Today I want to consider methods used to determine the amino acid composition of proteins, i.e., the number of each amino acid in a protein.

Determining the a.a. composition involves three basic steps as indicated in the handout.

The first step is hydrolysis of the protein to its constituent a.a..

Hydrolysis refers to cleavage of the peptide bonds that is catalyzed by strong acid, strong base or proteins called peptidases.

For acid hydrolysis, the protein is dissolved in 6N HCl, sealed in an evacuated glass tube and heated to 100° for 20 hrs-

Under these conditions trp is largely degraded. Asn and gln are converted to asp and glu plus NH_4^+ as a result of the hydrolysis of the amide group-

Base hydrolysis is carried out in 2-4 N NaOH at 100° for 4-8 hrs. Under these conditions cys, ser, thr and arg are destroyed. Base hydrolysis can be used to measure the trp content.

A mixture of various endo- and exopeptidases can also be used to completely hydrolyze a protein without a change in the a.a..

The conc. of peptidases must be kept low (<1% by weight) since the hydrolysis of a peptidase molecule by other peptidase molecules in solution will also contribute to the mixture of a.a..

The hydrolysis by peptidases is primarily used to determine the trp, asn and gln in a protein.

The second step in a.a. analysis involves the separation of the a.a. in the mixture following hydrolysis.

The first method developed used ion-exchange chromatography as described in the handout.

The mixture of a.a. in the acid hydrolysis solution is applied to the top of a glass column containing a cation exchange resin such as sulfonated polystyrene.

In acid solution, all of the amino acids will be positively charged and bind to the resin.

The a.a. are then separately eluted from the column by passing buffer solutions of increasing pH through the column.

As the pH of the eluting buffer increases, the positive charge on the a.a. decreases and the a.a. become less tightly bound to the negatively charged resin.

The acidic a.a., asp and glu, are least positively charged and tend to be eluted first, while the basic a.a., arg and lys, are most positively charged and tend to be eluted last.

The hydrophobic nature of the polystyrene also contributes to the separation; the more hydrophobic the a.a. the more tightly it is bound to the resin.

Fig T7.1 shows the elution pattern of the a.a. from the column. The a.a. corresponding to each peak was determined from the volume of effluent (or time) required to remove each a.a. in separate experiments.

Note as expected that asp is eluted first because it is the most acidic a.a. (lowest pI) and therefore the least positively charged at any pH.

Arg is eluted last because it is the most basic (highest pI) and therefore the most positively charged at any pH.

The third step in a.a. analysis involves quantitating the amount of each a.a. as it comes out of the column.

In automated a.a. analysis the effluent is continuously mixed with a solution of ninhydrin which reacts with a.a. and ammonia to form a purple-blue compound as shown in Fig T7.2.

The colored solution is passed through a flow cell of a spectrophotometer to record the absorbance which is proportional to the concentration of the a.a..

The amount of each a.a. is determined from the area under each peak of the absorbance vs time elution curve shown in Fig T7.1.

More recently a.a. are derivatized with o-phthalaldehyde and 2-mercaptoethanol as shown in the handout and then separated by reversed phase chromatography.

Separation is based on differences in solubility of the a.a. derivatives in a nonpolar solvent relative to a polar solvent.

The resin has nonpolar groups on its surface and will have nonpolar solvent molecules surrounding it forming a nonpolar stationary phase.

The moving phase which carries the a.a. through the resin is more polar.