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ANALYSIS OF WATER-INSOLUBLE PROTEINS OF FLESH.

An Application of Van Slyke's Method of
Analysis of Pure Proteins.

by

H. H. MCGREGOR

1912.

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Chemistry, in
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ANALYSIS OF WATER-INSOLUBLE PROTEINS OF FLESH.

An Application of Van Slyke's Method for Analysis of Pure Proteins.

"The fundamental importance of a definite knowledge of the quantity of each amino acid yielded by the several food proteins justifies the expenditure of much effort in studying the analytical methods in order that these may be improved or their limitations definitely ascertained." ¹

"From the standpoint of physiological chemistry it is highly desirable that the present very limited knowledge of the proteid substances of flesh be increased " ²

The desirability of a knowledge of the amino acid content of food proteins has led to much experimentation looking towards a method that will yield quantitative results without involving excessive time or manipulation in the analysis. An important method was elaborated by Van Slyke³ "enabling one to attain an insight into the composition of proteins by methods which require but small amounts of material and yield approximately quantitative results indicating the nature of all the nitrogenous products yielded by complete acid hydrolysis. The analysis is based not on the isolation of the amino acids but on determinations

¹ Osborne and Guest: Journ. Biol. Chem., xi, 333, (1911).

²Trowbridge and Grindley: Journ. Amer. Chem. Soc.,
xxviii, 470, (1906).

³Journ. Biol. Chem., ix, 185; x, 15, (1911).

of their characteristic chemical groups." The present paper describes the results of an attempt to apply the method devised by Van Slyke to the determination of the amino acid content of food proteins without a preliminary separation of the individual proteins of the material.

The later methods of protein analysis have been built up through the labors of many investigators. This paper reviews briefly the various analytical methods that have been used. The method of Van Slyke is then described. Then follow some considerations arising from the attempt to analyze a complex mixture such as flesh in which nitrogenous substances other than proteins are present. Limitations of the application of this method are pointed out. Finally, the experimental part of the present paper describes the method and the results obtained in the analyses of samples of (a) edestin, (b) dried beef and (c) dried pork.

PART I. ANALYTICAL METHODS.

That the simple proteins yield on decomposition crystalline end products was known as long ago as 1818 when Proust¹ discovered leucine in the decomposition products of cheese. In 1820 Braconnot² obtained glycocoll from gelatine and meat by boiling with sulphuric acid. Thirty years later Liebig³ and Hinterberger found tyrosine among the end products of hair by treatment with boiling sulphuric acid. As better

¹Proust: Ann. de Chim. Phys., 10, 40, (1818).

²Ann. Chim. Phys., 13, 113, (1820).

³Annalen, 71, 70, (1849).

methods were found for preparing and identifying the end products, other amino acids were slowly added to the list. Up to 1892, in addition to the substances mentioned above five other substances had been prepared, serine from silk glue, aspartic and glutamic acids from vegetable albumins, alanine from the fibroin of silk, and amino isovalerianic acid or valine prepared by Schulze¹ in 1892. In 1889 Drechsel² showed that a considerable proportion of the products of protein hydrolysis were strongly basic substances belonging to the class of diamino acids. He was the first to use phosphotungstic acid as a precipitant for protein cleavage products, and by its aid he found lysine, a diamino caproic acid in casein. Hedin³ prepared arginine and to these two basic compounds Kossel⁴ in 1896 added histidine discovered by him as a decomposition product of sturine, the protamine of the spermatic fluid of the sturgeon. Since then it has been shown by Kossel and his pupils and by E. Schulze to be a widely distributed end product. The three basic substances lysine, arginine and histidine were called by Kossel⁵ the hexone bases. He succeeded in working out methods for their quantitative estimation based on their precipitability with phosphotungstic acid. In 1901 Morner⁶ was able to demonstrate the wide distribution of the

¹Zeitschr. f. physiol. Chem., 17, 193, (1892).

²Ber. der Sachs. Ges. d. Wiss., 1889, 1890.

³Zeitschr. f. physiol. Chem., 21, 155 and 297, (1895).

⁴Ibid., 22, 176, (1896).

⁵Deutsche med. Wochenschr., 1898.

⁶Zeitschr. f. physiol. Chem., 34, 207, (1901).

the sulphur-containing amino acid, cystine, while an acid of an entirely new type - tryptophane, a derivative of indole - was isolated by Hopkins and Cole¹.

Regarding the development of analytical methods up to 1900, Mann² gives the following outline: The most important methods for determining quantitatively the decomposition products of proteins have been worked out by Schulze, Hausmann and others. Hausmann's method for determining the distribution of nitrogen in the protein molecule consists of the following operations:-

- 1) One gram of the substance under investigation is hydrolyzed with boiling hydrochloric acid.
- 2) The nitrogen which has been split off as NH_3 and which is present as NH_4Cl is distilled off with magnesia. This nitrogen is the so-called "amid-nitrogen", ammonia nitrogen, or readily displaceable nitrogen.
- 3) The fluid free from ammonia is precipitated with phosphotungstic acid and the nitrogen present in the precipitate is determined by Kjeldahl's method. This nitrogen is the diamino nitrogen or basic nitrogen of arginine, lysine, histidine.
- 4) The nitrogen which is not driven off by magnesia and is not precipitated by phosphotungstic acid is then determined by Kjeldahl's method as the mon-amino nitrogen.

It is seen that this procedure does not effect the complete separation of the end products, these substances being destroyed in the analysis. The first practical method for the isolation of amino acids was devised by Emil Fischer, whose phenomenal researches on this subject began to be published in 1901. In the Fischer method the amino acids are converted into their volatile ethyl-esters, separated

¹Journ. of Physiol., 27, 418, (1901).

