

and ignite until practically all the carbon is consumed. Transfer the remaining ash to the Erlenmeyer flask with hot water, using a policeman to loosen any particles that may adhere to the dish. Evaporate the moisture and dry the ash at 110° C. until thoroughly dry; weigh. The weight minus the weight of the flask represents the crude ash.

Connect the flask containing the crude ash to an apparatus¹ for determining the carbon dioxide, treat the contents of the flask with 80 cc. of distilled water free from carbonates and 20 cc. of dilute hydrochloric acid (1:10). Aspirate purified air through the apparatus while liberating the carbon dioxide. Boil for 30 minutes and absorb the gas in 50 cc. of a 4 per cent solution of sodium hydroxide. Drain the sodium hydroxide solution out of the absorption tower and wash the remaining caustic solution out of the tower with 250 cc. of CO₂-free water. Exactly neutralize with normal hydrochloric acid, using phenolphthalein as indicator. Add 2 drops of methyl orange solution (1 gram in 1000 cc.) and titrate with N/20 hydrochloric acid until the color of the methyl orange is just changed. From the number of cc. of N/20 hydrochloric acid used subtract blank: 1 cc. N/20 hydrochloric acid = 0.0022 gram carbon dioxide. The titration where phenolphthalein is used is ignored. The carbon, sand and silica are determined as outlined on page 22 in *Bulletin 107*, U. S. Bureau of Chemistry. The carbon dioxide plus the unburned carbon and sand is subtracted from the weight of crude ash. The remainder represents the amount of carbon-free ash.

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THE PRESENCE OF PROTEOSES AND PEPTONES IN SOILS

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Recent investigators concur in stating that the chemistry of soil nitrogen is essentially the chemistry of protein undergoing hydrolysis. Proteins find their way into the soil in the form of plant and animal débris, manures and fertilizers. As soon as these substances are incorporated in the soil, hydrolytic decompositions ensue.

The investigations of Shorey,² Jodidi,³ Robinson,⁴ Suzuki,⁵ Lathrop and Brown,⁶ and Kelley⁷ on the classification of the nitrogenous decomposition products as ammonia, monoamino acids, diamino acids, etc., and the work of Schreiner and Shorey,⁸ Shorey,⁹ Lathrop,¹⁰ and Robinson,¹¹ on the isolation of a number

of amino acids from soils indicate that the proteins are decomposed in a soil in much the same way as in acid hydrolysis or in animal digestion.

If this view is correct and the soil protein is hydrolyzed as in digestion by acids or enzymes we should expect to find in soils all of the intermediate products of protein hydrolysis provided the methods are sufficiently accurate for their identification. Up to the present time, however, only a few of the primary cleavage products have been found.

Although the complex, first stage, decomposition products of the proteins have not been found to occur or persist in soils, their presence has been anticipated. It is the object of this paper to report the presence of certain bodies, presumably proteoses and peptones, resulting either from partial hydrolysis of proteins or by the synthetic action of microorganisms.

The present methods for differentiating the various proteoses and peptones are unsatisfactory. These compounds are classified according to their varying solubilities, especially in ammonium sulfate solutions of different concentrations.

The differences in composition between the various members of the respective groups remain to be more accurately established. Because of the chemical similarity of these substances, the difficulty attending their separation, and the uncertainties in their identification, pure proteoses and peptones are not easy to procure, and in an investigation of this character where they are present in soil in comparatively minute quantities, only qualitative reactions can be employed to show their existence.

Numerous investigations¹ on the hydrolysis of proteins have shown that the protein molecule is gradually broken down into a series of long chains of amino acids. These chains are known as proteoses, peptones, and peptides according to the number of units in the chain. These still possess true protein characteristics. Further hydrolysis causes the ultimate splitting of these protein-like substances into amino acids of known chemical structure.

It would be beyond the scope of this paper to dwell upon the various proteoses and peptones which are described in detail in the literature. Only their general characteristic properties will be given.

The proteoses have been considered as the intermediate substances in the peptonization of proteins whose neutral or faintly acid solutions do not coagulate on boiling. They are subdivided into primary and secondary proteoses. The primary bodies possess a higher molecular weight and greatly resemble the proteins; they are precipitated and thus separated from the secondary proteoses by either completely saturating their aqueous solutions with sodium chloride or half saturating them with ammonium sulfate. According to Pick² and Haslam³ the primary proteoses are represented by at least three substances: hetero-proteose, α -proto-proteose, and β -proto-proteose.

