CHAPTER 2

MATERIALS AND METHODS

Materials :

1. The fresh lucerne plant material was supplied by the Agriculture College, Kolhapur.
2. L-arginine hydrochloride and other pure amino acids as well as N-Bromosuccinamide were the products of Sisco Reseach Laboratories.
3. Ninhydrin was obtained from Spectrochem Ltd. Bombay.
4. α - Napthol was the product of Merck (India).
5. Ion exchange resin Dowex 50X4, and DEAE cellulose were obtained from Serva.
6. Bio-Gel P-2 was obtained from Bio-Rad Laboratories.
8. All other chemicals used were of Analar grade.

Methods :

2.1 Preparation of Buffers :

1. Citrate Buffer : 20mM citrate buffer was prepared by mixing citric acid stock solution (A) and Sodium citrate stock solution (B) (96).

CHAPTER 2

2.1 Preparation of buffers

2.2 Extraction of Lucerne

2.3 Removal of protein and macromolecules by dialysis

2.4 Determination of free amino acids

2.5 Determination of carbohydrates

2.6 Quantitative determination of proteins

2.7 Isolation of potato starch

2.8 Fracrtionation of potato starch into amylose and amylopectin

2.9 Ion-exchange chromatography

2.10 Paper chromatography

2.11 Gel chromatography

Stock Solutions :

A : 0.421 gms of citric acid was dissolved in 100ml of distilled water.

B : (20 mM) 0.588 gms of sodium citrate was dissolved in 100 ml of distilled water.

Appropriate quantities of A and B were mixed to get the desired pH in the range of 3.6 - 5.6.

2. Phosphate Buffer : Stock solutions of sodium dihydrogen orthophosphate and disodium hydrogen phosphate were prepared (96). required quantities of the stock solution were mixed to obtain the necessary pH (range of 5.7 - 7.5).

3. Pyridine Acetate Buffer : The following stock solutions of pyridine and acetic acid were prepared.

A : Pyridine (10 mM), 1.65 ml of pyridine in one litre of distilled water.

B : Acetic acid, - 1.2 ml of acetic acid in one litre of distilled water.

Stock A and B were mixed in the required quantities (97) and diluted to one litre with distilled water. pH range obtained was 2.5 - 6.5.

4. Tris - HCl Buffer : 60.0 mg of Tris was dissolved in one litre of distilled water, the pH of which was adjusted to 8.3 with dilute HCl.

2.2 EXTRACTION OF LUCERNE

The fresh lucerne leaves and soft stems were weighed and washed. The extract was prepared by homogenizing it in a homomgenizer with high speed for 5 - 10 minutes using sufficient quantities of distilled water. The homogenate was filtered through cheese cloth and squeezed out. The squeezed material was again ground in a homogenizer with sufficient quantity of distilled water and fitered by the same procedure. The filtrate was then centrifuged at 6000 rpm for 20 min. The precipitate of chlorophyll and cell fibre was discarded. To the supernatent a few drops of Toluene or n - butanol was added to prevent bacterial growth.

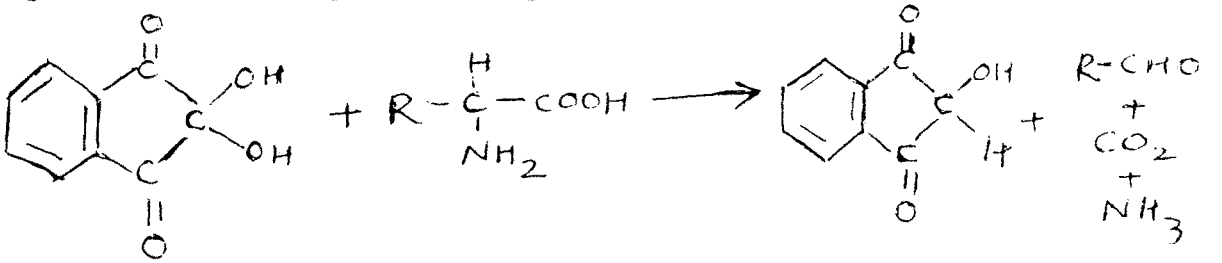
2.3 REMOVAL OF PROTEINS AND MACROMOLECULES BY DIALYSIS

The lucerne extract was dialysed using cellophane bag (23 x 32 pore size) against distilled water containing few drops of n - butanol. The dialysate so obtained was used for adsorption on amylose gel and ion - exchange

