no biuret reaction. This peptone shows a rotation in aqueous solution; $[\alpha]_{20}^{D} = +25.0$. A nitrogen determination by the Kjeldahl method gave 14.71 per cent. of nitrogen compared with 14.94 and 14.96 per cent. for the original silk and 14.88 and 14.89 per cent. for the residual silk after digestion.

Results and Conclusions.

1. It is possible to hydrolyze silk with trypsin.
2. Tyrosine is one of the products of this hydrolysis.
3. Tryptophan or some of its products were isolated from silk for the first time.
4. Tryptic peptones with a dextrorotation were obtained.

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[FROM THE LABORATORY OF FERTILITY INVESTIGATIONS, BUREAU OF SOILS.]

THE ORIGIN OF CREATININE IN SOILS.[1]

BY M. X. SULLIVAN.

Received September 28, 1911.

Creatinine has been found in soils. Its presence was demonstrated first by Dr. E. C. Shorey,[2] of this laboratory, who extracted it from various soils by means of dilute alkali. Subsequently, as shown in the present paper, it was found in the water and glycerol extracts of planted soils. The creatinine of the soils might have its origin (1) as a result of metabolic activity of microörganisms; (2) from stable manure introduced into the soil; (3) from the disintegration of plant debris and the direct passage from the living plant.

The particular phase of the question of the origin of creatinine in soils, which is considered here, is its presence in plants and consequently in plant debris and the passage of the creatinine into the soil either by the disintegration of the plant debris or as a result of cell sloughing or direct excretion from the living plant.

Creatinine, $C_4H_7ON_3$, is the anhydride of creatine, $C_4H_9O_2N_3$. Creatine is an ever present constituent of muscle and is found in addition in blood and urin. Creatinine is likewise found in muscle, blood and urin, particularly in the urin. The origin of these substances and their physiological relationship within the animal organism have long been under study.

Whatever may be the relationship between creatine and creatinine within the animal, it is certain that one can be converted into the other by appropriate means in the laboratory. Creatine, for example, goes to creatinine on boiling with acids, or, as shown by Folin and Denis,[3]

[1] Published by permission of the Secretary of Agriculture.

[2] *Sci.*, **33**, 340 (1910).

[3] *J. Biol. Chem.*, **8**, 399 (1910).

merely by heating crystallin creatine for three hours at a pressure of 4 or 5 kilograms per sq. cm. Creatinine is converted more or less to creatine on warming with alkali.

The literature[1] pertaining to the biochemistry of creatinine and creatine is rather extensive, but will not be reviewed in the present paper which deals only with the presence of creatinine in plants and the medium in which they grow.

Its occurrence in soils had already been reported[2] and will be presented more fully later by Dr. E. C. Shorey, of this laboratory. The effect of creatinine and creatine on plant growth, both of which are beneficial, will be presented by J. J. Skinner, of this laboratory.

Since the chemical properties of creatinine may be found in various text-books of physiological chemistry all that need be said in the present paper is (1) that creatinine is somewhat soluble in cold water and cold alcohol, more so in water and is rather soluble in hot water and hot alcohol, (2) that it is not precipitated by lead acetate, (3) that it gives certain well known color reactions, and (4) forms a characteristic crystallin salt with zinc chloride in neutral solutions.

Though the literature pertaining to the biochemistry of the animal organism is filled with references to creatine and creatinine, the possibility of the occurrence of these substances outside of animal products has not been investigated hitherto to any great extent. Zinno,[3] however, found that cultures of certain bacteria in peptone salt solution gave Weyl's, Salkowski's and Jaffé's reactions for creatinine and he isolated the creatinine as creatinine zinc chloride. Later, Antonoff,[4] relying merely on Weyl's reaction, reported the formation of creatinine by many bacteria in peptone salt solution. More recently Shorey[5] found it in the alkaline extracts of soils.

Previous to the present paper, however, creatinine has never been reported in vegetable matter, although it has been demonstrated often in the flesh of plant-eating animals.

Criteria for Creatinine.

In the present experiments the criteria for creatinine were (1) the color reactions,[6] especially Jaffé's—a red color with picric acid and a few drops

[1] See Magnus Levy, *Van Noorden's Handbuch der Pathologie des Stoffwechsels*, 1, 137 (1906). Hoogenhuyze and Verhloegh, *Z. physiol. Chem.*, 46, 415 (1905). Mellanby, *J. Physiol.*, 36, 447 (1908). Schaffer, *Am. J. Physiol.*, 23, 1 (1908).

[2] E. C. Shorey, *Science*, 33, 340 (1911).

[3] *Riforma Medica*, 9, [3] 806 (1893).

[4] *Centr. Bakt. Parasitenk.*, 43, 209 (1907).