¹ For general references see E. Abderhalden, "Biochemisches Handlexikon," Vol. IV; "Handbuch der Biochemischen Arbeitsmethoden," Vol. II and III; Gustav Mann, "Chemistry of the Proteids."

² E. P. Pick, *Ztschr. physiol. Chem.*, **24** (1897), 246.

³ H. C. Haslam, *J. Physiol.*, **32** (1905), 262.

¹ THIS JOURNAL, **4** (1912), 611.

² E. C. Shorey, U. S. D. A. Hawaii Sta., *Ann. Rept.*, **1905**, pp. 25-38 and **1906**, pp. 37-59.

³ S. L. Jodidi, Michigan Sta., *Tech. Bull.*, **4** (1909); Iowa Sta., *Research Bulls.* **I and III** (1911).

⁴ C. S. Robinson, Michigan Sta., *Tech. Bull.* **7** (1911).

⁵ S. Suzuki, *Bull. Coll. Tokio*, **7** (1907), 513.

⁶ E. C. Lathrop and B. E. Brown, THIS JOURNAL, **3** (1911), 657.

⁷ W. P. Kelley, *J. Am. Chem. Soc.*, **36** (1914), 429-444.

⁸ Oswald Schreiner and E. C. Shorey, U. S. Dept. of Agr., Bureau of Soils, *Bull.* **74** (1910).

⁹ E. C. Shorey, U. S. Dept. of Agr., Bureau of Soils, *Bull.* **88** (1913).

¹⁰ E. C. Lathrop, *J. Am. Chem. Soc.*, **34** (1911), 1260.

¹¹ S. C. Robinson, *Ibid.*, **33** (1911), 564.

This classification is based upon differences in solubility in various solvents.

The hetero-proteose has a tendency to pass into an insoluble form on standing or when heated to about 50°. This insoluble form Kühne¹ termed "dys-albumose" (dys-proteose)

The secondary proteoses (deutero-proteoses) are in many instances divided from the peptones only by arbitrary definitions.² Chittenden and Hartwell² have found that the primary proteoses, proto- and hetero-, are only slowly converted into peptone by proteolytic ferments, since they must first pass through the intermediate stage of deutero-proteose.

Proteoses and peptones may also be formed by the hydrolysis of proteins by dilute acids or alkalies³ and by the action of microorganisms.⁴

The proteoses in watery solution are precipitated at the ordinary temperature by nitric acid and by acetic acid and potassium ferrocyanide, and the precipitate has the peculiarity of disappearing on heating and reappearing on cooling. If a neutral proteose solution is saturated with sodium chloride, the proteose is partly precipitated, but on the addition of acetic acid it is more completely precipitated.

The proteoses give precipitates with ferric chloride, neutral and basic lead acetate, platinum chloride, phosphotungstic acid, phosphomolybdic acid, picric acid, tannic acid, trichloroacetic acid, and metaphosphoric acid. The precipitates are all more or less soluble; some redissolve on the addition of an excess of the precipitant, and some dissolve on heating and reappear on cooling, while others are permanent on heating. Copper sulfate and copper acetate precipitate the primary but not the secondary proteoses, and are sometimes used to separate these bodies.⁵ Acetic acid and potassium ferrocyanide precipitates all proteoses but the presence of peptone may interfere with the reaction. Nitric acid precipitates the primary proteoses in the absence of salts, the secondary proteoses only in the presence of sodium chloride, and the lowest members of the secondary proteoses only if the solution is saturated with salt. The precipitate is soluble in excess of nitric acid, especially on heating, but reappears on cooling.

The peptones are considered as the final products of the hydrolysis of proteins by means of proteolytic enzymes in so far as these products are still substances of a protein nature. They are readily soluble in water, glacial acetic acid, and in all salt solutions and are not coagulated by heat. The watery solutions are not precipitated by nitric acid, acetic acid and potassium ferrocyanide, picric acid, trichloroacetic acid, potassium mercuric iodide, nor by neutral salts and acids. These reagents, however, precipitate peptones in concentrated solutions of calcium chloride, calcium nitrite, and ammonium sulfate. They are precipitated by phosphotungstic acid, phosphomolybdic acid, mercuric chloride (in the absence of neutral salts),

absolute alcohol, and tannic acid, but the precipitate may redissolve on the addition of an excess of the precipitant. The tannic acid precipitate is soluble in acetic acid.

Proteoses and peptones give a red biuret reaction and the xanthoproteic reaction, while the other color tests may or may not be given according to the radicals contained in the individual molecules.