²Chemistry of the Proteids, 1906, 76.

into fractions by distillation, the fractions reconverted into amino acids by boiling with water or barium hydroxide and then separated by fractional crystallization. In practice the protein is hydrolyzed by boiling with hydrochloric acid for several hours at a temperature of 125° - 140° . The acid and water are evaporated off in vacuo, absolute alcohol added and the mixture saturated with HCl gas for esterification. To complete the esterification the alcohol and acid are evaporated in vacuo and a second or even a third treatment of alcohol-hydrogen-chloride given. The black pasty mass of esters is in the form of hydrochlorides. The esters are freed from hydrochlorides by barium hydroxide at low temperature and the mixture of esters extracted from the mass with ether. The esters are then fractionally distilled at less than one millimeter pressure. The distillate comes over from 80° to 200° , generally received in four fractions. These four fractions are then saponified separately and the resulting amino acids are isolated by crystallization. In a few cases other processes are used to isolate individual amino acids. Glutamic hydrochloride is formed in and, after cooling, precipitates from the hydrolyzed mixture, being insoluble in concentrated hydrochloric acid. Glycine ester hydrochloride precipitates out almost wholly from the alcohol-hydrochloric acid mixture. A large portion of the original material remains behind after the ether extraction of the esters as a carbonaceous mass of unknown composition; in fact at every stage of the process some material is lost.

The best results that have ever been obtained by this method are those of Osborne with vegetable proteins, where about 75 per cent of the products were recovered. Most of the results account for only about 60 per cent of the end products. Another objection to the Fischer method is the large quantity of material required for esterification, at least 100 grams and preferably 300 or 400 grams being used.

PART II. THE ANALYTICAL METHOD OF VAN SLYKE.

1. Determination of Aliphatic Amino Groups.

In 1910 Van Slyke published a preliminary report¹ of a method for the quantitative determination of aliphatic amino groups, showing applications to the study of proteolysis and proteolytic products. Later a complete method² appeared. Reference is made to the reaction of aliphatic amino groups with nitrous acid:

"As early as 1875 Sachs and Kormann made this reaction the basis of a method for quantitative determination of amino groups. Since then a number of methods based on this reaction have appeared, none of which have possessed such simplicity, rapidity and accuracy as would warrant their adoption for general use in chemical and biological problems."

The mechanical basis of the new method is an apparatus consisting in the main of a small reaction vessel from which a narrow bore glass tube, equipped with a stop cock, leads

¹Ber., xliii, 3170, (1910).

²Journ. Biol. Chem., ix, 185, (1911).

to a gas burette or nitrometer, bearing a leveling bulb. The burette is in turn connected through a stopcock with a Hempel absorption pipette filled with alkaline permanganate solution for the absorption of nitric oxide. The apparatus forms a closed system. In operation, acetic acid and a strong solution of sodium nitrite are introduced into the reaction bottle. Advantage is taken of the spontaneous decomposition of nitrous acid with formation of nitric oxide:

to displace all the air in the apparatus with nitric oxide. A solution of the amino substance is then with suitable precautions run into the nitrous acid solution, evolution of nitrogen mixed with nitric oxide resulting. The oxide is absorbed by the alkaline permanganate solution and the pure nitrogen measured in the gas burette. Using this apparatus Van Slyke made many determinations of separate solutions of the various amino acids and found that "every known amino acid obtained from proteins by acid hydrolysis reacts quantitatively with one and only one nitrogen atom, except lysine, which reacts with two, and proline and oxyproline which do not react at all. Or, all the amino acids react with all their nitrogen, except tryptophane which reacts with one-half, histidine with one-third, arginine with one-fourth and proline and oxyproline with none." The complete determination of the amino acids can be finished in a few minutes and the error kept within ± 0.05 mg. of nitrogen, equalling the accuracy of the Kjeldahl and Dumas methods.

2. The Analysis of Proteins.

Hausmann's method for determining the distribution of nitrogen in the protein molecule has been referred to. By a combination of this general method, much modified and improved, with the above-described process for estimation of aliphatic amino groups, Van Slyke has elaborated a method which "yields approximately quantitative results with small amounts of material and indicates the nature of all the nitrogenous products yielded by complete acid hydrolysis of proteins." The results show the distribution of nitrogen among the following substances or groups of substances: ammonia, melanin, cystine, arginine, histidine, lysine, the amino-nitrogen and also the non-amino nitrogen of acids not precipitated by phosphotungstic acid. The following is a summary of the steps in the Van Slyke analysis:

- a) Hydrolysis. The hydrolysis with 20 per cent hydrochloric acid is conducted in a tared flask. The progress of hydrolysis is followed by means of the method above described for determining aliphatic amino groups.
- b) Determination of NH_3 (Amid Nitrogen). The hydrolyzed protein, freed as far as possible from acid, is mixed with a ten per cent suspension of calcium hydroxide and the ammonia distilled off into decinormal acid under diminished pressure.
- c) Melanin Nitrogen. The melanin nitrogen is adsorbed by the undissolved lime during the ammonia distillation. This residue is submitted to Kjeldahl determination.
- d) Cystine, arginine, histidine and lysine are precipitated by phosphotungstic acid. These bases are redissolved and determined by reactions based on the following marked chemical differences:

LYSINE and CYSTINE contain only NH_2 nitrogen, which is liberated with HNO_3 .

ARGININE contains one aliphatic amino group and three nitrogen atoms in the guanidine group. HNO_3 therefore liberates one-fourth of its total nitrogen.

HISTIDINE contains one aliphatic amino group and two nitrogen atoms in the imidazole group. HNO_3 liberates one-third of its nitrogen.

ARGININE when boiled with alkali evolves one-half of its nitrogen in the form of NH_3 , the initial decomposition being represented, according to experiments by Schulze and Winterstein,

The urea then yields CO_2 and NH_3 , and the entire decomposition may be formulated,

CYSTINE contains one atom of sulphur which may be oxidized by ignition with $\text{Cu}(\text{NO}_3)_2$ and weighed as BaSO_4 .

