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I hereby recommend that the thesis prepared under my supervision by Lewis Edward Eilson *entitled* "Some Improved Methods for Separating Amino-Acids from the Hydrolytic Products of Proteins." *be accepted as fulfilling this part of the requirements for the degree of* Doctor of Philosophy

Approved by:

Albert P. Mathews

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SOME IMPROVED METHODS FOR SEPARATING AMINO ACIDS FROM THE HYDROLYTIC PRODUCTS OF PROTEINS

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C O N T E N T S

I N T R O D U C T I O N

Further experimental work with the individual amino acids of proteins is desirable but is hindered by the high cost of amino acids due to difficulties encountered in their preparation.

The aim of this work has been to develop methods of isolation that can be successfully followed by the average advanced student; at the same time keeping the cost to a minimum by using only cheap raw materials and relatively inexpensive reagents.

The use of unusual or complicated apparatus has been avoided so that the processes described can be carried on by a number of students simultaneously if desired. Fairly large quantities of material are to be used. In no case should less than 500 g. of protein be taken to start with, in most cases at least 1000 g. is to be used. Six-liter pyrex flasks are the largest pieces of apparatus called for.

The use of ethanol has been avoided wherever possible, methanol or the higher alcohols being used instead, so that most of the methods described can be used anywhere without restriction. The properties and uses of some of these solvents are discussed further on.

The optical rotations of the products made by the methods presented have not been determined. However, an effort has been made throughout the work to avoid operations likely to cause racemization.

More than twenty amino acids are now well known constituents of common proteins. Several others have been discovered during recent years. In this work the entire list has been considered. Simplified methods, tested by experiments, for preparing over half the list are discussed in detail. References and suggestions, for aiding the student to select a method of preparation, are given for the remainder of the list. All the methods described are suitable for large scale commercial manufacturing. Commercial production should not be undertaken, however, without first investigating the status of a number of patents that have been taken out by various workers on certain processes.

Some of the methods presented are original; others are modifications of well-known methods originally devised by various workers for the quantitative determination of amino acids in proteins, which have been simplified by omitting certain steps where this could be done without too great a decrease in the yield of the desired product. In a few cases already existing methods have been recommended without change.

Another feature of the work is the use of barium sulfide as a substitute for hydrogen sulfide for decomposing the amino acid compounds of the heavy metals. A disagreable feature of this kind of work has been thereby eliminated. A detailed discussion is given on a later page.

The bibliography is limited to references actually pertinent to the work.

P R O T E I N S

The following cheap and easily obtainable proteins are recommended for the preparation of amino acids.

CASEIN. The commercial dry powder is too well known to need description (8). It is especially suitable for tryptic digestion and the preparation of tryptophane.

LACTALBUMIN. This is now a cheap commercial product used in poultry foods. It comes in the form of a fine white powder containing 30% or more of calcium phosphate as a natural impurity precipitated with it in the process of making lactose from whey (8). It is unusually rich in tryptophane and aspartic acid.

BLOOD. All the blood proteins are comparatively rich in tryptophane. Sedimented blood corpuscles are an excellent source of histidine. Dry blood meal can be obtained from meat packers or dealers in animal foods if fresh blood is not available.

The KERATINS, horn, hoof, feathers, hair, etc., are discussed in connection with the preparation of cystine and tyrosine.

GELATIN, in pure form, is rather expensive. For most purposes commercial bone glue or hide glue, which are quite cheap, can probably be substituted.

WHEAT GLUTEN. This is discussed under glutamic acid.

CORN GLUTEN MEAL. This is a byproduct of the manufacture of corn starch (9)(10). It contains 40% protein, of which about one half is the alcohol soluble prolamine, zein. It is a cheap cattle food sold by all feed dealers. It should not be confused with "corn gluten feed" which is a commercial mixed cattle food containing about 22% protein, nor with "corn germ meal" made from corn germ oil residue and containing only 18% protein. While the zein can be extracted by ethanol or isopropanol and used in pure form, in this work methods have been devised of hydrolysing the whole substance just as received and separating amino acids from the mixture. Other organic acids, such as levulinic, formic, etc., are present in the hydrolysed mixture, being formed by the action of the acid on the carbohydrates present (starch and corn hulls). Furfural is also formed from pentosans present in the hulls and reacts with some of the amino acids, notably tyrosine (11) forming black "humin" products.

OIL MEALS. These are the residues from the extraction of various vegetable oils from seeds. They are used for cattle foods. If prepared by the hydraulic press or by the continuous expeller method they may contain some residual oil, if the solvent extraction method was used they may be nearly free from fatty material. The following may be obtained from almost any feed dealer:

Coconut copra meal, 20% protein.

Cottonseed meal, 41% protein.(12).

Peanut meal, 45% protein (13).

Linseed meal, 32% protein (14).

Soybean meal, 40% protein.

A soybean flour, "Farinette", 51% protein, intended for human consumption, is also on the market (15).

A number of other oil seed meals are available from special sources.

The protein contents given above are based on the conventional Kjeldahl N x 6.25 and are therefore somewhat too high since some nitrogen is present in non-protein form. Also, the factor 6.25 is too high for some proteins, for example, the factor for peanut protein has been estimated to be as low as 5.5 by some workers (13).

Copra meal, because of its low protein content, is of no importance for the preparation of amino acids by the methods discussed in this work; attention is directed, however, to a method recently described by Beckman (16) whereby the oil is separated and amino acids are liberated at the same time by the fermentation of ground copra or other seeds by Bacillus delbrueckia at 50° C.

The other seed meals may be used in their crude state for acid hydrolysis. The large amount of carbohydrates present complicates matters a bit. Tryptic

digestion is not to be recommended since the seed proteins digest with difficulty unless given a preliminary treatment with pepsin (3). Furthermore, the solutions obtained are slimy and impossible to filter. Hydrolysis by barium hydroxide also yields slimy solutions. Acid hydrolysis gives solutions that are readily filtered. Linseed meal requires a somewhat longer treatment with acid as it contains a gum which is rather resistant to chemical action.

By a preliminary extraction of the seed meals with a suitable solvent various other substances of biochemical interest may be obtained such as rare sugars, sterols, pigments, phospholipids, etc.; for example cottonseed meal yields among other substances 5% of raffinose and soybean meal 1% or more of lecithin. Isopropanol, discussed on page 20, is an excellent extracting medium for all such substances and also extracts any fixed oil remaining in the meal. By extraction of the lipoid matter from cottonseed meal by a solvent the protein content of the residue is raised to 60-65% (55). If soluble carbohydrates are also extracted the percentage is still higher.

A few other proteins are discussed in connection with some of the individual amino acids.

HYDROLYSIS OF PROTEINS

Four methods of hydrolysis of proteins were studied in connection with this work, namely, by sulfuric acid, by hydrochloric acid, by barium hydroxide and by tryptic digestion. Other methods are possible but are not convenient to use (3).

Acid hydrolysis is usually employed in large scale work. Much black humin is formed. Part of this is insoluble and part is soluble in the acid solution. Consequently the solution is always dark colored and sometimes this coloring matter is very difficult to remove. Humin formation is discussed in detail in Mitchell and Hamilton's book (3) and a good bibliography given therein. See also reference (11).

HYDROLYSIS BY SULFURIC ACID.

The original method of Kossel and Kutscher (17) called for 1 part protein, 3 parts by weight of sulfuric acid, and 6 parts water. After refluxing 14 hrs. the mixture was diluted so that each liter represented 50 g. protein and the sulfuric acid was removed by barium hydroxide. This amount of acid is excessive. After a few preliminary experiments the following proportions were adopted and used throughout the work:

Dry protein 1000 g., conc. sulfuric acid 1000 g., water 2000 g. These are mixed in a 6-liter pyrex flask and set on a steam bath overnight. In the morning the flask is set over a bunsen burner and refluxed all day or for 1 hr. after the biuret test shows negative. Usually 6 to 8 hrs. refluxing are sufficient.

For corn gluten meal, 40% protein, use 1250 g., equivalent to 500 g. protein, with conc. sulfuric acid 600 g., and water 1200 g., in a 6-liter flask. Larger amounts cannot be used as the mixture bumps and foams badly.

For fresh animal tissues, fresh blood or blood corpuscles, or fresh wet precipitated casein use 300 g. conc. sulfuric acid for each 1000 g. and no water added.

If a keratin is hydrolysed by sulfuric acid no cystine can be precipitated from the hydrolysate (1).

What becomes of the cystine is not known.

"Even after a prolonged hydrolysis by 20% sulfuric acid closed ring compounds or ketopiperazines are found in the hydrolysate, and their percentage increases if the hydrolysis is prolonged." (24). Prolylphenylalanine is found in hydrolysed zein (52).

Some workers advise the use of 20% or 25% sulfuric acid for hydrolysis. Takayama (91), however, reports that in preparing glutamic acid from soybean protein larger yields were obtained with 50% acid than with 25%. Still stronger sulfuric acid (10 N) split off some ammonia from pure glutamic acid at 100° and 135-45°.

HYDROLYSIS BY HYDROCHLORIC ACID.

Published directions usually call for diluted HCl. The constant boiling point mixture, 22% HCl gas by weight, is a favorite. For typical examples see the directions for preparing cystine or glutamic acid in "Organic Syntheses" vol. V (4).

Certain objections were found to the use of such dilute acid. A part of the HCl combines with the protein or liberated amino acids still further reducing the concentration unless a very large excess of the diluted acid is used. The contents of a 2-liter or larger flask on the ordinary steam bath seldom reach a temperature of 90° (1) and the time required for complete hydrolysis is greatly prolonged. Three days are often required; casein may require as long as 5 days (18). Even if refluxed the time is longer than need be. For this reason Folin (19) advocates the use of more concentrated acid.

The following modification of Folin's method was used throughout my work:

Dry protein 500 g., conc. HCl 1000 g., water 500 g., were used for a 2-liter or 3-liter pyrex flask, or double these quantities for a 6-liter flask. The flask is set on the steam bath over night and the next day boiled gently over a bunsen burner. No reflux condenser is used. Unless an extremely well ventilated hood is available

the fumes must be collected. A small inverted glass funnel loosely supported on the neck of the flask and connected to a filter pump is satisfactory. HCl gas or water vapor is boiled off until a constant boiling point mixture boiling at 110° is reached. The biuret test is often negative in less than 6 hrs. boiling. Boiling is continued 1 hr. longer. If during the boiling process the solution is concentrated to about half its original volume this is an added advantage.

For corn gluten meal, 40% protein, use 1250 g. meal, 1000 g. conc. HCl and 500 g. water for a 6-liter flask.

For fresh animal tissues, fresh blood or blood corpuscles or wet fresh precipitated casein use an equal weight of conc. HCl and do not add any water (80).

Hydrolysis by alcoholic HCl is only partial (3).

When hydrolysing by HCl metallic tin is sometimes added to decrease the amount of humin formed (23). This should not be done when cystine is to be prepared as it reduces this to cysteine.

HYDROLYSIS BY BARIUM HYDROXIDE.

This method is seldom used because it racemizes the amino acids. It is occasionally used for the preparation of tryptophane since this is destroyed by acid hydrolysis. No humin substances are formed and the hydrolysate is light colored.

The methods that have been published usually call for: dry protein 200 g., barium hydroxide crystals, $Ba(OH)_2.8H_2O$, 700 g., water 4 or 5 liters. This is an excessive amount of barium hydroxide. The proportion of protein can be nearly doubled and good results still obtained by a somewhat longer boiling time (1).

