates no further conformational change. Konev (1967) has reported that most of the proteins in SDS solutions show a maximum fluorescence around 330 nm. Shifrin et al. (1971) have observed that in the model compound, N-acetyl-L-tryptophanamide, whose fluorescence maximum is 350 nm in water shifts to 330 nm in 0.1% SDS solution. Also α-globulin in 6M urea and 5M GuHCl where the protein is denatured shows fluorescence maximum at 345 nm (see page No. 216) like any other tryptophan containing protein (Teale, 1959; Chen et al., 1969). These results indicate that at $\sim 5 \times 10^{-3}$M detergent concentration the tryptophan residues in α-globulin are in an environment which is more polar than the interior of the protein but

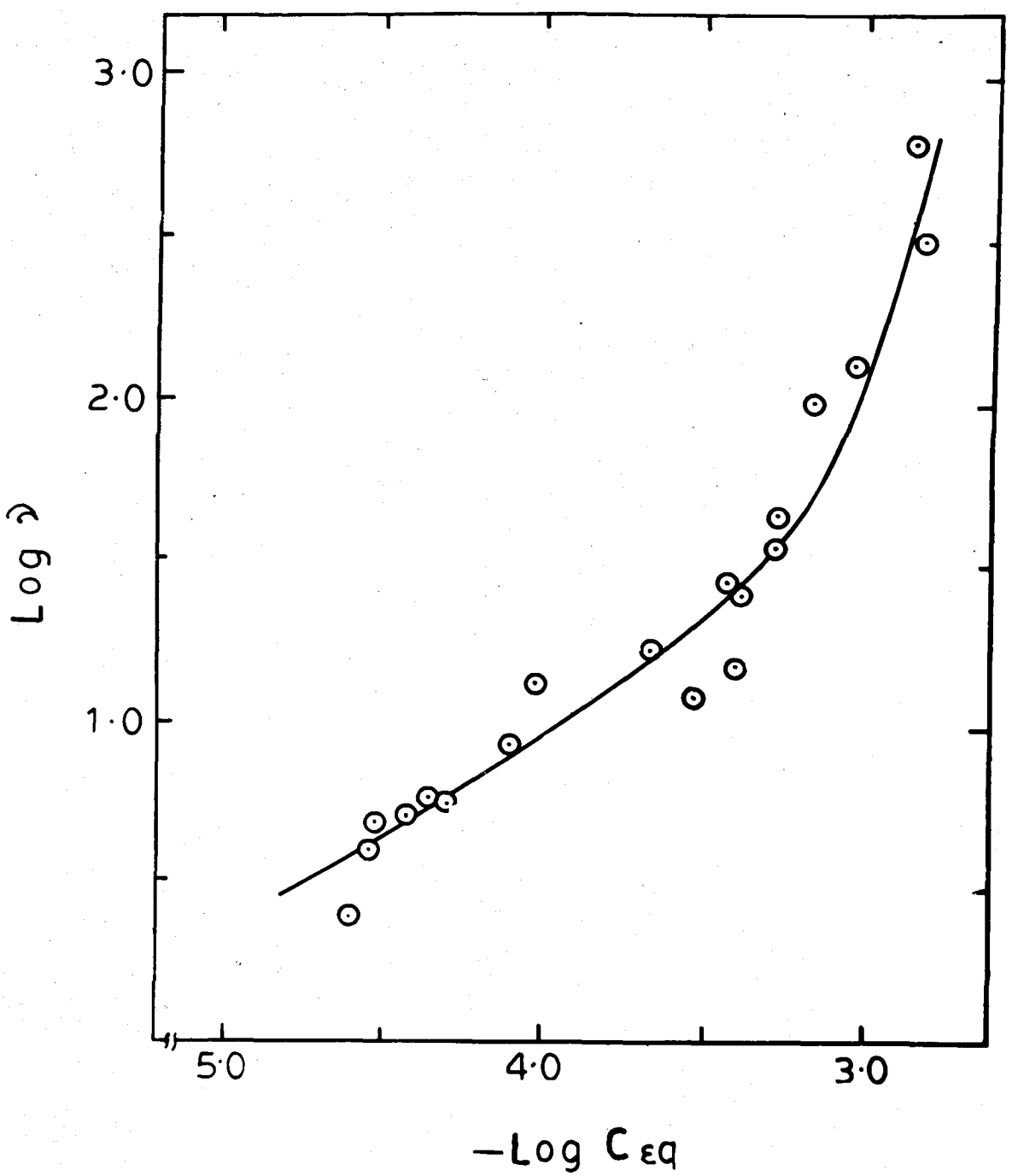
less polar than water. This has probably resulted from the binding of the detergent molecules near the fluorophors, offering a relatively nonpolar environment.

