DETERMINATIONS OF THE DIAMINO ACIDS IN VARIOUS WHEAT FLOURS

by

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A Thesis Submitted to the Faculty of
the Virginia Polytechnic Institute
in Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE
in
CHEMISTRY

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BLACKSBURG, VIRGINIA
JULY 18, 1947

150455

ACKNOWLEDGEMENT

The author wishes to express his sincere appreciation to Professor J.B. Lucas, whose many suggestions and invaluable aid made this thesis possible.

Blacksburg, Virginia
July 18, 1947

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INTRODUCTION

I

INTRODUCTION

Some years ago, Thomas, (37) in discussing the behavior of food and tissue proteins, summed up the knowledge at that time and stated that "We need to know which amino acids must be present in food, how much we require of each, and to what purpose". Today, a great deal is known as to which amino acids are essential dietary constituents. By determining the essential amino acid content of certain wheat flours and correlating this with the total nitrogen content of the particular wheat from which the flour was derived, and, if possible, with the nitrogen and mineral content of the soil in which the wheat was grown, it may some day be possible to develop a method whereby the maximum possible amounts of the essential amino acids will be produced in the wheat during growth. At present it is known that the amounts of these acids in the flour vary according to the quality of the flour and the protein content. The factors (51) which determine quality of wheat are (a) preharvest growth, (b) harvest and storage conditions, and (c) physical and chemical composition. The protein content of the flour is dependent upon (a) rotation of the wheat with other crops, (b) seed bed preparation, and (c) addition of food to the plant in the form of fertilizers.

The results of this work would enable nutritionists to have more exact information on the selection of wheat and wheat products for a properly balanced diet. Furthermore, by baking bread with the various flours and then analyzing the baked products it would be possible to tell if the amino acids are destroyed in the baking process, and if so, to what extent this occurs. Unfortunately however, much of the above is outside the scope of this work. This thesis is a continuation of several other investigations (2, 9, 15, 23, 27, 35, 37, 52) initiated in an attempt to determine the amount and ratio of the diamino acids present in wheat proteins.

The importance of this work lies in the fact that with the possible exception of glycine, the animal body, or at least the tissues of the higher animals, are incapable of synthesizing certain amino acids in quanities adequate for growth and maintenance.[58] Accordingly, the animal is dependent upon the plant for the amino acids which go to make up the molecule and vital proteins of the animal body. Consequently, if the animal organism is dependent on the vegetable proteins for the building blocks needed for its structural processes, it becomes of great importance in nutrition to consider the actual amino acid composition of dietary proteins, particularly since nine or more of the amino acids are, apparently, totally incapable of synthesis by the body.

As was previously mentioned, the purpose of this research was to determine the amount and ratio of the diamino acids (histidine, arginine, and lysine) in various wheat flours. Rose has defined an essential amino acid as one which cannot be synthesized by the animal body at a rate commensurate to meet its demands for normal growth. These essential amino acids are lysine, tryptophane, histidine, phenylalanine, leucine, isoleucine, methionine, threonine, valine, and arginine. Of these essential amino acids, determinations were made on the amounts of lysine, arginine, and histidine in wheat flours because these three acids are generally grouped together and are quantitatively separated with the greatest relative degree of accuracy of any amino acids.

II

REVIEW OF LITERATURE

II

REVIEW OF LITERATURE

A. HISTORICAL

1. General

Proteins are constituents of all living tissues, the amount and kind of protein varying with the particular tissue in question. Proteins are so universally distributed that it is not possible to ingest naturally occurring foodstuffs without including protein. It is not surprising then, that a great deal of research has been done on this subject in order to procure as much information as possible on the structure, composition, and utilization of proteins in the body.

Early attempts at protein analysis produced very little information due to the complexity of the protein molecules. All the known analytical procedures had been used in order to attempt to prepare characteristic derivatives by means of which the proteins could be differentiated and identified.

It was not until 1820 that Braconnet made the first great advance in the study of protein chemistry; he was able to obtain glycine from the acid hydrolysate of gelatin. This marks the beginning of modern protein chemistry.

It is now firmly established that the amino acids constitute the building blocks of proteins. A prerequisite to the elucidation of the structure of proteins is a knowledge of their amino acid content. There is still no generally accepted view as to the details of protein structure although the peptide bond as the mode of linkage between the amino acid residues seems to be firmly established.

The progress of advance in knowledge of the qualitative composition of proteins, with respect to the amino acids that they yield when subjected to appropriate hydrolytic action was slow at first, and, though rapid in recent years, the complete solution of the problem of protein composition has not yet

been achieved. At the present time twenty-three amino acids have been isolated from protein hydrolysates and were so thoroughly investigated that there is no reasonable doubt of their existence in these hydrolysates.(30)

Since the amino acids rather than the protein molecule itself are utilized in nutrition, the quantitative determinations of amino acids becomes extremely important. Very little was known of the quantitative relationship of the amino acids until Fischer[11] observed that the ethyl esters of the manoamino monocarboxylic and dicarboxylic acids could be distilled under reduced pressure without appreciable decomposition. At the present time almost all of the acids can be fairly accurately quantitatively separated, but since they are sufficiently alike in chemical properties, separation is difficult. For this reason the amino acids are separated as salts or esters.

2. The Diamino Acids

a. Lysine (40)

In 1889, Drechsel became interested in Schutzenberger's observation relating to the evolution of carbon dioxide when proteins were digested with alkali. First, he hydrolyzed casein by Hlasiwetz and Haberman's method. He observed that little, if any, carbon dioxide was formed during hydrolysis. He then removed the tin, concentrated his hydrolysate to a sirup and separated the crystallizable fractions. To the sirupy mother liquor he added phosphotungstic acid, a reagent long known to be an alkaloid precipitant. The heavy precipitate that formed was filtered, washed, and decomposed with barium hydroxide. The filtrate was acidified with hydrochloric acid and concentrated. On standing, a crystalline substance was deposited. It was recovered and crystallized from a mixture of water

and alcohol. Drechsel observed the strong basic properties of the new amino acid. He prepared the chloroplatinate and the silver salts, and noted that the amino acid was quite stable in the presence of strong acids; but on treatment with barium hydroxide it was decomposed with the liberation of barium carbonate. Drechsel named the new compound lysatine (later shown to be a mixute of lysine and arginine).

He repeated the experiments on the phosphotungstic acid fraction of protein digests. He obtained some perplexing results, particularly when he attempted the isolation of the silver salt of his lysatine.

The isolation and identification of lysine in a pure state were accomplished in Drechsel's laboratory by three assistants, Siegfried, Ernest Fischer, and S.G. Hedin. Fischer termed it lysine. Hedin isolated it from a pancreatic digest of fibrin and also prepared lysine chloroplatinate in accordance with Drechsel's pattern. None of these investigators were successful in crystallizing lysine as a free base. This was accomplished by Vickery and Leavenworth in 1928.

