NITROGENOUS COMPOUNDS- Ch.15,p.237

A-Methods for **Quantifying** Proteins

I. Methods based on elementary analysis of nitrogen (Kjeldahl, Dumas)

II. Methods Based on Direct Determination of Proteins (Biuret, Lowry, Dye-binding)

III. Indirect Methods (Turbidimetry, NIR, T.D)

B-Methods for Assessment of Protein **Quality**

I. In vivo tests (BV, PER, NPU etc.)

II. In vitro tests (non-protein nitrogen determination, amino acid composition, AA. availability).

C-Methods for elucidating protein **structure**

I. The sequence of amino acids

II. Conformational streochemistry of proteins.

Proteins are polymers of amino acids linked by “amide” linkages. They have significance in foods from:

-nutritional,

-rheological (functional)

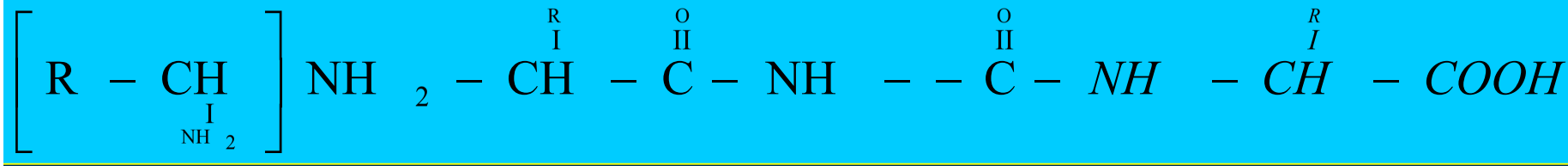
-organoleptic aspects,

Proteins are important for biological functions and cell structure; they include “essential amino acids” which cannot be produced in the human body; food texture and flavour are also very much influenced by protein contents. Therefore, protein analysis is important for biological activity determinations, functional property investigations and of course for food labeling.

MW of proteins :5000-10^6 daltons

omposed of H,C,N,O,S.

Proteins occur in foods often in combination with other macrofood components: as Lipoproteins (as in blood proteins);as Glycoproteins(as in egg white) ; as metalloproteins(as mineral complexes like Fe in hemoglobin, I in thyroglobulin). All proteins are made up of ≅24 amino acids, eight of which are "essential". Every a.a. has at least one amino and at least one carboxylic group in its molecule.



Amino acids come together first forming peptide chains(first oligopeptides;then the polypeptides) and finally the polymeric protein molecule. In all protein molecules, there is ~16% N present(%13-19).

Exact Nitrogen ratio in proteins ranges from13.4to 19.1%:

$$\frac{100}{16} \cong 6.25 \rightarrow \textit{protein factor}$$

Wheat protein factor : 17.55% N ; 100/17.55=5.7

Milk protein factor: 15.67%N; 100/15.67=6.38

Oilseeds protein factor: 18.87%N; 100/18.87=5.3

Protein contents of foods:

Beef:18% asis;80-90%d.m.; Fish:70-80%(d.m.)

Milk: 3.5%; Skimmed Milk powder:36%; Cheese:16-25%

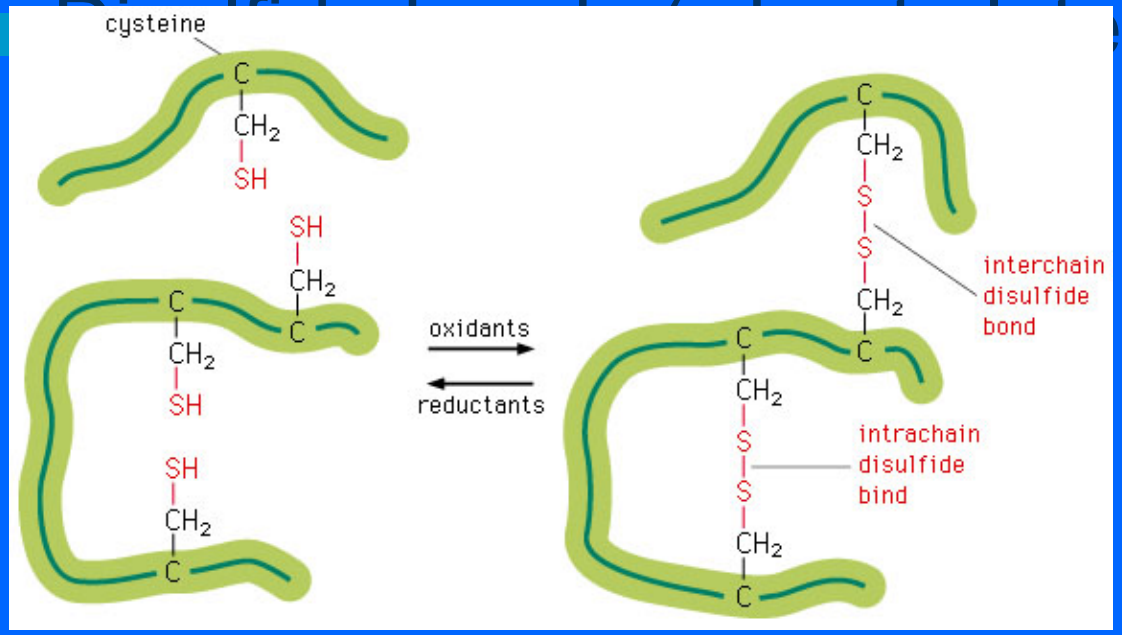
Bread:8%; Flour: 9-13%

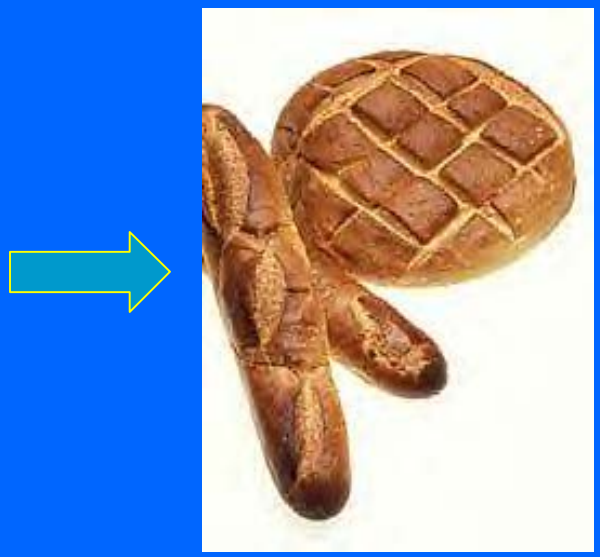
Fruits:0.2-1.5%

Vegetables:1-2%

PROTEIN ANALYSIS

- **Why are we interested in the overall protein content of a food?**
- Functionality (hydration , gellation, surface properties..)
- Disulfide bonds (gluten)





Protein Analyses

- Total protein content
- Amino acid composition
- Content of a particular protein or aa
- Protein isolations and identifications
- Nonprotein nitrogen
- Nutritive value (per,nitrogen balance etc.)

Methods based on Elementary Analysis of Nitrogen:

A. Kjeldahl Method for Crude Protein

Principle steps:

1-Digestion in concentrated H_2SO_4 + catalyst mixture:

$$n\left[-C-NH_2\right]+mH_2SO_4 \rightarrow (NH_4)2SO_4 + CO_2\uparrow SO_2\uparrow$$

N is converted to ammonium ion.

2-Neutralisation:Concentrated alkaline solution is added, followed by steam distillation of liberated NH_3, collecting into known amount of acid:

