one hand, and Θ,[1] U, on the other, it is to be borne in mind that whereas in laboratory or pot experiments one can easily get good average samples, it is quite different with field or plot experiments. In the first place it is not an easy task to obtain one or several tons of manure, hay or straw as a uniform and homogeneous mass. Nor is it possible absolutely evenly to distribute such organic materials throughout the plots. Likewise, it is very difficult, if at all possible, to get a truly average soil sample from a plot of the size of one-tenth of an acre. It is for these reasons that samples from the same plot, and still more so from different plots, though treated with the same organic materials, may in some measure differ from each other. This may account for some differences in the results reported, yet, on the whole, the data secured in the above experiments confirm the data presented in the former[1] publications.

Conclusions.

1. The principal portion of the acid-soluble organic nitrogen, contained in the soils investigated, is made up of acid amides, monoamino acids and diamino acids.

2. The larger part of the phosphotungstic acid precipitate, recorded in Table I as nitrogen of diamino acids, actually represents diamino nitrogen, the smaller part belonging to classes other than diamino acids.

3. In the case of the filtrate from the phosphotungstic acid precipitate, presented in Table I as nitrogen of monoamino acids, it was found that from 68.02 to 85.98 per cent. of that filtrate represent in fact monoamino nitrogen, the rest (from 31.98 to 14.02 per cent.) consisting of nitrogenous compounds other than monoamino acids.

[From the Laboratory of Fertility Investigations.]

THE ISOLATION OF CREATININE FROM SOILS.[2]

By Edmund C. Shorey.

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In the course of investigations into the nature of the organic constituents of soils a crystallin organic compound was isolated and identified as creatinine.

Reactions indicating the presence of this compound were first observed in a solution of a portion of the soil organic matter obtained in the following manner: The soil was treated with 2% sodium hydroxide solution for half an hour, and then without separation of the alkaline extract from the soil, acetic acid in slight excess was added and the acid solution filtered from the soil and precipitate formed. The acid filtrate was neutralized with sodium hydroxide and, without filtering, a solution of

[1] *Loc. cit.*

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

lead acetate added and the precipitate formed, separated by filtration. On the addition of ammonia to the filtrate a further precipitate was produced, which was filtered off, washed, and decomposed by hydrogen sulfide. The filtrate from lead sulfide was concentrated to a small volume and was found to give the Jaffé, Weyl, and Salkowski color reactions for creatinine.

It was further found that if the filtrate from the neutral lead acetate precipitate was made alkaline with sodium hydroxide instead of ammonia, and the resulting precipitate decomposed and concentrated, the color reactions were obtained more strongly, indicating a more complete precipitation of the creatinine by this means.

From the known behavior of creatinine when treated with lead acetate in alkaline solution it was evident that neither of the methods of precipitation mentioned would result in complete separation of the creatinine present, and for the isolation of this compound recourse was had to another method.

The method by which creatinine was first isolated from soils, and already noted elsewhere,[1] was to apply to an alkaline soil extract a method recommended by Balke[2] for the separation of purine bases, and used for that purpose in this laboratory.[3] This method depends on the fact that when there is precipitation of cuprous oxide from Fehling's solution by a reducing compound, purine bases and some related compounds combine with the cuprous oxide and are found in the precipitate. Creatinine behaves in this way, as was first pointed out by Masche.[4]

The method as applied to the isolation of creatinine from soils was carried out as follows: An alkaline extract of the soil, made by treatment for a short time with 2% sodium hydroxide, was made exactly neutral with acetic or sulfuric acid and filtered. The neutral filtrate was heated to boiling and a little glucose added and then Fehling's solution, slightly in excess of that required by the glucose present. The precipitate formed was separated by filtration, well washed, and decomposed by hydrogen sulfide. The filtrate from the copper sulfide was concentrated to a small volume under reduced pressure, a small quantity of a concentrated solution of zinc chloride and a little sodium acetate added, and the whole allowed to stand several days. Within a few hours crystals began to form, and in 48 hours these were observed to have the characteristic appearance of creatinine zinc chloride. The crystals were separated from the mother liquor by filtration, or, when the quantity of material was very small, by placing the whole mass on a porous plate. After separation the crystals