The elucidation of the chemical structure of lysine was finally accomplished by Fischer and Weigert, who synthesized it from the cyanopropylanalonic ester by treatment with nitrous acid and subsequent reduction.

b. Arginine (41)

In 1886, Schulze and Steiger discovered arginine in the aqueous extracts of etiolated lupine seedlings. They observed that the addition of phosphotungstic acid to the aqueous extracts gave a copious white precipitate. From this precipitate they separated a new crystalline compound which possesses basic properties and is precipitable at neutral or alkaline reactions by mercury salts in the presence of sodium carbonate.

Their first report was followed by another. They showed that arginine was stable when heated with strong acids and decomposed when heated with alkalies giving rise to carbon dioxide and ammonia. Nitrous acid removed only one-fourth of its nitrogen. It contained neither sulfur nor phosphorus. It was precipitable by most of the known alkaloid reagents.

Schulze and Steiger also investigated the amount of arginine produced during the sprouting of lupine seeds and concluded that proteins must have been converted (in part, at least) to arginine during the process of germination. They also demonstrated that arginine was decomposed by alkali with the production of urea. This latter observation led Schulze and Likiernik to associate Drechsel's discovery of lysine and lysatine with their new compound and its possible presence among the products of protein digestion.

Drechsel and his group, who had been diligently working on lysine and lysatine became interested in Schulze's arginine. However, it was Hedin who finally isolated the silver salt of arginine from the phosphotungstic acid precipitates of protein digests. He reinvestigated the whole of lysine and lysatine, and showed that the latter was actually arginine contaminated with lysine.

Following the solving of the mystery of lysine, lysatine, and arginine, Kossel demonstrated that the two amino acids, lysine and arginine, were found in abundance in the basic protamines he had obtained from fish sperms. In 1897, Schulze and Winterstein showed that ornithine and urea were among the decomposition products of arginine by alkalies. In 1910, Sorensen synthesized arginine from benzoylornithine by condensation with cyanimide and subsequent hydrolysis of the benzoyl group with a strong acid. In 1924 Kossel and Gross showed that arginine reacted with

flavianic acid to form a very insoluble compound. Vickery made use of this observation and developed an accurate quantitative method for its estimation.

C. Histidine (42)

Histidine was discovered independently by two investigators. On April 9, 1896 Kossel reported its isolation from the decomposition products of protamines; and on May 11, 1896 Hedin isolated it from the acid hydrolysates or proteins.

In 1894, Kossel began an investigation of protamines and observed that these products yielded heavy precipitates when added to solution of soluble proteins. Kossel subjected sturin to sulfuric acid hydrolysis and removed sulfuric acid from the hydrolysate with barium hydroxide. He next added mercuric chloride to the strongly alkaline solution. A heavy precipitate formed. This was recovered and treated with hydrogen sulfide. The mercury-free filtrate was concentrated. On standing, crystals of the chloride of a new base deposited. Kossel named this new compound histidine and accurately reported its chemical composition.

Hedin, on the other hand, was investigating the fraction of acid hydrolysate of casein that gave a copious precipitate with phosphotungstic acid and was studying its behavior toward silver nitrate. He decomposed the silver salts of the amino acid with a small amount of hydrochloric acid and removed the silver chloride formed. Following the concentration of the filtrate, he obtained a crystalline substance, the chemical composition of which was quite in agreement with Kossel's histidine. Hedin's method for the isolation of histidine is still in use.

Histidine is widely distributed in nature. This was demonstrated by the extensive work of Kossel and his co-workers and by Schulze. Some of the peculiar properties of histidine observed at that time are noteworthy.

Herzog shows that it gives a biuret test and, upon boiling in a strong alkali, it yields hydrocyanic acid, ammonia, and carbon dioxide.

Pauly demonstrated that histidine contains an imidozole ring which is responsible for its reaction with diazobenzensulfonic acid and the development of a highly colored solution. In 1911, Pyman synthesized histidine and worked out its structural formula.

B. HYDROLYSIS (17, 39)

The first step in the analysis of proteins is the breaking of the peptide linkages, by means of which the amino acids are connected together. These linkages are most easily split by means of hydrolysis. Amino acids may be obtained from proteins by the following hydrolytic methods; (1) by acids, (2) by alkalies, and (3) by enzymes. Each of these methods has its advantages and disadvantages. There is as yet no perfect method for hydrolyzing proteins.

1. Acid Hydrolysis

The most common hydrolyzing agents are sulfuric and hydrochloric acids. Hydrolysis with 3 to 5 parts of 20 per cent hydrochloric acid is generally carried out by refluxing for 6 to 24 hours, and according to Sullivan (50) the blackening due to humin formation can be prevented by the addition of titanous chloride instead of the stannous chloride used by the early workers. Humin, a black or brown substance, is said to form by the condensation of tryptophane with an aldehyde.

Hydrolysis with 30 per cent sulfuric acid is often employed and is generally used when the amino bases are to be isolated. This hydrolytic agent has the advantage of ready removal of the sulfate ion with barium hydroxide or carbonate.

Kossel and Kutscher (25), in an attempt to determine the best concentration of the most widely used acids for hydrolysis, obtained the

following results while working with casein:

	H_2SO_4 12 Hours	Conc. HCl 12 Hours	1:2 HCl 3 Hours
Histidine	0.90%	0.76%	1.56%
Agrinine	4.45	4.21	4.50
Lysine	1.84	2.29	2.00

Table 1: Completeness of Hydrolysis using various acids.

These results seem to indicate dilute hydrochloric acid as the best hydrolyzing agent.

The main drawback of using sulfuric acid is that the voluminous precipitates of barium sulfate formed upon neutralization retain or adsorb a certain amount of the constituents of the digested proteins. On the other hand, hydrochloric acid is difficult to use on account of the difficulties encountered in the removal of its anions.

Various other acids, including hydrofluoric, formic, hydriodic, phosphoric, and acetic, as well as acetic anhydride have been tested, but without revealing any particular advantages.

2. Alkali Hydrolysis

Hydrolysis of proteins by alkalies is very rarely used despite the fact that the proteins are completely hydrolyzed to their constituents. The main advantage that alkalies have over acids is that they do not cause the formation of humin, and therefore do not destroy tryptophane. However, when a protein is hydrolyzed by a base, its constituents, with the exception of glycine, are racemized. Alkali hydrolysis is generally used only in the isolation of tryptophane, since it is not destroyed by bases.

3. Enzymatic Hydrolysis

Hydrolysis of proteins by means of enzymes is exceedingly slow for it is carried out under mild conditions of acidity and temperature, and therefore the amino acids suffer little change of destruction. This method is particularly valuable when it is desired to obtain the natural unracemized amino acids without subjecting them to the drastic action of strong acids and alkalies.