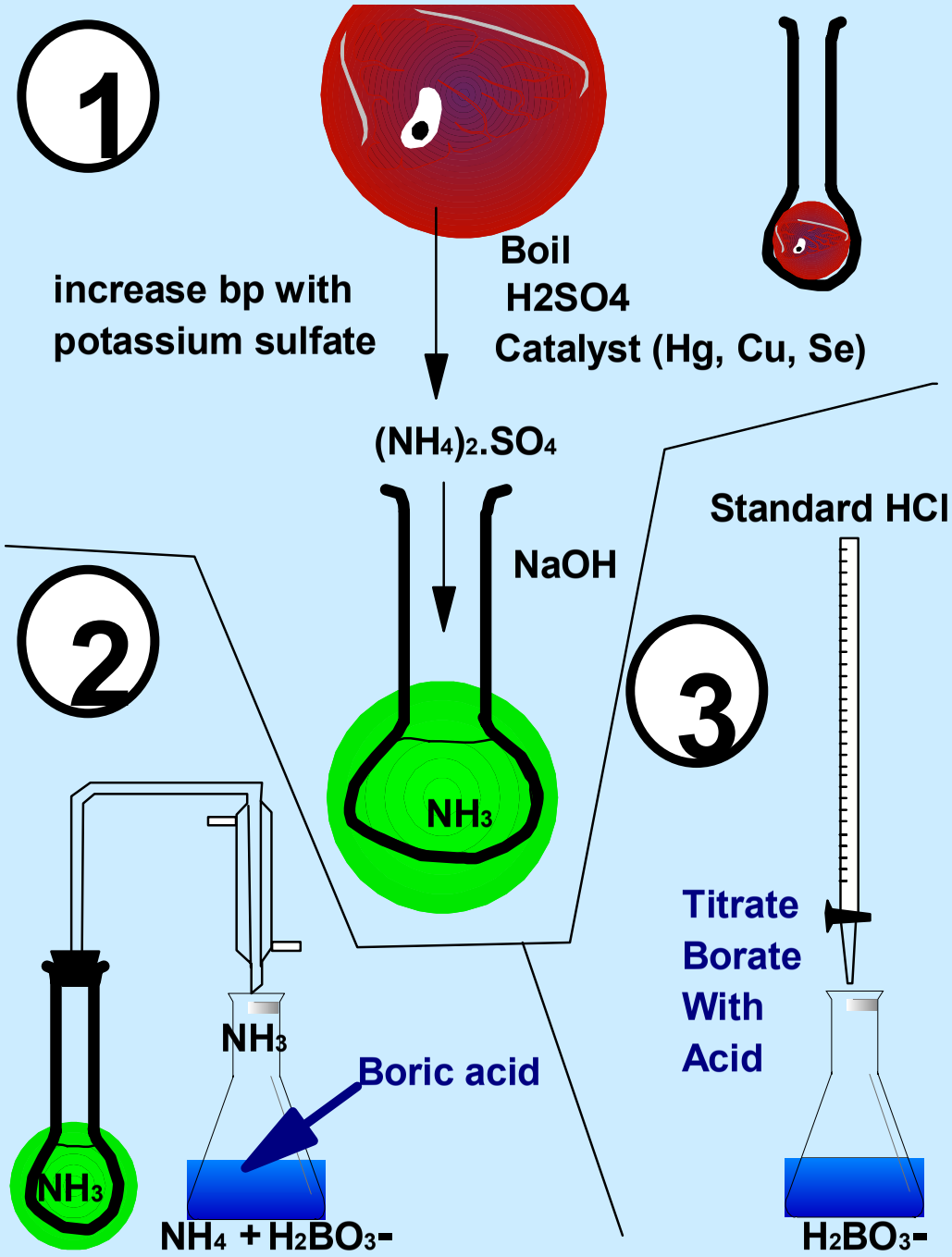
$$(NH_4)SO_4 + OH^- \rightarrow NH_3\uparrow$$

3-Titration of unreacted acid with standardized solution:

At turning point,molesHCl=molesNH$_3$

1ml of 0.1N HCl =1.4008 mg N

or 1ml 0.1253 N HCl =10 mg Protein


1
increase bp with
potassium sulfate
Boil
H2SO4
Catalyst (Hg, Cu, Se)
(NH4)2.SO4
NaOH
2
NH3
3
Standard HCl
Titrate
Borate
With
Acid
NH3
Boric acid
NH3
NH4 + H2BO3-
H2BO3-

Kjeldahl Method

Calculation:

Gram nitrogen/ gram of sample =

$$\frac{*(\text{ml of sample} - \text{ml of blank}) \times \text{N standard acid} \times 0.014\text{g/meq}}{\text{weight of sample}}$$

* ml of hydrochloric acid required to titrate sample solution.

KJELTEC: Automated device using Micro Kjeldahl principles(20 samples/hour)

Disadvantages: not all N is protein.

Purine

Pyrimidine DNA, RNA, etc.

Urea

Many plant tissues have > 50% non-protein N.

% N × 6.25 = % Protein

B. DUMAS Method(1830's) :

3 Steps:

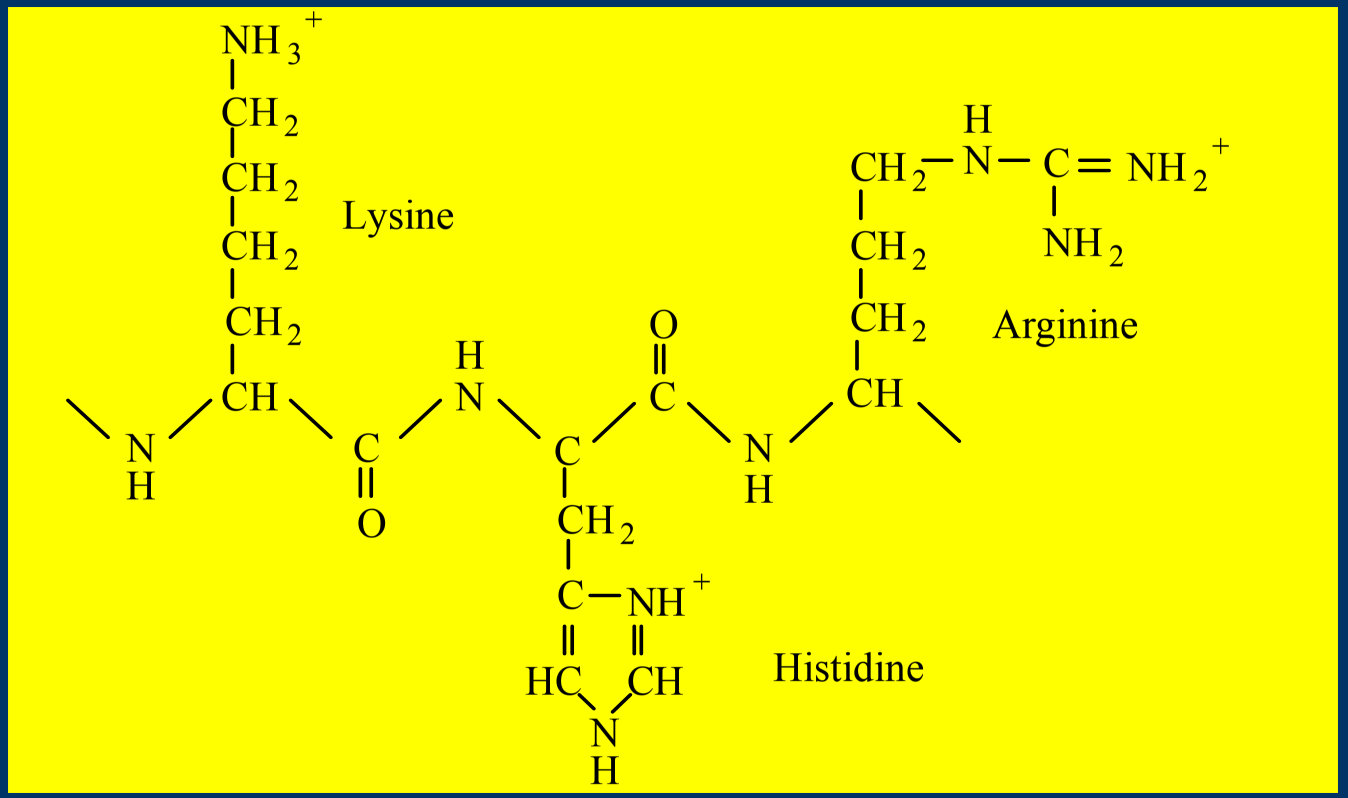
1. Pyrolysis: Nitrogen is freed from organic matter by pyrolysis(700-900°C), under a stream of O2 to yield a gas mixture(N2, NOx, , CO, CO2,CH4) and ash .
2. Reduction: Nitrogen oxides in the gas stream are reduced to N gas by heated metallic copper. NOx→N2
3. Quantification: The interfering gases (CO_2, CH_4, CO) are removed by special chemical adsorbants and N gas is determined either volumetrically or now by Gas Chromatography using TCD thermal conductivity detector.

Small sample sizes(0.1g) and takes 2 minutes; however homogenization is not easy.

2. Dye Binding Methods

Principle:At low pH, basic groups of protein are (+) charged. These will quantitatively bind a (-) charged dye.