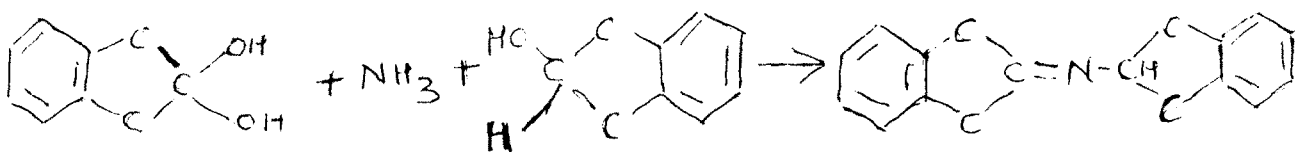
chromatography. The proteins and other macromolecules in the dialysis bag were discarded.

2.4 DETERMINATION OF FREE AMINO ACIDS :

(a) Ninhydrin Assay : The free amino acid concentration of lucerne dialysate was determined spectrophotometrically by the use of ninhydrin reagent (98) Ninhydrin reacts with ∝ - amino acid to cause decarboxylation and deamination. In the process it undergoes reduction to hydrindantin. One molecule each of ammonia, hydrindantin and ninhydrin combine to form purple coloured complex having absorbance maxima at 570 nm.



Ninhydrin Reduced Ninhydrin



Ruhemann's purple Complex

Reagents :

1. Ninhydrin Reagent : 3 gm of Ninhydrin was dissolved in 100 ml of distilled 2 - methoxy ethanol.

2) Acetate Buffer : 360 gm of Sodium acetate trihydrate was dissolved in 500 ml of water to which 66.6 ml of glacial acetic acid was added and it was further diluted to one litre with water to give a pH of 5.3.

3) Sodium Cyanide Solution : 45 mg of sodium cyanide was dissolved in 50 ml of distilled water. The solution was neutralized to pH 6.5 - 7.0 and diluted to 100 ml with water.

4) Cyanide Acetate Buffer : 20 ml of above sodium cyanide solution (0.01M) was diluted to one litre with acetate buffer.

5) Diluent : 500 ml of ethanol (distilled) was mixed with 500 ml of water.

6) Standard Leucine or Arginine Solution : 100 mg of l- leucine or l - arginine was dissolved in 100 ml of distilled water. A twenty fold dilution of this solution was used as standard leucine or standard arginine solution (10 - 50 U g / ml).

Experimental :

One ml of sample containing 0 - 50 U g of leucine, 0.5 ml of cyanide acetate buffer and 0.5 ml of 3% ninhydrin solution were mixed. The mixture was put in a stoppered tube and kept in a boiling water bath for 15 min The intense pink colour developed was immediately diluted by adding 5 ml of diluent. Each tube was shaken vigorously and allowed to cool to room temp. The optical density of the colour developed was recorded on spectronic 20. The calibration curve for leucine / arginine was obtained by plotting the concentration of the desired amino acid against the optical density recorded at 570 nm on spectronic 20. (Fig. VIII)

(b) Calibration Curve for Arginine by Sakaguchi Method : Arginine can be quantitatively estimated by various processes which include the use of diacetyl (99), 9,10 phenenthraquinone (100), automatic analyser (101) which form α - diketones; p - nitrophenyl glyoxal (102) and Sakaguchi reagent (98). The modified Sakaguchi reaction includes the use of 8 - hydroxyquinolene instead of α - naphthol (102).
The use of immobilized and free arginase and urease enzymes are also employed for detemining the arginine

level in biological samples (103). (Fig. XIX)

In the present study the modified Sakaguchi assay was conducted using N - Bromosuccinamide as oxidant (104).

