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**SCOPE AND OBJECTIVES OF THE PRESENT INVESTIGATION**

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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  $\alpha$ -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  $\alpha$ -globulin under various solution conditions e.g. acid, alkali, electrolytes, temperature, urea,  $\text{CaCl}_2$  and SDS solutions. These studies it is believed would help to understand the physico-chemical properties of  $\alpha$ -globulin, the major component of sesame seed protein.

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**PRESENT STATE OF KNOWLEDGE REGARDING  
DISSOCIATION AND DILUTION OF PRO-  
TEINS IN DIFFERENT SYSTEMS**  
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PROTEIN DENATURATION      KNOWLEDGE REGARDING DISSO-  
CIATION OF PROTEINS IN  
SOLUTION

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

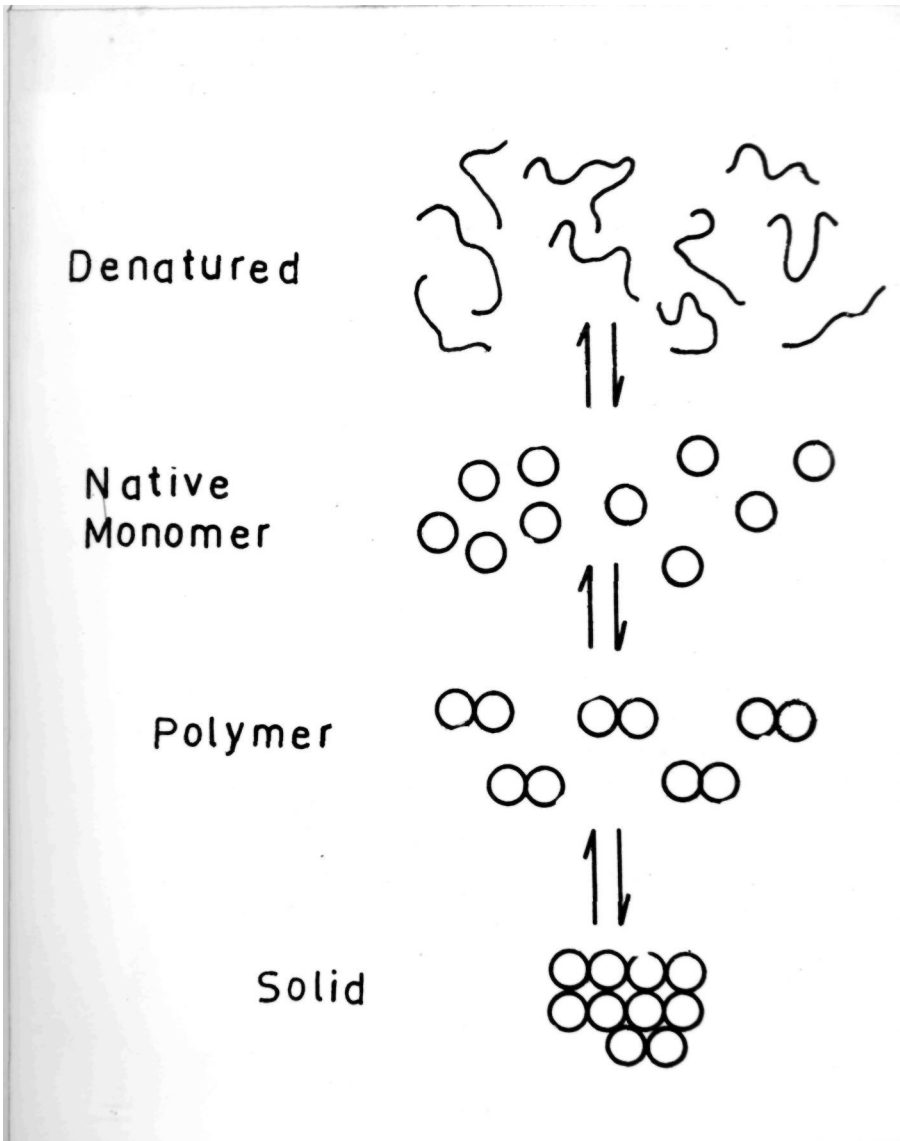
favourable than chain-chain contact in the molecule. Studies in the area of conformational change in the protein molecule have constituted a major area of physico-chemical studies of the proteins. Reports on the pre-denaturation transition and cis-trans isomerisation of the protein molecule before the onset of major structural change in it, are also available (Luary, 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 (Robinson 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; Cianagan 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 Schleich, 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 ( $\text{Li}^+$ ) in general has been observed to dissociate and denature proteins (von Hippel and Schleich, 1969; Jencks, 1969; Sawyer and Puckridge, 1973; Harrington and Herskovits, 1973; Nakashima *et al.*, 1973).