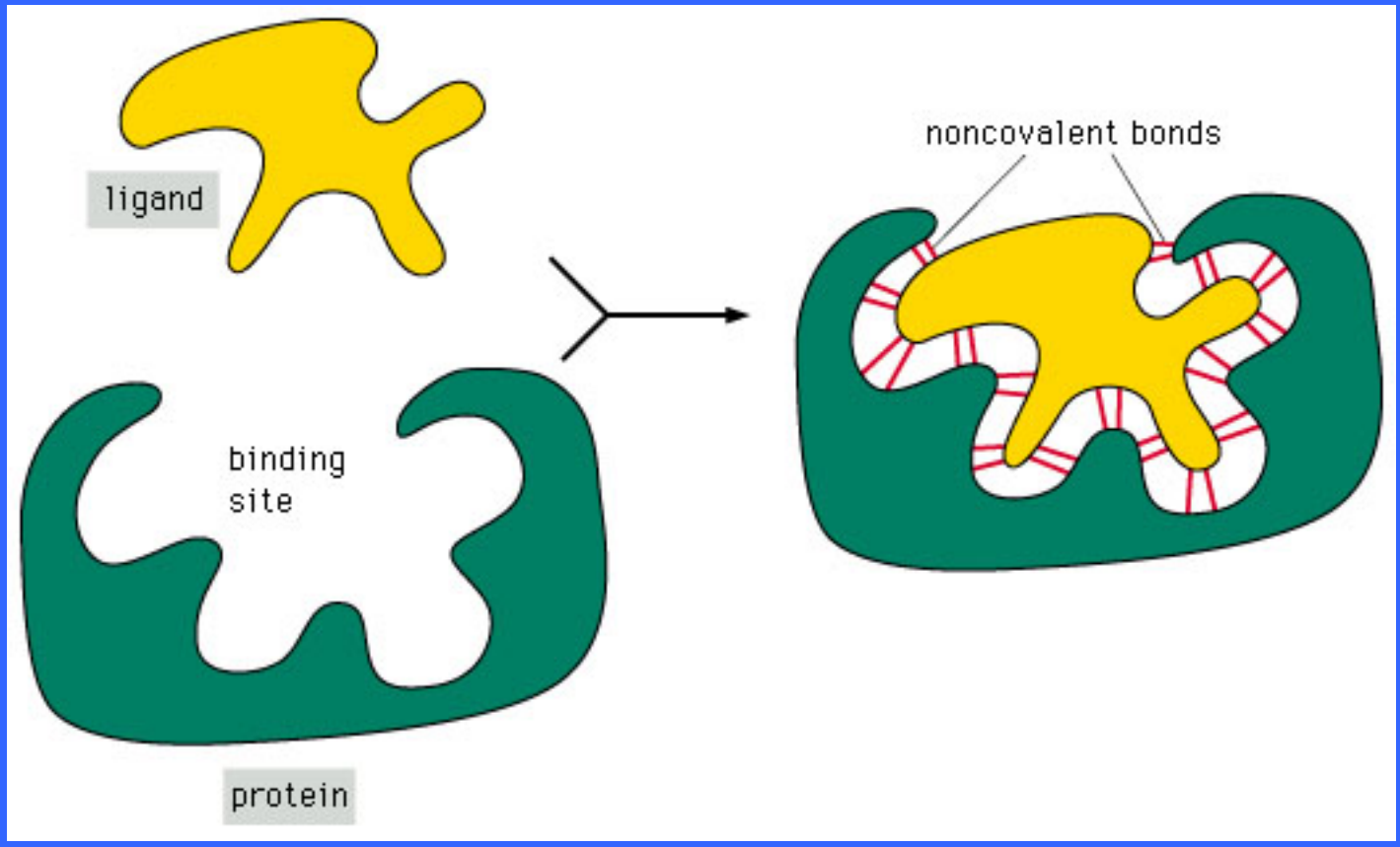
Examples of these basic groups are shown below:



“DYE BINDING METHOD”s, cont.

Under specified conditions, the total acidic or basic groups of proteins bind quantitatively with certain organic dyes, forming insoluble complexes. After separation of this complex, by determining amount of of unbound dye in solution , we can deduce protein contents. But all spectrophotometric evaluations need to be correlated with standardised methods and specific calibration graphs.

Binding selectivity-Example

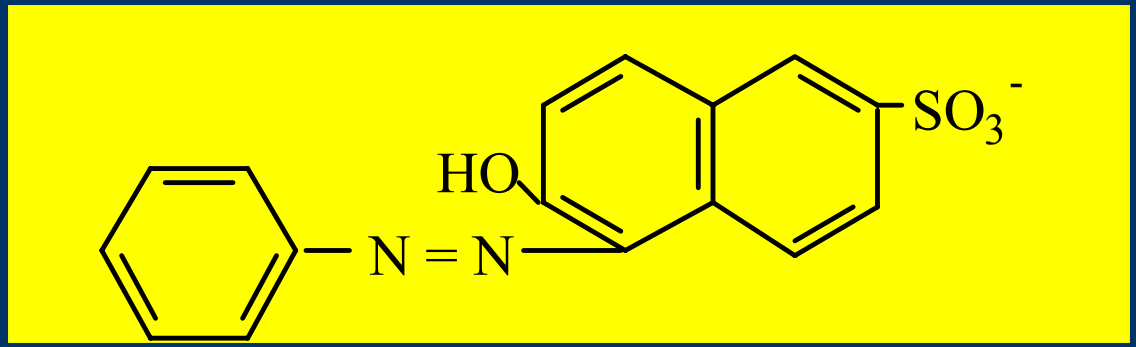


Cationic groups of the amino acids react with an anionic sulfonic acid dye (i.e Amido Black)

The more dye that was bound, the more protein present in the sample.

Example for Dye Binding Method

Acid Orange 12:

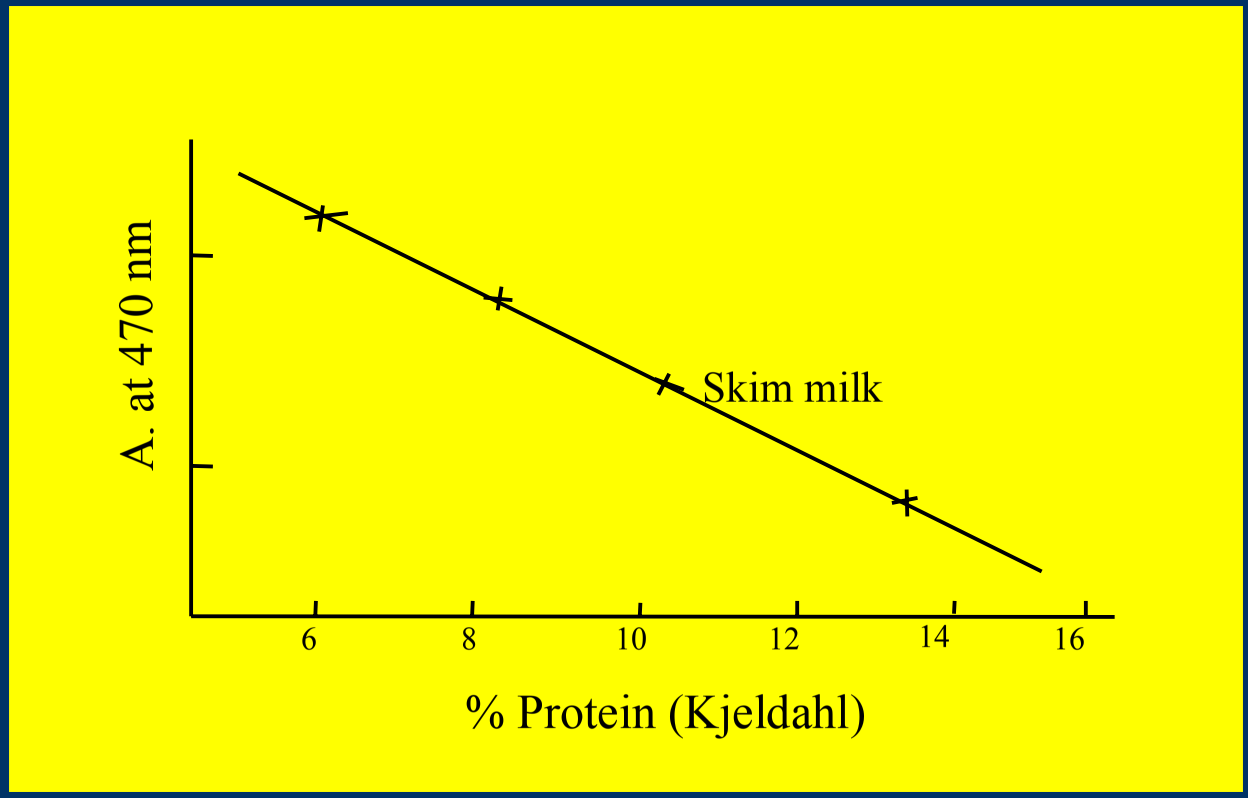


Procedure:

1. Mix protein, dye, buffer pH = 2.
2. Filter or centrifuge.
3. Measure optical density (O.D.) of filtrate.

Binding

Absorbance of dye bound by protein = (A dye initial - A filtrate)



Dye Binding Method

Factors Influencing Dye Binding determination:

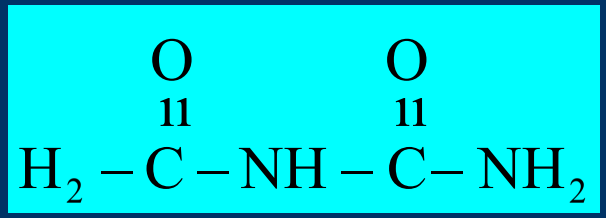
1. Temperature
2. Non-proteins.
3. Buffers systems.
4. Protein quality.