The following proportions are recommended: dry protein 300 g., barium hydroxide crystals 700 g., water 4 liters, in a 6-liter flask. Larger quantities are likely to bump badly. Two flasks should be run at the same time. No reflux condensed is used so that the ammonia liberated escapes as fast as formed. A mark is made on the flask at the initial liquid level and the water boiled o ff is replaced from time to time.

The boiling is continued until the biuret test gives only a faint pink color. Two days or longer may be required. Experiments on casein and lactalbumin showed that a negative biuret test could not be obtained even after 10 days boiling with additional barium hydroxide.

The usual way of removing the barium from the hydrolysate is to precipitate it with sulfuric acid. The large amount of barium sulfate formed, owing to its fine state of division, is difficult to filter off and almost impossible to wash. I have found that better results can be obtained by precipitating most of the barium as carbonate by bubling carbon dioxide from a commercial cylinder into the hot hydrolysate. The precipitate is coarse and easily washed. From the filtrate the rest of the barium is precipitated by sulfuric in the usual way.

It is possible that some aspartic acid is lost by being carried down with the precipitated carbonate. Barium aspartate seems to be only sparingly soluble in the presence of barium salts. This point needs further investigation.

By alkaline hydrolysis any arginine present in the protein is decomposed into ammonia and ornithine. No definite information has been found as to where ornithine appears in the subsequent separation of the amino acids. Presumably it accompanies the lysine as it is similar in constitution. Cystine is also destroyed, just what becomes of it does not seem to be well established although it has been discussed to some extent in several papers (21).

Calcium hydroxide only partially hydrolyses proteins even after 10 days boiling in a thick suspension (1).

HYDROLYSIS BY TRYPTIC DIGESTION.

This method is discussed further on in connection with the preparation of tryptophane.

Its use is not limited to the preparation of tryptophane, however. It may be used for the preparation of most of the amino acids provided the protein chosen is one that is readily digested by the trypsin mixture. Keratins are not digested and the seed products previously described are digested only to a limited extent (1), so its use is practically limited to casein, gelatin, blood and other animal tissues. The digested mixture probably always contains some peptides. Proline and phenylalanine are not liberated from casein (32).

Some compounds not free amino acids but soluble in butanol or ethanol are also formed. These are probably anhydrides or diketopiperazines (22). Some are soluble in ether. If the tryptic digestion is continued too long l-tryptophane is partly converted into d-tryptophane anhydride and l-tyrosine to a small extent into d-tyrosine anhydride (33).

USE OF BARIUM SULFIDE.

The decomposition of metallic compounds by hydrogen sulfide has always been a disagreable feature of the preparation of certain amino acids although the availability of compressed hydrogen sulfide in commercial cylinders makes this less true than formerly.

Ammoniumsulfide was tried but was not found satisfactory. While any excess of normal sulfide can be decomposed and expelled from the solution by boiling, any polysulfide present cannot be so removed an any reasonable length of time as it is fairly stable at boiling temperature.

Barium sulfide was tried and found very satisfactory. The ordinary reagent grade was used. This usually contains 80-85% of BaS, the remainder being a mixture of polysulfide, sulfite, thiosulfate, hydroxide and possibly salts of the complex sulfur acids, trithionic, tetrathionic and pentathionic. These impurities do not seem to interfere with its use.

The precipitated mercury, lead, copper, or zinc compound of the amino acid was suspended in water, and a hot concentrated solution of the barium sulfide, containing suspended insoluble material, added until a drop of themixture when placed on lead acetate paper made a black spot surrounded by a brown ring. The alkaline mixture

was then filtered on a suction filter and the precipitate washed with a little hot water.

To the filtrate enough sulfuric acid was added to give a blue color with congo paper. This liberates H_2S from the excess sulfide and H_2S plus free S from the polysulfide. Barium sulfite is insoluble in alkaline solution and should not appear in the filtrate, however, if it were present the SO_2 liberated by the H_2SO_4 would react with H_2S forming free S and H_2O. Barium thiosulfate is soluble in 480 parts water and is also capable of forming double salts which are more soluble. It reacts with H_2SO_4 to form $BaSO_4$, S, and SO_2 and the last named reacts with H_2S to form S and H_2O. The other complex sulfur acids probably react in a similar manner to thiosulfuric, if sufficient H_2SO_4 and H_2S be present, although positive information on this point is not available. The acid solution is warmed and air bubbled through until H_2S is removed, then filtered. The filtrate should then contain only amino acids and sulfuric acid.

To test the truth of this last assertion the following experiments were made: Three lots of barium sulfide were obtained from different supply houses. Twenty grams of each lot was dissolved in 500 cc. hot water, filtered, decomposed by a small excess of sulfuric acid, H_2S removed by a current of air, and filtered. The fil-

trate was made alkaline with barium hydroxide solution to remove sulfate ion and filtered. To the filtrate was added an excess of ammonium carbonate to precipitate barium and the solution again filtered. The filtrate was then evaporated to dryness. Two of the samples left no residue, the third sample left a very small amount of a white hygroscopic ammonium salt the identity of which was not learned as it did not respond to any of the tests for the ordinary acid radicles. It is recommended that each lot of barium sulfide be tested in a manner similar to that just given and that any lot which leaves an appreciable residue be rejected.

From the sulfuric acid solution of amino acids obtained by the above method the sulfuric acid may be precipitated by carefully adding just the right amount of barium hydroxide to precipitate all the sulfate ion. If too much is accidently added dilute sulfuric acid is added drop by drop until the right point is reached. This is a slow, tedious operation. Where the presence of a little ammonia will not interfere with subsequent operations the following method is much more convenient:

To the acid solution enough barium hydroxide is added to make it alkaline and the precipitate is filtered off. This removes all sulfate ion. If the barium salts of the amino acids are desired the solution is merely

evaporated at this point. If the free amino acids are desired excess of ammonium carbonate is added to precipitate all barium (also calcium, etc., if present) and the solution filtered. The excess ammonium carbonate is then expelled by boiling.

Mercuric sulfide though insoluble in ammonium sulfide is slightly in all other alkali sulfides. When the black precipitate is allowed to stand in the presence of an excess of barium sulfide it gradually changes over into the red microcrystalline form (artificial cinnabar). A small amount is also retained in solution in the alkaline sulfide filtrate but is all precipitated (black) when the filtrate is acidified.

ISOPROPANOL.

Isopropanol is now manufactured from propylene on a large scale by several concerns and the price of the commercial grade is at present only a few cents per gallon higher than tax-free ethanol. The commercial article is the constant boiling point (80.3°C.) mixture containing 91% by volume or 88% by weght of secondary propanol; the remainder is water. An absolute grade is also obtainable but the price is considerably higher. It boils at 83°. The term "isopropanol" as used throughout this work refers to the commercial c. b. mixture.

Isopropanol is destined to become an important laboratory reagent. It offers the following advantages:

There are no troublesome regulations governing its use. It may be bought, sold, redistilled and handled generally by anyone without restriction.

It is non-poisonous and its odor is slight and not disagreable.

When diluted it can be concentrated by shaking with sodium chloride. Enough water is removed as a separate brine layer to raise the concentration to nearly that of the c. b. mixture. About 2% of salt remains dissolved in the isopropanol layer. Shaking with dry potassium carbonate concentrates it still more, nearly to 100%.

Being a secondary alcohol it forms esters with

much greater difficulty than do primary alcohols. This makes it useful where it is necessary to heat alcoholic solutions of organic acids, especially in the presence of hydrochloric acid.

It is an excellent solvent for the extraction of dry animal or vegetable tissues. From the latter it extracts prolamines, sugars, chlorophyll, all yellow pigments, all lipoid material, and many other substances. Fixed oils and fats while only slightly soluble in isopropanol are completely extracted and form a separate layer in the Soxhlett flask. From dried hog brain it extracts everything that can be extracted by any other solvent or succession of solvents, a total of 60% of the dry brain tissue.

Isopropyl ether has been lately put on the market at the same price as ethyl ether. Biochemists should find it useful. It has the same solvent properties as ethyl ether but boils at 67.5°. It holds much less water in solution than ethyl ether and is also less soluble in water.

S E P A R A T I O N O F A M I N O A C I D S

I N T O G R O U P S

FISCHER'S ESTER METHOD.

This was the only method used until about 1920. It is too well known to need description. For a discussion see Mitchell and Hamilton's book (3). It is a very laborious method and requires special apparatus so will not be further considered here. (114).

THE SIGFRIED CARBAMINO METHOD.

For a description see references (3)(77). This method is hardly suitable for small scale laboratory use but for large scale operation in a factory equipped with a refrigerating outfit, filter presses and alcohol recovery stills would probably be a very convenient method to use.

DAKIN'S BUTANOL METHOD (35).

For a description see Mathews' text (2). The mixed amino acids are separated into the following groups:

1. Monoamino acids, insoluble in dry butanol but extracted by wet butanol.

2. Proline, soluble in dry butanol.

3. Peptic anhydrides (diketopiperazines) extracted by butanol and soluble in dry butanol but separated from proline by their sparing solubility in water and ethanol.

4. Dicarboxylic acids, not extracted.

5. Diamino acids, not extracted.

Groups 4 and 5 remain in the aqueous solution and are separated by other methods. Hydroxyproline if present goes into group 1. Serine and glycine are not readily extracted from aqueous solutions by butanol, at least 60 hrs. of continuous extraction is required (29).

While Dakin's method is suitable for small scale operations and for analytical work it can hardly be considered convenient for 1 kg., or larger, lots of hydrolysed protein because of the large volumes of solutions to be handled which would require special apparatus. Nor is the modification of Dakin's method described by Osborne and co-workers (34) any more convenient unless a suitable still is available for handling the large volumes of butanol used. This reference (34) also discusses

the solubilities of the diketopiperazines.

Dakin also states that propyl, isobutyl, and isoamyl alcohols can be used in a somewhat similar manner but are less satisfactory.

The presence of salts of any kind interferes more or less with the butanol extraction.

Butanol can be advantageously used in a few cases to effect a further separation of amino acids after they have been first separated into groups by other methods.

SEPARATION BY ORGANIC SOLVENTS.

A large number of volatile solvents are now readily available thanks to the lacquer industry. Samples of many of these were obtained and attempts made to separate mixed amino acids by their use. This work was soon abandoned as it did not seem to be leading anywhere. As a rough general rule it may be stated that most amino acids are insoluble in anhydrous solvents and are more or less soluble in solvents containing a little dissolved water.

Some work was done on the separation of amino acids by the varying solubilities of their salts in alcohols and ethers. The following alcohols can now be readily obtained in any quantity desired: Very cheap, methyl, ethyl, isopropyl, n-butyl and isoamyl. More expensive,

but still cheap enough to use, n-propyl, sec-butyl, n-amyl, sec-amyl, tert-amyl and cyclohexanol. The cheap ethers are ethyl, di-chloroethyl, isopropyl, and a number of monoalkyl ethers of several glycols. Specimens of all of these were obtained through the courtesy of the manufacturers.