Equilibrium dialysis:

The binding of the detergent to α-globulin was determined by equilibrium dialysis. The binding isotherm showing the number of moles of detergent bound per mole of the protein ($\bar{\nu}$) as a function of SDS concentration is shown in Fig. 62. The binding of SDS increases as the concentration of the detergent increases from $1x10^{-5}$M upto $1x10^{-3}$M. The increase in the $\bar{\nu}$ value is not very high over this concentration range of the detergent. Above this concentration of SDS, a sharp increase in the binding occurs and does not attain a constant value. The less amount of binding observed at $\leq 1x10^{-3}$M SDS concentration may be due to the less number of available sites for binding, as the protein is only dissociated at this concentration range of detergent as evidenced from sedimentation velocity, viscosity and optical rotation measurements. At higher concentration of SDS, at $> 1x10^{-3}$M the amount of binding is high due to more number of available sites for binding as the protein is in an unfolded state. At concentrations

Fig. 62 Binding isotherm for the SDS-α-globulin interaction in 0.05M TEA-HCl buffer pH 9.0 at 30°

Fig.62



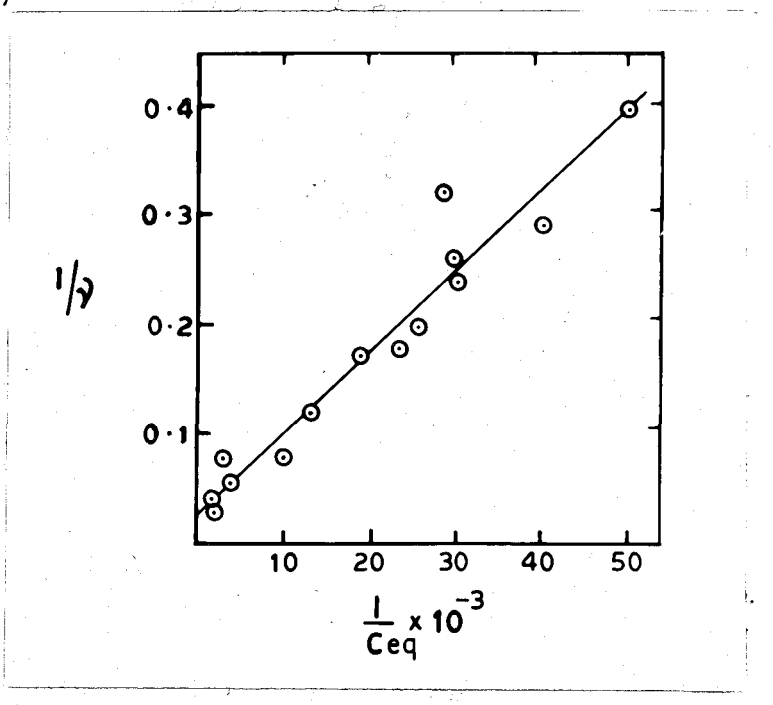
higher than this, considerable scattering in the value is observed.

The number of binding sites 'n' and the binding constant 'k' are determined from Klotz plot as shown in Fig. 63. The plot was linear over the concentration range from $1x10^{-5}$M to $1x10^{-3}$M SDS. From the plot the maximum number of binding sites n was $\sim$ 50 and the binding constant k was $3x10^{3}$.

These results indicate that SDS binds to α-globulin leading to dissociation as well as denaturation of the protein. At low concentrations of the detergent, only dissociation of the protein is observed above which, dissociation and denaturation take place simultaneously. The appearance of difference and fluorescence spectra in the range of detergent concentration $< 5x10^{-3}$M, where no major conformational change is observed by viscosity and optical rotation measurements, is due to binding of the detergent at site(s) near the chromophoric groups which alter their electronic properties. The appearance of the difference spectra in this range of detergent concentration is not due to the availability of the chromophores on the surface of the dissociated subunits (through which they might

Fig. 63 Klotz plot obtained for the binding between SDS and α-globulin.