C. TESTS FOR THE COMPLETION OF HYDROLYSIS

Hydrolysis of a protein is judged to be complete when the peptide linkages are entirely broken. This is rather difficult to determine accurately and is done by two methods:

1. Biuret Test (42a, 47a)

The biuret test, which is very useful and is extensively employed for the purpose of detecting peptide linkages, is not conclusive. When a dilute solution of copper sulfate, followed by a solution of sodium hydroxide, is added to a solution containing polypeptides, a blue-violet to pink color will be formed. The shorter the chain of polypeptides, the more the color grades into pink. Histidine, serine, and threonine give positive biuret tests; thus, it can be seen that a positive test is not specific for a peptide linkage. The color is due to the formation of a substituted biuret, RHN-CO-NH-CO-NHR, which reacts with the reagents to give the colored compound.

2. Ratio of Nitrogen Method (42a)

The best method for determining the completion of hydrolysis of a protein is the estimation of the total alpha-nitrogen and the total nitrogen, and the establishment of the ratio $\frac{\text{alpha-nitrogen}}{\text{total nitrogen}}$. As the

hydrolysis becomes complete, the ratio becomes constant. The primary amino nitrogen determination may be made by the Van Slyke method (56) or by Sorensen's method of titration in the presence of formalin (49).

D. DETERMINATION OF THE AMINO ACIDS (18)

The methods for the quantitative determination of the amino acids can be placed in two categories; (1) the methods for determining the groups of constituents, and (2) the determinations of the individual amino acids.

1. Group Analysis

a. Hausman Method (22)

It was stated by Hausman that the protein is characterized by the distribution of the nitrogen content of the hydrolysate into three parts; ammonia nitrogen, basic nitrogen, and non-basic nitrogen.

(1) The ammonia nitrogen: The hydrolysate is made basic with calcium hydroxide and the ammonia is distilled into a standardized acid. The ammonia is considered as coming from the amide linkages and is sometimes called "amide nitrogen".

(2) Basic nitrogen: The basic amino acids are precipitated by means of phosphotungstic acid and the nitrogen determined by the Kjeldahl method. This is taken as basic nitrogen.

(3) Non-basic nitrogen: The nitrogen is determined on the filtrate from the basic nitrogen determination and is recorded as the non-basic nitrogen.

Osborne (34) has added a fourth fraction which he called the humin nitrogen, determined by the Kjeldahl method on the humin. This method gives the ratio between the monoamino and diamino acids in the protein.

b. Dakin Butanol Method (6, 10, 31, 45)

The Dakin butanol method depends on the distribution of the amino acids between water saturated with butanol and butanol saturated with water. The protein is hydrolyzed with sulfuric acid and, after quantitative removal of the acid, the neutral aqueous solution is concentrated into a thin syrup which is then extracted in vacuo in a continuous liquid extractor. with butanol. Proline and practically all of the monoamino monocarboxylic acids are extracted. The solid cream-colored amino acids which separate out from the butyl alcohol are filtered off, and are washed with butanol and ether to remove pigment. The mother liquor contains all of the proline. The residue of non-extracted amino acids contains practically all of the basic amino acids and also the dicarboxylic amino acids. Several difficulties as to the complete separations have been found (25).

c. Electrical Transport Method (1, 6, 12, 13, 14, 21, 45)

This method is based on the influence of pH on the dissociation of amino acids. A direct current, passed through a protein hydrolysate at pH 5.5 in the center of a three-compartment electrodialyzing cell separated the amino acids into those which are predominately acidic (aspartic, glutamic, and hydroxyglutamic) and migrate to the anode; the basic amino acids (arginine, histidine, and lysine) which migrate to the cathode; and the non-dissociated monoamino monocarboxylic acids. pH can be controlled by addition of barium hydroxide to the center compartment and carbon dioxide to the cathode compartment.

Using the electrical transport method, at pH 7.5 the basic amino acids can be separated by transport of arginine and lysine to the cathode, and leaving histidine in the center compartment. Schmidt and Foster (13) used this procedure for the preparation of histidine.

d. Przylecki and Kasprzyk Fatty Acid Method (36)

The protein hydrolysate is freed of all inorganic ions and evaporated to dryness. The basic amino acids are soluble in anhydrous butyric and valeric acids. Glycine, alanine, valine, leucine, phenylalanine, proline, and hydroxyproline are soluble in either glacial acetic acid or propanoic acid. Tyrosine, cysteine, aspartic acid, and glutamic acid are insoluble.

e. Method of Schryver (1, 6, 8, 45)

The differential solubilities of copper salts of the amino acids in water and methyl alcohol was made the basis of a method for fractionation of amino acids by Schryver and his pupils. The success of the method depends upon the use of very dry solvents and thoroughly dry copper salts. The copper salts of the amino acids are dehydrated with absolute acetine. They are than fractionated as follows:

1. Water-insoluble fraction

   It contains the copper salts of leucine, phenylalanine, and aspartic acid.

2. Water-soluble fraction.

   It is fractionated with methyl alcohol into:

   (a) Copper salts which are insoluble in methyl alcohol. This fraction contains alanine, tyrosine, glycine, lysine, arginine, histidine, and glutamic acid.

   (b) Copper salts which are soluble in methyl alcohol. This fraction contains valine hydroxyvaline, proline, and prolylphenylalanine.

2. Methods for Isolation and Identification of the Individual Amino Acids

a. Solubility Method (3,4,6,32,33,46)

The solubility method, developed by Bergmann and Stein (4) is based upon the physical chemical principle that the solubility product of the ions of a saturated solution is a constant.

The process is carried out as follows: To an aliquot of the hydrolysate is added an excess of an amino acid salt. To a second aliquot are added the same reagents as the first, and also R moles of a precipitating agent (generally a sulfonic acid).

A = moles of amino acid in sample

R = moles of sulfonic acid added

S = moles of salt dissolved

The amount of amino acid present can be calculated from the following formulas:

$$K_1 = S_1 (A+S_1)$$

$$K_2 = (R+S_2)(A+S_2)$$

$$\text{If } K_1 = K_2, \quad A = \frac{S_1^2 - S_2(R+S_2)}{(R+S_2) - S_1}$$

$$\text{If } K_1 = FK_2, \quad A = \frac{S_1^2 - S_2(RS_2)}{F(R+S_2) - S_1}$$

b. Van Slyke Method (56)

Van Slyke precipitated the diamino acids with phosphotungstic acid. Cysteine is determined by the sulfur present. The ammonia and humin nitrogen are found as in the Hausman method, and total nitrogen is determined by the Kjeldahl method. Arginine nitrogen is determined by boiling the mixture with potassium hydroxide for six hours to decompose the arginine to ornithine and urea, the latter decomposing to give off carbon dioxide and ammonia. Histidine is calculated since three-fourths of the arginine nitrogen is equal to two-thirds of the histidine nitrogen

(18, 56). The lysine nitrogen is taken to be the remainder of the total nitrogen.

