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AND

# THEIR RELATION TO CORNSTALK DISEASE.

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# ENZYMES IN CORNSTALKS AND THEIR RELATION TO CORNSTALK DISEASE.

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The occurrence of substances which produce prussic acid in fodder plants was first noted by Dunstan and Henry.[1 a] These investigators have shown that the poisonous effects produced by Egyptian vetch are due to prussic acid, which is not present in the plant as such, but is formed by the hydrolitic action of an enzyme (lotase) on a glucoside (lotusin). Shortly afterwards similar observations on American sorghum were reported by Peters and Slade.[2] These authors found that prussic acid appeared in watery extracts of sorghum which were allowed to stand at room temperature for some time. Similar extracts which were first boiled and then set aside remained free from that poison. As a result of these experiments they concluded that the formation of prussic acid in the unheated sample was due to the action of an enzyme upon a glucoside, but did not succeed in isolating either of these bodies. Subsequently Brunnich [3] carried out a number of experiments for the Queensland department of agriculture to ascertain at what periods and under what conditions of growth sorghum and related fodder plants are most apt to contain substances producing prussic acid, and therefore when most dangerous and when they may be eaten with impunity. Brunnich came to the conclusion that all fodder plants related to sorghum should be fed with care in either the green or dried state; that they should not be fed in large quantities to animals which have fasted for some time, and also that they should never be fed in a very immature stage of growth.

In the corn district in the Middle West of the United States the common practice is to remove the ears of corn from the standing stalks and turn cattle into the stalk field to gather the ears left by the huskers and to consume what they will of the roughage. Frequently within a day or two after turning the cattle into the field they suddenly sicken and die. This sickness is known as cornstalk disease, and the annual loss of stock from this cause amounts to thousands of dollars. This disease has attracted much attention, and many theories as to the cause have been advanced. None of them, however, has proven satisfactory in explaining all outbreaks of the disease.

[a] The figures refer to the bibliography at the end of this article.

This uncertainty in regard to the cause of cornstalk disease is no doubt due to the fact that many different maladies have been included under this name. Some of them are probably true infectious diseases, while others appear to be simple intoxications due to some poisonous substance present in the food.

The symptoms seen in many outbreaks of cornstalk disease correspond so closely to those that would be expected after poisoning by prussic acid that, in the light of the previously quoted investigations concerning prussic acid in sorghum, it was considered advisable to conduct similar examinations of cornstalks.

During the winter of 1903–04 a number of deaths among cattle, supposed to be due to eating cornstalks, were reported to this Bureau from the State of Iowa. A representative of the Bureau in that State was requested to send samples of cornstalks obtained from the fields where the deaths occurred. These stalks were chopped up at the laboratory and a number of samples, which were placed in flasks with water, chloroform being added to prevent bacterial growth, were placed in the incubator at a temperature of 38.5° C. for periods of time varying from one to seven days. The contents of the flasks were then filtered, the filtrate acidified, and distilled until the distillate measured 50 c. c. The distillate was tested for prussic acid by Schönbein's paper, which is made by taking ordinary filter paper and dipping it into an alcoholic solution of guiacum and then allowing the paper to dry. If this paper is then dipped into a weak solution of copper sulphate and exposed to prussic-acid fumes it will turn a deep blue. Neither by this test nor by the well-known Prussian-blue test could any trace of prussic acid be noticed. Some of the finely chopped stalks were then subjected to a chemical examination for the presence of a glucoside which could be decomposed into prussic acid by means of an enzyme, the method of procedure being as follows: Extract the finely powdered material with methyl alcohol for several days, filter and distil off the solvent. The residue was warmed with water until nothing more dissolved. To this solution an aqueous lead acetate solution was added until no further precipitation occurred. The precipitate was removed by filtration and the filtrate treated with hydrogen sulphide until all the lead was precipitated. The precipitate was separated by filtration and the greater part of the hydrogen sulphide was removed from the filtrate by allowing a stream of air to pass through it for two hours. The liquid was then treated with powdered charcoal, the mixture being evaporated *in vacuo* at 40° C. until dry, when it was extracted in a Soxhlet apparatus with anhydrous acetic ether. This solvent should remove any glucoside, leaving behind nearly all glucose and extractive matter. On evaporating off the solvent any glucosides that are present will be in the form of a sirupy residue and may crystallize if allowed to stand in a vacuum

over sulphuric acid for several days. Neither by means of this method nor by several other methods which were tried could any trace be obtained of a glucoside that would break up into prussic acid.