Reagents :

1. Standard Arginine solution : As given before.

2. ∝- Naphthol (0.1%) : 0.1 gm of ∝- Naphthol dissolved in 100 ml of 95% ethanol (distilled) and store in brown coloured bottles.

3. 40% NaOH : 40 gm of NaOH dissolved in 100 ml of distilled water.

4. N - Bromosiccinamide (0.1%) : 0.1 gm of N - Bromosuccinamide in 100 ml of distilled water.

Experimental :

One ml of sample containing 10 - 50 µg of arginine was mixed with 1 ml of NaOH (40%), 0.2 ml of ∝- naphthol (0.1%) was added and the reaction mixture thoroughly mixed and incubated at room temp. for ten

minutes. N - Bromosuccinamide (1ml of 0.1%) was added. The resulted orange - red colour was measured at 470 nm. The colour is stable for six hours.

2.5 DETERMINATION OF CARBOHYDRATE

a) Estimation of Total Carbohydrate : (Phenol Sulphuric Acid Assay System) The polyhydroxy aldehydes and ketones containing 5 or 6 carbons undergo oxidative dehydration giving furfuraldehyde and hydroxymethyl furfuraldehyde which can form complex with phenol, orcinol, thymol, etc. The red colour is proportionate to the concentration of the sugar. The sample (1.0 ml, 20 - 100 U g) was mixed with 1 ml of 7% phenol, followed by 4.0 ml of concentrated H_2SO_4. The exothermal reaction develops red colour after cooling to room temperature (25° C), the colour is red at 470 nm. (Fig. XX)

b) Estimation of Amino Sugars by Ehrlich's Reagents : This procedure was used to carry out the qualitative extent of amino sugars. Ehrlich Reagent i.e. 10 percent p-dimethyl aminobenzaldehyde in Conc. HCl. The amino sugars give an intense red colour.

2.6 QUANTITATIVE DETERMINATION OF PROTEIN

Protein estimation was carried out using the method of Lowry et al (107). The intensity of colour is dependent on the biurate reaction of protein with copper ion in alkali, and the reaction of Phosphomolybdic - phosphotungstic reagent by tyrosine and tryptophan present in the proteins.

1. Lowry A - 10% Na_2CO_3 in 0.1NaOH.
2. Lowry B - 1% Copper Sulphate solution.
3. Lowry B_2 - 2% Sodium potassium tartarate.
4. Lowry C - It is prepared by mixing freshly 100 ml of Lowry A to 1 ml of Lowry B_1 and 1 ml of Lowry B_2.
5. Folin Phenol Reagent - Commercial preparation was diluted 1 to 3 times.
6. Standard Protein Solution - 20 U g / ml of Bovine Serum Albumin was prepared.

The standard protein volume was adjusted to one ml with varying concentration. Then 5 ml of Lowry C was added. After 15 minutes incubation at room temprature, 0.5ml of Folin phenol was added to each tube. The colour was allowed to develop for half an hour and read at 660 nm. (Fig XXI).

2.7 ISOLATION OF POTATO STARCH

The potatoes are washed peeled, sliced and ground in the waring blender with 2 to 3 volume of the distilled water for about 5 minutes, avoiding damage of starch granules. The slurry is screened through bolting cloth (97 mesh ± 9) with excess of water. The pulp is again ground in blender with sufficient water to form a thin slurry for 5 minutes, which is rescreened and pulp is discarded. The filtrate is allowed to settle for about one to two hours to form a firm dense deposit of starch. The supernatent is decanted. The soft top surface of the settled starch cake is rinsed with 0.14 M NaCl to remove persistant traces of fibre and contaminating proteins and repeatedly washed with water. The starch cake is then suspended in 85% methanol and refluxed for 3 hours to remove contaminated fatty material (97).