[1] *Science*, 33, 340 (1911).
[2] *J. prakt. Chem.*, [2] 47, 537 (1893).
[3] *Bull.* 74, Bureau of Soils, p. 41 (1910); *J. Biol. Chem.*, 8, 391 (1910).
[4] *Z. anal. Chem.*, 1878, 134.

were washed with a little cold water, suspended in water and boiled with some freshly precipitated lead hydroxide, filtered, and the filtrate concentrated to a small volume; on standing a short time crystals formed having the appearance, solubility, and color reactons of creatinine.

From another portion of the same soil from which creatinine had been obtained by the method just outlined, creatinine was also obtained by alcoholic extraction. About 100 grams of air-dried soil were extracted in a Soxhlet extractor for 7 hours with 95% alcohol. The alcohol was removed from the colored extract so obtained by evaporation, water being added to keep the volume constant. The aqueous solution was filtered from resinous and fatty matter and evaporated to a small volume under reduced pressure. The concentrated solution gave all the color reactions for creatinine strongly, and on treatment with zinc chloride gave, after standing two days, crystals of the characteristic creatinine zinc chloride.

Creatinine was also obtained from the same soil by simple extraction with water. About 5 kilograms of soil were extracted with cold distilled water in a percolator until 8 liters of extract had been obtained. This was concentrated to about 100 cc. by evaporation under reduced pressure, and filtered from a small quantity of insoluble matter present. The solution so obtained gave all the color reactions for creatinine strongly, and on treatment with zinc chloride, crystals of the characteristic creatinine zinc chloride were formed. From these, by treatment with lead hydroxide in the manner already described, crystallin creatinine was obtained.

Creatinine in water solution gives a number of color reactions that have been depended on largely as indicating or proving the presence of this compound in fluids such as urine.

Jaffé's Reaction.—If a solution of creatinine be made alkaline with sodium hydroxide and then a few drops of picric acid solution added, a red color is formed.[1] The color, which is similar to that of a concentrated solution of potassium dichromate, becomes orange on dilution. A quantitative colorimetric method for determining creatinine based on this reaction has been devised by Folin[2] and has been almost universally adapted in determining the quantity of this compound in urine. In the use of this reaction for either qualitative or quantitative purposes the absence of other compounds that give a similar color or that give a color that would obscure the one given by creatinine must of course be assured. In urine the only compound of this nature likely to be present is acetone, the presence of which can, of course, be easily determined. In solutions or extracts, however, containing soil organic matter there is the possi-

[1] *Z. physiol. Chem.*, 10, 399 (1896).

[2] *Ibid.*, 41, 223 (1904).

bility of other compounds that may simulate or obscure the color given by creatinine, but whose presence can not be definitely determined, because of lack of sufficient methods. Levulinic acid and furfural both give the Jaffé reaction strongly, and while neither have been definitely identified as present in soils, there are some indications of the presence of the former, and the latter might very easily be present or formed in soil extracts at some stage in the operation.

Weyl's Test.—A watery solution of creatinine to which a small quantity of a solution of sodium nitroprusside has been added gives on the addition of sodium hydroxide a red color which soon changes to yellow.[1] As with the Jaffé reaction, the presence of certain other compounds may interfere with the use of this test either by giving a color that obscures the one given by the test, or giving a similar one. Levulinic acid gives under the conditions of this test a color similar to that given by creatinine, and this test with others has been used to establish the presence of levulinic acid in the decomposition products of nucleic acid.[2] Furfural and the products resulting from heating pentose-yielding material with acid give a similar color, although in no case is the fading of the red color to yellow as pronounced as with creatinine. The dark purple color given by sulfides in the presence of sodium nitroprusside and sodium hydroxide would, of course, obscure the color given by creatinine, and, as was noted in the case of Jaffé's reaction, there are probably in soil extracts unknown compounds that give either a similar color or one that would obscure the one given by creatinine.