An examination was then made for enzymes. This was done by extracting some of the finely chopped stalks for several days with water saturated with chloroform. This extract was filtered and sufficient alcohol added to the filtrate to make a 60 per cent solution. The liquid was shaken for half an hour and set aside at room temperature for twenty-four hours, at which time an abundant flocculent precipitate had collected in the precipitating jar. The clear liquid was then siphoned off, the residue dissolved in water, and a solution of sodium phosphate and calcium chloride added until a heavy precipitate of calcium phosphate formed. This was allowed to settle, chloroform being added to prevent bacterial action. The clear liquid was again drawn off and the residue shaken up with water and filtered; this filtrate was dialyzed for twenty-four hours in running water; sufficient alcohol was then added to make an 80 per cent solution and the mixture shaken violently for half an hour. The precipitate was allowed to settle for twelve hours and then filtered off, the precipitate being washed with 95 per cent alcohol and dried. The powder thus obtained was of a light-brown color, soluble in water. The water solution was tested for enzymes as follows:

*Oxydase.*—To 5 c. c. of a 5 per cent solution of gum guiacum in alcohol was added, drop by drop, the solution to be tested, a boiled portion of the solution to be tested for enzyme being used as a check. No blue color was given by either the heated or unheated solution, showing the absence of oxydases.

*Peroxydase.*—To 5 c. c. of a gum guiacum solution a few drops of hydrogen peroxide were added and then drop by drop the solution to be tested. This gave a deep-blue color, indicating the presence of peroxydase, while some of the boiled solution when added to guiacum extract gave a brown color, showing that the peroxydase had been destroyed by boiling.

*Catalase.*—To 40 c. c. of a solution of cornstalk enzyme, containing 5 grams of enzyme powder to 100 c. c. of water, 5 c. c. of hydrogen peroxide solution was added, and the amount of oxygen given off was measured. In fifteen minutes 13½ c. c. of oxygen was given off. The check, which was a boiled solution of the enzyme, gave no oxygen when treated with hydrogen peroxide. This shows that the enzyme solution has the properties of catalase.

*Protase.*—A 10 per cent solution of gelatin in distilled water was prepared, thymol being added to prevent the interference of bacteria, and the solution was rendered slightly opaque by the addition of calcium carbonate. This was distributed in sterile tubes, approxi-

mately 5 c. c. in each tube, and cooled rapidly to prevent the calcium carbonate from settling. Five cubic centimeters of 0.5 per cent cornstalk enzyme solution, containing a few drops of toluol, was added to gelatin tubes, and these tubes set aside at room temperature for twelve hours. The gelatin had been acted upon, and at the end of twelve hours its height in the tubes had been lowered one-fourth of an inch. In the check, which contained some of the boiled enzyme solution, the gelatin had not been acted upon.

By acidifying a portion of the enzyme solution, so that it contained 0.27 per cent HCl, and by making another portion alkaline with sodium carbonate and then testing the action of these solutions on gelatin tubes, the acid solution was found to be the most active. These results indicate a proteolytic enzyme with properties similar to pepsin.

*Diastase.*—Five cubic centimeters of a 0.5 per cent solution of corn enzyme was added to a 1 per cent starch solution, toluol added, and the mixture set in the incubator at 38.5° C. for twenty-four hours. The solution was then tested for reducing carbohydrates by heating with Fehling's solution, with negative results. This shows that no diastatic enzyme was present.

