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ON THE OCCURRENCE OF CREATININ IN LEGUMINOUS SEEDS.

By

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INTRODUCTORY.

Creatinin is found in muscles and bloods of animals in varying quantities, accompanying creatin, though the former in much smaller quantity than the latter. In living muscles of many fishes it is present in a comparatively large amount. Together with creatin it forms one of the normal constituents of urine of mammals.

Concerning the origin of creatinin and its relation to creatin various views have been advanced. But it is a well established fact that creatin when administered either per os, or sub cutan, a part of it at least, is excreted in urine, as creatinin. Creatinin in food is resorbed from the intestinal walls and is excreted again, largely as such, in urine. Aside from these two sources, creatinin seems to be produced as the result of metabolism in animal bodies, with creatin as its intermediate form. While its physiological rôle in animal metabolism is not yet clearly understood, its sources have generally been considered as wholly confined to animal origin.

Though, the literature concerning the biochemistry of the animal organism is filled with references to creatinin, the possibility of its occurrence outside of the animal kingdom has not been investigated to any great

extent. Zinno¹⁾ found that cultures of a certain bacteria built creatinin in the nutrient medium. Antonoff²⁾ also reported the formation of creatinin by bacteria in pepton solution. Recently, E. C. Shorey³⁾ found it in the alkaline as well as alcoholic extracts of soils. He maintained that the considerable portion of the isolated creatinin has been present in the soil as such. According to him, nucleic acid and phytin in soils have some relation with the formation of creatinin. Shortly after the Shorey's study, M. X. Sullivan⁴⁾ has found it in the vegetable kingdom for the first time. He determined creatinin in wheat seeds, wheat seedlings and wheat bran, in rye, clover and alfalfa seeds, in mature cowpea plants and in potato tubers. He found also that both the planted soil and the soil which had not recently been cropped, contained creatinin, but it was present in larger amounts in the recently cropped soil. From this point of view, he asserted that the presence of creatinin in the soil is connected in some way with plant growth. He reported that creatinin seems to persist for a considerable time in soil and may increase in it by accumulation, and its presence in plants and in the medium in which plants grow has considerable bearing on soil fertility. J. J. Skinner⁵⁾ has shown that creatinin has a marked beneficial effect on plant growth by culture experiment. Under the direction of one of the authors K. Ebiko⁶⁾ determined the presence of creatinin in *Lespedeza bicolor*, Turcz., var. *Typica*. Max., which is used in Japan for fodder as well as for green manure.

In the following pages are presented the results of our experiments concerning the presence of creatinin in several leguminous seeds, which are generally consumed by Japanese as food stuffs. In all the legumes we have studied, the presence of creatinin was fully ascertained.

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- 1). *Reforma med.*, Roma, **9**, (1893) (3), p. 806; through *Jour. Amer. Chem. Soc.*, **33**, (1911), p. 2036.
 - 2). *Centrabl. Bakt.*, Jena, **43**, (1907), p. 209.
 - 3). *Science*, **33**, (1911), p. 340.
 - 4). *Jour. Amer. Chem. Soc.*, Easton, Pa. **33**, (1911), pp. 2035-2042.
 - 5). *Bot. Gaz.* **54**, (1912), pp. 152-163.
 - 6). *Jour. Soc. Agr. & Forest.*, Sapporo, No. 25, 1914.

EXPERIMENTAL.

As the materials for the present investigation the following leguminous seeds were selected.

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|---------------------|--|
| 1. Adzuki bean. | <i>Phaseolus radiatus</i> var. <i>aurea</i> Prain. |
| 2. Black soy bean. | <i>Glycine hispida</i> Maxim. |
| 3. Yellow soy bean. | ” |
| 4. Kidney bean. | <i>Phaseolus vulgaris</i> L. |
| 5. Green peas. | <i>Pisum sativum</i> L. |
| 6. Horse bean. | <i>Vicia Faba</i> L. |

The seeds were washed with water to remove dusts and other impurities, dried and ground in the mill as usual. To remove fats and oils contained in the seeds, about double volumes of ether were added to the ground preparation and allowed to stand a day under frequent shaking. The clear ether extract was then decanted and filtered. To the residue another portion of ether was added and after standing for a day under frequent shaking, it was filtered. The residue was then left at the room temperature until the smell of ether was no more appreciable. As the soy beans contained such a large amount of oil that the above process was not found satisfactory, they were extracted in the Soxhlet's apparatus with ether in the usual manner.

The sample thus prepared was boiled in a large flask provided with reflux condenser, with strong alcohol, for several hours. The alcoholic extract was filtered hot, and the residue was pressed to remove the extract well. The extract thus obtained was allowed to stand 24 hours, when some precipitates were found to be formed. They were separated by filtration and the clear filtrate thus obtained was concentrated by evaporating in a partial vacuum. As the solution became concentrated, some fatty substances seemed to separate out, the liquid was transferred to a porcelain basin and stirred with a glass rod under the addition of water, when fat-like substances adhered to the bottom of the basin as well as to the glass rod. The clear solution was decanted and evaporated on a

water bath at a low temperature, under constant stirring, until a syrupy solution was obtained. The syrup thus prepared was used for the identification of creatinin and is designated as syrup I in the statement beyond.