The HCl compounds of all the amino acids were found to be more or less soluble in the alcohols. Various proteins were hydrolysed with HCl, filtered without neutralizing, and evaporated to a thick syrup containing free HCl. In each case the syrupy solution dissolved readily and completely in 5-10 vols. of isopropanol, except that if the protein was rich in glutamic acid some glutamic acid hydrochloride crystallized out. It was found that by gradually neutralizing the warm isopropanol solution with powdered ammonium carbonate the amino acids could be precipitated out in several fractions. Proline and small amounts of several others remain dissolved and are left in the flask when the isopropanol is recovered by distillation. This residue also contains ammonium chloride since this salt is somewhat soluble in isopropanol. This method should be useful for working up the mother liquors from the preparation of glutamic acid from glutens by the HCl process.

The syrupy solution of HCl compounds of amino acids can also be dissolved in butanol which is nearly free from dissolved water.(34). In the case of some proteins the

mixture dissolves completely forming a clear solution, in other cases some dark colored material is left undissolved. When the butanol solution is neutralized with ammonium carbonate the ammonium chloride formed has a tendency to take up water and form a separate aqueous layer containing dissolved amino acids. Such a mixture is difficult to deal with and this line of investigation was abandoned.

No experimental work was done with the other alcohols and the HCl compounds of the amino acids.

Experiments on the separation of the metallic salts of the amino acids by alcohols and ethers are discussed later under the headings of the various metallic salts.

SEPARATION BY ELECTRODIALYSIS.

This method is not suitable for laboratory use because of the special equipment and careful control required. It might, however, prove to be a very convenient method for large scale factory operation. For a discussion and bibliography od the method see references (3)(56).

PRECIPITATION BY PHOSPHOTUNGSTIC ACID.

No work was done with the phosphotungstic acid compounds in connection with the preparation of this paper. The method was originated by Drechsel and perfected by Kossel in his work. Phosphotungstic acid precipitates histidine, lysine and cystine completely from acid solutions and arginine, tryptophane, proline (94), and aspartic acid more or less completely depending on conditions (3)(41). Ammonium, barium, calcium, magnesium and sometimes potassium are also precipitated as phosphotungstates.

The precipitation is often made with a 10% solution of phosphotungstic acid in the presence of 5% of sulfuric acid (42). A large amount of phosphotungstic acid is required, 10 to 15 times the weight of the amino acid precipitated (53). The precipitate is bulky and difficult to wash. The precipitate can be decomposed by barium hydroxide which forms a more insoluble precipitate of barium phosphotungstate (42). Probably the acid could be recovered for re-use by treating this barium precipitate with sulfuric acid; this has not been tried.

The precipitation can also be made in a hydrochloric acid solution, usually a solution containing 3.5% HCl. This method is discussed in detail by Van Slyke (43) and others (44). Precipitation is possible in 20% HCl (29).

The phosphotungstate precipitate can be decomposed and the acid recovered by wet extraction with ether or isoamyl alcohol or a mixture of the two (45)(71).

An up to date discussion with a complete bibliography is given by Thimann (53). A typical formula for a precipitated compound is $A_3.2H_3PO_4.24WO_3.H_2O$ where A stands for the amino acid.

SERARATION BY PERMUTIT.

Permutit will remove any fairly strong base from a neutral solution. Consequently it takes up lysine, histidine, arginine and presumably ornithine from solutions, also ammonia and amines. The monoamino acids are not adsorbed. The adsorbed compounds are liberated by shaking with saturated KCl solution (57). This method is hardly suitable for the isolation of the pure diamino acids but may have considerable value in connection with the analysis of proteins.

SEPARATION AS METALLIC SALTS.

While a single amino acid may readily form an insoluble metallic salt with a suitable reagent, in a mixture of amino acids this often does not occur, the compound being held in solution by the interfering action of the other amino acids present.

SALTS OF K, Na, Mg. These are all soluble in water. No information is available concerning their solubilities in alcohols. An objection to the use of such salts would be the difficulty of removing the base from the solution to get the free amino acids. Also the K and Na salts hydrolyse extensively. Ammonium salts of the amino acids either do not form or are unstable and decompose completely when the solution is evaporated.

SALTS OF Ca and Ba. These are very useful. They are discussed in a later section.

Chlorides of Au and Pt have been used by various workers. They are too expensive to be considered in this work.

SILVER SALTS. It still seems to be considered necessary to use the silver salts as a means of purify-

ing certain amino acids. Such methods are still too expensive for general use although the price of silver has been declining the last few years. Dangerously explosive compounds are sometimes formed when working with silver oxide and organic substances and several fatal accidents have been reported. The silver can easily and completely be recovered for re-use. A convenient method has been published by Robertson (46).

SALTS OF Fe, Al, Cr, Mn. These are not worth considering. The salts of the trivalent metals hydrolyse extensively of completely. Manganese has a tendency to oxidize.

SALTS OF Co, Ni, Cd. Preliminary experiments showed that these metals form complex ions with the amino group and cannot be made to form a precipitate in an aqueous or alcoholic solution of mixed amino acids. The ability of cobalt and nickel to form a large number of complex ammonium ions is well known.

ZINC SALTS. While some of the zinc salts are insoluble in water it seems to be very difficult to precipitate any amino acid as a zinc salt from a mixed solution. But zinc salts are frequently used in the final steps of purif-

ication of certain amino acids. These uses are discussed under the individual amino acids. The zinc salts seem to be quite stable. Little is known about their solubilities in alcohols or other organic solvents. Further research is needed on this point.

MERCURY COMPOUNDS. Mercuric sulfate or chloride reacts with the amino group forming insoluble precipitates with many of the amino acids under suitable conditions of concentration and pH. The mercury compounds of histidine and tryptophane are especially important and are discussed under the preparation of these amino acids. Mercuric reagents are not cheap and often a very large excess is needed to effect complete precipitation. The mercury is not readily recovered and in most, or perhaps all, cases is to be regarded as a total loss.

LEAD SALTS. Lead is a strong base compared to many heavy metals and forms stable salts with weak organic acids in general. The lead salts of tyrosine and aspartic acid are insoluble in water, all others are soluble (47)(48)(87). Nothing is known of the solubilities of the lead salts of amino acids in alcohols and ethers, but judging from the peculiar solubilities of other organic lead salts in these solvents, and the frequent use of lead salts for the separ-

ation of other organic acids, a study of the solubilities of the lead salts of amino acids in organic solvents might be worth while. A possible objection to the use of lead as a base is its tendency to form basic salts which might have different solubilities from the normal salts.

COPPER SALTS. These are very important and have been extensively studied by many workers. They are considered in the next section.

SEPARATION AS COPPER SALTS.

Copper forms stable crystalline salts with the carboxyl group (3)(49) and also complex dark blue compounds with the amino group (1).

Phenylalanine, the leucines and aspartic acid form salts which are practically insoluble in water and can often, but not always, be precipitated from a solution of mixed amino acids. The precipitates are insoluble in dilute acetic acid.

Tryptophane also forms an insoluble copper salt if pure, but in the presence of very small amounts of other amino acids the compound is soluble.

Cystine can be precipitated as a copper salt by boiling with copper hydroxide (40).

Certain copper salts are soluble in alcohols. Foreman states, "If the dry amino acids are boiled with copper hydroxide and absolute ethanol only the proline salt goes into solution." (50). If a little water is present others may dissolve (1). Other copper salts are soluble in methanol or other alcohols, in the glycol mono-alkyl ethers, and in mixtures of acetone and water but not in dry acetone (1).

Town (51) separated the mixed amino acids into groups through their copper salts in course of his work on the preparation of pure proline. Brazier (52) enlarged upon Town's

work and devised a complete method for the separation of all the amino acids of zein or similar proteins. Her first step was to prepare the dry copper salts of the mixture of amino acids. These were then separated into three fractions:

(a). Copper salts insoluble in water: Leucine, phenylalanine, aspartic acid.

(b). Copper salts insoluble in absolute methanol: alanine, tyrosine, glutamic acid, histidine, arginine, (also glycine and bases if present).

(c). Copper salts soluble in absolute methanol: valine, hydroxyvaline, proline, prolylphenylalanine.

Each fraction was then separated further by various precipitation methods. All students working on amino acids should study this method. Lysine and hydroxyproline are not mentioned in the above scheme as they do not occur in zein. Preliminary experiments have shown that this method would have to be modified somewhat before it could be used on hydrolysed blood corpuscles and some other proteins.(1).

In all the published work on copper salts the salts have been prepared by treating a solution of the amino acids freed from other compounds with copper hydroxide or basic carbonate. This is unnecessary. A simpler method is to hydrolyse the protein with sulfuric acid and neutralize with barium hydroxide in slight excess thus obtaining an alkaline solution of the barium salts . This is heated

and air bubbled through it until ammonia is removed. Then a solution of copper sulfate is added until the solution shows all barium precipitated and a slight amount of sulfate ion present. The mixture is then filtered and the filtrate evaporated. The excess copper sulfate present if sufficiently small in amount will not interfere with subsequent operations. Large amounts may interfere through the formation of complex double salts. The water insoluble copper salts precipitate more or less completely with the barium sulfate when the copper sulfate solution is added and the amino acids are recovered by treating the washed mixed precipitate with hydrogen sulfide or barium sulfide.

SEPARATION AS CALCIUM OR BARIUM SALTS.

The calcium or barium salts of aspartic and glutamic acids are insoluble in 80% ethanol or methanol consequently they can be precipitated out by adding several volumes of the alcohol to a concentrated aqueous solution of the calcium or barium salts of a mixture of amino acids. Details of the method are given under aspartic acid. It does not seem to be definitely known whether or not some phenylalanine is also precipitated at times. This point should be determined.

According to Kodama (79) aspartic and glutamic acids and phenylalanine all form "basic" calcium salts which are insoluble in water in the presence of excess calcium hydroxide. I have been unable to confirm this.

The amino acids are liberated from their calcium or barium salts by adding exactly the right amount of sulfuric acid in the case of barium or oxalic acid in the case of calcium. This requires careful work. Where ammonia will not interfere with subsequent operations a simpler method is to add an excess of ammonium carbonate. The calcium or barium carbonate is filtered off and the excess of ammonia and ammonium carbonate expelled by boiling (1).

In this work a new method of separating a mixture of amino acids into groups was developed based on the solubilities of the calcium or barium salts in various alcohols.

The following experiments will demonstrate the method:

(1). Calcium salts. Corn gluten meal was hydrolysed with sulfuric acid, filtered, made alkaline with slaked lime and again filtered. The solution of calcium salts was evaporated to a thick syrup a pinch of lime having been added to keep it alkaline. The mixture was then extracted with alternate 500 cc. portions of wet and dry butanol until nothing more dissolved. The undissolved residue was dissolved in a little water, filtered, a pinch of lime added, and again evaporated to a syrup to remove all dissolved butanol. Then it was extracted with isopropanol. The residue insoluble in isopropanol was dissolved in the minimum amount of water and 4 vols. of methanol (ethanol can be used equally well) added to precipitate calcium aspartate and glutamate. Four fractions have now been obtained:

(a). Calcium aspartate and glutamate, insoluble in all alcohols.

(b). Calcium salts soluble in 80% methanol but insoluble in isopropanol or wet butanol.

(c). Calcium salts soluble in isopropanol but not in wet butanol. (These salts are also soluble in 80% methanol or ethanol.) This fraction was small.