Four determinations suffice to distribute the nitrogen of the bases among the four amino acids:

A. Distillation with alkali	One-half arginine N.
B. Kjeldahl determination	A + B = Total nitrogen
C. Determination of amino groups	One-fourth arginine N.
	One-third histidine N.
	Total cystine N.
	Total lysine N.
D. Oxidation of cystine sulphur	Cystine N.

Therefore,

$$\text{Arginine N} = A \times 2$$

$$\text{Histidine N} = \frac{3}{2}(A + B - C - \frac{3}{2}A)$$

$$\text{Cystine N} = D$$

$$\text{Lysine N} = \text{Total N} - (\text{arg.N} + \text{cyst.N} + \text{hist.N})$$

e) Amino acids not precipitated by phosphotungstic are determined in two groups:

- 1) Group reacting with all nitrogen in amino form, - Glutamic acid, aspartic acid, tyrosine, phenylalanine, serine, leucine, isoleucine, valine, alanine, glycine.
- 2) Heterocyclic amino acids, - proline, oxyproline and tryptophane.

The principal uncertainty of the Van Slyke method as above outlined appears to be connected with the determination of cystine and tryptophane. Van Slyke has shown by control experiments¹ that about one-half of the cystine originally present is altered during the hydrolysis with acids; further,

¹Journ. Biol. Chem., x, 1, 38, (1911).

an amount of cystine containing 0.5 per cent. of the total cystine nitrogen remains in solution when the bases are precipitated. In view of these facts it would appear that by the method used accurate estimation of cystine is impossible, although Van Slyke has obtained fairly satisfactory results by an empirical method of calculating a correction for the cystine figure, from experimental data showing the cystine decomposed by hydrolyses of varying durations. The nitrogen compounds resulting from the decomposition of the cystine will introduce errors into the other determinations. This liability to erroneous results receives no emphasis in Van Slyke's discussion. It may be suggested here that a determination of the cystine sulphur present immediately after hydrolysis would furnish data which could serve as a basis for the distribution of the nitrogen due to cystine at the various stages in the analysis.

Tryptophane undoubtedly interferes with the accurate partition of nitrogen among the amino acids. Van Slyke's experiments with pure tryptophane show the possibility of its partial precipitation with the "base" fraction. Of the two nitrogen atoms in tryptophane, one forms part of the indol ring and therefore does not react with nitrous acid: on the other hand, in the Van Slyke analysis no assignment is made for the nitrogen resulting from the nitrous acid decomposition of the α -amino group of tryptophane. The fact is that tryptophane is largely altered during the acid hydrolysis¹ and consequently only traces appear among the

¹Mann: Chemistry of the Proteids, 1906, 54.

end products. Osborne and Guest¹ conclude that "no method exists whereby tryptophane can be estimated with any approach to accuracy. It may well be that tryptophane forms a not inconsiderable part of most of the proteins and that much of the part still unaccounted for may be made up of this amino acid.

PART III. THE ANALYSIS OF FLESH.

In attempting to apply the above method of nitrogen distribution to the analysis of the cleavage products of flesh the problem of assigning the nitrogen to the compounds in which it originated becomes more complex. Two facts confront the investigator:

1. Approximately 12.5 per cent of the nitrogen content of dead muscle is in the form of non-protein substances.²
2. The cold water extract of raw beef contains about 13.56 per cent of the total protein of the flesh.³

If water be used to remove the non-protein bodies the washings contain some of the protein. If the water extractives are not removed their presence vitiates any approach to accuracy in the analysis.

Nitrogenous Substances of Flesh.

The composition of muscles of different animals varies greatly, although it should be stated that the nitrogen percentage shows remarkable constancy. The average amount of nitrogen in fresh lean meat is 3.4 per cent.⁴

¹ Journ. Biol. Chem., ix, 350, 1911.

² Hammarsten: Text Book of Physiol. Chem., 1911, 572.

³ Trowbridge and Grindley: Journ. Amer. Chem. Soc.,
xxviii, 480, 1906.

⁴ Hammarsten: Text Book of Physiol. Chem., 1911, 571.

Elementary analyses of flesh made with great care by Argutinsky¹ show the average nitrogen in fat-free dry ox-flesh to be 15.3 per cent. Salkowski² finds that 87.52 per cent of the nitrogen of beef-flesh is present in the form of protein, the balance as "other soluble bodies". The non-protein nitrogen exists partly as nitrogenous extractives, consisting chiefly of creatine, also the purine bases hypoxanthine and xanthine besides guanine and carnine, but chiefly hypoxanthine.³ These nitrogenous but non-protein constituents may be separated from the main protein mass by suitable solvents but as above stated, soluble proteins are removed in the process.

Cold Water Extracts of Raw Flesh.

It has been shown by Trowbridge and Grindley⁴ that "the cold water extract of raw flesh contains a marked quantity of the total protein of the flesh, consisting chiefly of proteins coagulable by heat together with some which do not coagulate by heat but are precipitated by saturating their solutions with zinc sulphate. These investigators found that the water extract of raw beef from which all visible fat and connective tissue were removed, has the composition, expressed in percentage of the fresh substance, shown in the following table prepared from figures presented in their report:

¹Hammarsten: Text Book of Physiol. Chem., 1911, 571.

²Ibid., p. 572.

³Ibid., p. 545.

⁴Journ. Amer. Chem. Soc., xxviii, 469, (1906).

Water		73.86
Protein		
Insoluble in cold water	16.45	
Soluble in cold water	<u>2.51</u>	18.96
Organic extractives		
Nitrogenous	1.23	
Non-nitrogenous	<u>1.80</u>	3.04
Ash		.92

The method of analysis of flesh that this paper describes must be carried out after thorough extraction of the material to remove all non-protein nitrogen. The considerable amount of protein in the extract should be examined also, after separation by coagulation, saturation or other suitable means. In the following analyses the determinations were made only upon flesh washed with cold water, the extract being discarded owing to insufficient time for its analysis.