It is obvious that the names proteose and peptone do not indicate any definite compounds. They represent stages of decomposition between that of true proteins and amino acids. These terms will undoubtedly disappear, as acquisitions to our knowledge concerning the chemical nature of the polypeptides establish a more complete relationship to these cleavage products. In a few instances this relationship, both chemical and biological, has already been indicated for the lower members of the proteose-peptone group. The most interesting analogy in this connection is the fact that the tetrapeptide obtained from silk-fibroin by Fischer and Abderhalden,¹ consisting of two glycine molecules, one of alanine and one of tyrosine, shows properties with which many proteoses correspond.

EXPERIMENTAL

In a recent investigation of a sample of Norfolk sandy loam soil from Virginia a solution was obtained which responded to many of the color and precipitation reactions given by proteoses and peptones.

The solution was obtained in the following way: 50 lbs. of soil were extracted with a 2 per cent solution of sodium hydroxide for 24 hours. The alkaline extract was separated from the insoluble residue by decantation and made acid with sulfuric acid and filtered from the so-called humus precipitate. The acid filtrate was shaken out with ether to remove compounds soluble in this solvent and a solution of phosphotungstic acid in 5 per cent sulfuric acid added after removal of the dissolved ether, until no further precipitate was formed. After several days the phosphotungstic acid precipitate was filtered off and washed thoroughly with a dilute solution of sulfuric acid and phosphotungstic acid. The precipitate was then suspended in water and an excess of barium hydroxide added to precipitate the phosphotungstic and sulfuric acids. The excess of barium in the filtrate from the barium phosphotungstate and sulfate was removed by carbon dioxide and the barium carbonate filtered off.

It frequently has been found in dealing with solutions obtained in a similar manner from many other soils that the barium is very difficult to remove by treatment with carbon dioxide.

At this point it is necessary to consider somewhat in detail the carbamino reaction of Siegfried.² This investigator has found that on saturating a mixture consisting of equal volumes of equinormal solutions of glycocoll and barium hydroxide at a low temperature (0° C.), an alkaline solution is obtained which remains clear when carbon dioxide is passed through it, and this continues to be the case till, for each volume

¹ W. Kühne and R. H. Chittenden, *Ztschr. f. Biol.*, **20** (1884), 11.

² R. H. Chittenden and J. A. Hartwell, *J. Physiol.*, **12** (1891), 12.

³ Gustav Mann, "Chemistry of Proteins," p. 199.

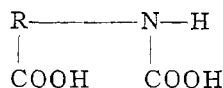
⁴ W. Kruse, "Allgemeine Microbiologie," p. 490.

⁵ O. Folin, *Ztschr. physiol. Chem.*, **25** (1898), 152.

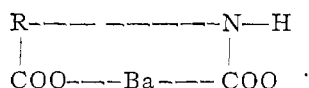
¹ E. Fischer and E. Abderhalden, *Ber.*, **39** (1906), 752.

² M. Siegfried, *Ztschr. physiol. Chem.*, **44** (1905), 85.

of glycocoll, nearly two volumes of equivalent barium hydroxide have been added. This solution gives off barium carbonate slowly on standing in the cold but quickly on boiling. Siegfried¹ and his students have subsequently obtained analogous results for several of the amino acids and peptones. An analysis of some of these compounds has shown that the amino acids contain the carbamino acid radical



and the barium salt would have the formula



These compounds are formed from the amphoteric amino acids by simply adding carbon dioxide.

This reaction has been recognized in dealing with the soil solution described above. However, it was found that these carbamino compounds are stable only at low temperatures (Siegfried's work was carried out at 0°) and that the barium could be completely removed by adding a slight excess of sulfuric acid after treatment with carbon dioxide and then boiling the solution with a slight excess of solid barium carbonate.

When a soil is treated with a dilute solution of sodium hydroxide a very dark-colored solution, the so-called humus extract, is obtained. When this extract is treated with an excess of acid a dark-colored precipitate is formed, the so-called humic acid. This precipitate usually contains the greater portion of the organic matter of the soil. The filtrate from this precipitate still contains a portion of the organic matter and is usually dark colored but much lighter than the original alkaline extract.

The extract obtained as described above, however, was a very light-colored solution which could be used very satisfactorily in any of the color and precipitation tests characteristic of the proteins, proteoses, and peptones.