Fig.63



remain associated in the parent protein molecule) as evidenced from the inaccessibility of these chromophores to sucrose. At higher detergent concentrations, the tryptophan moieties might have been enveloped by the detergent molecules. The binding of the detergent molecules in large numbers possibly increases the hydrodynamic volume of the protein which is observed by continuous increase in the η_{red} value even after denaturation is complete as is evidenced from optical rotation measurements.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

The present investigation on "Studies on the Physico-Chemical Properties of Sesame (*Sesamum indicum*) Seed Proteins" has been carried out as follows. We have concentrated our study on the major protein fraction α-globulin of sesame seed, its isolation, determination of some of its chemical and physico-chemical properties and studies on the association-dissociation and denaturation phenomena under various solution conditions, e.g. acid, alkali, electrolytes, urea, GuHCl and SDS which have been presented here. These studies on the association-dissociation and denaturation phenomena under the above solution conditions have been helpful in understanding the structural aspects of the protein.

(1) The protein α-globulin isolated by the procedures of Nath and Giri (1957b) and Ventura and Lima (1962) contains (contaminations both low, 2S component and high molecular weight, 16S component) ∽ 15% and ∽ 25% respectively as revealed

by sedimentation velocity experiments. The α-globulin isolated in the present investigation was homogeneous, > 95% by the techniques of gel filtration, polyacrylamide gel electrophoresis, DEAE-cellulose chromatography and sedimentation velocity experiments. The yield of α-globulin also increased by the procedure adopted here.

(2) The protein isolated was practically free from nucleic acid and carbohydrate contamination as revealed by the low values of phosphorus and carbohydrate.

(3) Amino acid analysis of the protein indicates that it is rich in acidic, sulphur containing and aromatic amino acid residues.

(4) The protein has a molecular weight of 2,30,000±15,000 daltons as determined from Archibald's approach to sedimentation equilibrium method.

(5) It is an oligomeric protein containing at least 11 subunits.

(6) The value of intrinsic viscosity of 0.03 dl/gm indicates the protein to be globular.

(7) In acid solution, in the pH range of 4.2-1.5, the results of gel filtration, fluorescence and viscosity measurements indicate that α-globulin

dissociates and denatures upto pH $\backsim$ 3.

(8) The difference spectrum in this range of pH is characterised by troughs at 280, 287 and 292 mμ and arises from a combination of dissociation, denaturation and charge effect on the chromophore.

(9) In still stronger acid solution (pH < 3) reassociation of the dissociated fraction takes place as indicated by difference spectra , fluorescence spectra and sedimentation velocity measurements.

(10) The temperature effect on the association-dissociation phenomena of α-globulin at pH 1.5 indicates that the reassociation of the dissociated components takes place probably by hydrophobic interaction.

(11) In alkaline solution, in the pH range 7-12, α-globulin dissociates around pH 8, and above pH 10, dissociation and denaturation proceed simultaneously as have been evidenced by sedimentation velocity, fluorescence spectra, ultraviolet spectral changes, optical rotation and viscosity measurements.

(12) The change in the shape of the ultraviolet spectrum with pH in alkaline solution indicates that both tyrosyl ionisation and conformational changes are taking place simultaneously in the protein.

The constancy of absorbance at 242 nm after tyrosyl ionisation is complete, can be taken as an evidence that no other reaction which might result from alkaline hydrolysis of disulfide groups is taking place.

(13) The phenolic group in the protein is abnormal (pK_{Int}=10.6). In 6M GuHCl the pK_{Int} of tyrosyl groups is 9.6 which is the expected value for the pK_{Int} of tyrosyl groups. The denaturation in alkaline solution is irreversible.

(14) The characteristic pH values of transition from 10.6-10.8 indicates that the transition of the protein involves a single step in alkaline solution.

(15) Effects of various sodium salts on the association-dissociation phenomena of α-globulin show the following effectiveness towards dissociation of the protein:

$$SO_4^{--} < Cl^- < Br^- < ClO_4^- < SCN^- \leq I^- < CCl_3COO^-$$

the first two members reduce the extent of dissociation. CCl_3COONa has been found to be the most effective amongst the series in inducing dissociation in the protein.