PART IV. EXPERIMENTAL.

1. Apparatus.

a) Aminometer. An essential part of the Van Slyke method is the mechanical device for measuring the nitrogen evolved in the nitrous acid decomposition of aliphatic amino groups. This apparatus may be termed an "aminometer". A brief description of it is given in Part II of this paper. In the present analysis it was not, however, found expedient to purchase a duplicate of the original apparatus. Various articles of assorted glassware were therefore brought into requisition. Semi-disabled burettes, a short-stemmed tap funnel, a graduated levelling bulb, with other appropriate vessels were assembled, connected as in the Van Slyke apparatus with small bore tubing, and mounted with the aid of numerous

clamps on an iron ring stand. The aminometer thus constructed is shown in the figure. The reaction bottle D is of 40 cc capacity. It is removable. A rubber stopper which fits securely into the neck of D connects the bottle with burettes A and B and with tube C from the gas burette. Burette A is connected with D by bent glass tubing of 6 mm bore, the tubing passing to the bottom of D. A contains water and serves the double purpose of (a) displacing air or other gas from D and forcing it into gas burette F, and (b) receiving solution forced back from D by the nitric oxide generated in the reaction bottle. Burette B has a capacity of 10 cc and contains the amino solution to be analyzed. Tube C from D leads through a paraffined rubber tube equipped with a pinch cock, to the gas burette. Gas burette F consists of a graduated levelling tube, to the base of which levelling bulb L is connected by flexible rubber tubing. Into the top of F is fitted a three-holed rubber stopper, admitting (a) gas from D, (b) gas from absorption bulb H, and (c) a bent tube M of 1 mm bore, tightly fitted outside with a removable rubber plug. M serves as an outlet for air expelled from D in the flushing of the apparatus preparatory to the collection of the nitrogen. Tube G connects F directly with absorption bulb H, which consists of a dropping funnel used in lieu of a Hempel pipette. K is a heavy side-necked flask connected with the leg of H, and contains permanganate solution. All the rubber connections are well, paraffined. The gas burette was recalibrated by the aid of a corrected burette: a table of corrections for the gas burette is appended to this paper.

In operation, tube G is filled with permanganate

forced through H by lung pressure on the tube leading from K. The water in the gas burette is raised by the levelling bulb until it is within one centimeter of the top and is held in this position by pinching the rubber tube attached to the levelling bulb. A is half filled with nitrogen free water, the outlet tubes from A and B being filled with water below the stop cocks. The amino solution diluted to 10 cc is placed in B. All the cocks are closed except c and O. 25 cc of a 30 per cent solution of sodium nitrite are placed in the reaction bottle D with 7 cc glacial acetic acid, and D is fitted securely into the rubber stopper. (It was found unnecessary to wire it in.) Mild evolution of nitric oxide ensues. This is allowed to escape through C and M for five minutes. It is found that by this time all the air has been displaced from the nitrite solution. Cock A is now opened and by pressure on the water in A it is forced into D where the liquid is allowed to rise through tube C until a few drops have passed into F. Cock c is promptly closed, cock A being left open to receive solution from D. By raising the levelling bulb the gas burette is freed from all gas through M, this tube being allowed to fill with water from F. M is then closed securely with the rubber plug. During the expulsion of air from the burette the nitric oxide has generated in sufficient amount to provide space in D for the reception of the amino solution. Cock A is now closed, c opened, the levelling bulb lowered to lessen the pressure, and the amino solution slowly run into D as far as the lumen

of the cock. A is rinsed twice with small amounts of water and these are run into D. Cock H may now be opened so that the nitric oxide may be absorbed continuously. After the reaction has continued twenty-five minutes D is shaken gently for one minute. The gas is then driven from D and C into F as in the initial flushing of the apparatus, c is closed and A reopened. Complete absorption is accomplished by forcing the gas from F into H and gently shaking H by swaying the whole apparatus. The gas may be tested for complete absorption by repeated measurement.

To test the efficiency and accuracy of the apparatus a determination of urea was made. Van Slyke's statement that urea reacts in eight hours with all of its nitrogen was confirmed. .2034 grams of Merck's urea were dissolved in water and made up to 100 cc. 10 cc of this solution were used for determination in the aminometer, the method above outlined being followed with the exception of the time of reaction, which was extended to exactly ten hours. A blank experiment was performed under identical conditions, substituting 10 cc nitrogen-free water for the urea solution. The results were calculated, as were the results of all subsequent determinations with the aminometer, from the general equation:

$$N = \frac{v(b - c - w) \cdot 0.001256}{760(1 + .00366t)}$$

N = weight nitrogen
 v = observed volume
 b = barometric reading
 c = barometer correction for expansion mercury and glass
 w = aqueous tension
 t = observed temperature

UREA - 19.5 cc gas - temp. 24° - barometer 755 mm.

$$N = \frac{19.5(755 - 2 - 22) \cdot 0.001256}{760(1 + .00366 \times 24)} = .02178 \text{ g.}$$

BLANK - 2.3 cc gas - temp. 24° - barometer 758 mm.

$$N = \frac{2.3(758 - 2 - 22) \cdot 0.001256}{760(1 + .00366 \times 24)} = .00257 \text{ g.}$$

.01921 g.

Nitrogen in .02084 g. urea = .01921 ÷ 2 = .0096 g.

Calculated for $\text{CO}(\text{NH}_2)_2$: N, 46.67 per cent.
Found N, 46.11 per cent.