Color Reactions.

The following three reactions were made use of for the determination of creatinin.

a) *Jaffé's reaction.* An intensive red color is produced when to the aqueous solution of creatinin are added, first, a little picric acid and, then, a few drops of caustic alkali. The red color disappears on adding excess of alkali or on acidifying the solution with acetic acid or with hydrochloric acid. The red color appears soon after the addition of caustic alkali even in cold. The intensity varies from orange red to dark blood red according to the amount of creatinin present. The characteristic red color will soon change into yellow if too much alkali is used and particularly when exposed to light. The presence of glucose does not interfere with this reaction, since the similar red color produced by glucose itself appears much more slowly than in the case of creatinin. It should be remembered in this connection that acetone, laevulinic acid and furfural give the *Jaffé's* reaction.

b) *Weyl's reaction.* When a freshly prepared, very dilute solution of sodium nitroprusside is added to the aqueous solution of creatinin until it gives a distinct yellow color, and then a few drops of dilute caustic alkali are added, the solution gives a ruby red color. The color becomes lighter as the time passes on until after a short time it assumes a straw-yellow color. *Salkowski*¹⁾ asserted that ruby red color can not be considered as characteristic because this color easily changes into yellow. In the course of our investigation we have observed the same fact, in confirmation of *Salkowski's* view. Furfural gives the same color with sodium nitroprusside and caustic alkali as in the case of creatinin, the only difference being that the red color given by furfural remains unchanged for a much longer time. Laevulinic acid gives a color similar to that of creatinin. Sulphide gives purple color under the same conditions and makes the creatinin reaction obscure if both are present at the same time.

c) *Salkowski's reaction.* If the yellow solution obtained in testing *Weyl's* reaction, is acidified with acetic acid and heated, it turns first green, then blue and finally the precipitate of Prussian blue is separated. Hydantoin gives the same reaction but not creatinin. Laevulinic acid and furfural give the same final blue color and precipitation, but the solution is turned purple directly after acidifying with acetic acid and before heating. The reagents alone will give the same final color and precipitation if heated before acidifying.

1) *Hoppe-Seyler's Zs. Physiol. Chem., Strassburg*, **4**, (1880), p. 133.

A) About 1 cc. of the syrup I was taken and the color reactions were tested, according to the methods already described. The results obtained are shown in the following table.

Table I.

Syrup from	Reducing sugar	Jaffé's reaction		Weyl's reaction		Salkowski's reaction	
Adzuki bean	—	+	+	+	+	+	+
Black soy bean	—	+	+	+	+	+	+
Yellow soy bean	—	+	+	+	+	+	+
Kidney bean	+	+	+	+	+	+	+
Green peas	—	+		+		+	
Horse bean	—	+		+		+	

B) The syrup I was diluted somewhat with water and then neutral lead acetate was carefully added, until no more precipitate was formed and after allowing to stand 24 hours it was filtered. The excess of the lead in the filtrate was removed by hydrogen sulphide. The lead-free filtrate was concentrated to a syrup (syrup II) under the reduced pressure. The results were as follows:

Table II.

Syrup from	Jaffé's reaction		Weyl's reaction		Salkowski's reaction	
Adzuki bean	+	+	+	+	+	+
Black soy bean	+		+		+	+
Yellow soy bean	+		+		+	+
Kidney bean	+		+		+	+
Green peas	+		+		+	
Horse bean	+		+		+	

Isolation of Creatinin as Creatinin Zinc Chloride.

If the solution under examination contains compounds giving similar color reactions as creatinin, the color reaction alone can not be depended upon as the criterium for the presence of creatinin in it. Hence, when the indications for the presence of creatinin were obtained by the color tests, it was confirmed and established by the preparation of the characteristic crystals of creatinin zinc chloride. Creatinin zinc chloride forms prisms or slender needles, grouped in rosettes, clusters and stars. The crystal is formed when concentrated solutions of creatinin and of zinc chloride are mixed in the absence of free mineral acids, a condition usually obtained by the addition of a little sodium acetate. When a large quantity of creatinin is treated in the manner above described there is immediate precipitation, but when small quantities are being treated, the precipitation does not begin for several hours and not complete for several days. The time required for crystal-formation seems also to be greatly influenced by the amount of impurities present in the syrup.

A. From impure syrup.

To the syrup II the solution of sodium acetate and of zinc chloride were added and the mixture was kept in a desiccator over concentrated sulphuric acid. Gradually the solution became concentrated and the formation of characteristic crystals of creatinin zinc chloride was observed, but not in all cases. The time required for crystal-formation, the forms of crystals etc. are described in the following.

Adzuki bean. The characteristic crystals appeared after 10 days in the forms of stars, crossed needles and clusters, and after 16 days they grew larger and well developed monoclinic plates were observed.

Black soy bean. Crystals were not observed.