[5] *Loc. cit.*

[6] In the case of soil or plant extracts, the occurrence of the color reactions is but a presumptive test that creatinine is present, since other substances than creatinine give these reactions. The absence of these color reactions, however, is a sure test

of sodium hydroxide, a color which is discharged on adding excess of alkali and on acidifying—and Salkowski's—a green color or precipitate of Prussian blue on adding sodium nitroprusside, sodium hydroxide to alkaline reaction and subsequently acidifying with acetic acid and warming; (2) the formation of the characteristic double salt of creatinine and zinc chloride; (3) the regaining of creatinine from the zinc salt by boiling with lead hydroxide with a resulting solution which gave the color reactions.

Creatinine in Planted Soil.

When the concentrated water extracts and water-glycerol extracts of a recently cropped soil were boiled with Fehling's solution, there was more or less reduction of the copper with the formation of a greenish white precipitate. The character of the precipitate suggested the presence of purine bases and creatinine in the soil solution and it became of great interest to determin (1) whether or not the water and glycerol extracts of the cropped soils contained these compounds; (2) whether or not there was a relation between the content of these compounds in the soil and the fact that the crop had grown thereon. As regards the purine bases, it may be said in passing that by appropriate means their presence in the extracts was demonstrated. The main interest, however, was in creatinine for which the water and glycerol extracts of several recently cropped soils were tested.

Arlington Clay Loam.—A sample of Arlington clay loam, upon which cowpeas had been grown for four years, was planted with wheat in paraffin wire pots and the cultures kept in the greenhouse for three weeks. Then the wheat seedlings were cut close to the soil. The samples from the pots were combined and sieved through a one-eighth-inch mesh sieve. For the water extracts, three kilograms of the soil were extracted with six liters of water for twenty-four hours, with frequent stirring, especially in the early stages of extraction. For the glycerol extracts, 3 kilograms of soil were treated with 1200 cc. of pure glycerol, for twelve hours, when 4800 cc. of water were added and the mixture stirred and allowed to stand twelve hours longer. The glycerol itself gave no creatinine test.

The water extract was filtered through paper and 2500 cc. were concentrated to a small bulk. The solution was then made slightly acid with hydrochloric acid and was treated with a large volume of alcohol. After a time a slight precipitate formed. The filtrate, concentrated to about 20 cc., gave Jaffé's and Salkowski's tests for creatinine. The glycerol extracts similarly concentrated to a small bulk gave stronger tests for

that creatinine is absent or present only in traces. When, however, no other substance which gives these reactions is present in a solution, as in the urin, for example, the color reactions are decisive tests for creatinine and the Jaffé reaction has been made good use of as a quantitative test by Folin (*Z. physiol. Chem.*, 41, 223 (1904)) and others.

creatinine. The concentrated glycerol extract of 2500 cc. of the original solution was syrupy from the glycerol. The syrupy, concentrated glycerol extract was boiled with Fehling's solution and the mixture was filtered. The precipitate was then suspended in water and freed from copper by means of hydrogen sulfide. The filtrate from the copper sulfide was concentrated as far as possible on the steam bath and was still found to contain some glycerol. To the concentrated solution, zinc chloride and sodium acetate were added with the formation of a white precipitate. In twenty-four hours, colorless rosets of creatinine zinc chloride formed.

Dunkirk Clay and Duchess Silt Loam.—In the glycerol extract of a recently cropped sample of Dunkirk clay and in the water and glycerol extracts of a recently cropped sample of Duchess silt loam, creatinine was demonstrated in the same way by means of the double salt of zinc chloride and creatinine.

The creatinine zinc chloride crystals from the Arlington clay loam, the Dunkirk clay and the Duchess silt loam were boiled separately with freshly precipitated lead hydroxide. The filtrates were concentrated to a small bulk. All the concentrated solutions gave Jaffé's and Salkowski's tests. How much of this creatinine, which exists in the concentrated extracts in but small quantities, is present as such in the original extract can not be told, since in the process of concentrating some would be formed from creatine, if present.

Creatinine was also found in the glycerol extract of Arlington clay loam as it came from the field. As judged by the quantity of the double salt of creatinine zinc chloride obtained from the glycerol extracts of Arlington clay loam, planted and unplanted, similarly treated as regards concentrating, precipitating by Fehling's solution, freeing from copper by hydrogen sulfide, concentrating the filtrate from the sulfide and adding zinc chloride and sodium acetate, the extract of the planted soil contained more creatinine than did the extract from the unplanted soil. The increased amount of creatinine in the planted soils must have come (1) from an increased activity of microörganisms on the plant debris and plant constituents and on the organic matter of the soil or (2) directly from the growing plants.

Creatinine in the Water in which Seedlings had Grown.