Dissociation and denaturation of the protein in high concentrations of urea and  $\text{CuHCl}$  solutions have been extensively studied (Tanford, 1968; Jencks, 1969; McKenzie and Balston, 1973; Wallevik, 1973; Knapp and Pace, 1974; Touwissen *et al.*, 1974). A few instances are also available where the proteins are found to be resistant to urea or  $\text{CuHCl}$  denaturation even in concentrated solutions of these reagents (Leonis, 1956; Buckley *et al.*, 1963; Stauffer and Sullivan, 1971; Asuma *et al.*, 1974).  $\text{CuHCl}$  is more effective in dissociating and denaturing proteins than urea with minor exceptions (Tanford, 1968; Jencks, 1969; Raymond and Pace, 1974; Tsong, 1973). 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  $\text{CuHCl}$  concentration (Imai *et al.*, 1963; Takagi and Isemura, 1966; Tanford, 1968; dekok and Ravitch, 1969; Aoki *et al.*, 1974). There are a few evidences available in literature

where the denatured protein in urea and GuHCl undergoes aggregation reaction (Frensdorff *et al.*, 1953; McKensie *et al.*, 1955; Gutter *et al.*, 1957; McKensie *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; Miniyall, 1950; Jirgensons, 1952; Lytvynenko, 1960; Riddiford, 1966).

Detergents, particularly ionic, at very low concentrations are effective, in dissociating and denaturing proteins (Decker and Foster, 1966; Tanford, 1968). The measurement of different physico-chemical properties of certain proteins indicate that their  $\alpha$ -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 Kausman, 1962; Jirgensons, 1967; Rosenberg *et al.*, 1968). Uricase 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 adled 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).

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PRINCIPLES OF THE EXPERIMENTAL APPROACHES  
EMPLOYED IN THE PRESENT INVESTIGATION  
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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  $\alpha$ -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-1-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 (Deternann, 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 the acrylamide gel polymerised in tubes and will migrate 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  $\beta$ -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  $\beta$ -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:

(1) 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),

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

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 the different schlieren peaks obtained. The phenomena of dissociation and denaturation can also be studied by molecular weight determinations from various sedimentation equilibrium methods.

(ii) Molecular weight determination from  $S_{20,w}$  value:

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

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

where  $[\eta]$  is the intrinsic viscosity (dl/gm),  $\bar{v}$  is the partial specific volume and  $\rho$  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,  $M_w$  is given by (Schachman, 1959).

$$M_w = \frac{RTF \left( \frac{dc}{dx} \right)_{x_0}}{(1 - \bar{v}\rho) \omega^2 x_0 \Delta x \left[ \sum \frac{dc}{dx} - \frac{1}{x_0^2} \sum x^2 \frac{dc}{dx} \right]} \dots (3)$$

where  $R$  is the gas constant,  $T$  is the absolute temperature,  $F$  is the <sup>lens</sup> magnification factor,  $\frac{dc}{dx}$  the concentration gradient,  $\bar{v}$  is the partial specific volume,  $\rho$  is the density of the solution,  $\omega$  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

$$[\eta] = \lim_{c \rightarrow 0} \left( \frac{\eta_{sp}}{c} \right) \dots (4)$$

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

centration of the protein in gm/dl.  $\eta_{sp}$  is defined as

$$\eta_{sp} = \frac{\eta - \eta_0}{\eta_0} \dots (5)$$

where  $\eta$  and  $\eta_0$  are the viscosities of the protein solution and solvent respectively.

Globular proteins in general, have low values of intrinsic viscosity  $\sim 0.03$  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  $[\eta]$ ,  $\eta_{sp}$  or  $\eta_{red}$  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, even though the chemical nature of the molecule remains unaltered (Kausmann, 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 150-600 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 (Kausmann, 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 the protein 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 fluorophores. 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).

$$I = I_0 e^{-Jx} \quad \dots\dots (6)$$

where  $I_0$  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  $J$  is called the turbidity of the medium. The turbidity  $J$ , 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., 1958).

#### 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.

(1) NBS method: In this method the indole chromophore of tryptophan 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 trye-

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

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

where  $\Delta \text{Abs}$  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  $\Delta \text{Abs}$ .

(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,  $M_{\text{Try}}$  determined from the equation (Edelhoch, 1967).

$$M_{\text{Try}} = 10^{-3} (0.322 \epsilon_{288} - 0.0969 \epsilon_{280}) \quad \dots (8)$$

where  $\epsilon_{288}$  is the extinction coefficient of the protein at 288 nm and  $\epsilon_{280}$  is the extinction coefficient of the protein at 280 nm.

(111) 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 organisms 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 m $\mu$  region. The pH of the experimental solution is varied and the increase in absorbance at 293-295 m $\mu$  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 of sulphuric 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 (Montgomery, 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-

standard 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, 1953).