Salkowski's Reaction.—If the yellow solution resulting from Weyl's test be acidified with acetic acid and then heated, the solution turns green, then blue, and Prussian blue is precipitated, if much creatinine is present.[3] Both levulinic acid and furfural give the final blue color and precipitate, but in the case of levulinic acid the solution after the addition of acetic acid and before heating is purple. The reagents alone will give the final blue color and precipitate if the solution before acidifying be allowed to become warm.

Since soil extracts or solutions prepared from them may contain compounds that are known to give color reactions similar to those given by creatinine, and since there is the possibility of the presence of others as yet unknown, it is evident that results obtained with the color tests usually considered indicative of the presence of creatinine can not be considered conclusive proof of the presence of this compound in the soils from which the soil extracts were prepared.

When indications of the presence of creatinine are obtained by any

[1] *Ber.*, 11, 2175 (1878). Arnold, *Z. physiol. Chem.*, 49, 397 (1906).

[2] Inouye, *Z. phsyiol. Chem.*, 42, 116 (1904).

[3] *Z. physiol. Chem.* 4, 133 (1880); 9, 127 (1885).

of the color tests just described, this can best be confirmed and established by the preparation of the creatinine zinc chloride $(C_4H_7ON_3)_2ZnCl_2$. This salt, almost insoluble in alcohol and difficultly soluble in water, is formed when concentrated solutions of creatinine and zinc chloride are brought together in the absence of free mineral acids, a condition usually obtained by the addition of a little sodium acetate. When any large quantity of creatinine is treated in this way there is immediate precipitation, but when small quantities are being dealt with precipitation does not begin for several hours and is not complete for several days. The crystals of this salt are quite characteristic in form, although this form is so modified by the concentration of the solution, the presence of other substances, and other conditions that they appear to differ widely. A study of the form assumed when the crystals first appear, and of their growth, and of the form assumed on recrystallizing, soon results in familiarity with the characteristic appearance, so that the compound can be readily identified, no matter what form it may assume. Usually it appears in balls with radiating structure, due to their being made up of fine needles. Under other conditions its first appearance may be in star-like plates which eventually assume the form of bunches of radiating needles or plates. If the crystallization is slow, the plates may grow to a well-developed form. Under still other conditions the radiating needles are bunched in tufts rather than balls. This last form is the one assumed by the pure compound when a concentrated solution is cooled rapidly.

When sufficient creatinine zinc chloride can be prepared pure, its identification by the method of preparation, solubility, and crystallin form may be supplemented by analysis, although once familiarity with the characteristic behavior and appearance of this compound is acquired, this is not essential. A preparation of creatinine zinc chloride made from an alkaline soil extract in the manner already described was purified by recrystallization, dried on a porous plate, and then in a desiccator, and nitrogen and zinc oxide determined in the following manner: A portion of the creatinine zinc chloride, 0.3600 gram, was digested with a small quantity of sulfuric acid and a little potassium sulfate, as in the Gunning modification of the Kjeldahl method for total nitrogen. The resulting solution was made alkaline with sodium hydroxide and distilled into standard acid in the usual manner. After the removal of the ammonia by distillation the solution was made acid with acetic acid and the zinc in solution precipitated by hydrogen sulfide, the zinc sulfide collected, the precipitate and filter paper treated with nitric acid, dried, and carefully ignited and weighed as zinc oxide. The analysis gave the following figures:

Calculated for $(C_4H_7ON_3)_2ZnCl_2$:	N, 23.16; ZnO, 22.45
Found:	N, 23.22; ZnO, 22.31

The formation of free creatinine from creatinine zinc chloride by boiling with lead hydroxide in the manner already described, the crystallization of the creatinine so obtained, and observations regarding its crystallin appearance, solubility, and color reactions may be resorted to in further confirmation of the identity of the compound. The crystallin appearance of creatinine, monoclinic plates or prisms, is, however, not characteristic enough to form the basis of identification.