This solution gave the following reactions: a red biuret reaction; Hopkins-Cole reaction; Millon reaction; Molisch carbohydrate reaction; a precipitate was formed with hydrochloric, nitric, and sulfuric acids which dissolved on warming and reappeared on cooling; a precipitate was formed upon the addition of an equal volume of absolute alcohol; a precipitate was formed when the solution was saturated with ammonium sulfate; a precipitate was formed in saturated sodium chloride containing acetic acid; and the tests for the presence of sulfur were negative.

On carefully evaporating the soil solution to dryness a yellow, sticky, gelatinous residue was obtained. No crystalline material could be observed. As there was not enough of this solution, upon which further tests could be made with any degree of certainty, a second sample of soil was obtained three months later and the solution was obtained again as described above.

This solution gave the following reactions: a red

biuret reaction; Hopkins-Cole reaction; Millon reaction; Molisch carbohydrate reaction; Liebermann reaction; a precipitate was formed with hydrochloric, nitric, and sulfuric acids which dissolved on warming and reappeared on cooling. The solution containing the nitric acid changed from a light yellow to an orange upon the addition of an excess of potassium hydroxide; a precipitate was formed in 50 per cent alcohol; a precipitate was formed in saturated sodium chloride solution containing acetic acid; a precipitate was formed with picric acid, phosphomolybdic acid, acetic acid and potassium ferrocyanide, trichloroacetic acid, silver nitrate, copper sulfate, copper acetate, ferric chloride (slight), tannic acid (soluble in acetic acid) and neutral lead acetate; a precipitate was formed when an equal volume of acetone was added to the soil solution; a slight precipitate was formed with mercuric chloride. The precipitate which was formed in half-saturated ammonium sulfate was filtered off and the filtrate saturated with ammonium sulfate when a further precipitate was formed.

Absolute alcohol was added to a portion of the soil solution until no further precipitate was formed. After standing for 24 hours the precipitate was filtered off and washed with 95 per cent alcohol.

The precipitate was then dissolved in warm water. This solution did not give the Hopkins-Cole reaction, thus indicating the absence of the tryptophane group. No precipitates were formed with acetic acid, picric acid, copper acetate, or mercuric chloride. All of the other reactions observed on the original solution were positive.

The filtrate, after removal of the alcohol, gave all of the reactions obtained on the original solution.

Weyl¹ has found that certain proteins, polypeptides, and amino acids are precipitated by acetone in certain concentrations.

To another portion of the test solution acetone was added until no further precipitate was formed. After standing for 24 hours the precipitate was filtered off and washed with a small quantity of acetone. After careful drying the acetone precipitate was dissolved in warm water. This solution gave the following reactions: A red biuret reaction; Millon reaction; Molisch test for carbohydrates; precipitates with tannic acid, picric acid, phosphotungstic acid, acetic acid and potassium ferrocyanide, lead acetate, and half-saturated and saturated ammonium sulfate solution. The Hopkins-Cole reaction was negative and no reactions could be observed with hydrochloric acid, nitric acid, or saturated sodium chloride and acetic acid.

The filtrate after removal of the acetone gave all of the above tests with the exception of the Hopkins-Cole reaction.

In order to determine whether or not these protein-like substances existed as such in the soil and were not entirely formed by hydrolysis of the protein by the dilute alkali used in making the extract, a water extract of the soil was obtained. This extract was made

¹ E. Abderhalden, "Biochemisches Handlexikon," Vol. 4, p. 198.

¹ Theodor Weyl, *Ztschr. physiol. Chem.*, **65** (1910), 246; *Ber.*, **43** (1910), 508.

acid with sulfuric acid to about 5 per cent and a slight excess of phosphotungstic acid added in the usual way. The phosphotungstic acid precipitate was treated exactly as described above and the solution finally obtained gave the following reactions: a red biuret reaction; Millon reaction; Molisch test for carbohydrates; the usual precipitates with hydrochloric, nitric, and sulfuric acids; precipitates with acetic acid and potassium ferrocyanide, saturated sodium chloride and acetic acid, half-saturated and saturated ammonium sulfate and a precipitate when an equal volume of alcohol or acetone was added.

A portion of the soil solution kept sterile by a few drops of toluol was observed after several days to contain a slight precipitate. It may be that this substance is similar to the dys-proteose which Kühne obtained from the hetero-proteose.

In an extensive examination of the nitrogen compounds of processed fertilizers, Lathrop¹ has reported the presence of certain protein-like bodies similar to those described above.