II.Methods Measuring Proteins Directly

These depend on characteristic reactions of proteins.

Example 1:

BIURET METHOD: Colorimetric (1914) method depending on the principle that proteins (or any molecule with more than 2 peptide bonds) form a purple-coloured complex with Cupric salts(i.e. CuS04) in alkaline solutions. It is used mainly for proteins in solutions and is simple, rapid and inexpensive.

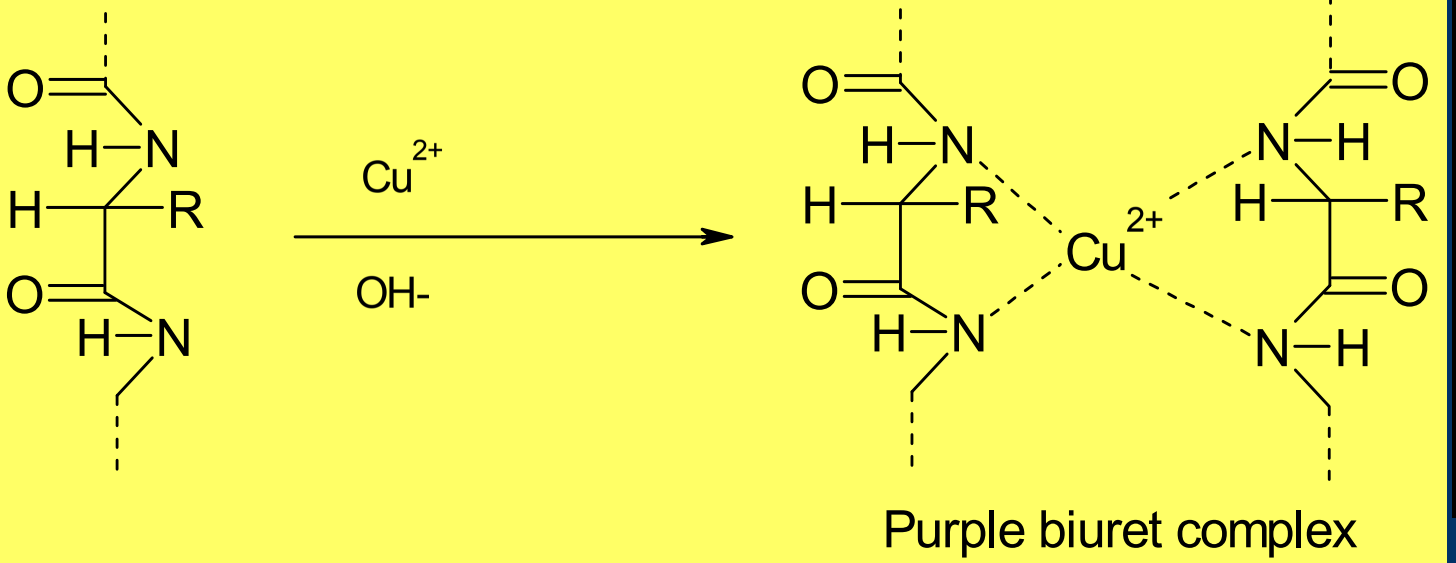


urea urea

"biuret"

The Biuret Method:

- Cupric ions react with peptide bonds under alkaline conditions
- (copper sulfate + K-Na-tartrate + alkali)
- Measure color in SPEC at 540 nm

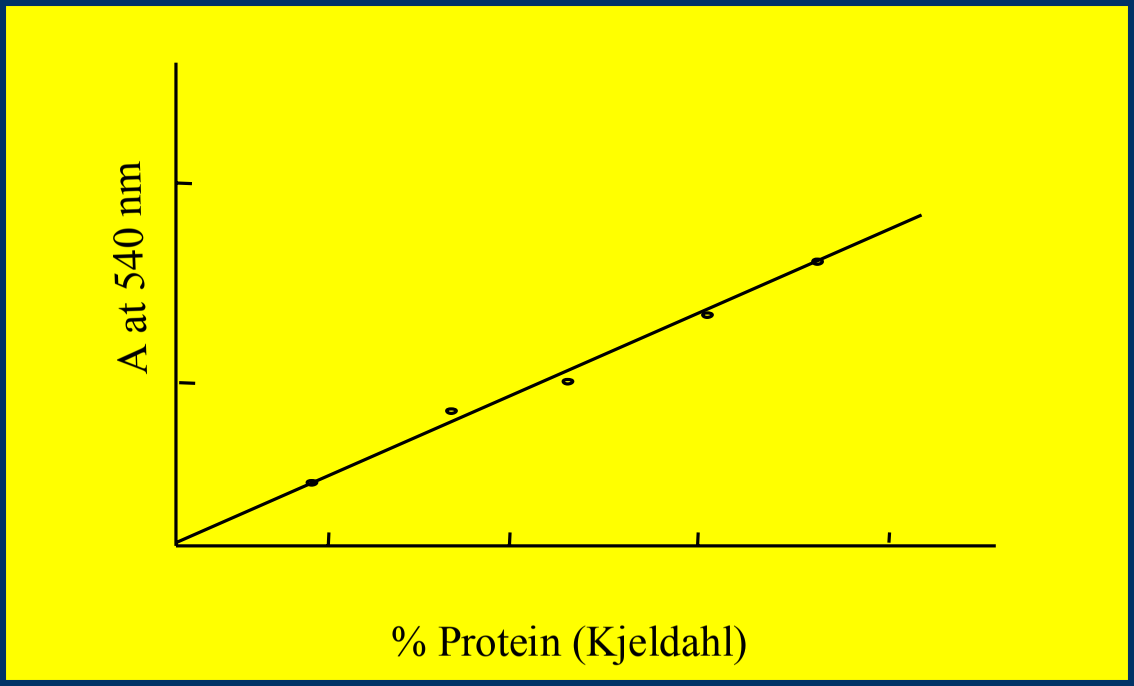


Purple biuret complex

Biuret Method (cont.)

Principles: Cu++ in alkaline solution form complexity with peptide bonds - giving pinkish-purple color.

Measure the intensity of color at 540 nm.



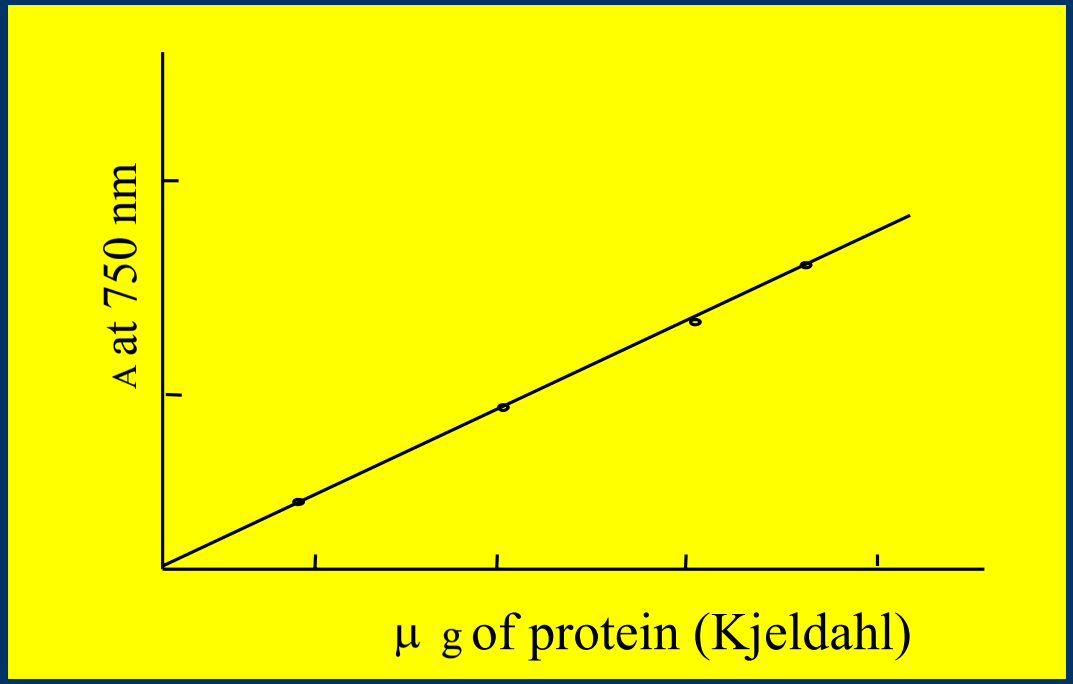
Ex 2:LOWRY METHOD

Principle: Proteins interact with phenol reagent* and copper under alkaline conditions, oxidizing the aromatic amino acids.