Lastly, calcium salts dissolved in wet butanol. These are further separated as follows: The butanol solution is heated on the steam bath and ammonium carbonate

lumps added until all calcium has been precipitated and ammonia is freely evolved. The precipitate is separated, after cooling the flask, by decantation or filtration (fraction d). The butanol solution is distilled to a boiling point of 115°, which removes nearly all the dissolved water, and cooled. A quantity of mixed amino acids separates out (fraction e). The proline fraction remains in solution as in the Dakin method . Summarizing this last paragraph we now have:

(d). Diamino acids, mixed with calcium carbonate.

(e). A mixture of monoamino monocarboxylic acids soluble in wet butanol but precipitated out when the water is removed.

(f). Proline and other compounds (anhydrides) soluble in anhydrous butanol.

Thus by a very simple method the mixture of amino acids has been separated into six fractions. Each fraction can then be further separated into its components by suitable methods. The identity of the amino acids in fractions (b), (c), and (e) has not yet been fully established. Work is still in progress.

2. Barium salts. Casein was hydrolysed with barium hydroxide, the excess of hydroxide reduced to a small amount by carbin dioxide, and the solution evaporated to a syrup. This was then treated according to the above scheme and the

six fractions obtained. These have not all been identified as yet. Nor is it yet known whether the corresponding fractions contain the same amino acids when barium is used as when calcium is the base employed. A large amount of work remains to be done on this. The tryptophane was not found in fraction (e) as expected but in fraction (f). Apparently it had been changed into an anhydride or peptide soluble in dry butanol. Some tyrosine was found in several fractions.

3. The calcium salts from 4 kg. of fresh blood corpuscles were treated in a similar manner and the six fractions obtained. In this case fraction (c) was very small and deliquescent due to presence of calcium chloride.

MISCELLANEOUS PRECIPITATION METHODS.

Many amino acids form insoluble precipitates with certain organic acids or other compounds such as picric acid, picrolonic caid, beta-naphthalene sulfochloride, flavianic acid and others. Such precipitation methods are usually employed after the crude mixture of amino acids from the hydrolysed protein has been separated into fractions by other methods. No use of such methods was made in this work. For discussion see reference (3).

Picric acid (61) has been used by many workers in the isolation and purification of amino acids. While this is suitable for small scale experiments it should not be forgotten that picric acid is a dangerous explosive and that its use in 100 g. or larger lots may eventually through carelessness or otherwise lead to serious accidents. Now it is known that in some cases the corresponding chloro- and nitro- benzene derivatives have similar properties and the suggestion is offered here that it might be worth while to investigate the properties of trichlorophenol to see if it could be substituted for picric acid in certain procedures.

Many amino acids form difficultly soluble double salts. Extensive study of these has been made by Pfeiffer (3). They are not of interest here.

OTHER POSSIBLE METHODS.

Formaldehyde reacts with the amino group forming a methylene disubstituted group which is inactive toward acids and metals (2)(58)(62). Only the carboxyl group of the amino acid is then reactive. If a mixture of amino acids as obtained from a hydrolysed protein were treated with formaldehyde the mixture of new acids formed might be converted into suitable metallic salts and separated by various solvents. If the methyleneimino group could then be reconverted into the amino group, regenerating the original amino acid, a separation of the amino acids could thus be effected. How readily this could be done is not known, if indeed it be possible at all.

In a recent paper by H. S. Fry and P. E. Bowman (59) isoamylamine was converted into isoamylmethyleneimide by formaldehyde and the isoamylamine quantitatively regenerated by dilute sulfuric acid. This would suggest that the method is worth trying.

Possibly it might be found difficult to completely remove the liberated formaldehyde from the acid solution so that it could not recombine with the amino acid when the solution was neutralized. In one method of preparing synthetic glycine (60) methyleneiminoacetonitrile is first prepared from formaldehyde and ammonium chloride followed by potassium cyanide. The nitrile is boiled wuth barium

hydroxide whereby it is hydrolysed and the ammonia liberated expelled from solution. After removal of barium as sulfate the methyleneiminoacetic acid is boiled for 4 hrs. with 3% sulfuric acid which is said to completely remove the formaldehyde leaving only glycine and sulfuric acid in solution.

PART II

THE INDIVIDUAL AMINO ACIDS

Each is treated in this order:

Natural occurrence.

Synthesis, if any.

Properties.

Preparation from protein.

For tables of physical properties, solubilities, etc., see reference (3).

For a historical introduction see reference (20).

CYSTINE - SOURCES.

The most convenient method of preparing cystine so far published is that given by Gortner and Hoffmann in "Organic Syntheses" vol. V (4). Students get excellent results following this method. However, the method calls for human hair (barber shop sweepings) and this is an inconvenient raw material to use when large quantities are required. For this reason a survey of possible sources of cystine was made. The only proteins high in cystine are the keratins and not all of these are suitable.

HUMAN HAIR. This has been estimated by various workers to contain 8% to 18% of cystine depending upon the method of determination used. Similar uncertainties as to actual cystine content exist in the case of all the other proteins rich in cystine. In any case the amount actually isolated is much less than that determined by colorimetric tests. Students usually obtain 5-8% of pure white cystine from human hair.

HAIR FELT. Cow hair felt from tannery hides. This is sold in large rolls of soft felt 3 ft. wide and 1 in. thich by dealers in heat insulating materials. Two samples were hydrolysed but no cystine was obtained from either. Evidently it had been destroyed by the alkali soaking used at the tannery to loosen the hair from the hides. A mixed

solution of lime and sodium sulfide is often used for this purpose. Alkali sulfides are known to react with the cystine of keratins (54). Large yields of leucine were obtained from hair felt.

HATTERS' FELT SCRAP. Made from rabbit hair. One sample (dyed dark gray) was examined. No cystine was isolated. Probably it had been destroyed by the nitric acid solution of mercury used in the felting process.

HOG BRISTLES. These were obtained from a farmer at "hog killing time". One kg. was hydrolysed by the slow dilute HCl method described in "Organic Syntheses" vol. V and 1 kg. by the quick concentrated HCl method described in the first part of this thesis. Fourteen grams cystine was obtained in each case showing that either method of hydrolysis is satisfactory. The low yield, 1.4%, makes hog bristles too expensive a raw material to use although they can be purchased in large quantities from the meat packing houses. Good yields of tyrosine and leucine were obtained from hog bristles.

HOG BRISTLES. Bristle brush trimmings. Two samples were obtained from a brush manufacturer. They consisted of pieces 1 to 5 mm. long. One sample was found to be mixed with artificial bristles made from a cellulose preparation and was discarded. The other sample (100 g.) yielded less than 1% of cystine. The bristles had been cleaned by

washing with a trisodium phosphate solution and also bleached, probably with an alkaline peroxide solution. Evidently the treatment had destroyed part of the cystine.

WOOL. Raw degreased wool. This is too expensive to use. Furthermore the yield os cystine is low. While 13% has been reported, determined by a colorimetric method (39), the most any one has has reported isolating is less than 3%. Wool contains sulfur in other compounds than cystine (67)(119).

WOOL RAGS. All-wool scraps dyed various dark colors. One lot of 200 g. was hydrolysed. Three grams cystine or 1.5% was isolated.

WOOL FELT. A sample of a very pure white wool felt scrap (German hammer felt) was obtained from the Baldwin Piano Co. and 185 g. hydrolysed. Eight grams cystine or 4.2% was obtained in pure white form free from tyrosine. This is hard to believe. Some tyrosine and 13 g. leucine were also obtained. The hydrolysate was unususlly light colored.

HORN. This has always been regarded as an excellent source of cystine. The preparation of cystine from horn has been described by A. P. Mathews (2)(96). If the quick process of hydrolysing with concentrated HCl is used the horns need only be broken into pieces small enough to slip through the neck of the large flask used. They dissolve and hydrolyse in a very few hours. The yield of finished

pure white cystine is 5 to 8%. Old moldy horns stored for a long time in a wet cellar gave equally good yields. Horn actually contains 16% cystine.(40). Large yields of leucine are also obtained from horn.

HOOF MEAL. This is sold in 125 lb. bags by the large meat packing companies. It contains 16% of nitrogen and is used for fertilizer. Its low price, 4 ¢ lb. makes it the cheapest of pure proteins obtainable. From its apparent resemblance to horn one would expect it to be a good source of cystine but such is not the case. Two lots were examined and found to be free from bone and other tissues except a few hairs. A little sand was present, probably originally embeddedin the bottoms of the hoofs. According to the manufacturer the material consists of only the hoofs of cattle, sheep and hogs, loosened from the feet by boiling, then dried and ground to a coarse powder.

The material hydrolysed readily but no cystine could be isolated, although qualitative tests showed some reduced sulfur present. Good yields of leucine were obtained. Apparently hoof differs materially from horn in composition. Indeed thre is reason to believe that hoof, in part at least, consists of mucoprotein since a volatile acid, possibly, acetic, can be distilled out of the hydrolysed solution. Further work on this point is planned. Because of its cheapness hoof meal may prove to be a good source of

other amino acids than leucine. This has not yet been investigated.

COW HOOF. To obtain further information concerning the cystine content of cattle hoofs, the feet of a freshly killed cow were cut off, washed, and boiled 3 hrs. in distilled water so that the hoofs could be slipped off the bones free from other tissues. After drying, the set of hoofs weighed just 500 g. After hydrolysis only 0.5 g. cystine or 0.1% was isolated. A foot from a year old bull calf was left out in warm rainy weather until the hoofs separated by decomposition of the connective tissue. About 80 g. of dry hoof was obtained which yielded almost 1% of cystine.

HORSE HOOF. This was obtained from decomposed horse feet found on the dump of a local fox farm. One kg. of air dry hoof yielded 22 g. purified cystine or 2.2%, also a large amount of leucine and some tyrosine.

FISH SCALES. Scales of Lake Erie whitefish were procured from a local fish market. They were rubbed with water until all the silvery coating was removed leaving them clean and transparent. A 100 g. portion was soaked in 2% HCl overnight to remove the calcium carbonate and phosphate present, then washed and hydrolysed with HCl. The hydrolysate was almost colorless, no humin whatever being formed. Tests showed cystine or reduced sulfur compounds entirely absent. Nor could any tyrosine be detected

in the solution although the washed raw scales themselves quickly turn brick red when dropped into cold Millon's reagent. Another 100 g. portion of scales, washed with water but not with acid, was hydrolysed with barium hydroxide. No tyrosine, tryptophane, nor sulfide ion from decomposed cystine could be detected in the solution. Fish scales do not contain a keratin. Properly cooked they yield a high grade of gelatin (A. P. Mathews).

WHALEBONE, the horny material lining the upper jaw of the Greenland whale, formerly used for corset stays etc., is said to be a keratin rich in cystine (36).

Shed snake skin, from python, is a keratin low in cystine (37).

FEATHERS. Feathers are probably the most convenient raw material for the preparation of cystine in large quantities. At first glance this may not seem possible. But it should be borne in mind that there are many poultry dressing establishments where wet freshly plucked feathers can be had for the asking. A fwe firms dry and sell the feathers but even so the price of certain grades of coarse chicken feathers is very low, sometimes only 4¢/ lb. A full grown hen carries about 100 g. of feathers and it is not unusual for even a very small establishment to dress a hundred hens in the course of a forenoon, as well as a much larger number of smaller chickens, so it will be seen

that the total amount of such material available is very large indeed.