*Invertase.*—Ten cubic centimeters of a 10 per cent cane-sugar solution was added to 5 c. c. of 0.5 per cent enzyme solution, some toluol added, and the solution examined with polariscope, then set aside at 38.5° C. for twelve hours, again tested with polariscope, which showed an inversion. A check containing boiled enzyme solution showed no inversion.

*Lactase.*—Ten cubic centimeters of 10 per cent lactose solution was added to 5 c. c. of 0.5 per cent enzyme solution, some toluol added, and the solution examined with polariscope, then set aside at 38.5° C. for twelve hours, again tested with polariscope, which showed no inversion, showing the absence of lactase.

*Maltase.*—A solution of maltose tested in the same manner as the cane sugar and lactose showed the absence of maltase.

*Cytase.*—By taking a thin section of a carrot and dissolving the starch by means of saliva, then staining the cellular membrane with methyl green and putting the section on a thin cover glass with a few drops of enzyme solution and examining by the ordinary hanging-drop method, the slide being maintained at 30° C., no action was noticed on the cellulose.

*Lipase.*—To 10 c. c. of ethyl butyrate solution was added 5 c. c. of cornstalk enzyme solution containing some toluol, this mixture set aside at 38.5° C. for twelve hours and the acidity determined, using litmus as indicator. The acidity was not increased. The enzyme solution was therefore lacking in ability to break up fats.

*Glucoside-splitting action.*—When the glucoside amygdalin is decomposed by the enzyme emulsin, both of which occur in bitter almonds, the glucoside breaks up by hydrolysis according to the following reaction:

$$C_{20}H_{27}NO_{11}+2H_2O=2C_6H_{12}O_6+HCN+C_6H_5CHO.$$

This property of amygdalin was utilized to determine whether the cornstalk enzyme possessed the power of producing prussic acid from that glucoside.

Ten cubic centimeters of a 0.5 per cent solution of enzyme from cornstalks containing some toluol was added to a solution of amygdalin in water, the mixture was set aside at room temperature, and at the end of half an hour tested for prussic acid. A strong odor of oil of bitter almonds was noticed and the solution gave strong reaction for prussic acid. A boiled sample of the enzyme solution showed no action on the amygdalin. These tests show that the cornstalks contained an enzyme similar to the emulsin enzyme of bitter almonds. To determine the difference between emulsin enzyme and the cornstalk enzyme, some emulsin was isolated from bitter almonds and tested according to the method used in testing the character of the cornstalk enzyme.[a]

| | Bitter almond enzyme. | Cornstalk enzyme. |
|---|---|---|
| Oxydase | − | − |
| Peroxydase | − | + |
| Catalase | − | + |
| Protase | − | + |
| Diastase | − | − |
| Invertase | + | + |
| Lactase | + | − |
| Maltase | − | − |
| Lipase | − | − |
| Cytase | − | − |
| Glucoside-splitting enzyme | + | + |

The enzyme solution from cornstalk having shown the properties of several enzymes, it would indicate that we had a mixture to deal with rather than one enzyme with these various properties. The following method was used to determine whether or not the supposition was correct: Five cubic centimeters of a 0.5 per cent solution of cornstalk enzyme was put in each of ten test tubes and brought rapidly to the temperatures indicated below. On reaching these temperatures they were immediately cooled by the addition of some amygdalin solution and placed in the incubator for twelve hours, when they were tested for prussic acid, with the following results:

[a] The sign + indicates a positive reaction; the sign −, a negative reaction.

| Tube No.— | Temperature. | Reaction. |
|---|---|---|
| | ° C. | |
| 1 | 60 | + |
| 2 | 62 | + |
| 3 | 64 | + |
| 4 | 66 | + |
| 5 | 68 | + |
| 6 | 70 | + |
| 7 | 72 | + |
| 8 | 74 | + |
| 9 | 76 | + |
| 10 | 78 | − |

A temperature of 78° C. destroys the glucoside-splitting action of the cornstalk enzyme.