Since creatinine was found in the extract of soil on which wheat had grown for several weeks, it seemed possible that it might exist in water in which wheat seedlings had grown. Wheat was soaked for about five hours in water and was then placed with a little water in an enameled pan to germinate over night. The seeds with the plumule just showing were then placed on a perforated aluminium disk which was floated on water by means of four air-tight glass tubes. In two days

the water was changed. The water collected from six pans, about 12 liters in all, was concentrated on the steam bath to 300 cc. To the concentrated solution a few cubic centimeters of concentrated hydrochloric acid and one liter of alcohol were added. In a short time a flocculent precipitate settled out. This precipitate gave a good pentose reaction with orcin and hydrochloric acid, contained phosphoric acid, and gave a slight xanthine test and probably contained nucleic acid. The filtrate from this precipitate was concentrated to about 25 cc. and was then tested for creatinine by means of picric acid and sodium hydroxide. It may be said that many compounds such as levulinic acid, tyrosine, pentose sugars, gallic acid, etc., which might be in the extract of plants, give the red color reaction with picric acid and sodium hydroxide, the red changing to yellow on adding acetic acid, so that this reaction was taken merely as a suggestion that creatinine was present in the water in which the seedlings were growing. The alcohol solution, which gave the color test such as creatinine would give if present, was neutralized and brought to dryness, and was then treated with boiling, absolute alcohol and filtered. The filtrate was again brought to dryness and the process of taking up with absolute alcohol and drying was repeated several times. The final slightly syrupy residue was treated with zinc chloride and sodium acetate in the usual manner and was put in a desiccator. In a few days, stars, radiating clusters of needles, and rosets of creatinine zinc chloride appeared. To make a further test the mass containing the crystals was boiled with freshly prepared lead hydroxide and filtered. The filtrate concentrated to a small volume gave Jaffé's test for creatinine, and also Salkowski's test with the formation of Prussian blue. The original non-acidified solution, when strongly concentrated, likewise gave Salkowski's and Jaffé's tests for creatinine. The amount of creatinine in the water was, however, very small.

Creatinine was likewise found in the water in which the seedlings were growing, with merely the roots in contact with the water.

That most of the creatinine, at times at least, is not present as such in the culture water is shown by the fact that if the water without acidification be concentrated to a small volume in a vacuum, at a temperature between 50° and 60°, Jaffé's test is very slight and at times negative. By warming on the steam bath, especially in the presence of dilute hydrochloric acid, the creatine or other antecedent body is converted to creatinine.

Whether the presence of creatinine or creatine in the medium in which the plants are growing is to be attributed to the action of microörganisms on material sloughed from the roots or to direct osmosis from the intact seed and roots is a question that is rather hard to settle. The following experiment, however, shows that creatinine or creatine may pass from

seeds by direct osmosis and may pass in the same way through the roots in the normal growth of plants.

Creatinine in the Water in which Seeds had been Soaking.

One kilogram of carefully selected, unbroken wheat seeds, were placed in a flask with two liters of distilled water and the flask was kept in an ice-box at 5–6° for five days. The liquid to the quantity of about 1500 cc. was then poured off the slightly swollen but entirely ungerminated seeds. In the liquid was a medley of organic substances among which a small amount of creatinine was detected by the creatinine zinc chloride method. The creatinine must have come from the ungerminated seeds either as creatinine or as creatine or some other antecedent body which readily changes to creatinine.

Creatinine in Vegetable Material.

Wheat Seedlings.—About five thousand wheat seedlings, nine to eleven days old, were ground and extracted for several hours with boiling alcohol. The hot alcohol was poured off and pressed out of the pulp and allowed to cool. The solution filtered through filter paper was concentrated to a small volume in a vacuum. The concentrated solution was filtered and treated with lead acetate to free it from coloring matter, etc. The fairly colorless filtrate was freed from lead by hydrogen sulfide. The filtrate from the lead sulfide was then concentrated to a small volume. The concentrated solution gave Jaffé's test. Accordingly, zinc chloride and sodium acetate were added to the solution. In the course of several days creatinine zinc chloride appeared in the mass, in the form of stars, crenated hexagonals, needle clusters, and rosets. Treated with lead hydroxide in the customary manner a solution was obtained which gave Jaffé's and Salkowski's tests for creatinine.

Wheat Seeds.—By the creatinine zinc chloride method, creatinine was also demonstrated in the alcohol and water extracts of ground wheat seeds.

Wheat Bran.—In a similar manner, wheat bran was tested for creatinine and on determining its presence a large amount of the bran was used for preparing creatinine zinc chloride. The syrupy mass containing the creatinine zinc chloride was placed on a porous plate and treated with alcohol and water until white. The residue on the plate was dissolved in boiling water and the solution was filtered. The filtrate was brought to dryness on the steam bath in a weighed porcelain dish. To the weighed residue, nitric acid was then added and the solution was brought to dryness and carefully heated in a free flame until the nitric acid was all driven off. The residue was weighed as zinc oxide. The first weighing gave 22.6 per cent. of zinc oxide. On reheating, this was reduced to 21.8, which thereafter remained practically constant. Creatinine zinc chloride requires 22.4 per cent. of ZnO.