2.8 FRACTIONATION OF POTATO STARCH INTO AMYLOSE AND AMYLOPECTIN

A 2% suspension of the defattened potato starch was dispersed in water. Then n - butanol was added till the effective concentration was 8% by volume. The mixture was then allowed to cool slowly to room temperature

after heating constantly for three hours at 80° C with continuous stirring. It was subsequently kept for refrigeration for a week. The amylose gel was then seperated by centrifugation.

Adsorption of L-Arginine on Amylose : The basic amino acids especaially arginine, are specifically adsorbed on the amylose gel. To optimise the conditions for better and quantitative adsorption the following conditions were observed.

1. Adsorption at different pH
2. Adsorption at different pH and treatment with 70 percent alcohol.

1. Adsorption at different pH :

The amylose gel was stirred with 20 mM citrate buffer of different pH, from 3.0 to 5.5, individually and centrifuged. The process was repeated till the supernatent of the gel was adjusted to the pH under consideration. The arginase solution (1 mg / ml) was stirred with 1.5 ml of amylose gel.

The adsorption process was carried out for one hour with stirring. The amylose gel was then seperated by centrifugation washed again with buffer of pH 4.6 and centrifuged. The process was repeated thrice, and the combined supernatent was analysed for amino acids using the ninhydrin reagent.

2. Treatment of Amylose with 70% Alcohol

The amylose gel was washed with 70% alcohol, the lucerne filtrate was also given the same treatment and the precipitated proteins were seperated by centrifugation. The adsorption of arginine was carried out at pH 4.6 in 20 mM citrate buffer. The gel was washed with alcoholic buffer until free of contaminating amino acids.

3. Adsorption with change in Buffer

The adsorption and desorption process of amino acids was carried out by following similar conditions as given above except the buffers are different. The buffers were 20 mM citrate and 20 mM phosphate buffer, the pH of [illegible] was 4.6.

4. Selective adsorption of Arginine HCl

The adsorption of pure arginine HCl was carried out at different pH using 20 mM citrate buffer. The gel was washed by a similar procedure i.e usding buffer without alcoholic treatment and with alcoholic treatment. The pH of arginine HCl, amylose gel and buffer was same throughout the adsorption process.

5. Desorption of Adsorbed Amino Acids from Amylose gel

The best procedure for the effective desorption was the change in pH value. Desorption occured towards alkaline side viz. pH 7.5 and above. The gel was washed three times and the combined supernatent, on centrifugation, was used for amino acid analysis by the ninhydrin reagent.

2.9 ION - EXCHANGE CHROMATOGRAPHY

The seperation of a amino acid from a mixture of amino acids and related compounds is possible using [illegible] methods. Ion exchange chromatography is one of

the best methods for seperation of amino acids. It was developed by Moore and Stein (110). The elution of the amino acids was effected by change in strength and pH value of buffer or by the use of increasing strengths of HCl (1.5 M - 4.0 M). The use of volatile buffers including pyridine acetate buffer, ammonium formate, ammonium acetate, etc., can be done for the elution of amino acids. About 50 - 300 mg of an amino acid mixture can be separated by this procedure.

Column Preparation :- The chromatographic column of dimension 30 x 1.2 cm was used. For the preparation of resin Dowex 50 X 4 was washed with 4 M HCl (1 litre). The excess of acid is removed by several washes with distilled water until the filtrate is neutral. The resin was then suspended in 2 M NaOH. The sodium salt of the resin is then washed with large excess of water to remove excess of sodium hydroxide to bring the filtrate to neutrality. The resin can be stored in this form for prolonged periods.

The resin (Na^+ form) was then suspended in the volatile buffer, 0.2 M pyridine acetate buffer of pH 2.5. About 2 litres of the buffer was required. The suspended

resin (deaerated) was transfered to the chromatographic coloumn by means of funnel. The gel was allowed to settle down, the required length of 20 cms was obtained and the excess buffer was withdrawn, leaving a liquid level of 10 cms above the resin. The buffer was passed through the column at the rate of 200 cc/hr. The pH of the effluent and inffluent was checked to see that they were the same.