It is not the purpose here to enter into a discussion of the availability of the various forms of organic nitrogenous compounds. It may be stated, however, that in the light of recent extensive researches by Schreiner and Skinner,² which show clearly that most of the simple primary cleavage products of proteins, the amino acids, are readily available to green plants, that perhaps some of the simpler peptones and polypeptides (such as the tetrapeptide alluded to above) may also be used by green plants to a limited extent as a direct source of nitrogen.

From the nature of the reactions obtained in the various solutions described above it is clear that they contain a mixture of the various proteoses and peptones resulting from the hydrolysis of proteins and that they exist and persist in the soil as such for a considerable period, or, the original protein is only very slowly hydrolyzed. It is also clear that an attempt to make any further separation of these bodies, according to our present knowledge, would lead us beyond the limits of the ordinary laboratory equipment.

The experiments described above indicate that proteins undergo hydrolytic decompositions in the soil in much the same way as in digestion by enzymes, acids, or alkalies, in the laboratory.

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THE DETERMINATION OF NITRATES IN SOIL

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C. B. Lipman and L. T. Sharp³ have shown that such substances as alum, aluminum cream and bone-black for coagulating the clay and organic matter in obtaining the soil extract to be used for the determination of nitrates cause a decided loss in the amounts of nitrates found. They then recommend the use of quicklime for this purpose. It was found by them

that no loss was induced when amounts of lime, varying from 1 to 5 g., were shaken with solutions (volumes not given) containing 1 and 5 mg. of nitrate nitrogen. Practically the entire amount of nitrate nitrogen in a soil of known¹ nitrate content was found by the use of 2 g. of calcium oxide with 100 g. of soil. Working with a soil whose content of nitrate nitrogen was not known, it was found that it made no difference whether the lime was added before or after the water.

The above investigators point out the fact that J. G. Lipman and P. E. Brown, in their "Laboratory Manual on Soil Bacteriology," give directions for the use of 2 g. of lime with 100 g. of soil in preparing the extract for nitrate determination, but Dr. Brown informs us that he has always used lime in the form of the carbonate. Since, during some preliminary work, the results with calcium oxide seemed inconsistent, it was decided to compare the action of these two flocculating agents. At the same time parallel tests were made with the colorimetric method and the aluminum reduction method as modified by Burgess,² who recommends the following procedure: 100 cc. of the soil extract are placed in a casserole, 2 cc. of 50 per cent sodium hydroxide added, the mixture boiled down to about 50 cc. to remove the ammonia, the residual solution washed into a large test tube, and made up to 100 cc. A strip of aluminum is then added and the test tube closed with a stopper bearing a glass tube drawn out to a capillary. By this means, with the amount of nitrate nitrogen less than 100 mg., Burgess states the loss of ammonia by diffusion is insignificant. After allowing the reduction to go on for 11 to 14 hours at about 20°, the solution is transferred to suitable flasks and the ammonia is distilled off into standard acid. The only difference from this of the procedure used by us has been to transfer from the casserole directly to the Kjeldahl flask of the aeration apparatus described by us elsewhere.³ With the volume at 100 cc. aluminum strips were added; the flask was then connected to its absorption bottle which contained a suitable quantity of 0.02 N acid in 200 cc. of water. Either a slow stream of air may be run through the series for the required length of time, or the long tube in the Kjeldahl flask may be closed with a glass plug or a pinch cock. In either case, after 11 to 14 hours the pump is opened to give the maximum current of air. At the end of an hour, if the air current is sufficiently strong, the aeration will be complete, and the residual acid can be titrated. The use of this procedure effects a decided saving of time if the apparatus is at hand. Another advantage is that all danger of loss of ammonia by diffusion is avoided. As many as fifteen determinations have been run on one series. The colorimetric method used by us was the modification of the phenoldisulfonic acid method proposed by Chamot and collaborators.⁴ Lipman and Sharp do not state just which modification of this method

¹ E. C. Lathrop, U. S. Dept. of Agr., *Bull.* 158 (1914).

² Oswald Schreiner and J. J. Skinner, U. S. Dept. of Agr., Bureau of Soils, *Bull.* 87 (1912).

³ *Univ. Cal. Pub. Agr. Sci.*, 1 (1912), 21.

¹ How the amount of nitrate nitrogen was found is not stated.

² *Univ. Cal. Pub. Agr. Sci.*, 1 (1913), 51.

³ *THIS JOURNAL*, 7 (1915), 221.

⁴ *J. Am. Chem. Soc.*, 33 (1911), 381.