The values obtained for arginine nitrogen, histidine nitrogen, and lysine nitrogen do not represent the actual amounts of the corresponding compounds present, for any compound which breaks down under the influence of strong alkalies to give ammonia would be reported in the calculations as arginine nitrogen. This would not only make the arginine content inaccurate, but also the histidine content. The reported cysteine content is generally low because cysteine is decomposed when boiled with acid, and that which is not attacked is either racemized or converted into an isomer, the phosphotungstate of which is appreciably soluble (19).

c. Isotope Dilution Method (43, 47)

The isotope dilution method makes use of a principle that if a known amount of an isotopically labeled amino acid is added to a mixture, and then some of the amino acid is isolated, the percentage of isotope in the isolated amino acid will bear a quantitative relationship to the total amount of the amino acid in the mixture. The quantity can be calculated from the equation $Y = (\frac{C_o}{C} - 1)\, x$.

where Y = quantity of amino acid in the mixture

x = amount of labeled amino acid added

C_o = isotope concentration above normal of the amino acid added

C = isotope content of isolated amino acids

At present this tool is restricted because of the limitations that exist for the isolation and measurement of isotopes. In the work carried out by Rittenberg and Foster, heavy nitrogen, N^{15}, was used to label the amino acids. In some instances deuterium, which is much easier to obtain and to estimate, has been employed for this purpose. For the sulfur-containing acids, S^{35}, could be used.

d. Microbiological Assay (26, 48, 47)

Biological methods for the estimation of amino acids that appear to hold great promise are assays with the lactic acid producing organisms, lactobacillus arabinosus, lactobacillus caser, and with mutants of the ascomycete Neurospora crassa.

The growth and lactic acid production of the lactobacillus organisms are functions of certain essential nutrilites. These include some of the amino acids. By preparing media in which only one of the essential amino acids for the microorganism is made the limiting factor, the growth, and more particularly the amount of lactic formed, can be used to estimate the content of the amino acid. By this procedure it is now possible to carry out assays for arginine, glutamic acid, leucine, isoleucine, phenylalanine, tryptophane, tyrosine, and valine. The method appears to be accurate within 2 per cent.

e. Boyd's Uramine and Hydantoin Method (6, 7, 20)

The neutralized hydrolysate is treated with KOCN to from uramic acids:

$$RCH(NH_2)COOH + KOCN \rightarrow RCH(NH_2)CONHCOOH$$

The mixture is brought to the end point with congo red. The uramino acids of leucine, isoleucine, and phenylalanine precipitate out and are converted to their respective hydantoins

$$RCH(NH_2)CONHCOOH \xrightarrow{HCL} RCH\text{—}CO\ /\ NH\ /\ HN\text{—}CO$$

which are than separated by means of their differential solubility. The uramino acids remaining in solution are soluble in ethanol; the remaining hydantoins are insoluble. The individual hydantoins are separated on the

basis of their solubility in ether and chloroform.

f. Selective Adsorption Methods and Ion Exchange Resins (43, 57)

The research for materials which will exert a selective adsorption effect on amino acids has been carried on for some time. This method is not at present developed to the extent of offering a practical means of isolating amino acids, but shows some promise. Whitehorn (57) has reported that Permutit selectively adsorbs the basic amino acids. Calcium hydroxide was used as the eluting agent.

The commercial availability of synthetic ion-exchange resins such as the "Amberlites" has stimulated interest in their use for amino acid separations. Bloch (43) reported that these resins have proved useful for large-scale separation of the basic amino acids from protein hydrolysates. Other materials have been used with good success (43).

g. Precipitation of Insoluble Amino Salts (44)

It is well known that several of the amino acids form insoluble salts with various types of reagents and many of these salts have proven useful for isolation purposes. Mercury, silver, copper, phosphotungstic acid, and phosphomolybdic acids have all been used.

Among the most useful reagents for isolation of amino acids as insoluble salts are the aromatic sulfonic acids. One of the first of these to be used was flavianic acid (1-naphthol-2, 4-dinitro-7-sulfonic acid). This is used extensively as a precipitant for arginine. Another reagent is picric acid which is used in the isolation of lysine.

III

EXPERIMENTAL

III

EXPERIMENTAL

A. PRELIMINARY DETERMINATIONS

The amounts of the diamino acids were determined quantitatively on several varieties of flour, namely, flour produced from V.P.I. wheat No. 131, V.P.I. dining hall flour (Dainty Maid, milled by Roanoke City Mills), and Pillsbury's Best. The V.P.I. wheat No. 131 was milled for this study from certified seed wheat by the United States Agricultural Experiment Station at Beltville, Maryland.

Preliminary to the actual amino acid determinations, runs were made on the flour samples to find moisture content, ash, protein nitrogen, total protein and fat; and the probable ash in the whole wheat was calculated according to Bailey (3) from the ash in the flour. All preliminary determinations were made according to "The Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists", and the method for each follows.(53)

1. Moisture

A weighed quantity of the sample (about 2 grams) was dried in an oven at 100°C to constant weight. The percentage loss in weight is moisture.

2. Ash

3-5 grams of the sample were weighed in a previously ignited crucible which had been cooled in a desiccator and weighed soon after attaining room temperature. The sample was incinerated in a furnace at 550°C (dull red) until no further loss in weight occurred. It was then cooled in a desiccator and weighed soon after attaining room temperature.

3. Protein Nitrogen

3.0 grams of the sample was placed in a digestion flask. 10 grams of anhydrous sodium sulfate, 20cc. of concentrated sulfuric acid, and 0.2

gram of crystallized copper sulfate were added. The flask was placed in an inclined position and was heated below the boiling point of the acid until frothing ceased. The heat was increased and the mixture digested for a time after the mixture became colorless. The digestion required about two hours. After cooling, the mixture was diluted with 200 cc. of water and a few glass beads were added to prevent bumping. Sufficient sodium hydroxide solution was added to make the reaction strongly alkaline. The flask was connected by means of a Kjeldahl connecting bulb with the condenser, the tip of which extended below the surface of the standard acid in the receiver. The contents were mixed by shaking and the mixture distilled until all ammonia had passed over into a measured quantity of standard sulfuric acid. The acid was titrated with a standard sodium hydroxide solution using methyl red indicator. The amount of nitrogen in the flour could easily be calculated from the amount of ammonia which passed into the sulfuric acid.

4. Total Protein

The total protein was calculated from the protein nitrogen by multiplying by the factor 5.7.