Operation of Column :- The sample solution to be chromatographed was adjusted to pH 2.5. One ml. of sample (100 mgs) was applied to the surface of the resin with the help of a pipette. It was washed three times with 1 ml of buffer of pH 2.5. The column was mounted over a fraction collector and fractions of 2 ml each were collected at the rate of 8 ml/hr so as to get a good resolution.

The buffer of pH 2.5 was then replaced by pH 3.1, followed by the buffer of pH 4.5 and the buffer of 2 M pyridine acetate of pH 5.5 was used after that.

After completion of the run the resin is removed from the column and regenerated for future use.

Sample Preparation :- The extract of lucerne was applied to the amylose gel for selective adsoption of arginine and other guanidino compounds. The desorbed supernantent was concentrated and the small impurity of other amino acids was removed by ion-exchange chromatography. The sample containing 100 mg of amino acid was loaded on to the column and eluted out using buffers of different strengths as given above.

Analysis of Effluent Fraction :- The amino acid analysis of the eluted fractions was carried out using the ninhydrin reagent using 20 Ul aliquotes. No prior neutralisation of the sample is necessary as the same buffer of pH 4.6 is essential for the assay. The blank for the assay is taken from the tube before the peak appears or after the elution of the peak.

However, for arginine analysis using Sakaguchi reaction, preliminary removal of pyridine is essential as it forms a precipitate with the reagent. For this purpose 20 - 30 Ul aliquots of the eluted amino acid were pipetted and evaporated in a descicator under vaccum or by heating in a water bath at 50° C for a minute. It was again brought to the original volume and then used for guanidine analysis.

The result of the ion-exchange chromatography using volatile buffer are shown in Fig.(XII)

Normally increase in temperature increases the resolution as seen on the Dowex column (111). However, when volatile buffers are used there is no such improvement and hence in the present experiments the operations were carried out at room temperature.

Finally, the fraction tubes containing the necessary amino acid ~~peak~~ were combined, rinsed with 5-10 ml of distilled water, evaporated under reduced pressure in the descicator and reduced to a small volume. It was again diluted with 50-100 ml of distilled water and evaporated under reduced pressure to remove excess of free acid and pyridine.

2.10 PAPER CHROMATOGRAPHY

The amino acids can be identified and characterised by paper chromatography. The procedure of Consden et al (112), is used to effect a separation of the components of a mixture by liquid-liquid partition. The procedure was carried out as follows.

Application of Sample to the Paper :- The Whatman paper No. 1 was cut to get a dimension of 34 x 4 cms. The amino acid sample (10-15 Ug) was spotted at the origin which is 2 cms apart from the lower end of the paper.

Development :- The paper was developed by ascending chromatography in a glass chamber using solvent system of Patride i.e. Butanol : Acetic acid : Water in the ratio of 4 : 1 : 5 for 18 hours.

Detection :- The developed chromatogram was dried and sprayed with ninhydrin reagent to detect the amino acids. The amino acids form a bluish coloured spot on heating the chromatogram at 105° C for 2 - 5 minutes.

The spraying reagent was prepared by mixing 0.25 % of the ninhydrin in acetone (95%) with 5 mM cadmium acetate. This gives a stable and quick colour.

The Rf value of the amino acid is given by the ratio of the distance travelled by the amino acid (solute) to the distance travelled by the solvent.

$$\text{Rf Value} = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

Rf values of known and unknown amino acids were calculated using above equation and compared to characterise them.

2.11 GEL CHROMATOGRAPHY

Gel chromatography separates components based on their size and weight. The larger molecules will emerge first from the column while the smaller molecules are retarded.

The chromatographic gel, in gel filtration usually does not need regeneration and can be used repeatedly. The polyacrylamide gel (Bio-gel) employed for gel chromatography, is a zero gel, and is stable in the pH range 2-10 due to its inert matrix chain. It is marketed with different types of swelling characteristics and can be used for a wide range of molecular mass separation. Operation range of Bio-gel P-2 which was used in the present study has a range of 100-1800 molecular mass.