The error here is approximately 0.6 per cent for urea. The time for reaction, ten hours, increases the amount of foreign nitrogen evolved. The error is therefore likely to be very much less when the aminometer runs for the regular period of one-half hour.

b) Barometer. The barometer used was the ordinary siphon form made by boiling mercury which had been introduced into a 7 mm bore U-tube sealed at one end. The constant correction made use of in the above equation for calculating nitrogen is not exact. Theoretically the reading corrected for cubical expansion of mercury and linear expansion of glass would be represented

$$\text{corrected height} = \text{observed height} \frac{1 + .0000035t}{1 + .0001818t}.$$

For readings between 20° and 25°, and with barometer height between 755 mm and 760 mm, the reduction is approximately 1.5 mm. For the present analysis a reduction of 2 mm is sufficiently accurate.

c) Diminished Pressure Distillation Apparatus. This apparatus is similar to that used in the original Van Slyke

method, with the exception that the double-necked distilling flask was replaced by a one liter ordinary balloon flask, with a three holed rubber stopper. Through one hole, 1 mm glass tubing passes to the bottom of the flask through which air bubbles into the solution when the flask is evacuated. The air facilitates the distillation and effectually prevents bumping. The tube from a glass stop cock enters through another hole to allow the free ingress of air after the reaction is complete and before the suction is removed. A bent glass tube of 1 cm. bore connects the distillation flask with the receiver, the tube terminating in the body of the latter.

2. Analysis of Edestin.

a) Preparation. About 300 grams of crushed hemp seed were extracted with five per cent salt solution for three hours at 60° . The hot extract was filtered through cloth and again heated up to 60° . A large Buchner funnel was prepared using as filtering material filter paper pulp saturated with the saline solution. The extract was filtered through this by suction three or four times, being heated up to 60° before each filtration. The final filtrate was clear and colorless. The vessel containing the liquid was placed in a large basin containing hot water and was allowed to stand over night, thus insuring slow crystallization. The supernatant liquid was poured off as completely as possible and the white suspension taken again into solution with hot salt solution, the volume at this time being about one liter. This was diluted with two volumes of distilled water and in about two hours a

perfectly white precipitate had settled. This was filtered through a Buchner funnel and washed with water until free from chlorides. Before the powder became dry it was removed from the funnel and shaken with alcohol, again filtered and sucked nearly dry. At this stage the mass was snow white. The material was placed on a watch glass in a vacuum desiccator over sulphuric acid. After standing for twelve hours a slightly yellow layer had formed on the surface. When ground the material furnished a nearly white, fine, dusty powder.

b) Hydrolysis. 3.0208 grams of the edestin as above prepared were placed in a small tared flask with 15 cc 20 per cent hydrochloric acid. This was connected with a Hopkins reflux condenser and placed in a boiling water bath. The boiling was continued for two periods of seven hours each. By this time the mass had become black. The flask and contents weighed 52.79 grams. One cubic centimeter was removed and the flask reweighed, showing 1.1 grams of the material withdrawn. This was determined in the aminometer. 39.33 cc gas were evolved at 24° and 758 mm. Hydrolysis was continued for seven hours more, the solution cooled and the flask weighed, showing, with the constant tare of the flask deducted (34.36 g), 17.21 grams. 1.1 grams were withdrawn, which yielded 39.13 cc gas at 24° 758 mm. It was therefore concluded that the edestin was completely hydrolyzed.

c) Total Nitrogen. The hydrolyzed material was transferred to a distilling flask and as much as possible of the HCl distilled off at 30 mm pressure. The residue, a dark colored liquid containing some black spongy flocks, was

taken up with warm water and diluted to 200 cc in a measuring flask. Two 10 cc portions were removed for Kjeldahl determination, using 25 cc H_2SO_4 and 10 grams K_2SO_4 for the digestion. A blank experiment was also run.

No. 1	neutralized	18.32 cc	N/10 acid	
" 2	"	18.11	"	
	Average	18.22	"	
Blank	neutralized	2.18	"	
		16.04	"	x .0014 = .02245 g. nitrogen.

The remainder of the solution, 180 cc, therefore contained .4041 grams nitrogen which must be accounted for in the analysis.

d) Amid nitrogen. The 180 cc of hydrolyzed protein solution was transferred to the one liter flask of the distillation apparatus already described. 25 cc of N/10 acid were placed in each of the receiving flasks, with alizarin as indicator. To the protein solution were added 100 cc alcohol and a ten per cent suspension of calcium hydroxide to alkaline reaction. The distillation was continued thirty minutes from a water bath, at 40° and 30 mm. 25.9 cc N/10 acid neutralized indicated .0362 grams AMID N. A blank determination showed no correction necessary for this figure.

e) Melanin Nitrogen. The lime residue in the reaction flask became dark from adsorption of melanin. It was filtered off, washed free from chlorides, and the filter paper with the lime and melanin digested in a Kjeldahl flask for nitrogen estimation. A blank test was also conducted.

N/10 acid	neutralized	=	7.89 cc
"	"	(blank)	= 4.91 cc
			2.98 cc or <u>.0042 grams MELANIN N.</u>

The high nitrogen content of the blank in this determination was probably due in part to the filter paper used which was of poor grade. It may be noted that the blanks on each of the three melanin determinations reported in this paper show high nitrogen, pointing to impurities in the reagents.

f) Filtrate from Melanin. The filtrate was neutralized with hydrochloric acid, concentrated to 100 cc in vacuo and washed into a 200 cc Erlenmeyer flask. 18 cc concentrated hydrochloric acid were added, then an aqueous solution containing 15 grams phosphotungstic acid. A heavy grayish precipitate of the bases was immediately thrown down. The flask was placed in a boiling water bath until nearly all of the precipitate had dissolved, fifteen minutes heating being required before solution occurred. The flask was then allowed to stand for upwards of 100 hours. The precipitate was washed and filtered, Van Slyke's procedure being followed exactly, a three inch Buchner funnel being fitted with a hardened filter paper lining the bottom and sides, and the precipitated bases washed repeatedly with a solution of 3.5 per cent hydrochloric acid and 2.5 per cent phosphotungstic acid until the calcium test became negative.