5. Fat

5 grams of the ground sample was placed in a 200 cc. Erlenmeyer flask and a mixture of 10 cc. of 95 per cent alcohol, 2 cc. of strong ammonium hydroxide, and 3 cc. of water was added. The mixture was placed on a steam bath and the contents maintained at the boiling point for 2 minutes. After cooling, the mixture was extracted with 3 successive 25 cc. portions of ether, the material being kneaded and tamped down each time with a stirring rod flattened at one end. The ether layer was decanted off into a 250 cc. beaker and the last 25 cc. portion of ether was drawn off

as completely as possible. Another 15 cc. portion of ammoniacal alcohol solution was added to the extracted residue in the flask and the matted material was disentegrated as thoroughly as possible by means of the flattened glass rod which was left in the flask for that purpose. The flask was returned to the steam bath as before and care was taken that no loss of material occurred through bumping, due to the presence of ether. The contents were boiled for 2 minutes and again extracted with 3 successive 25 cc. portions of ether, adding the ether extracts to those obtained in the first extraction. The combined extracts were evaporated to dryness on a steam bath and the fatty residue extracted with 5 or 6 successive 15 cc. portions of a mixture of equal volumes of ether and petroleum ether. The extracts were collected in a weighed dish and were evaporated to dryness on a steam bath. The residue was dried to constant weight in an oven at the temperature of 100°C, cooled in a desiccator, and weighed. The percentage of fat was then calculated.

B. DETERMINATION OF THE DIAMINO ACIDS

The diamino acids in the flour samples were determined by the silver precipitation method as outlined by Bloch and Bolling (5). Determinations were first run with gelatin, for which the amino acid content is known, so that the author could familiarize himself with the technique. The same procedures were followed for both the gelatin and flour determinations, the original procedure being followed as closely as possible.

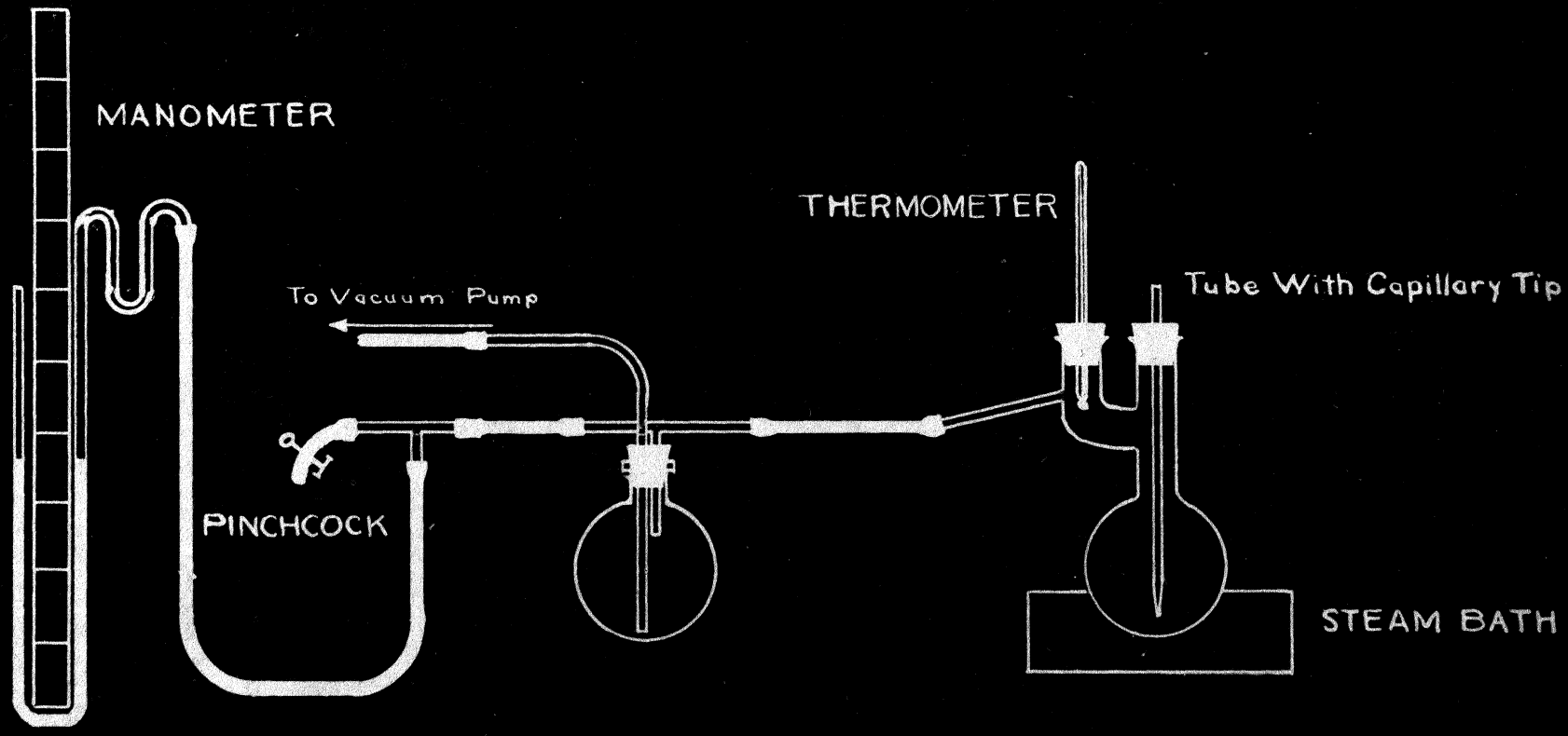
Briefly, arginine, histidine, and lysine were separated from each other and from the non-basic amino acids by precipitating histidine silver at pH 7.4, arginine silver between pH 8.5 and 14, and lysine with phosphotungstic acid. The procedure is as follows:

1. Determination of Histidine

2.500 grams of lipid free flour were hydrolyzed with 25 cc. of 8N sulfuric acid under reflux from 18 to 24 hours. A few glass beads and a cc. of caprylic alcohol aided in easy boiling and in the prevention of foaming. At the end of this period the protein hydrolysate was transferred to a 250 cc. centrifuge bottle and the excess acid was neutralized with 30 grams of barium hydroxide dissolved in to to 100 cc. of hot water. The amino acid solution was adjusted to pH 3.5 at which point it turns congo red paper black. All test papers were carefully washed to reduce losses. Dilute barium hydroxide or sulfuric acid were employed to bring the amino acid solution to the required pH.

The precipitate was removed by centrifugation and the supernatant liquid was filtered through Whatman filter paper into a 500 cc. Claisson flask. The apparatus shown in Diagram 1 was used for all vacuum evaporations in this procedure. The barium sulfate precipitate was washed three times with hot water and added to the filtrate in the flask. A few cc. of caprylic alcohol were added to prevent foaming, and the clear filtrate and washings were concentrated to approximately 75 cc. and transferred quantitatively into a 250 cc. centrifuge bottle.