***Folin-Ciocalteau Reagent: Mixture of Phosphotungustic and phosphomolybdic acid solutions, yielding a blue colour following copper-catalyzed oxidation of a.a.,which is read spectrophotometrically.**

Lowry Method

- Cu^{++} in alkaline solution to form complexity with protein.
- Cu++ catalyses oxidation of phenol group of tyrosine with phosphomolybdic-phosphotungstic acid.



III. INDIRECT Methods

1.Nephelometry and Turbidimetry:
Turbidity resulting from precipitating proteins with a precipitating agent(Ex: 3-10%Trichloroacetic acid, sulfosalysilic acid, potassium ferricyanide) will be proportional to protein content and is to be measured by reduction in transmittance values at 600nm or direct turbidometer readings Most suitable for nitrogen compounds in solution.

2.Thermal Analysis Techniques: Relying on measuring the heat changes in thermal decomposition of proteins under controlled conditions. Neutron activation and proton activation analyses are also being used.

Ultra-violet Absorption (UV) at 280 nm

1. Chromophoric side chains of aromatic amino acids (Trosine, Tryptophan).
2. Absorption at 280 nm. "Non-destructive means to determine protein".
3. Calculation protein conc. based upon absorption value.

Fluorescence Method

Tyrosine is a fluorescent compound.

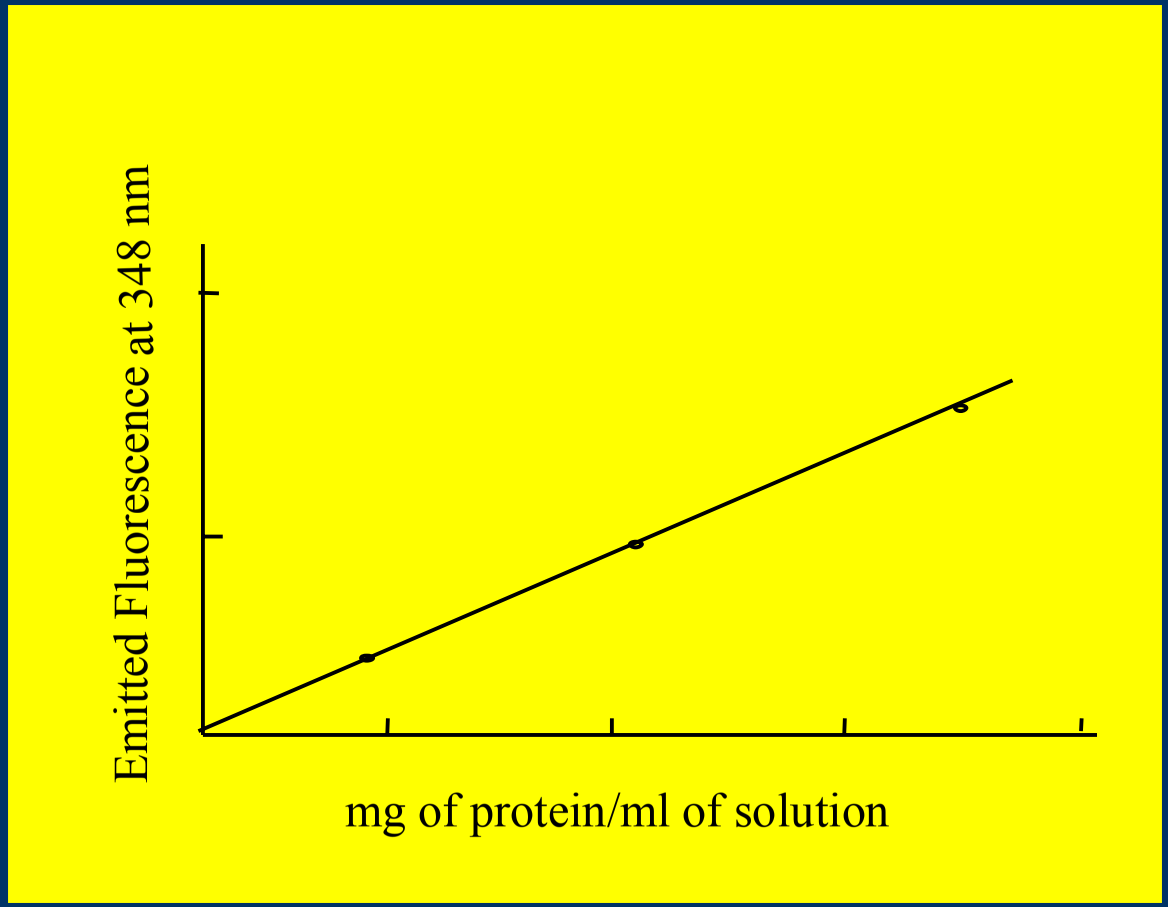
Tryptophane is a fluorescent compound.

Excite these amino acids at 280 nm.

Measure emission at 348 nm.

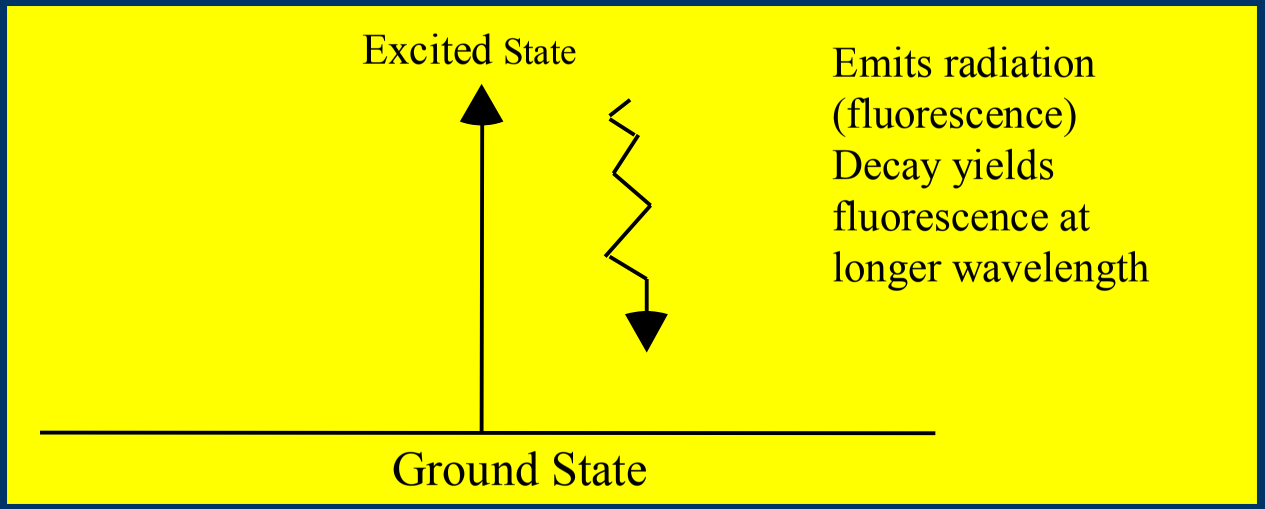
Advantage: more sensitive than UV absorption.

Fluorescence Method



Principle of Fluorescence Methods

What is fluorescence and how to measure it?



By using specific λ (wavelength) to excite and measure output at a specific λ. It is rather specific.

Problems: Turbidity/Quenching (self or others)/Expensive/ Quantitation is difficult.

Infrared SPECTROSCOPY

- IR spectroscopy measures the absorption of radiation in near or mid IR regions by molecules in foods.Different functional groups absorb different frequerncies of radiation: for proteins,6.47μm,1560-1670 μm and 3300-3500 μm are characteristic for the peptide bond. Therefore, irradiating a sample with the characteristic wavelength and measuring the energy that is reflected or transmitted by sample (which in turn is inversely proportional to the energy absorbed and protein content) can lead to quqntitative estimations of protein content.

B-Methods for assessment of protein quality

B.I. In vivo test: Ratio of the regression coefficient of growth of Protoza of genus *Tetrahymena*, Bacteria like *Streptococci*, *Leuconostoc*, Fungi like *A. Flavus* (i.e. Dry mycelial weight after 72 hours) on N content of test material to the regression coefficient of the growth of same organism on the N content of casein gives "nutritional index of protein quality". (Study pp.268-276 in your book)

Essential amino acids?

Amino acids which the body cannot make (or make enough of) for protein synthesis due to lack of enzymes.

Essential Amino Acids:

Histidine,	Isoleucine,	Leucine
Lysine,	Methionine,	Phenylalanine
Threonine,	Valine	

Limiting amino acid is the essential amino acid which is lacking in the protein to have a balanced protein.

Product	Limiting Amino Acid
Corn	Lysine
Oats	Lysine
Rice	Lysine
Wheat	Lysine
Sesame Seed	Lysine
Cow's Milk	Methionine
Potato	Methionine
Chick Pea	Methionine
Green Pea	Methionine
Cotton Seed	Isoleucine
Beef	Valine
Chicken	Tryptophan

PROTEIN QUALITY DETERMINATION

1. Protein Efficiency Ratio.
2. Biological Value.
3. Net Protein Utilization.

What are the measurements of protein quality?