At this point the question naturally arises whether the isolation of creatinine by the methods described indicates or proves the presence of creatinine in the soil. In deciding this question in the case of other organic compounds isolated from soil the conclusion that the compounds in question must be in the soil as such was based on the fact that all the knowledge of the behavior of the compounds and their antecedents indicated that they could not be formed by any of the treatments to which the soil or soil extracts had been subjected. In the case of creatinine the question is complicated: first, by the fact that nothing is definitely known regarding its antecedents, *i. e.*, what complex molecule, if any, it is derived from; and, second, by the fact already mentioned that creatinine is readily changed to creatine and *vice versa* according to the conditions imposed. Consequently, from general considerations only, it might seem that the creatinine isolated by methods which involve chemical reagents and heat might be derived from creatine or some unknown more complex compound from which creatinine was easily split off. More detailed consideration, however, of the methods involved effectually disposes of the possibility that the creatinine is wholly derived from creatine. While the transformation of creatine and creatinine back and forth into each other is comparatively easy, Folin has shown[1] that it is not so easy as many investigators have assumed. All the operations in the methods used, except the final concentration, were such as would result in the change of creatinine back to creatine rather than otherwise. The alkaline extraction and boiling with Fehling's solution would no doubt bring about this change to some extent so that the creatinine finally obtained was that which had resisted this treatment. The final concentration of the aqueous solution at a low temperature might form some creatinine from creatine, but investigation has shown that this change could be but slight and could never involve the whole of the creatine if it were present. Treatment of these solutions by boiling for several hours with acid resulted in but slightly increased colorimetric readings for creatinine. In other words, the small quantity of creatine present shown by this method was out of all proportion to what would

[1] "The Chemistry and Biochemistry of Creatine and Creatinine," Festkrift, Olof Hammersten, III.

remain after evaporation under reduced pressure, if creatine only had been present in the original solution.

The question of the possibility of the creatinine obtained being formed during the treatment from some more complex antecedent easily broken down, unfortunately can not be disposed of until some definit information regarding such a complex, if there is one, is available.

In the light of all the knowledge available and on consideration of the bearing of the methods on the final product it seems safe to conclude that a considerable portion at least and probably all of the creatinine isolated from soils is present in the soils as such.

The possible relation of creatinine to more complex compounds found in the soil or added to soil in dead vegetation is still obscure, but a few observations made in this connection are worthy of record. Nucleic acids of unknown constitution have been found in several soils.[1] Both nucleic acid from soil and yeast nucleic acid prepared by Merck have been found to give the color reactions for creatinine after heating or even warming for a few minutes with dilute hydrochloric acid. From a solution of yeast nucleic acid treated in this way crystallin creatinine zinc chloride was prepared. It was found, however, that on washing the nucleic acid with cold dilute hydrochloric acid the nucleic acid no longer had the property of giving creatinine on heating with acid. The hydrochloric acid washings, however, after heating showed the creatinine reactions. Whether the explanation of this lies in the inclusion of some creatine in the nucleic acid or whether creatinine is actually split off from the nucleic acid or some complex included with it is as yet unknown. Phytin, or hydroxymethylene diphosphoric acid anhydride, is an organic compound that occurs in the seeds of many plants and must find its way into the soil. So far, this compound has not been isolated from any soil, but it was found that a crude preparation prepared from wheat bran gave on heating with hydrochloric acid a solution which gave the color reactions of creatinine, and from this solution crystals of creatinine zinc chloride were prepared. On purifying the phytin, however, in the usual way by precipitating it several times as the barium salt[2] it likewise no longer had this property.

The relation of the creatinine to plants and plant products generally has been already treated in papers from this laboratory[3] and the only further observations that will be made here bearing on the relation of creatinine to soil organic matter have to do with that added in agricultural practice, *viz.*, organic manures. Creatinine has been found both in stable manure and in fresh cowpea vines as used in green manuring.

[1] The results of the investigation on nucleic acids in soils will be reported later.

[2] Patten and Hart, *Am. Chem. J.*, 31, 564 (1904).

[3] Sullivan, THIS JOURNAL, 33, 2035 (1911) and Skinner, *Bot. Gaz.* (1911).