The gel was swollen in hot distilled water for 2 hours. After deaeration of this slurry under vaccum the column of dimensions 78 x 0.8 cms was packed at room temperature.

The sample volume used was 1-2 percent of the bed volume (i.e. 2 mgs of the guanidine fraction concentrate) was loaded on the column.

The Bio-gel P-2 was equilibrated and eluted with distilled water at the rate of 4 ml/hr at room temperature. Fractions of 1 ml were collected and aliquotes of 10 Ul were analysed for carbohydrates using Phenol Sulphuric Acid Method, amino acids were analysed by ninhydrin method and arginine was detected using Sakaguchi Reagent. The tubes of peak fraction containing particular amino acid in large concentrations were combined and rinsed with distilled water. The result of the elution on Bio-gel P-2 is shown in the Fig. XIII The combined fraction with large amino acid value was concentrated and 0.1 ml was rechromatographed on Bio-gel P-2 using the same conditions. The result of which is reflected in Fig.XIV. A single fraction having the major amino acid activity from the rechromatographed sample was again loaded on to the same column, elution was effected under the same conditions as before. The result of this gelfitration column is shown in figure XV.

2.12 ARGINASE ENZYME

The mammalian liver is the main source of the enzyme arginase (L - Arginine amidino hydrolase, E.C. 3.5.3.1). Its metabolic role was established by Krebs and Henseleit (113). It is the terminal enzyme of the urea cycle and hydrolyses arginine specifically into ornithine and urea. The molecular weight of the purified arginase is 115,000. It has an oligomeric structure and exists in a tetrameric form. The optimum pH for liver arginase is 9.3 (114) and a optimal concentration of 2 mM/l of $MnCl_2$. Recorded Km values for arginase are 10.5 (115), 5.3 (116), and 7.4 (117).

Enzyme Effectors :- L - ornithine (Ki = 1.3 mM) and adenine (Ki = 0.7 mM) are competetive inhibitors, while borate is non-competitive inhibitor of bovine liver arginase. Activation and stabilization of arginase is achived by bivalent metal ions such as Mn, Mg, Ni, and Cd. The reaction products such as ornithine, lysine, adenosine and inosine inhibit the enzyme competitively (116, 117). The optimum pH of human platelet arginase is 10.5 (118).

Isolation of Enzyme Arginase :- The liver homogenate was prepared by homogenising 10 gms of fresh

sheep liver in a waring blender at low speed for 3 minutes in 100 ml of 5 mM tris-HCl buffer. The pH of the buffer was 8.3 and it contained 5 mM $MnCl_2$ at chilled conditions. The extract was centrifuged at 5000 g and the precipitate formed was discarded. The opalescent supernatant was stored at 4° C under toluene. It was diluted 10 to 15 times as required before incubation. The semi-purified enzyme was obtained by passing the crude enzyme solution on DEAE cellulose anion exchanger.

Arginase Chromatography on DEAE cellulose : Five grams of DEAE cellulose was weighed and brought to the H^+ form using 0.2 M HCl. The acid was immediately washed out with water till the supernatant reached the pH 6.5. The DEAE cellulose was further treated with 0.5 M NaOH for 1 hr. Excess of NaOH was removed by several washes with distilled water till the pH was neutral.

The regenerated DEAE cellulose was treated with tris-HCl buffer (5 mM) at pH 8.3. The column of dimensions 15 x 0.9 cms was filled with the above DEAE cellulose. The crude enzyme preparation (3 ml) was applied to the column. Washing was done with tris-HCl buffer at pH 8.3. Fractions of 5 ml at the rate of

5 ml/hr were collected. The entire process was carried out at 4° C. Aliquots of the fractions were used to determine arginase activity as follows.

Arginase Assay :- The catalytic activity of the enzyme arginase can be determined in two ways.