g) Treatment of Precipitate of Bases. The precipitate was carefully transferred to a liter beaker, the last traces of precipitate being removed from the hardened filter paper by dissolving in 20 per cent sodium hydroxide. The bases were then brought into solution by cautiously adding 50 per cent sodium hydroxide, phenolphthalein being used in the beaker to prevent any great excess of alkali being added. A 20 per

cent solution of barium chloride was used to precipitate the phosphotungstate. It is important at this step that the barium chloride added be not excessive: its presence causes a most undesirable bumping in the arginine determination. The precipitated barium phosphotungstate is filtered off on the same hardened filter paper used for the bases, washing being continued until chlorides are absent. The filtrate is concentrated in vacuo and some precipitated barium phosphotungstate filtered off; The filtrate was made up to 100 cc.

h) Cystine Nitrogen. Benedict's solution was used to determine the sulphur content of the bases, which represents the cystine present. This solution contains 200 grams crystallized copper nitrate and 50 grams potassium chlorate made up to one liter. 10 cc of the solution of the bases and 5 cc of Benedict's solution were placed in a quartz evaporating dish, evaporated to dryness on a water bath and finally heated to redness four or five minutes with the blast. The residue was extracted with 10 cc of 10 per cent hydrochloric acid. A residue (of silica, probably) remained undissolved. This was filtered off and washed to remove all traces of soluble material. The oxidized sulphur is now present as sulphate and was determined in the usual manner by precipitation as barium sulphate. Each milligram of barium sulphate represents .06 mg. of cystine nitrogen, or, as one-tenth of the solution of the bases was used, .6 mg. in the total solution.

$$\text{BaSO}_4 = .0014 \text{ grams} = \underline{\underline{.00084 \text{ grams CYSTINE NITROGEN}}}$$

i) Arginine Nitrogen. The Van Slyke apparatus for arginine

nitrogen consists of a Kjeldahl flask in which are placed 25 cc of the solution of the bases and 12.5 grams potassium hydroxide. This is connected with an upright condenser, in the top of which a Folin absorption tube containing 15 cc N/10 acid is inserted. Van Slyke had found that under these conditions one-half of the arginine nitrogen is evolved as NH_3 after six hours gentle boiling. At the end of six hours 100 cc of water is added and the remaining ammonia boiled off through the regular Kjeldahl condenser and received in a flask which contains the acid from the Folin bulbs. In the edestin determination this apparatus was prepared and the mixture of bases and alkali were boiled in an oil bath at 125° . During the reaction the bumping became so severe as to cause some of the acid from the Folin apparatus to back-flow into the Kjeldahl flask, spoiling the determination. A modification of the method was attempted: for the Folin bulbs were substituted 7 mm bore glass tubing, leading through the Kjeldahl condenser to the standard acid. The alkali solution was boiled exactly six hours from an oil bath, when the reflux was removed and the Kjeldahl flask, after addition of 100 cc water, connected directly with the condenser. Not more than 50 cc of water were distilled over. 2.33 cc of the N/10 acid were found to have been neutralized; that is, one-fourth of the total solution of the bases had yielded one-half of its arginine nitrogen, neutralizing 2.33 cc acid. If all the arginine had reacted, the solution therefore contained nitrogen equivalent to 18.64 cc decinormal acid, or

.02609 grams ARGININE NITROGEN.

It is obvious that this figure for arginine nitrogen, which

k) Total Nitrogen of the Bases. To the residue from the arginine were added 35 cc sulphuric acid and 25 grams copper sulphate. This was digested and distilled, 19.84 cc N/10 acid being neutralized. This, added to 2.33 cc acid neutralized by part of the arginine nitrogen, shows 22.17 cc acid neutralized by one-fourth of the solution of the bases.

Amino Nitrogen of the Bases. 10 cc of the solution bases were determined in the usual way, yielding 8.45 nitrogen at 23.5° and 760 mm.

m) Filtrate from the Bases. This filtrate is strongly acid and is therefore neutralized with 50 per cent sodium hydroxide, then slightly acidified with acetic acid. The solution was concentrated under diminished pressure until salt began to crystallize in the flask. It was then made up to 200 cc in a measuring flask.

No. 1 yielded 17.29 cc gas - 26° - 755 mm = .1898 g. nitrogen.
 No. 2 " 16.89 " " - 23° - 760 mm = .1898 g. "
 .1898 grams AMINO N IN THE FILTRATE.

o) Total Nitrogen of the Filtrate. Kjeldahl determinations were made on two 25 cc portions.

No. 1	=	21.84 cc N/10 acid
No. 2	=	21.96 "
Average	=	21.90 "
Blank	=	2.46 "
<u>19.44</u> x 8 x .0014 = <u>.2177 g. N IN FILTRATE.</u>		

p) Histidine Nitrogen. Calculated by difference, equation given in Part II, 2, d) = .0856 g. HISTIDINE NITROGEN.

q) Lysine Nitrogen.

By difference, .1241 - (.0261 + .0856 + .0008) =

.0116 grams LYSINE NITROGEN.

Reference was made in connection with the arginine results to the large error apparent. As the arginine figure is used as a basis for the calculation of histidine and lysine, it is evident that the above determinations of these two bases are not correct.

The following summary compares the results of the edestin analysis with the figures obtained by Van Slyke. Comparison is made with Van Slyke's "uncorrected" data inasmuch as the above determinations are not corrected for the solubility of the bases.

Results of Edestin Analyses in Percentages of Total Nitrogen.