Experience has shown that the feathers need not be washed or even dried. Wet feathers freed from as much water as possible by squeezing in balls between the hands retain about an equal weight of water. For hydrolysis one first places 2 kg. concd. HCl in a 6-liter pyrex flask and then adds 2 kg. of squeezed wet feathers or 1 kg. dry feathers and 1 kg. water. By placing the acid in the flask first, the feathers wilt down rapidly as added and it is possible to add the whole quantity of feathers at ane time, otherwise it would be necessary, to add them in installments as a 6-liter flask will not hold 1 kg. of crude feathers.

Students have never obtained less than 4% of pure white cystine from feathers and if not too much difficulty is encountered in decolorizing the crude cystine the yield is often abow 5%.

The color of the feathers used is immaterial, a very black hydrolysate is obtained in any case.

Denis (38) prepared cystine from feathers in 1911 and reports a yield of 3.2% from chicken feathers. No references later than this have been found in the literature.

CYSTINE - PREPARATION.

The literature on the preparation and determination of cystine if collected would make a large volume. No attempt is made to give a review here.

Cysteine can be made synthetically and cystine can be made from cysteine.

Cystine is soluble in 8840 parts water at 19° (Beilstein), and is almost as insoluble in boiling water. It is insoluble in alcohols and ethers (1).

Cystine can be precipitated as a copper or silver salt or by mercuric sulfate or phosphotungstic acid (40), but is usually precipitated as the free amino acid.

The protein must be hydrolysed by HCl. Hydrolysis by sulfuric acid yields no cystine (1). Alkaline hydrolysis destroys the cystine and trypsin does not digest keratins. Horn or feathers are the preferred sources.

A little cystine is destroyed by HCl hydrolysis and its optical rotation is decreased by slight racemization.(97)(116)(117)(118).

The methods usually recommended for students wishing to prepare cystine are those given by A. P. Mathews (2)(96) or in "Organic Syntheses" vol. IV (4) or slight modifications of these methods. Mathews's method has the advantage that other amino acids besides cystine are obtained from the same hydrolysate. The alcohol can be omitted with

only a slight decrease in yield.

In the method given in "Organic Syntheses" vol. V the hydrolysate is partly neutralized by sodium hydroxide, then a very large amount of sodium acetate is used to precipitate the cystine. The final solution contains much free acetic acid. This prevents the precipitation of calcium phosphate, iron and aluminium hydroxides and other mineral impurities derived either from the protein or the decolorcarbon and also holds some of the tyrosine in solution. The filtrate from the cystine is almost saturated with sodium salts and cannot conveniently used for the preparation of other amino acids. Even most or all of the tyrosine is lost. The one advantage of the method is that it is impossible to overshoot the neutral point and make the solution alkaline with consequent destruction of cystine.

I have worked out the following method which I believe to be superior to any yet devised:

The feathers or horn are hydrolysed by HCl by the rapid method described on page 11. During the hydrolysis the solution is allowed to concentrate by evaporation to about 60% of its original volume. It is then cooled, partly netralized with 40% NaOH solution, but still leavit acid enough to turn congo a bright blue, again cooled, and filtered to remove humin. No attempt is made to

decolorize the solution at this stage. The filtrate is then further neutralized with 40% NaOH, testing frequently, until it turns congo purple instead of blue, then 10% NaOH is added cautiously, with rapid stirring and frequent testing, until the solution is still acid to litmus but has no effect on red congo paper. This is not as difficult as it may seem. Should by any chance the solution become alkaline a little acid is quickly added and the neutralization repeated. A slight alkalinity for a short time will do no harm as there is enough NH_4Cl present in any such protein hydrolysate to react with a considerable quantity of NaOH forming NH_4OH which is without effect on cystine. Some of the amino acids present would probably act in a similar manner as buffer compounds.

The neutralized solution is allowed to stand for at least 48 hrs. at room temperature as in such a mixture tyrosine sometimes crystallizes out very slowly. Cystine, tyrosine, calcium phosphate, hydroxides or basic salts of iron and aluminium and possibly other mineral impurities, as well certain unidentified organic substances, usually very dark in color, all precipitate out together. The precipitate is collected on a suction filter and washed with a little cold water. The filtrate is saved for the preparation of other amino acids. The precipitate is dissolved in 5% HCl and decolorized with charcoal. Decolorizing is

is often difficult. It is best accomplished by digesting the warm solution 2 hrs.at a time with several small successive portions of the charcoal, filtering each time. Carboraffin is superior to Norite (115). Most charcoals need not be purified (HCl washed) for use at this stage but bone charcoal should be because of the large amount of calcium phosphate it contains. All decolorizing charcoals adsorb some tyrosine (95) but probably do not adsorb cystine (115).

To the colorless or pale yellow solution add a little acetic acid or sodium acetate, and neutralize with 10% NaOH as done previously but using greater precaution to avoid an alkaline reaction than in the first precipitation since ammonium salts are now absent. Or, for safety, some NH_4Cl or NH_4OH can be added before the NaOH. An alternative method of neutralizing the HCl solution is to sift into it dry $NaHCO_3$, having added 20 cc. or more of isoamyl alcohol to prevent foaming. Normal sodium carbonate cannot be used as it forms lumps when it strikes the solution and these lumps dissolve slowly. Properly neutralized the solution is neutral to congo red but contains a little acetic acid which tends to hold some, but not necessarily all, of the mineral impurities in solution. Filter, and wash with cold water, followed by a little acetone to facilitate quick drying. Discard the filtrate.

SEPARATION OF CYSTINE AND TYROSINE. Cystine and tyrosine are both insoluble in neutral solutions but very soluble in strongly acid or alkaline solutions. Their isoelectric points do not exactly coincide so they can be separated by solutions of proper pH. A number of such methods have been published but are all more or less unsatisfactory so will not be discussed here. Acetic acid can be used but is not very satisfactory. Tyrosine is slightly soluble even in 5% acetic acid and very soluble in 25% or more (1) although insoluble in glacial acetic. Cystine is only very slightly soluble in any concentration of acetic acid.

I believe the following original method, using ammoncarbonate, to be the most satisfactory. The reagent grade of ammonium carbonate in lumps is used. If the lumps are hard and translucent a 5% solution is used. If the lumps are chalky on the surface a 6% solution is used. The solution should make a faint pink but not a red spot when a drop is placed on phenolphthalein paper. If the ammonium carbonate lumps are soft and crumbly it means much NH_3 has volatilized and the material is largely bicarbonate. Such material gives a solution which is neutral to phenolphthalein and should not be used as it will not dissolve cystine. Or a 5% solution can be made and NH_4OH added until the solution gives the proper color with phenolphthalein.

About 100 cc. of the ammonium carbonate solution is used for each gram of cystine estimated to be present. This large excess is necessary so that the pH of the solution is not appreciably changed by the dissolved cystine. The mixed cystine-tyrosine precipitate is digested for at least 2 hrs. with the reagent, stirring frequently. The solution may be warmed to 35-40°C. but it is best not to go higher than this. The tyrosine and mineral impurities remain undissolved and are collected on a suction filter.

To the cystine solution add a little isoamyl alcohol to prevent foaming, partly neutralize with HCl, then make acid to litmus with acetic acid. Let stand over night to insure complete precipitation, collect on a suction filter, and wash with hot distilled water followed by a little acetone. Cystine thus prepared is quite free from ash or tyrosine. As a check on the method 20 g. pure cystine was dissolved in 2 liters of reagent and over 19 g. recovered.

The impure tyrosine remaining undissolved by the ammonium carbonate reagent is treated with a hot 5-10% sodium carbonate solution, boiled a few minutes to decompose any traces of cystine, and filtered. The mineral impurities remain undissolved. A little acetic acid or sodium acetate is added to the solution, then dilute HCl is added until the solution is acid to litmus but neutral

to congo. At least 10 cc. of isoamyl alcohol should be added to prevent foaming. The mixture is allowed to stand 24-48 hrs. then filtered and the tyrosine washed with cold water followed by a little acetone.

CYSTEINE.

It is not known to what extent, if any, cysteine occurs in proteins. When needed for experimental work it has always been made by reducing cystine, usually by tin and hydrochloric acid (2).

Optically inactive cysteine has also been prepared synthetically (5).

Cysteine is quite soluble in water.

TYROSINE.

Tyrosine occurs in all the common proteins except gelatin.

Synthetic tyrosine has been prepared (108), also the corresponding ortho and meta compounds, which do not occur in proteins (120).

Tyrosine is soluble in 2490 parts water at 17° or 150 parts at 100°. Insoluble in ether, almost insoluble in alcohols (3).

Its lead salt is soluble in 2600 parts water (47). All other common salts are quite soluble in water.

It is not precipitated by phosphotungstic acid (6).

It is precipitated by mercuric sulfate reagent but the precipitate is soluble in dilute sulfuric acid.

In the acid hydrolysis of proteins not all of the tyrosine is liberated (93). A part of that liberated goes into the humin and if carbohydrates or other aldehyde forming substances are present nearly all of it may be so lost (11). When the solution is decolorized an additional quantity is lost since decolorizing carbons adsorb appreciable amounts (95). Nevertheless it can still be prepared in quantities by the acid hydrolysis of keratins. Beyer published a method of preparing it from hoof as early as 1867. As prepared from keratins it is usually to be considered as a byproduct of the preparation of cystine.

This has already been discussed under cystine. For the preparation of tyrosine by acid hydrolysis of casein see Cole's book (6). Tyrosine prepared by acid hydrolysis may be partly racemized.

Tyrosine is also obtained by the tryptic digestion of animal proteins. Being insoluble in neutral or only slightly alkaline solutions it precipitates as the digestion proceeds. Marshall (109) prepared it by digesting a mixture of casein and ground hog pancreas.

Tyrosine is obtained as a byproduct in large amounts in the preparation of tryptophane by tryptic digestion of casein. Casein contains about 6%. This method of preparation is described in "Organic Syntheses" vol. X (4). However, the method as there given is unsatisfactory and the following modification is recommended: The casein is digested as directed and after adding plenty of diatomaceous earth the digestion mixture is filtered using a suction filter and changing the paper frequently. The collected precipitate need not be removed from the paper. Paper and precipitate are digested cold with 1 liter of 5% HCl. The original directions say to boil the HCl solution but this should not be done. If boiled the solution turns inky black and is extremely difficult to decolorize. The HCl solution is filtered on a suction filter. If digested cold the filtrate is colorless or nearly so. A little acstic

acid or sodium acetate is added, then 40% NaOH solution is stirred in until the solution is neutral to congo but acid to litmus. The tyrosine precipitates pure white. It is not pure but contains a white organic substance and perhaps also calcium phosphate, both of which are soluble in acids but not in alkaline solutions. After standing at least 24 hrs. the precipitate is collected on a suction filter and washed with cold water. The precipitate is then stirred with 1 liter of 5% sodium carbonate solution, boiled a few minutes to decompose any cystine present, and filtered. The filtrate is nearly colorless. After adding a little isoamyl alcohol to prevent foaming the solution is nearly neutralized with HCl, then acetic acid is added until the solution is just acid to litmus. If too much HCl is accidently added sodium acetate is used instead of acetic acid. After standing 24 hrs. or longer the precipitate is collected on a suction filter and washed with cold water followed by a little acetone to speed drying.

GLYCINE.

Glycine is naturally abundant only in silks and in gelatin. Many proteins contain none at all.