1) By measuring the arginine concentration which steadily decreases.
2) By measuring an increase in the concentration of urea and ornithine .

In the present work arginase assay was carried out by measuring the arginine concentration by Sakaguchi Reaction. Arginase is very specific to l-arginine as a substrate. It hydrolyses arginine at alkaline pH (9.5) to yield l-ornithine and urea.

$$\text{l-arginine} + H_2O \xrightarrow[Mn^{2+}]{\text{Arginase}} \text{l-ornithine} + \text{urea}$$

The manganese ions (5 mM) activate the enzyme action. To the arginine (or substrate) 20 mM solution (1 ml), 0.1 ml of enzyme (10 fold diluted), 0.1 ml of glycine buffer of pH 9.5 and 0.1 ml $MnCl_2$ was added and the mixture was incubated for half an hour.

The enzyme was deactivated by adding 5 ml of perchloric acid (·5 %) which precipitated the enzyme protein. Assay mixture was centrifuged to remove the precipitate and 1 ml of the supernatant was assayed for arginine by the Sakaguchi Reaction. The fraction containing the highest arginase activity was used to hydrolyse the guanidine compound previously purified using gel chromatography. Assay conditions were the same throughout. On precipitation of the arginase enzyme, the hydrolysed fraction was utilised for ion-exchange chromatography to remove impurities using volatile buffer.

Ion-exchange Chromatography of Hydrolysed Guanidine Fraction :- The column was prepared by the procedure described earlier. The hydrolysed guanidine compound was dissolved in the smallest quantity of pyridine acetate buffer at pH 2.5 and loaded on the Dowex column of dimensions 30 X 1·2 cms.

The column was developed successively with 2 lts of 0.2 M buffer of pH 2.5, 3.1, and 4.5 at the flow rate of 100 ml/hr. The column was then eluted with 2 M buffer at pH 5.5 and fractions of 5 ml were collected and aliquots were analysed for arginine by Sakaguchi Reaction.

Finally the column was washed with the 2 M pyridine buffer. The arginine positive peak fraction tubes were combined and rinsed with water. The total volume was reduced by evaporation under reduced pressure. The fractions were then diluted with water, re-evaporated to remove excess pyridine and acetate.

Paper Chromatography of Hydrolysed Purified guanidine compound :- The arginase assay using 10 fold diluted enzyme was carried out with guanidine compound as a substrate. After removal of enzyme, paper chromatography of the sample was carried out using the solvent system - butanol : acetic acid : water in the ratio of 4 : 1 : 5 by ascending method for 18 hours. The detection of the amino acid was done by spraying the paper with ninhydrine reagent.

Characterisation of Amino Sugar :- The purified compound was subjected to vaccum drying and the reaction with Erlich's Reagent was subsequently carried out to test the presence of amino sugar. A small quantity of the purified guanidine compound was added to 0.5 ml of Erlich's Reagent which immediately gave intense red colouration. This test is confirmative of the presence of amino sugar.

2.13 HYDROLYSIS OF OLIGOPEPTIDE

The presence of protein as given by the Lowry method, in the purified compound suggested its nature as an oligopeptide. The hydrolysis of this oligopeptide was carried out.

The acid hydrolysis of proteins or peptides include hydrolysis with 6 M HCl - 90% formic acid (v/v), 8 M H_2SO_4, 80 percent tri fluoro acetic acid, formic acid, etc. The most common procedure for total hydrolysis of protein or peptide employed is by heating the peptide with an excess of 10 M HCl (1 : 30 to 1 : 100 w/v) at 105° C for a period of 18 to 24 hours. This procedure was used in the present study and the oligopeptide was hydrolysed for about 20 - 24 hours in sand bath.

The excess of hydrochloric acid was evaporated and under reduced pressure in a descicator. The hydrolysate was moistened with water and again evaporated to dryness. This procedure was repeated twice, after which the residue was dissolved in a small volume of distilled water and centrifuged to remove charred sugar particles. The supernatant was reduced to small volume and subjected to paper chromatography.