For labeling purposes, one needs to know the protein efficiency ratio.

1. If PER = casein (2.5), the RDA = 45 g/day.
2. If 0.5 < PER < 2.5, then RDA = 65 g/day.
3. If PER < 0.5 (20% of casein), then “not a significant source of protein”.

How does one determine PER?

1. Male lab rats ≥ 21 days, ≤ 28 days of age, at least 10 rats/group.
2. Feed a standardized diet containing salt mix, vitamins, cotton seed oil, cellulose, starch or sucrose + water for 28 days.
3. Measure weight gain and food intake at regular intervals, not > 7 days.
4. PER = Weight Gain/Gram of Protein in Diet.
5. Usually normalized for casein = 2.5.
6. Determine protein quality of sample as ratio of sample PER to reference casein PER.

Protein Efficiency Ratio = Gain in weight per gram protein taken.

Product				PER
Rice	100%			2.30
Rice	70%	Black Beans	30%	2.70
	50%		50%	2.60
	20%		80%	1.30
			100%	NIL
Corn				
	+ 0.4% Lysine			
	+ 0.07% Tryptophan			2.14
Corn (50%) + Black Beans (50%)				2.05

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Product	PER
Soybean	2.32
Cotton Seed Meal	2.25
Egg	3.90
Chick Peas	1.68
Peanuts (ground nuts)	1.65
Kidney Beans	0.88

OTHER PROTEIN QUALITY DETERMINATION

Biological Value (BV)

Net Protein Utilization (NPU)

BV = Retained Nitrogen (nitrogen intake - fecal & urinary nitrogen)/Absorbed Nitrogen (nitrogen intake - fecal nitrogen)

NPU = Retained Nitrogen/Intake Nitrogen = BV × Digestibility

In-vivo Indices of Protein Quality: Index→indices

$$\text{Biological value} = 100\,\frac{B^{*}-B_{0}}{A^{**}}$$

B = Nintake - Nexcreted by test animals,

B_0 = B for animals fed a nonprotein diet,

A = Nintake - (Nexcreted by test animals - Nexcreted by animals fed nonprotein diet)

NPRatio=[Gain in b.w.with test protein+loss in b.w. With N-free diet]/Protein intake

$$\text{PER (Protein efficiency ratio)} = \frac{\text{gain in body weight}}{\text{protein intake}}$$

$$\text{NPU(Net protein utilization)} = \frac{\text{Body N content of animal fed with test protein} - \text{Body N cotent of animal on N free diet}}{\text{N intake of test animal}}$$

PROTEIN QUALITY TESTIN (Nutritional Value)

- **In vivo tests – monitor** animal growth, nitrogen balance – measure how well a protein is metabolized and used by body. These tend to be expensive and time consuming.

Adult - 0.75g/kg body weight (ca. 52.5g/70 kg) must be equivalent to egg or milk proteins – standards of quality.

Biological Tests

- Measures the growth rate of young rapidly growing rats relative to casein (not a great protein as reference)
- Over estimates the value of some animal proteins relative to humans.
- Under estimates the value of some plant proteins.
- Problem with an animal model: rats during rapid growth need higher levels of certain amino acids than humans.
- Costly and time consuming

B.II. In vitro tests for protein quality :

A. Quantification of individual essential a.a. is done mainly by HPLC or Automated aminoacid analyzer.

Sample preparation involves 3 stages:

1. Hydrolysis of protein(breaking down the peptide linkages) which is done either with 6N HCl at 110°C or with enzymes.
2. Separation by ion-exchange chromatography: The hydrolysed amino acids are eluted from an ion-exchange resin column,by stepwise changing the pH and ionic strenghth of eluent and each a.a. being eluted at a specific rate correlated with its molecular size and charge.

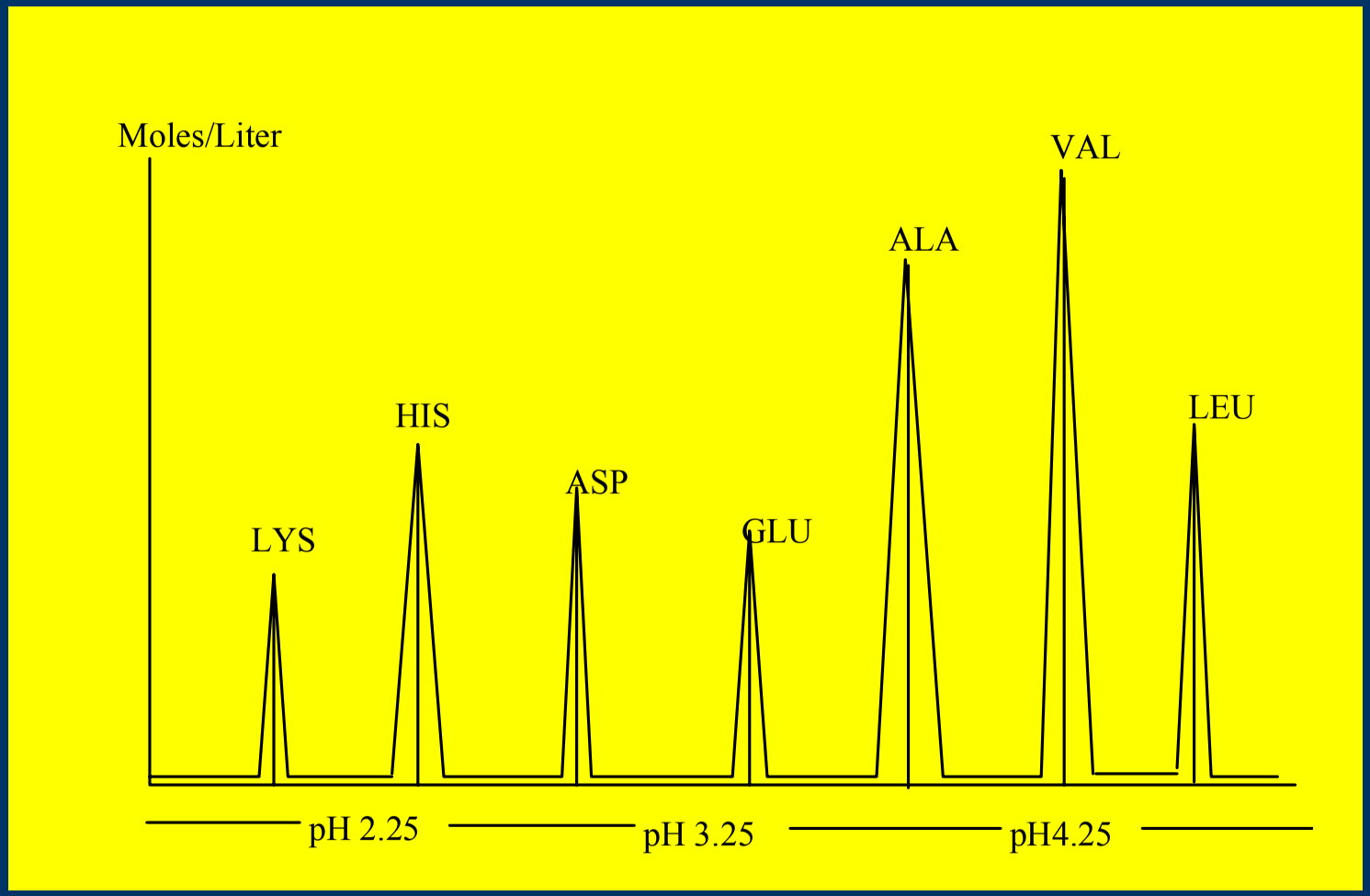
3. Quantification with ninhydrin: The Automated a.a. analyzer uses a Ninhydrin reagent, which together with each eluting a.a., form a pinkish - violet colour, the intensity(or Absorbance value at 570nm) of colour being correlated with a.a. concentration.

In parallel, a "standard a.a. mixture" (2.5μmoles of each a.a./5 ml) is also injected into the liquid column.