50 per cent aqueous silver nitrate was added until a drop of the amino acid solution added to a small amount of cold saturated barium hydroxide, held in a porcelain spoon, gave a copius precipitate of brown silver oxide. Cold saturated barium hydroxide was added to the amino acid solution to pH 7.4 as indicated by a distinct blue color with bromthymol blue. This test was also made in a porcelain spoon. The change in the pH as the barium hydroxide was added was followed by litmus paper. As the end point was approached, the precipitate of histidine silver settled very rapidly. When



VACUUM DISTILLATION APPARATUS

DIAGRAM I

NOTE: This apparatus is most conveniently used with a water aspirator.

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the solution was adjusted to blue with bromthymol blue, the reaction was tested with phenolphthalein to be sure the solution was definitely acid to this indicator. The precipitate was centrifuged and the clear filtrate poured into the same 500 cc. flask as employed for the original concentration. The same apparatus was used wherever possible to reduce slight mechanical losses. The histidine silver precipitate was washed once or twice with 200 cc. of water. The combined filtrate and washings, which contained arginine and lysine, were acidified to pH 3-4 with 2cc. of 1:3 sulfuric acid (approximately) and concentrated to approximately 50 cc. in vacuo. Caprylic alcohol was used to prevent foaming.

The histidine silver precipitate was suspended in about 100 cc. of water and acidified with 1:3 sulfuric acid to pH 1 to 2 (distinctly blue to congo red paper). The precipitate was decomposed with hydrogen sulfide. The decomposition is complete shortly after the silver sulfide coalesces into fairly large clumps. The precipitate was removed by centrifugation and the filtrate poured into a 500 cc. Claisson flask. The precipitate was suspended in a little water, again treated with hydrogen sulfide, centrifuged, and the filtrate added to the original. The silver sulfide was washed a second time with water containing a few drops of 1:3 sulfuric acid. The histidine sulfate solution was concentrated in vacuo to approximately 200 cc. and it was transferred to a 250 cc. centrifuge bottle without rinsing the still or the flask. The excess sulfuric acid was then removed by the careful addition of warm aqueous barium hydroxide until the solution turned Congo red paper black. The barium sulfate was removed by centrifugation and the supernatant liquid was returned to the same flask as used above. The precipitate was then washed with 200 cc. of hot water containing a drop of 1:3 sulfuric acid and a few drops of caprylic alcohol.

The combined filtrate and washings, which contained histidine sulfate, were concentrated in vacuo to approximately 10 cc.

The solution was filtered into a 125 cc. Erlenmyer flask using Whatman filter paper. The flask was carefully rinsed with 10 to 15 cc. of water followed by two 15 cc. portions of methanol. Each washing was passed through the filter paper in succession. The histidine sulfate solution sometimes became cloudy after the addition of the methanol. The solution was cooled to room temperature and an excess of nitranilic acid dissolved in a small quantity of methanol was added. The precipitation of histidine nitranilate began upon scratching with a glass rod. The solution was placed in the refrigerator overnight to complete the precipitation, and the precipitate was filtered on a sintered glass filter. The histidine nitranilate, after washing with methanol and ether, was dried at 105°C and weighed.

Histidine = 0.403 x weight of histidine nitranilate

2. Determination of Arginine

The amino acid solution containing arginine and lysine, which had been concentrated to approximately 50 cc., was transferred to a 250 cc. centrifuge bottle and was tested for the presence of silver by the brown spot test (cold saturated barium hydroxide). If the test was not strongly positive more silver nitrate was added. The arginine silver was precipitated by the addition of hot saturated barium hydroxide solution. The solution was made distinctly purple to phenolphthalein and a few cc. of hot saturated barium hydroxide added to insure complete precipitation. The arginine silver precipitate was removed by centrifugation and washed carefully with cold saturated barium hydroxide. The filtrate and washings were kept below 250 cc. A correction, based on the solubility of arginine silver, of 3.6 mg.

of arginine per 100 cc. was applied at this point. The arginine silver filtrate contained the lysine. This was poured into a 250 cc. centrifuge bottle and immediately acidified to below pH 1 with 1:3 sulfuric acid.

The arginine silver precipitate was suspended in 200 cc. of dilute sulfuric acid, the solution being rendered distinctly blue to Congo red paper. The arginine silver salt was decomposed by passing in a stream of hydrogen sulfide. The end point of the reaction was easily determined by the clumping of the silver sulfide. The precipitate was removed by centrifugation and was suspended in 100 cc. of water. Hydrogen sulfide was passed into the suspension a second time to insure complete precipitation of the salt. The precipitate was centrifuged off and washed once more with a little hot water containing a few drops of 1:3 sulfuric acid. The combined filtrate and washings were concentrated in vacuo to 200 cc. and the solution was transferred to a 250 cc. centrifuge bottle without washing the flask. The excess sulfuric acid was removed by the careful addition of warm aqueous barium hydroxide. The barium sulfate was removed by centrifugation and washed twice with hot water containing a drop of 1:3 sulfuric acid, and a few drops of caprylic alcohol. The filtrate and washings were concentrated to about 15 cc. in vacuo and were filtered through Whatman paper into a 125 cc. Erlenmeyer flask. The flask was thoroughly rinsed with small portions of water which, in turn, were used to wash the filter paper.

The combined filtrate and washings, which were kept below 50 cc., were heated on the steam bath to 90°C and an excess of flavianic acid (2,4-dinitro-1-naphthol-7-sulfonic acid) dissolved in a small amount of warm water was added. Within a few minutes the shining plates of arginine

flavianate appeared in solution. At times it was necessary to seed the solution with a crystal of pure arginine flavianate to start precipitation. The flask was placed in the refrigerator for a day to insure complete precipitation. The arginine flavianate was filtered on a sintered glass filter and was washed with cold water, acetone, and ether, respectively. The precipitate was dried at 105°C and weighed.

Arginine = 0.357 x weight of arginine flavianate

3. Determination of Lysine

The acidified arginine silver filtrate containing the lysine was saturated with hydrogen sulfide and the precipitate of barium sulfate and silver sulfide was centrifuged off. The precipitate was suspended in hot water and again treated with hydrogen sulfide. The precipitate was then washed with a little hot water. The combined filtrates were concentrated in vacuo in a 500 cc. Claisson flask to 100 cc. One to 2 cc. of a 0.1 per cent solution of phenolpthalein in alcohol were added. Just enough 10 per cent sodium hydroxide solution was added so that the solution remained red after the addition of 75 cc. of methyl alcohol. The solution was then concentrated in vacuo to approximately 10 cc. and sufficient 1:3 sulfuric acid added to discharge the red color of the phenolphthalein. Sometimes during the concentration the red color disappeared due to insufficient alkalinity as a result of the distillation of ammonia or destruction of the indicator. When this occurred some indicator was added at the completion of the concentration.