It is difficult to isolate so is usually prepared synthetically. Synthetic amino acids are usually racemic but this is impossible in the case of glycine since it has no asymmetric carbon atom. Hence the synthetic is identical with the natural. Methods of synthesis from chloroacetic and ammonia are given in many laboratory manuals of organic chemistry. Due to formation of complex byproducts the yield of pure glycine is always low, about 20% of the theoretical. Robertson (46) has studied this reaction and found that by using an enormous excess of ammonium hydroxide the yield can be increased to 80%. A modified method, simple in execution, and giving a yield of 50-60%, is given by Boutwell and Kuick (63). It is here recommended for student use. Other methods have been published (60)(64)(82).

Glycine is soluble in 4 parts water, insoluble in absolute ethanol, methanol, or acetone. It can be precipitated from water by addition of 3-4 vols. ethanol (65). Butanol extracts it from aqueous solution with difficulty. About 75% is extracted after 23 hrs. in a continuous extraction apparatus, 100% in 65 hrs. (29).

Its ethyl ester hydrochloride is insoluble in

absolute ethanol or ether and a method, of preparation from gelatin, through this compound, is given in Cole's book (6). In the original Fischer ester method "glycine ethyl ester hydrochloride can be made to crystallize almost quantitatively from a mixture of the remaining amino acid ethyl ester hydrochlorides by concentrating the ether solution in a vacuum and allowing to stand at 0°." (66).

Glycine may be purified by converting it into its picric acid compound and recrystallizing this from water (61)(86).

By the copper salts method described on page 34 glycine is obtained mixed with alanine. These are readily separated by the carbamate method (121).

SERINE.

Serine occurs in many proteins but only in small amounts. Most proteins contain less than 2%, or perhaps because of the difficulty of its isolation the results obtained are lower than the true values. Even silk gum, or sericin, for which it was named, contains only 6.8% (25). Sericin is a waste product not usually recovered from the silk washing solution in which it is dissolved.

Serine is difficult to isolate (26). For this reason various methods of preparing it synthetically have been devised (27), and a method of separating the racemic product into its optically active constituents (28).

It is not readily extracted from aqueous solution by butanol. In a continuous extraction apparatus at least 60 hrs. is required (29). It is not precipitated by any of the reagents ordinarily used in the preparation of amino acids. It is decomposed by boiling NaOH or KOH solutions but not by calcium hydroxide (31). The levo form is much more soluble than the racemic (31).

No convenient method for its preparation can be offered at this time. For possible methods see references (29)(30)(31).

ALANINE.

The most convenient sources are zein (10%), gelatin (8.7%), and hemoglobin (4.2%). Other common proteins concontain smaller amounts, except the silks, which are very rich in alanine. Alanine anhydride has been isolated from Liebig's beef extract (73).

Racemic alanine can be prepared synthetically by several methods (72).

Alanine is only moderately soluble in cold water but very soluble in hot. Insoluble in ethanol, methanol or ether.

Its copper salt is very soluble in water, insoluble in absolute ethanol or methanol.

For convenient methods of preparation see references (52)(121).

Alanine is obtained as a byproduct of the preparation of proline from the mother liquors from the preparation of glutamic acid hydrochloride from the glutens. This is discussed under proline.

BETA-ALANINE.

Beta-aminopropionic caid. This compound is known to exist but it is doubtful if it ever occurs in unaltered proteins. No literature references have been located.

AMINOBUTYRIC ACID.

This is said to have been found in a protein by Foreman in 1913 (20). No literature references have been located other than (76).

The synthetic compound is well known (5).

AMINOISOBUTYRIC ACID.

This has not yet been found in protein.

A method of synthesis is given in "Organic Syntheses" vol. XI (4).

VALINE.

Most of the common proteins contain less than 5% of valine and some contain very little or none at all. Linseed meal globulin has probably the highest percentage, 12.7% (14), but linseed meal is difficult material to work with because of its gum content. Casein, which contains about 8%, is probably the most convenient source.

Valine is readily synthesized (5).

Valine is soluble in about 10 parts water.

Its copper salt is only slightly soluble in water but is soluble in methanol (88). Insoluble in absolute ethanol or nearly absolute isopropanol (1).

Valine is readily isolated by the copper salts method.(52)(121). This is discussed under proline.

NORVALINE.

This has been detected in globin (122). From casein 0.2% has been actually isolated, about 1% is probably present (123).

For synthesis and separation of optically active components see (124).

HYDROXYVALINE.

This has been isolated from the proline fraction of zein by the copper salts method by Brazier (52). The method is not difficult.

LEUCINE.

Beta-isopropyl-alpha-aminopropionic acid. Leucine was prepared by Proust in 1818. Until recently the word leucine as used in the literature referred to the mixture of leucine and isoleucine isolated from proteins. This mixture is difficult to separate. Leucine probably occurs in all the common proteins. Hemoglobin, zein and the keratins are the richest sources.

Leucine has been synthesized (3)(82).

Leucine is soluble in 46 parts water at 18° or 45 parts at 20° (Beilstein). It is soluble in 800 parts hot ethanol (5). Soluble in glacial acetic acid (3).

Leucine copper salt is soluble in 3045 parts cold water or 1640 parts boiling water (Beilstein).

l-leucine is not racemized by heating with acids (87).

It is claimed that if a solution of amino acids such as a protein hydrolysate be nearly neutralized and then boiled with an excess of zinc oxide and cooled the zinc salts of luecine and glutamic acid precipitate out (3). I have found this reaction very unreliable. The zinc salts are soluble in acetic acid. According to Brazier (52) leucine zinc salt is insoluble in water only after drying at 110°.

Under some conditions leucine may be partly precipitated as a lead salt (47)(48)(87).

A mixture of leucine, isoleucine and valine cannot be separated by fractional crystallization (87). Their copper salts can be separated by alcohols (52)(87)(88)(121).

Since leucine is not very soluble in cold water it can be partially crystallized out of a solution of mixed amino acids, provided it be present in sufficient amount, by simply concentrating the solution and cooling. Such a method is described in Cole's book (6) and in many other laboratory texts. Unless previously removed, tyrosine separates with it.

As a method of purification Cole (6) recommends recrystallization from 70% ethanol. If tyrosine is absent leucine may be purified by recrystallization from water.

It is a peculiar fact that leucine, if once thoroughly dried, sometimes will not redissolve in water even if boiled (1). I have found that the addition of a few drops of ammonium hydroxide causes it to dissolve quite readily. It does not form a stable ammonium salt and all the ammonia can be expelled by boiling.

From a number of keratin hydrolysates, after the cystine and tyrosine had been removed as described under cystine, page 53, leucine, phenylalanine and aspartic acid were prepared in the following simple manner:

The dark colored filtrate from the cystine and tyrosine was made just barely alkaline to phenolphthalein

with NaOH and filtered if any precipitate formed. Sixty grams of copper sulfate crystals per liter of solution was then added and stirred until dissolved. When all had dissolved the mixture tested slightly acid to litmus. The mixture was allowed to stand over night then the precipitate of copper salts was collected on a suction filter and washed with cold water until the washings were almost colorless. A colorless filtrate cannot be obtained in washing since the copper salts are not completely insoluble. It is necessary to use a large amount of copper sulfate since a large proportion of it goes into soluble dark blue complex ions before precipitation occurs. The filtrate and washings were discarded. The mixed precipitate is insoluble in dilute acetic acid.

A precipitate of copper salts was obtained in a similar manner from a hair hydrolysate from which the cystine had been precipitated by sodium acetate. Similar precipitates were also obtained from hydrolysed casein and hog brain protein, but from hydrolysed corn gluten or blood corpuscles no precipitate of copper salts could be obtained. This is strange considering that hemoglobin is the richest in leucine of all proteins.

The three amino acids were then prepared from the mixed insoluble copper salts by Brazier's method (52) with slight modification. From the copper salts a solution of

the free amino acids was prepared, in some cases by H_2S, and in other cases by the barium sulfide method given on pages 16-19. This was done in quite dilute solution lest some leucine separate out. Since the amount of leucine present was believed to be large in proportion to the other amino acids it was thought advisable to remove some of it first before attempting separation by Brazier's method. Accordingly the dilute solution was concentrated by evaporation until a few scales of leucine were seen floating on the surface then allowed to cool and crystallize. The separated leucine was collected on a suction filter and washed with cold water.

The combined filtrate and washings were made alkaline with barium hydroxide and evaporated to a syrupy solution of the barium salts. The barium aspartate was precipitated by adding alcohol until no further precipitation occurred. Various odd lots of recovered ethanol or methanol were used. Some contained acetone and some ether and in two cases denatured alcohol of the radiator anti-freeze variety was used. Good results were obtained in all cases. The precipitated barium aspartate was redissolved in the minimum quantity of water and reprecipitated in a similar manner. The different lots of barium aspartate were combined and worked up together so it was not possible to determine the yield from each source. The yield was

small in any case. The barium aspartate was dissolved in water, the barium precipitated by just the right amount of sulfuric acid, and the filtered solution of aspartic acid evaporated to crystallization. The wet crystals were digested with warm wet butanol to remove traces of other amino acids and washed with acetone.

From the alcoholic solutions of the barium salts of leucine and phenylalanine the free amino acids were separated as directed by Brazier. If one wishes to recover part of the alcohol special treatment is necessary. The solution of barium salts cannot be distilled directly even if diluted as a film forms on the interior of the flask and violent bumping takes place. If it is attempted to remove the barium before distillation by precipitation by sulfuric acid in the alcoholic solution the precipitate formed is slimy and cannot be filtered. Best results are obtained by adding an excess of dry ammonium carbonate. The barium carbonat precipitate is easily filtered off. The alcohol can then be distilled off but it contains ammonia which distils with it, and this limits its subsequent use.

The above method of isolating phenylalanine and aspartic acid is not believed to be by any means quantitative but it is an excellent method of getting some value out of amino acid solutions that would otherwise be discarded.

A possible alternative method for separating the three amino acids is as follows:

The aqueous solution of the free amino acids is evaporated to a pasty mass, then triturated to break up the lumps and extracted several times with alternate portions of wet and dry butanol. Phenylalanine is very soluble in wet butanol, leucine but slightly, and aspartic acid not at all. By this means the mixture is separated into two fractions, a solution of phenylalanine and some leucine, and a residue containing all the aspartic acid and the remainder of the leucine. Further separation is then effected by appropriate methods.

For preparing leucine, phenylalanine, and aspartic acid from casein the filtrate from the tryptophane-mercuric sulfate compound obtained in the preparation of tryptophane from casein by tryptic digestion was used. This solution contained 5-6% sulfuric acid by volume and a considerable amount of mercuric sulfate. A 40% NaOH solution was stirred in cautiously until the solution was acid to litmus but neutral to congo. A flocculent grayish white precipitate of mercury compounds was formed. This precipitate is soluble in excess of NaOH. According to Onslow (93) mercuric sulfate may precipitate from a casein hydrolysate leucine, cystine, tyrosine, glutamic and aspartic acids, histidine and proline but not lysine and arginine. The precipitate

was discarded as there appeared to be very little of it once it had been consolidated on a suction filter. If tryptophane were being prepared on a large scale the precipitate could be saved until enough had accumulated and then worked up for amino acids, especially histidine.

To the filtrate enough more sodium hydroxide was added to make the solution barely alkaline to phenolphthalein, then 60 g. copper sulfate crystals added to each liter and the remainder of the process carried out as for the keratin hydrolysates above mentioned. From 600 g. commercial casein 12 g. aspartic acid was obtained or about half that supposed to be present. Thirty g. leucine were obtained and no phenylalanine. It is stated by Fischer and Abderhalden that trypsin does not liberate phenylalanine from casein (32). The copper salt of the peptide prolyl-phenylalanine is soluble in water (52), presumably the same is true of other peptides.