Yellow soy bean. „

Kidney bean. The characteristic crystals appeared after about 3 weeks. They were radiated needles or crosses.

Green peas. The characteristic crystals appeared after about 10 days. Crystal forms were the same as in the case of kidney bean.

Horse bean. The characteristic crystals appeared after about 3 weeks in the form of radiated needles or crosses.

B. From purified syrup.

The syrup II seemed to contain still some impurities which prevented more or less the crystallisation of creatinin zinc chloride. The syrups obtained from both kinds of soy bean which showed apparently strong color reactions of creatinin did not show any sign of forming characteristic crystals of creatinin zinc chloride under the similar conditions in which the syrups from other legumes formed the crystals. The syrups from soy beans evidently contained some impurities which had preventive influence on the formation of the crystal. Starting from this point of view we have attempted first to separate creatinin from admixed substances and then to try the isolation of creatinin as its zinc chloride salt. For the separation of creatinin from the syrups of leguminous seeds, we have adopted the following procedure.

The alcoholic extract of the seed was concentrated to a small volume under reduced pressure and treated with neutral lead acetate to remove impurities. The excess of lead in the filtrate was removed by hydrogen sulphide. The lead-free filtrate was concentrated to about 30 cc. and a small quantity of glucose was added. The hot solution thus prepared was then poured into 50 cc. of the boiling Fehling's solution. The solution was kept boiling for 2 minutes and then allowed to cool, when a greyish white gelatinous precipitate of creatinin cuprous oxide, mixed with brownish red precipitate of cuprous oxide was separated. The precipitate formed was filtered, well washed with 95 % alcohol, suspended in water and decomposed with hydrogen sulphide. The copper sulphide was separated by filtration and the filtrate was concentrated to a small volume under reduced pressure and, with a small portion of it, color reactions were tested, after the complete removal of the hydrogen sulphide by careful evaporation, since its presence makes the Jaffé's and Weyl's reactions obscure. The results are shown in the following table.

Table III.

Syrup from	Jaffé's reaction	Weyl's reaction	Salkowski's reaction
Adzuki bean.	+	+ +	+ +
Black soy bean.	+ +	+ +	+ +
Yellow soy bean.	+	+	+ +
Kidney Bean.	+	+	+
Green peas.	+ +	+ +	+ +
Horse bean.	+ +	+ +	+ +

From the results obtained, it is seen that in all cases the creatinin was precipitated with cuprous oxide and recovered again by hydrogen sulphide.

To the concentrated solution obtained in the above treatment, saturated solution of zinc chloride and a little sodium acetate were added and the whole was allowed to stand several days. Within a few hours crystals began to form, and in a few days they were observed to have the characteristic appearance of creatinin zinc chloride. The time required for crystal-formation and the forms of crystals are shown in the following.

Adzuki bean, black soy bean and yellow soy bean. Crystallisation completed in 24 hours. Crystal-forms were radiating needles, crosses and clusters.

Kidney bean. Crystallisation completed in 24 hours. Crystal-forms were radiating needles, crosses, clusters and plates.

Green peas. Crystallisation completed in 48 hours. Crystal-forms were stars, rosettes and plates.

Horse bean. Crystallisation completed in 5 days. Crystal-forms were radiating needles, crosses and clusters.

It is thus seen that by separating creatinin with cuprous oxide from the admixed impurities, the crystallisation could be effected much sooner than in the case of direct treatment. Even in the extract of soy bean with which we were unable to obtain the characteristic crystals of creatinin zinc chloride by direct treatment, the crystallisation was completed within 24 hours. In every case, the crystal-forms were very distinct and free

from impurities when seen under the microscope.

Regaining of Creatinin.

The regaining of creatinin from creatinin zinc chloride was undertaken in the following manner.

The crystals obtained from the extract of Adzuki bean were separated from the mother-liquor by filtration and well washed with strong alcohol. The residue on the filter was dissolved in boiling water and filtered hot and the filtrate was allowed to recrystallise after concentration. The operation was repeated once more. The crystals thus obtained were white in color and when observed under microscope, they were somewhat oblong hexonal plates. They were dissolved in water and boiled with some freshly prepared lead hydroxide, filtered, and the filtrate was concentrated to a small volume. The concentrated solution gave Jaffé's reaction so distinctly that there is no doubt of the presence of creatinin in it. Unfortunately from the lack of the material, other reactions could not be observed.

The crystals of creatinin zinc chloride from the extract of legumes other than Adzuki bean were so little that they did not allow further working up.

Summary.

1) The color tests, the formation of characteristic double salt of creatinin zinc chloride and lastly, the regaining of creatinin from the double salt, were applied to determine the presence of creatinin in legumes.

2) The presence of creatinin was confirmed for the first time, in the seeds of Adzuki bean, black soy bean, yellow soy bean, kidney bean, horse bean and green peas.

3) The amounts of creatinin in the seeds of Adzuki bean, kidney bean and soy beans are apparently in much larger quantity than in horse bean and green peas, though, its absolute amount seems to be very small.

(March, 1914)