(16) The cations Li^+, Na^+, K^+ and Cs^+ induce association, the effectiveness being

$$Cs^+ \leqslant K^+ \geqslant Na^+ \geqslant Li^+$$

(17) Minor discrepancy from the Hofmeister series has been observed in the present investigation.

(18) The low concentration of salts (anions) necessary to induce dissociation does not involve any detectable change in protein conformation.

(19) The temperature effect on the association reaction 7S $\rightarrow$ 11S, indicates that the subunits of α-globulin are associated predominantly by hydrophobic interaction.

(20) The dissociating effect of the electrolytes may be due to a combination of favourable energetics of the chaotropic ions with the amide dipole against a low positive unfavourable free energy of interaction of the nonpolar groups with the same ions.

(21) α-globulin undergoes dissociation, aggregation and denaturation in urea and GuHCl solutions. The protein precipitates at low concentration of these reagents.

(22) The aggregation phenomenon is maximum at a critical concentration of either of the denaturants.

(23) The protein precipitated at low concentrations of these reagents goes back into solution at higher reagent concentration.

(24) The soluble aggregate having a sedimentation coefficient of ~12S is the precursor of this insoluble aggregate.

(25) Viscosity results indicate that the denaturation of the protein is complete at 8M urea and 6M GuHCl.

(26) The difference spectrum of the protein is characterised by troughs at 280, 287 and 293 nm indicating that both tryptophan and tyrosine groups have been effected by the denaturants. A red shift at 300-305 nm is also observed in both the reagents and is attributed to the denaturation of the protein and anomalous tryptophan absorption change.

(27) Fluorescence measurements indicate a red shift of the spectrum from 325 to 345 nm with increasing concentration of the denaturant. This indicates that the tryptophan fluorophor is experiencing increasing polar environment with increasing concentration of urea and/or GuHCl. The fluorescence

transition is complete at a lesser concentration of both urea and GuHCl as compared to viscosity and difference spectral results.

(28) The simultaneous dissociation and aggregation reactions have been explained by considering two types of subunits present in the protein molecule, one leading to smaller sedimenting component (4S) and the other producing the aggregate (∽12S).

(29) The amino acid analysis of the aggregated fraction indicates that it is rich in aliphatic amino acid residues.

(30) The endothermic nature of the aggregation process has been explained as probably arising from hydrophobic interaction of aliphatic side chains of the relevant subunits i.e. which are rich in aliphatic amino acid residues.

(31) α-globulin exists in a more denatured state in GuHCl than in urea solution. The extent of denaturation in these solutions are reduced in the presence of 0.5M KCl.

(32) α-globulin undergoes stepwise dissociation initially and then denaturation in SDS solution.

(33) Gel filtration, polyacrylamide gel electrophoresis and sedimentation velocity measurements indicate the dissociation of the protein. The dissociation is complete at $5x10^{-3}$M SDS and four components are observed in the sedimentation velocity pattern with $S_{20,w}$ values of 28, 48, 73 and 118.

(34) The specific rotation measurements indicate a cooperative transition between $3x10^{-3}$M to $8x10^{-3}$M detergent concentration, suggesting conformational change.

(35) The difference spectra of the protein is characterised by blue shift with troughs at 280, 287 and 292 nm indicating that both tryptophan and tyrosine groups have been effected by the detergent.

(36) The appearance of a blue shift in the difference spectra of protein below $3x10^{-3}$M arises from the binding of the detergent near the tyrosine and tryptophan chromophores. Above $3x10^{-3}$M SDS, the conformational change contributes to the observed blue shift in the difference spectra.

(37) In the presence of detergent, the fluorescence intensity decreases and a red shift in the fluorescence maximum occurs resulting in an 'isoemissive point' at 335 nm. The red shift in the fluo-

rescence maximum at the highest concentration of the detergent used is 330-335 nm. This red shift is less as compared to urea and GuHCl solutions where it is 345 nm.

(38) The binding studies of SDS to protein indicates a steep binding curve above 2.5×10^{-3}M SDS. Analysis of the binding data in the range 1×10^{-5} to 1×10^{-3}M SDS indicates the presence of ~30 binding sites in the protein and a binding constant of 3×10^{3}.

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