ISOLEUCINE.

Beta-methyl-beta-ethyl-alpha-aminopropionic acid. Isoleucine probably occurs in many proteins but to what extent is not known since in most cases leucine and isoleucine have been determined together and all reported as leucine.

Isoleucine has been synthesized (5).

Themcopper salt of isoleucine is somewhat more soluble in water than that of leucine.

A possible method of separating leucine and isoleucine has been suggested by Levene and Van Slyke (87).

According to Ehrlich (88) separation can be made through the copper salts. The copper salt of isoleucine is soluble in methanol, that of leucine is not.

NOR-LEUCINE.

Alpha-amino-n-caproic acid. Very little is known about the occurence of this amino acid in proteins. It is found in brain protein (2) but to what extent has not been determined.

For the synthesis of racemic nor-leucine see "Organic Syntheses" vol. IV (4). Also (5)(82).

Its copper salt is insoluble in water.

Just how it can be separated from the other leucines is not known.

PHENYLALANINE.

Phenylalanine occurs in all the common proteins in small amounts. Zein contains 7.6%, much more than most proteins.

A review of seven methods of synthesis is given in reference (78). Also see (82).

Phenylalanine is best separated from a mixture of amino acids by precipitating it together with leucine and aspartic acid as copper salts. This method is described under leucine. Copper phenylalanine is insoluble in water, alcohols or dilute acetic acid(1).

The zinc salt is very soluble in water.

According to Kodama (79) phenylalanine is precipitated from concentrated aqueous solution by excess of calcium hydroxide but I have not been able to confirm this.

It may be partially precipitated with phosphotungstic acid (5) or picrolonic acid (3).

For a complete method of isolation see (52).

HISTIDINE.

Most of the common proteins contain less than 3% of histidine. Hemoglobin contains 11% so most of the methods published have been based on the use of blood corpuscles as a raw material. The literature of histidine is extensive and no review is attempted here. References to methods using silver salts are omitted.

Blood corpuscles are the preferred source where large quantities are to be prepared. Small amounts are obtained as a byproduct when preparing arginine or lysine from other proteins.

The student should first read the method given by Jones (80) and if possible also that given in Cole's book (6), and then read the discussion and criticism of this method given by Hanke and Koessler (81) before starting the work. HCl hydrolysis is used and considerable histidine, sometimes as much as one-sixth of that present, is lost in the humin formed (11). See also (125). Mercuric chloride in the presence of sodium carbonate is the precipitating agent and the quantity required is many times the theoretical amount. It is to be hoped that a cheaper method can be discovered.

Mercuric sulfate reagent also precipitates histidine quantitatively (3).

LYSINE.

All the common proteins of animal origin are good sources of lysine. Zein contains none, other vegetable proteins contain various amounts.

No work was done on lysine in connection with the preparation of this paper.

For possible methods of preparation see references (3)(103)(104).

ORNITHINE.

Ornithine does not occur in proteins, so far as known. It is prepared by splitting off urea from arginine by strong alkalis (or by the enzyme arginase).

A method of preparation and purification is given by Herzog (70).

ARGININE.

Arginine occurs in all proteins. According to Cahn (69) it plays an important part in the structure of animal proteins, each protein molecule containing 16 or a multiple of 16 arginine molecules. The most convenient source for its preparation is probably gelatin or glue which contains about 9%. Small amounts can be obtained as byproducts in the preparation of other amino acids from various proteins.

Arginine is very soluble in water but insoluble in alcohols.

Boiled with strong alkalis it splits into ornithine and urea. The urea may in turn split into ammonia and carbon dioxide.

Arginine forms an insoluble compound with flavianic acid (3). Flavianic acis is 1-naphthol-2,4-dinitro-7-sulfonic acid.

No work was done on the preparation of arginine.

For possible references see (3)(104)(105)(106).

TRYPTOPHANE.

Tryptophane and ite preparation by both alkaline hydrolysis and tryptie digestion have been discussed in a previous paper: L. E. Gilson, "The Separation of Tryptophane from an Amino Acid Mixture", M. Sc. thesis, Univ. of Cincinnati, Dept. of Biochemistry, 1930.

Only additional material not included in this previous paper is discussed in the present work.

The student will find a suitable method of digesting casein with trypsin described in "Organic Syntheses" vol. X (4). Where ither amino acids than tryptophane are to be prepared from casein by tryptic digestion the use of sodium carbonate in the mixture may be objectionable as it is impossible to remove the sodium from the solution later. Other bases can be used. Walters (98) states: "The nature of the base combined with casein has little or no influence on the process of hydrolysis. Basic caseinates of Li, Na, K, NH_4, CA, Sr, and Ba, hydrolyse with approximately equal velocities." Ammonium and barium are especially suitable since they can easily be removed from solution. In connection with the present work ammonium carbonate was substituted for sodium carbonate with excellent results. About 50 g. is used in place of the 60 g. anhydrous sodium carbonate called for in preparing 600 g. of dry casein for digestion.

The pH of the casein solution should be adjusted so

that the reaction is strongly alkaline to litmus but not alkaline to phenolphthalein. If too much alkali is added it is best not to add an acid to correct the pH since this precipitates large lumps of casein which redissolve with exasperating slowness. It is preferable to add more casein. Wet fresh precipitated casein is difficult to dissolve, because of its lumpiness, wothout getting the solution too alkaline. Dry powdered casein is much to be preferred.

Tryptophane can also be readily prepared from other proteins of animal origin. Lactalbumin (8) is the richest in tryptophane of all easily obtainable proteins. However, the commercial dry lactalbumin is not readily digested by trypsin.

Blood proteins are all rich in tryptophane. Hemoglobin contains 6.1%. According to Fischer and Blankenstein (99) the six proteins of blood serum average between 2 and 3% tryptophane, over 1% cystine and over 7% tyrosine. According to Ohlsson (100) blood albumin contains about 1% tryptophane and the serum globulins contain 2 to 6%. It is interesting to note in this connection that the Bence-Jones protein found in certain pathological urines contains 4.2% tryptophane (101), indicating its probable derivation from one of the blood globulins.

Either blood corpuscles or whole blood can be readily digested with trypsin. Two liters sedimented blood

corpuscles are mixed with 3 liters water in a 6-liter flask, allowed to stand 2 hrs. for hemolysis to take place, then an alkaline solution is added until the proper pH is reached, then the solution is treated exactly like the usual sodium caseinate solution. If whole blood or blood serum is used it is diluted with an equal volume of water. During digestion the hemin is convereted into a hemin-proteose (102). This precipitates when the digested mixture is exactly neutralized with sulfuric acid (1), together with the cystine and tyrosine. In actual practise 4 g. tryptophane was obtained from each liter of horse blood corpuscles. Some tyrosine was obtained as a byproduct.

Brain protein is also rich in tryptophane. The residues from the preparation of cholesterol and phospholipids from dry hog brain were further extracted in a Soxhlett apparatus with isopropanol. Considerable extract was obtained. Experiments showed that nothing more could be extracted by ethanol, ether, benzene, acetone or chloroform. Further experiments on whole dried brain showed that isopropanol alone extracts everything that can be extracted by any or all of the above mentioned solvents. The residual protein amounted to 40% of the original dried brain.

The dry protein was finely powdered in a mortar, then suspended in water and enough water and sodium carbonate solution added to make a mixture of the proper pH

containing about 100 g. protein per liter and digested in the usual/way. The yield of tryptophane was 2%. A small amount of tyrosine was also obtained. The residual solution was lost by accident so other amino acids could not be separated.

It is difficult to get tryptophane perfectly white. Decolorizing charcoals should be used very sparingly as they adsorb appreciable quantities of tryptophane (95).

HYDROXYTRYPTOPHANE.

6-hydroxy-2,3-dihydroindolyl-3-alanine. The only references found were:

Abderhalden and Kemp, Z. physiol. Chemie 52, 207 (1907), or C. A. 1, 3016.

Abderhalden and Sickel, Z. physiol. Chemie 138, 108(1924), or C. A. 18, 3188.

From 1 kg. casein 0.7 g. was obtained. It was precipitated as a mercury compound.

PROLINE.

Proline occurs in nearly all proteins. In animal proteins proline and hydroxyproline usually occur together which makes the preparation of proline from such sources more complicated. The common vegetable proteins used for foods, and possibly all vegetable proteins, contain no hydroxyproline.

Proline has been synthesized (84).

Proline is soluble, though not very (51), in absolute ethanol or ether. It is the only amino acid soluble in anhydrous butanol.

It is the only amino acid which forms a copper salt soluble in absolute ethanol (50)(76). The copper salt is soluble in methanol, isopropanol or water.

Proline can be readily recrystallized from hot ethanol or isopropanol (51).

Before attempting to prepare proline the student should read references (30)(51)(52)(83). Complete directions are given in (52) and (83).

The mother liquors from the preparation of glutamic acid from wheat gluten or corn gluten contain much proline but preparation from this source is difficult on account of the large amount of organic material present derived from the non-protein part of the crude gluten mixture.

HYDROXYPROLINE.

Hydroxyproline has not been found in any of the common vegetable proteins (68). It occurs in many, or perhaps all, proteins of animal origin although the amount is often very small.

It is usually prepared from gelatin or glue which contains about 14 % together with about 10% of proline. Complete details of its preparation are given by Klabunde (83).

Hydroxyproline is insoluble in absolute ethanol whereas proline is soluble.

Proline is somewhat soluble in ether, hydroxyproline nearly insoluble (3).

The copper salts of both hydroxyproline and proline are soluble in absolute methanol (51)(83) but only proline copper salt is soluble in absolute ethanol (76).

ASPARTIC ACID.

Lactalbumin, containing 9.3%, is the best source, other common proteins contain 5% or less. However, it is so easily isolated it may be prepared from almost any protein although the yield may be very small.

It may be prepared synthetically from fumaric acid and ammonia (5). Fumaric acid is now a cheap commercial product. Other syntheses have been published (74). All methods give low yields. A method of preparation by hydrolysis of asparagine by HCl is given in Cole's book (6). Asparagine is an expensive raw material.

The synthetic product is of course racemized and it is said that the natural l-aspartic acid is also easily racemized. The racemic acid is soluble in 360 parts of cold water or 19 parts boiling water (6), while the l-aspartic acid is soluble in 200 parts cold water or 18 parts boiling water (3).

Asparatic acid is practically insoluble in alcohols and can be precipitated from aqueous solutions by the addition of several volumes of ethanol (42). It is not extracted by wet butanol in the Dakin method. It is insoluble in cold glacial acetic acid (76).

If a mixture of amino acids be converted into their barium (75) or calcium (30)(76) salts and the solution concentrated sufficiently the addition of several volumes

of ethanol will precipitate all of the aspartic and glutamic acid salts but not other amino acid salts. The precipitate should be dissolved in the minimum amount of water and again precipitated by adding 3 vols. of ethanol to eliminate small amounts of other salts mechanically carried down by the first precipitation. Methanol works equally well but isopropanol precipitates the salts of some other amino acids.

Boiling a solution of amino acids 15-20 min. with lead oxide precipitates aspartic acid and tyrosine as lead salts (47). A large amount of lead oxide must be used as all the other amino acids present combine with it forming salts soluble in water. Ammonia is also liberated from some of its salts by the lead oxide (1). Lead aspartate is soluble in about 7700 parts water (47).