	THIS PAPER	VAN SLYKE	
		NO. 1	NO. 2
	<u>Grams</u> <u>N found</u>	<u>Percentage N</u>	<u>Percentage N</u>
Ammonia N	.0362	9.80	10.17
Melanin N	.0042	1.83	2.12
Cystine N	.0008)	.71)	.82)
Arginine N	.0261)	26.85)	25.45)
Histidine N	.0858).1241	3.63)30.71	5.63)35.8
Lysine N	.0116)	3.58)	3.93)
Amino N			
Filtrate	.1898)	49.4)	48.6)
Non-Amino N	).2177	)52.9	)51.4
Filtrate	.0279)	3.5)	2.8)

Even if the wide divergence of the figures for the individual bases is left out of consideration, it is evident that other discrepancies are altogether beyond the limits of experimental error. Lack of familiarity with the method is responsible for much of the divergence. Also, the relatively enormous corrections sometimes found necessary for impurities in the reagents have to a large extent affected the accuracy of the results.

3. Analysis of Beef Flesh.

a) Preparation of the Material. The beef used was from the rump of a steer estimated to weigh 1200 pounds and to be five years old. The meat was freed from all visible fat and connective tissue, then weighed and ground in a meat grinder. The finely macerated material was mixed with about two liters of cold water. This was thoroughly stirred and then filtered through cloth, most of the water being squeezed out by the hand. A second similar treatment was given the mass, as much water as possible being expressed. The residue was free from blood and possessed a grayish white color. It was dried at 60° for ten hours in an air bath, then ground, desiccated over sulphuric acid and weighed. The ground material lacked the true powdery quality of the edestin, and the mass possessed some slight resiliency. It was gray in color. While weighing a sample of the dried meat, it was found to be extremely hygroscopic.

Weight lean meat before washing	=	251.5 grams
Weight after washing and drying	=	45.2 grams
Water and extracts	•	82. per cent.

b) Ash. 2.2946 grams of the beef powder were incinerated slowly in a porcelain crucible. A few crystals of ammonium nitrate were added during the heating. The pure white

residue weighed .1036 gram, or 4.51 per cent of the dry substance.

c) Fat, or Total Ether Extract. Much of the fat separated out in the washing. 13.3088 grams of the dried powder were extracted for four hours with ether in a Soxhlet apparatus. The residue after distilling off the ether weighed 2.0331 grams, or 15.26 per cent of the dried material.

d) Hydrolysis. 4.7078 grams of dried beef (containing fat and ash) with 40 cc of 20 per cent hydrochloric acid were heated in a tared flask to 120° on an oil bath for twelve hour and eight hour periods with the following results:

Twelve hours hydrolysis: weight of solution, 49.03 grams.
2.29 grams in aminometer yielded 42.63 cc N, 23°, 760 mm.

Twenty hours hydrolysis: weight of solution, 46.49 grams.
1.08 grams in aminometer yielded 21.0 cc N, 23°, 760 mm.

The hydrolysis was therefore complete.

e) Total Nitrogen. After distilling off the hydrochloric acid the solution was diluted to 250 cc and two 10 cc portions withdrawn for Kjeldahl determination.

No. 1	=	17.66	cc N/10 acid	
No. 2	=	16.95	"	
Average		17.3	"	
Blank		2.0	"	
		<u>15.3</u>	"	
				= .02142 g. N in 10 cc.
				= <u>.49266 g. N in 230 cc USED FOR ANALYSIS.</u>

At this point the percentage nitrogen in the dried material can be shown. Deducting the figures for ash, 4.51 per cent, 15.26 per cent fat, together with the amounts of solution removed during the hydrolysis and in the total nitrogen estimation, from the weight of the sample taken for analysis:

$$\frac{4.7078}{4903} \times \frac{.9549}{4649} \times \frac{.8474}{250} \times \frac{4674}{4541} \times \frac{4541}{250}$$

= 3.2355 grams dry protein substance.

This 3.2355 grams dry protein substance contained .49266 grams

nitrogen, or 15.23 per cent. This is in exact agreement with the percentage as found by Argutinsky referred to earlier in this paper.

f) Amid Nitrogen. This determination was conducted in the same manner as the edestin, with the exception that barium hydroxide was substituted for calcium hydroxide. It was thought that the calcium hydroxide might be responsible for the high nitrogen figure in the blank experiment controlling the melanin determination. Later experiments, however, showed that this was not the case. 50 cc N/10 acid were used. 12.64 cc acid were neutralized, indicating .0177 grams AMID NITROGEN.

g) Melanin Nitrogen. The baryta residue was filtered off through filter paper of good quality and digested in a Kjeldahl flask, a blank being run at the same time.

$$\begin{array}{rcl} \text{N/10 acid neutralized} & = & 12.17 \text{ cc} \\ \text{" " (blank)} & = & 3.22 \text{ cc} \\ \hline & & 8.95 \text{ cc} = \text{.0125 grams MELANIN NITROGEN.} \end{array}$$

h) Cystine Nitrogen. 10 cc of the solution of the bases were treated with Benedict's solution. A trace of barium sulphate was obtained which was not appreciable.

j) Arginine Nitrogen. 25 cc of the solution of the bases were used, the determination being carried out in the same way and at the same time as for the edestin arginine, and a similar error appeared in the results. 3.59 cc decinormal acid were neutralized. Therefore, $100 \text{ cc} = 4 \times 3.59 \times .0014 \times 2 =$

$$\text{.0402 grams ARGININE NITROGEN.}$$

k) Total Nitrogen of the Bases. The residue from arginine yielded nitrogen = 23.46 cc N/10 acid
 arginine = 3.59 "
 $27.05 \times .0014 \times 4 = \text{.1515 grams N IN BASES.}$

by three treatments with cold water, one liter of water being used each time and the meat thoroughly mixed to a suspension, then filtered through cloth. Most of the water was pressed out and the material dried for fifteen hours in an air bath at 65°. The dried meat weighed 51 grams, or 20.52 per cent of the raw flesh. The dry material was slightly yellow in color, very hygroscopic, and possessed a faint meaty odor.

b) Ash. 1.8193 grams were determined for ash, the latter weighing .0710 grams, equal to 3.9 per cent of the dry substance.

c) Fat, or Total Ether Extract. 15.3488 grams of the dried meat were extracted for four hours with ether in a Soxhlet. The fatty residue weighed 2.8724 grams, or 13.71 per cent.

d) Hydrolysis. 4.8769 grams of unextracted dried meat were hydrolyzed with hydrochloric acid by heating to 130° on an oil bath.