A sample of well-rotted stable manure was extracted with water and the solution allowed to stand until further fermentation had ceased. From this extract creatinine was isolated by precipitation with Fehling's solution in the same manner as from soil extracts. The resulting solution gave the color reactions for creatinine and crystals of both creatinine zinc chloride and creatinine were prepared from the material isolated. Green cowpea vines were crushed and extracted with cold alcohol, the alcohol evaporated at a low temperature, and water added to keep the volume constant and the solution filtered from insoluble matter. From this solution creatinine zinc chloride was prepared by precipitating the creatinine with Fehling's solution and subsequent treatment with zinc chloride and also by treating the concentrated aqueous solution directly with zinc chloride.

The work so far done on the quantitative determination of creatinine in soils is but preliminary and no definit statement regarding the quantity present in soils can be made other than that it is small and apparently but a small portion of the total organic matter. The following facts bearing on this question were established. It is much more easily extracted from soil by alcohol than by water. Usually enough could be obtained by extracting 100 grams of soil with alcohol to establish its identity, but to accomplish this by aqueous extraction several pounds of soil were usually necessary. In spite of this fact, alcohol extraction does not offer an available method for quantitative determination, as is shown by the following experiment: One hundred grams of soil were extracted with alcohol until the concentrated extract no longer gave any reaction for creatinine. Ten milligrams of creatinine were then added and the soil extracted with alcohol again, and the creatinine determined colorimetrically in the extract obtained. After 14 hours of continuous extraction only three-tenths of a milligram of creatinine was found in solution, and a second extraction of 7 hours gave an amount too small to be determined. The color obtained was not greater after heating these extracts with acid, showing that the creatinine had not been extracted and changed to creatine. Since both water and alcohol extraction seemed unavailable as the basis of a method, attention was given to alkaline extraction. In attempting to carry this out quantitatively several difficulties are met. The complete extraction of the organic matter from soil with dilute alkali is slow and results in a very large volume of solution. Again this solution is darkly colored and the use of a colorimetric method is out of the question unless there is removal of the color and concentration of the solution. It is possible that alkaline extraction and precipitation of the creatinine from the solution with Fehling's solution or in some other way will ultimately afford a means of determining this compound in soils, but so far these attempts have not given satisfactory figures.

The quantity of creatinine that has been found in soils by any of the methods tried, while small, and representing but a small portion of the organic matter, is by no means negligible, being usually several parts per million of soil and usually equal to and sometimes in excess of the quantity of nitrates normally present.

There is every indication that in no case was there complete extraction of the creatinine, and it is moreover possible that this organic soil constituent is a fluctuating quantity, being generated by bacterial or other biological agencies in the soil under certain conditions, and being changed or removed from the soil by growing plants under other conditions.

The initial work on the isolation of creatinine from soil was done with a sample of Volusia silt loam from New York. The sample was from a field that had been in cultivation for many years. At the time the sample was taken and for several years previous the crops on the field had been very poor. So far as could be ascertained the field had never had any application of either commercial fertilizer or stable manure.

After the identity of the compound isolated from the Volusia silt loam was established as creatinine a number of other samples of soil were examined for the presence of this compound. Comparatively few soils have been examined, but so far no soil has been found in which the presence of creatinine was not indicated by the color reactions given by the extract.

Creatinine zinc chloride in addition to color reactions was obtained from samples of the following soils:

Frankstown stony loam from Pennsylvania. This sample was taken from a field that had been in cultivation 30 years, but had recently lain idle for 3 years. When put in cultivation again the crops were still very poor.

Clarksville silt loam from Kentucky. This sample was from an area where the crop yields were fair and the soil responded well to applications of stable manure.

Dunkirk clay from New York. Fair crop yields had been obtained on the field where the sample was taken.

It would seem, then, that creatinine is probably a normal and constantly occurring constituent of soils. While the soils examined have all been under cultivation some years there is no theoretical reason for concluding that virgin soils differ from cultivated ones in this respect except perhaps in quantity.

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