Aspartic acid, leucine and phenylalanine can be more or less completely precipitated from aqueous solution as copper salts. This method is discussed under leucine. Copper aspartate is not completely insoluble in water so in the copper salts method of separating amino acids described on page 34 while most of the aspartic acid is found in fraction (a), a small amount may also be found in fraction (b).(121).

Zinc aspartate is very soluble in water (85).

Kodama (79) states that calcium aspartate is precipitated from aqueous solution by excess of calcium hydroxide

but I have not yet confirmed this. I have reason to believe however, that barium aspartate is only sparingly soluble in the presence of other barium salts or barium hydroxide and that some is precipitated out when solutions are treated with an excess of barium hydroxide to remove sulfate ion or when copper aspartate is decomposed by barium sulfide. How much may be lost this way has not been determined.

GLUTAMIC ACID.

Nearly all proteins contain glutamic acid. These ordinarily used for food contain a relatively large proportion. Wheat gliadin is the richest, containing 44%. For laboratory preparation wheat gluten, corn gluten or casein (18) are the usual sources.

Several workers have prepared synthetic glutamic acid (3).

Glutamic acid is insoluble in alcohols or ethers. According to Beilstein it is soluble in 100 parts water at 16° or 300 parts 32% ethanol or 1500 parts 80% ethanol. The zinc salt is soluble to the extent of 0.064 g. per 100 cc. water at room temperature.

Glutamic acid is insoluble in glacial acetic acid, hydroxyglutamic acid and pyrrolidone carboxylic acid are soluble (75).

While it is claimed (3) that if a slightly acid solution of mixed amino acids is boiled with an excess of zinc oxide and cooled the zinc salts of leucine and glutamic acid are precipitated, experiment has shown this method to be highly unreliable (1).

Some workers have advocated adding metallic tin to the hydrolysis mixture before boiling. The amount of humin formed is said to be decreased (23) and the yield of glutamic acid appreciably increased, often as much as one-fifth

(90). On the other hand Fong (89) prefers to add an oxidizing agent such as nitric acid or manganese dioxide. The student is advised to do neither. According to Roxas (11) none of the glutamic acid goes into the humin.

Two methods are commonly used for separating glutamic acid. In the first method the protein (usually gluten) is hydrolysed with HCl and the glutamic acid separated as its hydrochloride. This method is so well discussed in "Organic Syntheses" vol. V (4), and also in Cole's book (6), that it needs no discussion here. Students get good results with this method. Sometimes the glutamic acid hydrochloride does not promptly crystallize out of the syrupy solution obtained. In such a case the solution should be allowed to stand, for several weeks if necessary, when crystals will eventually form. In one case reported (29), where cocomut globulin was the protein used, the salt crystallized out very completely only after standing three months at room temperature. Although the concentrated hydrolysate is very dark colored the crystals obtained are almost white in the case of corn gluten, though dark when wheat gluten is used as a raw material (1). From the combined mother liquor and washings other amino acids can be prepared.

In the second method of isolating glutamic acid, devised originally by Foreman (76), the protein is

hydrolysed with HCl, filtered, evaporated to a syrup to remove excess of HCl, then made alkaline with lime, filtered, and again evaporated to a small volume to remove ammonia. Several volumes of alcohol are added and the calcium salts of glutamic and aspartic acids precipitated together. Either ethanol or methanol can be used (1). Isopropanol and the other higher alcohols precipitate other salts as well (1). The precipitate should be redissolved in the minimum volume of water and reprecipitated by addition of 3 or 4 volumes of the alcohol to free it from small amounts of other salts carried down in the first precipitation.

A modification of this method by Jones and Moeller (75) uses the barium salts instead of calcium. Five volumes of ethanol are used for the first precipitation and 2 or 3 volumes for the second.

I have used both the calcium and barium salts and find either satisfactory. I prefer to hydrolyse with sulfuric acid instead of hydrochloric, filter, neutralize with an excess of slaked lime, filter, and evaporate the filtrate to a concentrated solution of the calcium salts. This is a cheap and convenient method, although some of the glutamic acid may be lost in the bulky precipitate of calcium sulfate.

A possible objection to the use of calcium salts in the preparation of amino acids is that practically all lime

contains some magnesia. Magnesium once introduced into the solution is difficult to remove completely, although most of it is precipitated by an excess of calcium hydroxide unless ammonium chloride is present in large amount. In such a case an excess of calcium hydroxide is added, the solution heated, and the ammonia removed by a current of air. Under such conditions removal of ammonia is slow, requiring 2 hrs. or longer. To what extent amino acids may be racemized by boiling with calcium hydroxide is not known.

According to Kodama (79) glutamic and aspartic acids (also phenylalanine) form "basic" calcium salts insoluble in the presence of calcium hydroxide. I have not been able to confirm this. According to Takayama (91) the solubility of dibasic calcium glutamate, $C_5H_7NO_4Ca.3H_2O$, in grams per 100 cc. solution is:

0° -- 1.322	61° -- 3.943
19° -- 1.928	100° -- 5.698
21° -- 1.979	

From this compound and the corresponding aspartate one-half the calcium is removed as carbonate by carbon dioxide.

Takayama (91) used 50% sulfuric acid (by weight) for hydrolysis of soybean protein and obtained a higher yield of glutamic acid than with 25%. Heating pure glutamic acid with 10 N sulfuric acid split off some ammonia at 100° and 135-45°.

The concentration of aqueous solutions ot temperatures above 50° slowly converts free glutamic acid into the lactam, pryyolidone carboxylic acid (75). Boiling with HCl regenerates glutamic acid (76). For a discussion of pyrrolidone carboxylic acid see (2). Pyrrolidone carboxylic acid is not poisonous to rats (92) although some compounds of this type are said to be. Some pyrrolidone carboxylic acid is also formed when solutions of certain salts of glutamic acid are boiled (75).

When glutamic and aspartic acids have been precipitated together as calcium or barium salts separation is effected by precipitating the barium from the aqueous solution by sulfuric acid or the calcium by oxalic acid in just the right amount. The filtrate is acidified with HCl and evaporated to a small volume and glutamic acid allowed to crystallize out as the HCl salt. The mother liquor is then neutralized with dry basic copper carbonate and the aspartic acid precipitated as the copper salt. After filtering, the copper can be removed from the filtrate by H_2S and a small additional amount of glutamic acid hydrochloride obtained by evaporating the copper-free solution with a little HCl (75). Methods of separating glutamic and aspartic acids based on the different solubilities of their lead or zinc salts are not recommended.

Directions for preparing free glutamic acid from its

hydrochloride are given in "Organic Syntheses" vol. V. The free acid can also be liberated by adding aniline. A method has been published (113).

Monosodium glutamate is used in large quantities in Asiatic countries as a flavoring for foods because of its meat-like taste. Its manufacture is carried on on a large scale. This is discussed by Han (110). It is usually produced by the HCl hydrolysis of wheat or soybean proteins.

Glutamic acid and some other amino acids (111) occur in beet sugar molasses and in the waste liquors from the fermentation of molasses and numerous processes for preparing glutamic acid from these sources have been patented within recent years (3)(110)(111)(112).

HYDROXYGLUTAMIC ACID

Beta-hydroxyglutamic acid. This was discovered in casein by Dakin (35). Casein and lactalbumin contain 10% or more, gliadin and zein about 2.5%. To what extent it occurs in other proteins has not yet been determined.

Hydroxyglutamic acid is soluble in glacial acetic acid, glutamic is not, pyrrolidone carboxylic acid is (75). Separation can thus be effected.

For method of isolation see Dakin's paper (35).

MISCELLANEOUS RARE AMINO ACIDS.

METHIONINE.

Methylthioaminobutyric acid. For discovery and preparation see, J. H. Mueller, J. Biol. Chem. 56, 157(1923).

Preparation, constitution and synthesis, G. Barger and F. P. Coyne, Biochem. J. 22, 1417(1928).

Synthesis, Windus and Marvel, J. Am. Chem. Soc. 52, 2575(1930).

DIHYDROXYPHENYLALANINE.

3,4-dihydroxyphenylalanine. "Dopa." From Vicia faba, Guggenheim, Z. physiol. Chemie 88, 276(1913).

Miller, J. Biol. Chem. 44, 481(1920).

Synthesis, C. R. Harrington, Biochem. J. 22, 407(1928). or C. A. 22, 2745.

THYROXIN.

Bibliography, C. R. Harrington and W. T. Salter, Biochem. J. 24, 456(1930).

DIIODOTYROSINE.

See (2) or (20).

HYDROXYLYSINE.

See (3), page 191.

CITRULLINE.

From watermelon juice. C. A. 24, 2721 and 5335.

VARIOUS OTHERS.

See (2) pages 123, 318-9, 870. Also see (20).

Also C. A. 24, 340 and 25, 1247.

PART III

NOTES AND REFERENCES

No attempt has been made here to present a complete bibliography of the amino acids. Only references pertinent to the problem of preparing amino acids in large quantities are given. By consulting the references cited additional references can be easily located.

(1). This statement was verified by experiment.

(2). A. P. Mathews, "Physiological Chemistry", 5th. ed., 1930. Wm. Wood & Co., New York.

(3). A discussion and references will be found in the book by H. H. Mitchell and T. S. Hamilton, "The Biochemistry of the Amino Acids", Am. Chem. Soc. Monograph No. 48, Chemical Catalog Co., New York, 1929.

(4). "Organic Syntheses", a series of annual volumes published by John Wiley & Sons, Inc., New York. Vol. I appeared in 1921.

(5). References to the older literature will be found in V. von Richter's "Organic Chemistry".

(6). S. W. Cole, "Practical Physiological Chemistry", 5th. ed., 1919. C. W. Mosby Co., St. Louis.

(7). For additional data consult Beilstein's "Organische Chemie" or Abderhalden's "Biochemisches Handlexikon".

(8). For additional information see, F. P. Nabenbauer, "Manufacture of casein and lactose from skim milk", Ind. Eng. Chem. 22, 54-6(1930). The lactalbumin used in this work was obtained from the California Milk Products Co., Gustine, Calif., for whom Smith, Kline and French, Philadelphia, act as selling agents.

(9). For process of manufacturing corn gluten and a discussion of maize proteins see Osborne and Mendel, J. Biol. Chem. 18, 1(1914); also ref. (10).

(10). Johns, Finks and Paul. J. Biol. Chem. 41, 391.

(11). M. L. Roxas, J. Biol. Chem. 27, 71(1916). Gives other references.

(12). Cottonseed proteins. D. B. Jones and F. A. Csonka. J. Biol. Chem. 64, 673(1925).

(13). Johns and Jones, J. Biol. Chem. 28, 77(1916); 30, 33(1917).

(14). Linseed meal proteins. F. W. Foreman, C. A. 5, 1297 and 2508(1911).

(15). Sold by the American Lecithin Co., affiliated with the American Mills Co., Atlanta, Ga.

(16). J. W. Beckman, Ind. Eng. Chem. 22, 117(1930).

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(18). Osborne and Guest, J. Biol. Chem. 9, 333(1911).

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(20). R. A. Gortner, "Outlines of Biochemistry", 1929, John Wiley & Sons., Inc., New York.

(21). See J. Biol. Chem. 80, 191; 83, 681; 87, 681.

(22). Hunter, C. A. 17, 1970.

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