	Period of hydrolysis	Weight solution	Grams withdrawn	cc nitrogen	
I	9 hours	36.14 g.	1.15	27.85	760 mm, 25.5°
II	18 hours	34.84 g.	1.13	29.25	755 mm, 26.5°.

The hydrolysis is shown to be complete.

e) Total Nitrogen. The solution was diluted to 200 cc and two 10 cc portions determined.

No. 1 21.19 cc N/10 acid

No. 2 21.2 "

Blank 2. "

19.2 "

= .0269 grams N in 10 cc.

= .4838 grams N in 180 cc USED FOR ANALYSIS.

The weight of dry protein substance used for analysis is therefore,

$$4.877 \times .7739 \times \frac{3499}{3614} \times \frac{3371}{3484} \times \frac{18}{20} = 3.1815 \text{ grams.}$$

The percentage nitrogen in the dry extracted material is 15.27.

f) Amid Nitrogen. Calcium hydroxide was used to distil over the ammonia. 22.79 cc N/10 acid were neutralized, indicating, .0319 grams AMID NITROGEN.

g) Melanin Nitrogen.

$$\begin{array}{rcl} \text{N/10 acid} & = & 10.76 \text{ cc} \\ \text{Blank} & = & \frac{3.18}{7.58} \text{ cc} \\ & & = \end{array} \quad \underline{.0107 \text{ grams MELANIN NITROGEN.}}$$

h) Cystine Nitrogen. No sulphur was found.

j) Arginine Nitrogen. This determination was carried out at the same time and under the same conditions as the other arginine experiments and is therefore subject to the same error. 4.38 cc N/10 acid were neutralized. Therefore,

$$100 \text{ cc} = 4 \times 4.38 \times .0014 \times 2 = \underline{.0491 \text{ grams ARGININE NITROGEN.}}$$

k) Total Nitrogen of the Bases.

$$\begin{array}{rcl} 25.55 \text{ cc N/10 acid} & & \\ \frac{4.38 \text{ cc}}{29.93} & \text{ " } & \text{from arginine} \\ & \times .0014 \times 4 = & \underline{.1676 \text{ grams NITROGEN IN BASES.}} \end{array}$$

l) Amino Nitrogen of Bases.

$$\begin{array}{l} \text{I. } \frac{16.01(747 - 2 - 26) \cdot .001256}{760(1 + .00366 \times 27)} \times \frac{10}{2} = .0865 \text{ grams N} \\ \text{II. } \frac{16.01(749 - 2 - 24.5) \cdot .001256}{760(1 + .00366 \times 26)} \times \frac{10}{2} = .0882 \text{ grams N} \\ \quad \underline{.0873 \text{ grams N OF BASES IN AMINO FORM.}} \end{array}$$

m) Amino Nitrogen of the Filtrate. The solution was made up to 200 cc.

$$\frac{24.35(753 - 2 - 23.5) \cdot .001256}{760(1 + .00366 \times 25)} \times \frac{20}{2} = \underline{.2682 \text{ g. AMINO N IN FILTRATE}}$$

n) Total Nitrogen of the Filtrate. 25 cc portions used.

$$\begin{array}{rcl} \text{No. 1} & = & 31.02 \text{ cc N/10 acid} \\ \text{No. 2} & = & 30.98 \text{ " } \\ & & \frac{31.02}{31.02} \text{ " } \\ \text{Blank} & = & \frac{4.5}{26.5} \text{ " } \\ & & \times .0014 \times 8 = \end{array} \quad \underline{.2968 \text{ grams NITROGEN IN FILTRATE}}$$

o) Histidine Nitrogen. By calculation, .0652 GRAMS.

p) Lysine Nitrogen. By calculation, .0553 GRAMS.

Summary of Results. - Analysis of Dried Pork Flesh.

Nitrogen in sample taken, .4838 grams.

	<u>Grams Nitrogen</u>	<u>Percentage of .4838 g.</u>
Amid N	.0319	6.59
Melanin N	.0107	2.21
Cystine N	--)	--)
Arginine N	.0491)	10.15)
Histidine N	.0652) .1676	13.47) 34.64
Lysine N	.0553)	11.02)
Amino N		
Filtrate	.2382)	55.43)
Non-Amino N	) .2968	) 61.35
Filtrate	.0236)	5.92)
	-----	-----
	.5070	104.79
	=====	=====

A comparison of the tables summarizing the results of the beef and pork analyses shows close similarity of composition with respect to the groups of nitrogenous constituents.

It is reasonable to expect, in view of the above analyses, that this method is capable of general application to the estimation of the amino acids of food proteins.

APPENDIX.

Table of corrections for gas burette of Aminometer.

Graduation	Actual Volume
48.6	48.43
48.5	48.42
48.	47.91
47.5	47.4
47.	46.9
46.5	46.4
46.	45.9
45.5	45.4
45.	44.9
44.5	44.4
44.	43.9
43.5	43.4
43.	42.9
42.5	42.4
42.	41.9
41.5	41.4
41.	40.89
40.5	40.39
40.	39.88
39.5	39.38
39.	38.88
38.	37.86
37.	36.86
36.	35.89
35.	34.90
34.	33.87
33.	32.91
32.	31.93
31.	30.92
30.	29.94
29.	28.94
28.	27.95
27.	26.94
26.	25.93
25.	24.93
24.	23.94
23.	22.98
22.	21.96
21.	21.
20.	19.98
0.	0.
-100.	-99.94