Four cc. of 1:3 sulfuric acid were added and the solution of the amino acid was transferred to a 250 cc. centrifuge bottle. The flask was washed 3 or 4 times with small portions of water. The final volume of the amino acid solution was kept below 40 cc. The solution was then heated

on a steam bath to 90°C and 10 grams of phospho-24-tungstic acid dissolved in 50 cc. of 5 per cent sulfuric acid were added. The solution was cooled in ice water and stirred for a short time. Crystallization of lysine phosphotungstate began immediately. The centrifuge bottle was kept in the ice bath for 45 minutes. At the end of this time the lysine phosphotungstate was removed by centrifugation and was washed 3 times with small portions of 2 per cent phosphotungstic acid dissolved in 5 per cent sulfuric acid. A correction of 14 mg. of lysine per 100 cc. of filtrate was made at this point, due to the slight solubility of the phosphotungstate.

The crystalline lysine phosphotungstate was suspended in 50 cc. of water containing 4 cc. of 1:3 sulfuric acid. Fifty cc. of amyl alcohol-ether mixture was than added and the solution stirred until the lysine phosphotungstate was completely dissolved. If not all of the barium was precipitated before removal of the silver it appears as barium sulfate at this stage. When this occurred, it was removed by centrifugation. The water and solvent were transferred to a 500 cc. separatory funnel and the aqueous layer was drawn off into a second funnel of the same size. The solvent layer was poured into an Erlenmeyer flask and temporarily retained there. The separatory funnel was rinsed with 10 to 15 cc. of water containing a few drops of sulfuric acid. The aqueous solution containing lysine was again extracted with solvent and after separation was returned to the first funnel, which had been cleaned in the meantime. The solvent was combined with the original solvent in the Erlenmeyer flask, and this funnel, in turn, was rinsed with dilute acid. The extraction of phosphotungstic acid was carried out a third time in the same manner. The lysine sulfate solution was then transferred to a 500 cc. Erlenmeyer flask. The combined solvent solutions were poured

into a separatory funnel and the aqueous layer drawn off. The solvent layer was discarded. The aqueous layer was extracted with another 50 cc. portion of solvent and the aqueous solution combined with the lysine sulfate in the Erlenmeyer flask. The solvent layer was discarded. The combined aqueous solutions were again extracted with solvent in a separatory funnel and the aqueous layer transferred to a 250 cc. centrifuge bottle. The solvent was treated with water containing a few drops of 1:3 sulfuric acid. This served to rinse out the flask, etc. The aqueous solution was combined with the lysine sulfate in the centrifuge bottle and the reaction was adjusted to approximately pH 5 with warm dilute barium hydroxide (5-6 grams dissolved in 25 cc. of water). Two to 3 grams of barium carbonate were added and the suspension stirred thoroughly. The reaction of the lysine solution was then alkaline to litmus paper. The precipitate of barium sulfate and barium carbonate was removed by centrifugation and the residue was washed several times with hot water. The filtrate and washings were poured into a 500 cc. Claisson flask containing 500 to 100 mg. of barium carbonate and 5 to 10 cc. of caprylic alcohol. The lysine carbonate solution was concentrated to 150 cc. and the contents were transferred to a 250 cc. centrifuge bottle. The precipitate in the flask was also washed into the centrifuge bottle. The barium carbonate was removed by centrifugation and the clear solution was returned to the same flask. The precipitate was washed twice with hot water and the washings added to the filtrate in the flask.

The lysine carbonate was concentrated to about 20 cc. in vacuo. The solution was filtered in a 100 cc. round-bottomed flask and the apparatus rinsed with several small portions of water. The lysine carbonate

solution, which was now in the round-bottomed flask with the washings, was concentrated in vacuo to about 5 cc. The apparatus was rinsed with a few drops of water and then with absolute alcohol until the lysine solution became cloudy. An excess of purified picric acid dissolved in absolute alcohol was added. Lysine picrate crystallized out. The flask was placed in the refrigerator for a day to insure complete precipitation. The precipitate was filtered on a sintered glass filter, washed with cold absolute alcohol and ether and dried at 105°C.

Lysine = 0.39 x weight of lysine picrate

Solution of Arginine, Histidine, and Lysine and other amino acids in H_2SO_4. Add $Ba(OH)_2$ until pH 3.5 is reached. Filter.

Ppt: $BaSO_4$ Discard

Solution: Arginine, histidine, lysine, and other amino acids. Bring solution to pH 7.4; add excess $AgNO_3$. Filter

Ppt: Histidine Silver

Suspend in H_2SO_4 at pH 1-2, add H_2S ~~at pH 3.5~~

Arginine, lysine, and other amino acids in $Ba(OH)_2$ solution. H_2SO_4 solution is added until pH 5 is reached, then solution is evaporated.

$AgNO_3$ added. Solution brought to pH 13 with $Ba(OH)_2$. Filter.

Ppt: $BaSO_4$ Ag_2S Discard

Filtrate: Histidine Sulfate.

Add Nitranilic Acid.

Histidine Nitranilate crystallizes.

Wash, dry, & weigh

Ppt. $BaSO_4$, Ag_2SO_4, Arginine Silver.

Suspend in H_2SO_4 at pH 1-2, add H_2S.

Ppt: $BaSO_4$ Ag_2S Discard.

Filtrate: Arginine Sulfate.

Add flavianic acid.

Arginine Flavianate crystallizes.

Wash, dry, and weigh.

Filtrate: Lysine. H_2SO_4 added to pH 1. H_2S added. Filter.

Ppt: $BaSO_4$ Ag_2S Discard.

Filtrate: Lysine Sulfate

Add NaOH. Evaporate

Residue: Sodium salt of Lysine.

Add excess phospho-24-tungstic acid. Filter

Decompose Lysine Phosphotungstate with amyl alcohol, ether, H_2O. Separate the two solution layers.

Solution: Lysine. Make basic with $BaCO_3$. Filter.

Solvent: Phosphotungstic acid.

$BaSO_4$ ppts.

Filt: Lysine Carb.

Isolate as Picrate

Flow Sheet 1

OUTLINE OF THE SEPARATION OF THE DIAMINO ACIDS USING THE BLOCK-BOLLING SILVER PRECIPITATION METHOD.