Results can be expressed in either of the following basis:

- [moles a.a./100 g sample]
- [mg a.a./100 g sample]
- [mg a.a./g N in sample]
- [mg a.a./100 gr N in sample]
- [mg a.a./16 g N in sample]
- [mg a.a./100 g dry sample-d.m.basis]

CHROMATOGRAM OF AMINO ACIDS



Calculations for converting a.a. content from one basis to another:

Given a food sample with following composition:
N%= 1.571; Protein%= 1.571x6.25=9.82%
Dry matter%= 86.48;
Glutamic acid= 4436 mg/100gr gr of sample
(MW of glutamic acid= 147.13).
Express results of glutamic acid analyses in 4 different bases:

2823 x 16= 45168 mg glutamic acid/16 g N

$$\frac{4.436}{1.571} = 2323 \text{ mg glu/g.N}$$

$$\frac{4436}{147.13} = 30 \text{ moles glu./100 gr sample}$$

$$\frac{4436}{86.48} \text{x} 100 = 5129 \text{ mg glu./100 g dry matter}$$

B. Bioavailability of Aminoacid : During processing, a.a. side-chains may be degraded or may interact with sugars to form typical aromas.For example, in baking or cooking for long durations,lysine very easily binds to glucose, becoming unavailable nutritionally. To quantify the bioavailability, following reagents may be used:

1. DNFB (dinitrofluoro benzene) reagent at alkaline pH reacts only with free lysine,producing yellow compounds.

2. Dansyl chloride in borate buffer at 40°C reacts with free but not with bound lysine. The didansyl lysine peak gives indication of the amount of available lysine.

C. Non-Protein Nitrogen Determination

These include free amino acids, nitrosamines, nitrites and nitrites, ammonia and ammonium compounds. These can be separated from the proteins by either one of the following protein separation methods: Dialysis, ultrafiltration with special membranes, precipitation with heat or by protein-precipitants like trichloroacetic acid, picric acid, tannic acid, sulfosalysilic acid, phosphotungstic acid.

1.Ammonia and ammonium compounds can be detected with "Nessler Reaction or Nesslerization": Nessler reagent [KOH+HgI+KI] reacts with NH_3 yielding a red-orange to brown colored complex, which can be quantified spectophotometrically at 440nm(p.754-5).

$$NH_3 + K2HgI_4 + 3KOH \rightarrow 7KI + 2H_2O + Hg_2OINH_2$$

(red-colored complex)

2.Nitrates,nitrites:
Spectrophotometric determination after reducing; Griess-Ilosway Procedure: Interaction with primary aromatic amines, like Sulfanilic acid, in acid solution, to form a diazonium salt, which is then coupled with an aromatic compound (i.e.naphtylamin) to from an azo-colour(p.767).

3.Free aminoacids and a.a. salts (i.e.MSG):
2-dimensional TLC. Individiual a.a. can be qualitatively determined by TLC and then be subjected to specific colour reactions (Ex. With ninhydrin reagent) Free a.a. can also be quantified by Formol titration where the amino group is blocked with formaldehyde, and then the free COOH group is titrated with NaOH.
4.Nitrosoamines

C-Methods for elucidating protein structures:

1. A.a. sequence deciphering or determination; This has made insulin(51 a.a.in insulin molecule) synthesis possible(1950's-SANGER). The principle behind these methods involves stepwise degradation of peptides by specific chemical reagents:

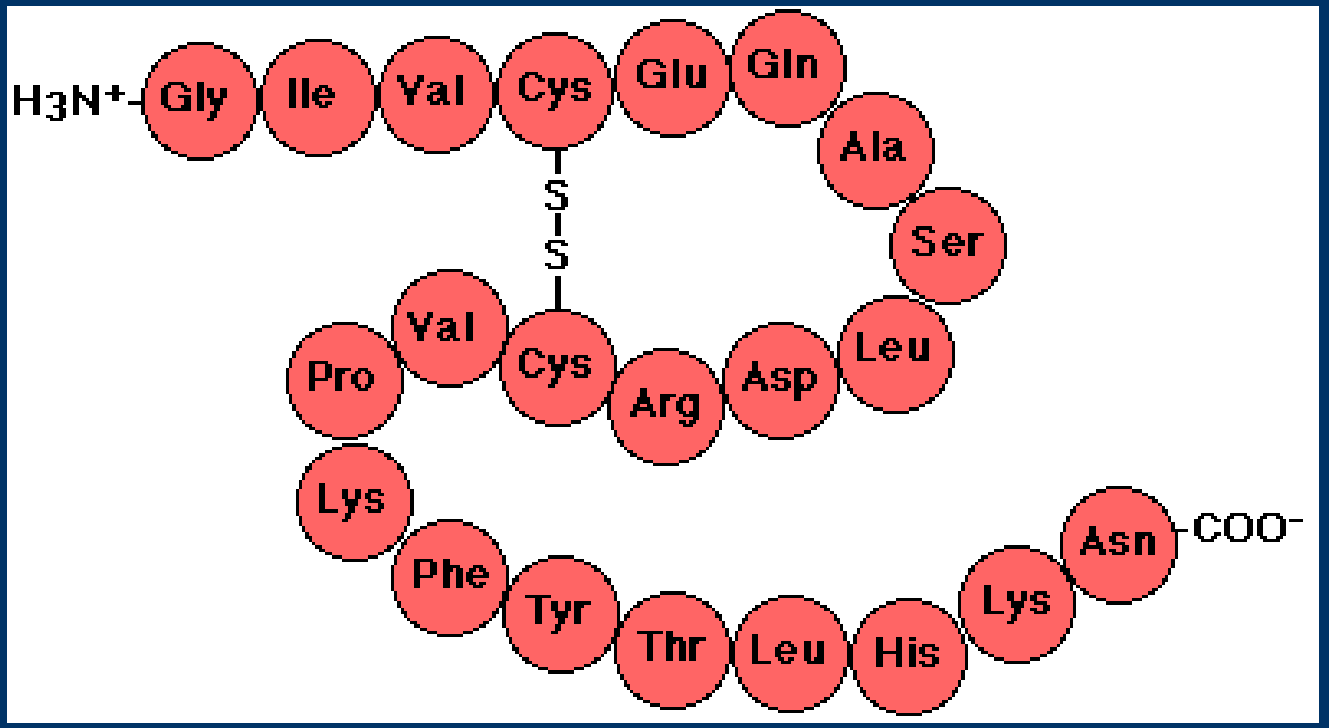
a. Sanger method:The α-amino group forms a yellow complex with FNDB(fluoronitrodibenzene) reagent which is soluble in nonpolar solvents. The Dinitrophenyl complex formed is then eluted with CHCl3 and subjected to a.a. analysis.

b. Edman degradation: involves formation of "a.a. Hydantoins" with phenylisothiocyanates.

2. Conformational analysis for determining stereochemistry. Primary, secondary, tertiary and quaternary structures (α-helix shapes, pleated sheets, s-s and s-h linkages etc.) are being determined mainly by X-RAY crystallography.

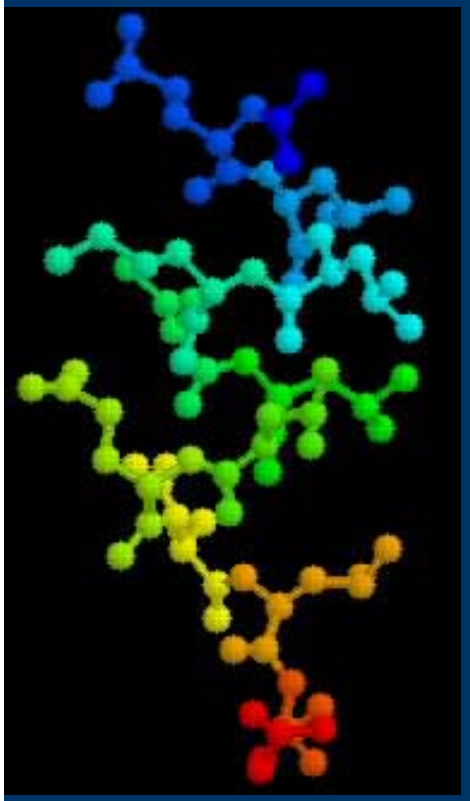
Protein Structures

Primary Structure of Protein



Primary Structure: due to covalent peptide bonds of individual amino aci

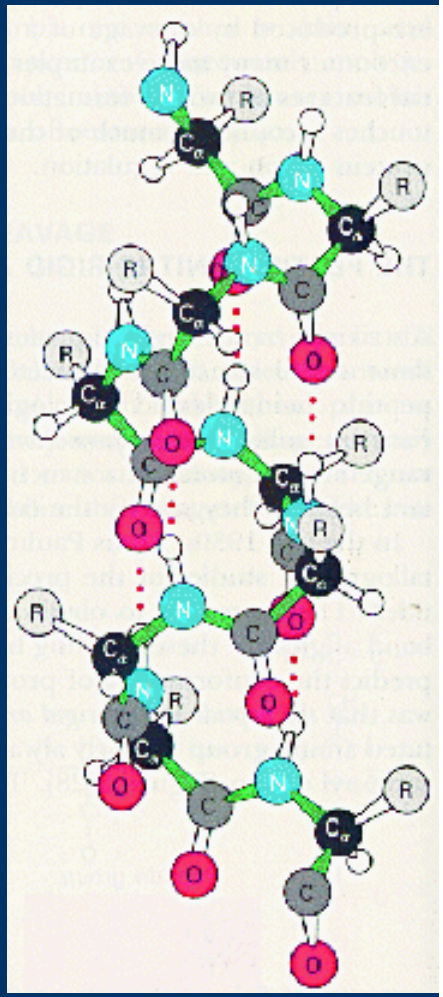
Secondary Structure of Protein



Secondary Structure: due to hydrogen bonding between peptide bonds.