Wheat Straw.—In alcoholic extracts of wheat straw no creatinine could be detected until the concentrated extracts were slightly acidified and boiled. Apparently, creatinine does not exist as such in the wheat straw, while its congener creatine or other antecedent substance does exist therein.

Wheat Protein.—On boiling a water extract of ground wheat seeds a heavy precipitate formed. This precipitate, which gave the protein color reactions, was washed with water, dilute hydrochloric acid, and alcohol until the washings on neutralizing and concentrating gave no tests for creatinine with picric acid and sodium hydroxide. The protein material was then boiled for thirty minutes with 3 per cent. hydrochloric acid. From the filtrate, neutralized and concentrated and taken up with absolute alcohol, a solution was obtained which gave Jaffé's reaction and also crystals of creatinine zinc chloride on treating with zinc chloride and sodium acetate. Since the washings from the protein material also gave creatinine on heating with hydrochloric acid, the protein undoubtedly contained creatine.

Though there is some possibility that creatine and creatinine can be formed from the protein molecule or less complex compounds containing nitrogen, the origin of creatine and creatinine in the plant tissue is still an unsettled question. Whatever the origin of creatinine, it exists in the wheat seed and in the wheat seedlings and is left more or less in the medium in which the seeds have germinated and the seedlings have grown.

Rye, Clover, Alfalfa, Cowpeas and Potatoes.—Creatinine was also found in the alcohol extract of rye, clover and alfalfa seeds, in mature cowpea plants and in potato tubers. The identification in each case was made by the color reactions and the preparation of the characteristic creatinine zinc chloride.

Summary.

Creatinine was found in planted soils as well as in soil which had not been recently cropped, but was present in larger amounts in the recently cropped soil, thus indicating that the appearance of creatinine was connected in some way with plant growth. Creatinine was also found in the water in which wheat seedlings had grown. It was found also in wheat seeds, wheat seedlings, and wheat bran, in rye, clover alfalfa, cowpeas and potatoes. If, as is suggested by several investigators, creatinine in the animal arizes as the result of the breaking up of albumin, then it seems reasonable to expect that creatinine would be found in practically all plants.

Though the amount of creatinine and creatine in vegetable matter is small, it is worthy of attention, since by the decay of plants, green manures, etc., and by direct cell sloughing or even by osmosis, the creatinine and

creatine are left in water and soil where they exercize an effect on subsequent plant growth. Creatinine seems to persist for a considerable time in soils and may indeed increase in the soil by accumulation. Since both creatine and creatinine have a favorable effect on plant growth, their presence in plants and in the medium in which plants grow has considerable bearing on soil fertility.

WASHINGTON, D. C.

[FROM THE WOODS HOLE LABORATORIES OF THE U. S. BUREAU OF FISHERIES.]

COMPARATIVE PROTEOLYSIS EXPERIMENTS WITH TRYPSIN.

BY GEORGE F. WHITE AND WILLIAM CROZIER.

Received October 20, 1911.

Considerable work has been done on the artificial digestion of various proteins in an attempt to establish their relative values as food. The proteins are digested with either pepsin or trypsin and the rate at which solution takes place is determined. No attempt will be made to review the large amount of literature on this subject, as the present investigation is entirely independent of previous results. These former experiments are of somewhat limited value, as no idea is obtained of the extent to which the soluble products have been hydrolyzed, and the degree of cleavage of the proteins is of great importance, naturally, in a study of comparative digestive properties.

In an article entitled "A Method for Quantitative Determination of Aliphatic Amino Groups," D. D. Van Slyke[1] has described a convenient and generally accurate process by which the amino nitrogen in proteins and their cleavage products can be readily determined. An aqueous solution of the protein is treated with sodium nitrite and glacial acetic acid and the gases evolved passed into alkaline permanganate solution, in which solution nitric oxide is completely absorbed. There is left free nitrogen, which corresponds to the amino nitrogen in the original protein, and the volume of which is measured by passing the gas into a suitable buret. Van Slyke has suggested his method for studying the rate and course of hydrolysis of various proteins by either acids, alkalies, or enzymes, and it is the object of this article to apply it to a study of the comparative digestibility of beef, the edible fish, cod (*Gadus callarias*), and the unutilized dogfish (*Mustelus canis*).

Trypsin was chosen instead of pepsin as the proteolytic enzyme acting on the meats, as the hydrolysis proceeds much farther and a greater insight into the complexity of the end-products of digestion can be obtained.

The meats were boiled with a little water for about fifteen minutes, allowed to drain, and kept ice-cold for analysis and the digestion ex-

[1] *J. Biol. Chem.*, 9, 185 (1911).