CONSTITUENTS	VPI WHEAT FLOUR NO. 131	DAINTY MAID FLOUR	PILLSBURY'S BEST FLOUR
	Percent	Percent	Percent
Moisture	9.05	11.96	12.83
Ash*	0.503	0.477	0.522
Probable ash in wheat*	1.60[a]	1.46[a]	1.68[a]
Fat*	1.17[b]	2.31	1.73
Protein nitrogen*	1.86[c]	1.96	2.43
Total protein*	10.57[d]	11.19[d]	13.83[d]

Table II

Results of Preliminary Analyses

* Calculated on a moisture-free basis

a Calculated according to Bailey (3)

b Determined by Clark (9)

c Determined by Clark (9)

d Calculated from protein nitrogen (53)

Diamino Acids	GELATIN*		FLOUR					
			VPI No. 131		Dainty Maid		Pillsbury's Best	
Sample No.	1	2	1	2	1	2	1	2
Wt. Sample	2.5000	2.5000	3.0000	5.0000	3.0000	3.0000	5.0000	5.0000
HISTIDINE: Wt. Nitranilate	0.1489	0.2778	0.1561	0.2855	0.1352	0.1268	0.1739	0.1983
Wt. Histidine	0.0599	0.1120	0.0629	0.1151	0.0543	0.0510	0.0699	0.0992
Percent Histidine	2.40	4.48	2.30	2.50	2.05	1.93	1.61	1.84
Average Percent Histidine	~~3.44~~		2.40		1.99		1.72	
ARGININE: Wt. Flavianate	0.4551	0.4062	0.2019	0.4627	0.2001	0.2293	0.3223	0.3546
Wt. Arginine	0.1591	0.1449	0.0721	0.1653	0.0715	0.0819	0.1076	0.1311
Percent Arginine	6.90	5.82	2.42	3.30	2.71	3.10	2.64	2.98
Average Percent Arginine	6.36		2.86		2.91		2.81	
LYSINE: Wt. Picrate	0.2479	0.2212	0.0000	0.1538	0.1008	0.0391	0.1412	0.1563
Wt. Lysine	0.0967	0.0864	0.0000	0.0601	0.1170	0.0457	0.0551	0.0611
Percent Lysine	3.87	3.45	0.00	1.22	1.31	1.52	1.26	1.49
Average Percent Lysine	3.66		~~0.61~~		1.61		1.38	

Table III

Experimental Results of Diamino Acid Determinations
(Percentages on a moisture-free basis)

*Gelatin runs made only to improve technique

SUBSTANCE	V.P.I. No. 131 FLOUR	DAINTY MAID FLOUR	PILLSBURY'S BEST FLOUR
		Ratios	
Protein	1.00	1.06	1.31
Histidine	1.39	1.16	1.00
Arginine	1.02	1.03	1.00
Lysine	1.00	1.32	1.13

Table III a

Distribution Ratios of Protein, Histidine, Arginine, and Lysine in Three Wheat Flours

IV

DISCUSSION OF RESULTS

IV

DISCUSSION OF RESULTS

The method outlined by Bloch and Bolling (5) appears to be much more time consuming than stated by him. The time for a complete analysis is considerably more than 12 working hours, although the author discovered that as more runs were made the improvement in technique enabled the analyses to be carried out much more rapidly. The most time-consuming operations were the many vacuum evaporations and the long periods of time required for complete crystallization of the amino acid salts.

The many precipitates which formed during the procedure undoubtedly trapped some of the desired products, as carefully washed as they were. Other losses probably occurred during the many filtrations, evaporations, and crystallizations involved in the procedure. Another fact to be considered is that the quantitative precipitates are all somewhat soluble, and the refrigeration temperature was never low enough to completely precipitate all of the desired products. All of these facts, coupled with the consideration that only 2.5 grams of sample are used in the analysis would indicate that the method is not as accurate as would be desired unless the worker was well-versed in the technique of the analytical procedure.

Several precipitates came down which were not previously reported in the literature. These, in all cases, were found to be due to excess barium or silver salts and were generally brought down during or after boiling. These did not affect the determinations since they were precipitated in fairly small amounts, so would not be capable of trapping any appreciable quantity of amino acids. Nevertheless, they were carefully filtered off and washed, the washings being added to the solutions from

which the precipitations occurred.

The wheat granule is composed of different layers, each one containing a certain amount of each of the five chief wheat proteins.(59). These proteins are gliadin, 40-45 per cent of the total protein, glutenin, about 40-45 per cent, leucosin, 3-4 per cent, edestin, 6-7 per cent, proteose, 3 per cent. No matter how carefully the wheat is milled, it is practically impossible to get the same ratio of the proteins in two separately milled flours from the same wheat; therefore it can be readily observed that the amounts of the diamino acids would vary somewhat in each particular sample of flour from the same wheat. Accordingly, in order to get truly accurate results, it would be necessary to analyze a great many samples of flour, each sample produced by a separate milling of the same wheat. Since the author had only time enough for the analysis of two samples of each flour, the results obtained in this thesis must be taken as being approximate only, and are merely an indication of the probable results which would be obtained for a large number of samples.

V

SUGGESTIONS FOR FUTURE WORK

V

SUGGESTIONS FOR FUTURE WORK

The author strongly believes that since the amount of the diamino acids in wheat flour is actually quite small, and since the wheat sample is so small, more accurate results could be obtained by using the same method as outlined in this paper if from 25 to 50 grams of flour are taken for analysis. Obviously, such a large amount of flour could not easily be adapted for use in this semi-micro method. However, it is suggested that the flour be taken in a quantity of about 50 grams and hydrolyzed as usual. The next step would be to pass it through an ion-exchange resin such as "Amberlite" and by eluting at the correct pH, the diamino acid group could be removed. This could be concentrated to a reasonably small volume and separated by Bloch's procedure. Of course, much larger quantities of reagents would have to be used, but the results should prove decidedly more accurate than those in this paper.

Another suggestion would be to use a completely different and less time-consuming method which would enable the worker not only to carry out more determinations, but would also serve as an excellent check on the original method. The microbiological methods could well serve this purpose since the procedures are said to be accurate within 2 per cent and consume very little time in comparison with the procedure used here.

In future determinations it would be advisable to determine the content of the other essential amino acids in wheat samples grown in different types of soils, and to correlate the amino acid content with the organic and mineral content of the soils. Further, it would be well to have breads baked from the analyzed flours and perform amino acid analyses on the baked products; in this manner the loss on baking could be determined.

VI

SUMMARY

VI

SUMMARY

Three samples of flour were quantitatively analyzed for histidine, arginine, and lysine. The three diamino acids were separated from each other and from the non-basic amino acids by precipitating histidine silver at pH 7.4, arginine silver between pH 8.5 and 14, and lysine with phosphotungstic acid. The histidine was isolated as the nitranilate, arginine as the flavianate, and lysine as the picrate. Analyses were also carried out for moisture, ash, fat, protein nitrogen, and total protein; the probable ash in the whole wheat was also calculated. It was concluded that the method was not too accurate due to the many possibilities for error and the exactness of technique necessary, and also the procedure was too long for the accuracy obtainable. Improvements in the method and further work were suggested for the future.

VII

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