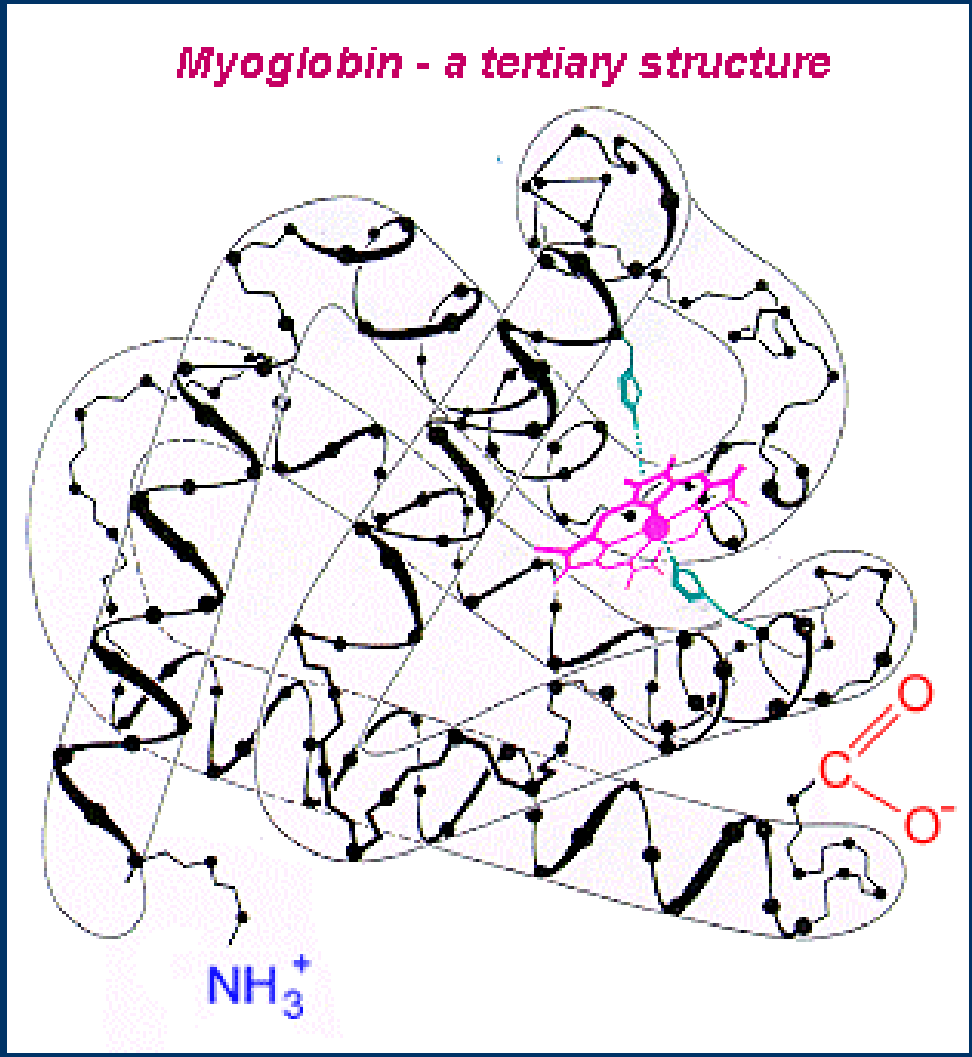
Kinds of Secondary Structure:

1. a - Helix
2. Pleated sheets structure
   - A. Parallel
   - B. Anti-parallel



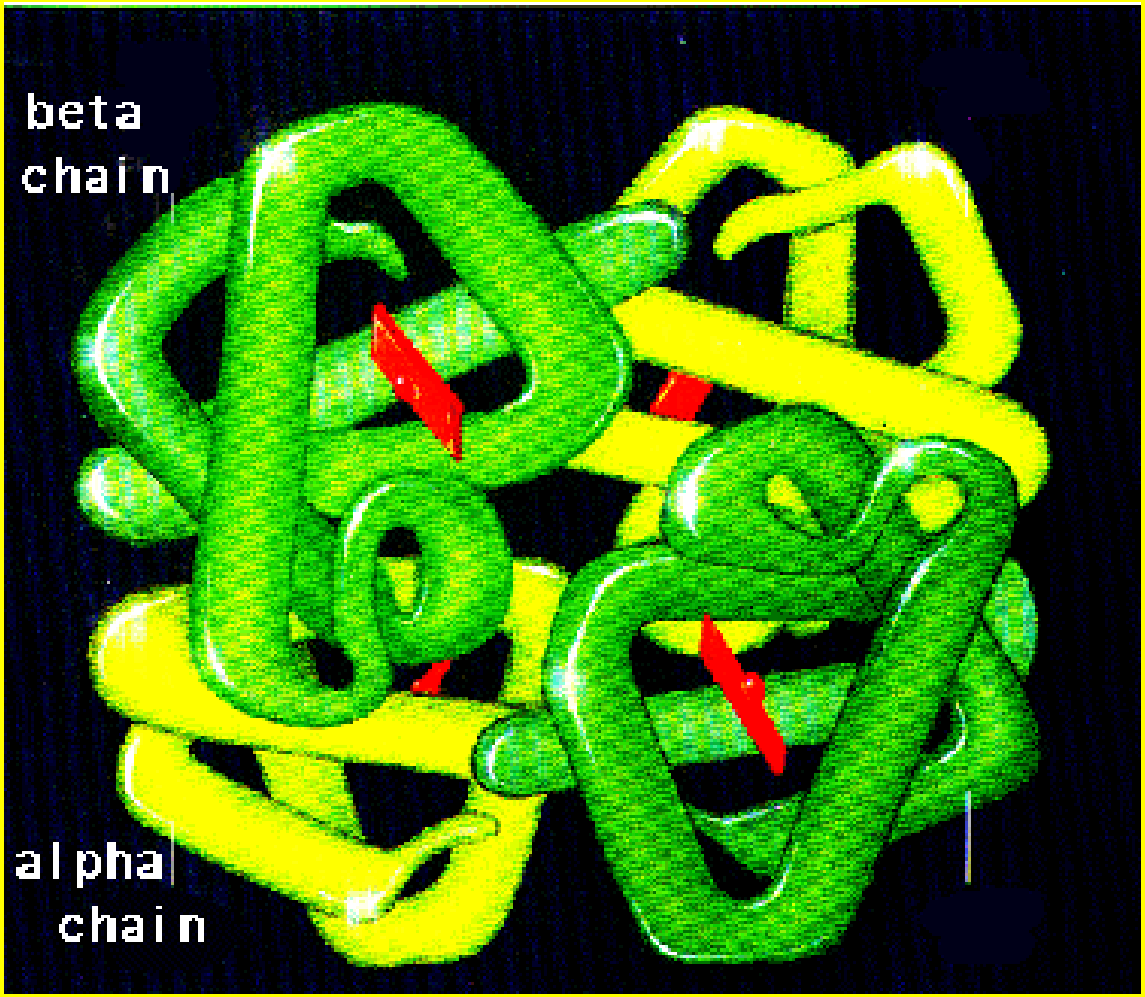
Tertiary Structure of Protein

Tertiary Structure: aggregation of individual protein through hydrogen, ionic, hydrophobic and disulfide bonds



Quaternary Structure of Protein

Quaternary structure
Aggregation of several peptide chains to form a definite molecule by ionic bond, hydrogen bond, and/or hydrophobic bond



Separation techniques for proteins

1. Separation by differential solubility characteristics:isoelectric precipitation

2. Separation by adsorption:ion exchange chromatography and HPLC

3. Separation by size: Ultrafiltration and dialysis

4. Separation by electrophoresis

Electrophoresis:

Definition: "Migration of charged molecules in a solution through an electrical field"

A. In polyacrylamide gel electrophoresis, proteins are forced to migrate in aqueous buffers through a solid matrix(polyacrylamide gels) under the influence of an electrical field. Migration or mobility of specific proteins is a function of the net charge on molecule, as well as molecular size, shape.

B.In isoelectric focusing, ph gradients are formed using ampholytes(buffer solutions), and proteins migrate to the location in the gradient at which ph=their isoelectric point.

C.Capillary eletrophoresis:capillary tubing is used in place of gels

Visualisation of Proteins

Fluorescence microscopy after reacting with a dye(ANS-anilineonaphtalene sulfonic acid) which renders only proteins fluorescent is used. Staining intensity is influenced by compositional differences in protein and also by structural changes due to processing.