Some more of the enzyme solution was heated in a similar manner, cooled immediately and tested for its proteolytic action.

| Tube No.— | Temperature. | Reaction. |
|---|---|---|
| | ° C. | |
| 1 | 50 | + |
| 2 | 52 | + |
| 3 | 54 | + |
| 4 | 56 | + |
| 5 | 60 | + |
| 6 | 64 | + |
| 7 | 68 | − |
| 8 | 72 | − |
| 9 | 76 | − |
| 10 | 78 | − |

A temperature of 68° C. destroys the proteolytic action of cornstalk enzyme.

The temperature which destroyed the inverting action of the cornstalk enzyme was next determined.

| Tube No.— | Temperature. | Reaction. |
|---|---|---|
| | ° C. | |
| 1 | 50 | + |
| 2 | 52 | + |
| 3 | 54 | + |
| 4 | 56 | + |
| 5 | 58 | + |
| 6 | 60 | + |
| 7 | 64 | − |
| 8 | 68 | − |
| 9 | 70 | − |
| 10 | 74 | − |

A temperature of 64° C. destroys the inverting action of cornstalk enzyme.

The emulsin enzyme of bitter almonds was heated and its destruction point noted.

| Tube No.— | Temperature. | Reaction. |
|---|---|---|
| | ° C. | |
| 1 | 60 | + |
| 2 | 62 | + |
| 3 | 64 | + |
| 4 | 66 | + |
| 5 | 68 | + |
| 6 | 70 | + |
| 7 | 72 | + |
| 8 | 74 | + |
| 9 | 76 | + |
| 10 | 78 | − |

The glucoside-splitting property of emulsin enzyme from bitter almonds and that of the cornstalk enzyme were destroyed at the same temperature.

A physiological test was made with the cornstalk enzyme by feeding some to guinea pigs and rabbits in the following manner:

Guinea pig No. 1, fed 5 c. c. of 0.5 per cent enzyme solution plus 0.04 gram amygdalin.

Rabbit No. 1, fed 5 c. c. of 0.5 per cent enzyme solution plus 0.04 gram amygdalin.

Both of the above animals died within twelve hours.

Guinea pig No. 2, fed 5 c. c. of 0.5 per cent boiled enzyme solution plus 0.04 gram amygdalin.

Rabbit No. 2, fed 0.5 per cent boiled enzyme solution plus 0.04 gram amygdalin.

Neither guinea pig No. 2 nor rabbit No. 2 showed any ill effects as a result of the feeding.

Duplicates of these feeding experiments gave similar results. After having tested each sample of cornstalks that had been forwarded from the West and having found this emulsin-like enzyme present in large quantities in each sample, two samples of cornstalks that had been grown in Maryland were examined. One of these samples was obtained from the field before the corn was harvested and was quite green, while the other was obtained from a quantity of stalks that had been lofted the previous year. Both samples showed equally as large amounts of this enzyme as were found in the cornstalks from the Western States.

The presence in cornstalks of this enzyme, which has the property of decomposing amygdalin into prussic acid, and the absence of any glucoside in the stalks, which could be decomposed into prussic acid, leads us to believe that, if some cases of so-called cornstalk disease

are caused by prussic acid through the action of an enzyme upon a glucoside, the glucoside probably occurs only in certain stalks, and then only when the conditions are especially favorable for its development, or else that it is at times present in other field plants. It will be seen from some of the histories of characteristic cases of cornstalk disease given below that it is not unlikely that prussic-acid poisoning may be the cause of some cases of the disease; that other plants in the cornfield, or in other fields to which the animals had access either at the same time that they pastured in the cornfield or previously, could have furnished the glucoside necessary for the formation of prussic acid.

A case from Kansas, quoted from Bulletin No. 49 of the Kansas Agricultural Experiment Station, has been reported, where a farmer drove his cattle, numbering 12, from his pasture to the barnyard about 5 o'clock in the evening. The pasture was very dry and short, and, as the cattle were taken up earlier than usual, they were fed some cornstalks taken from the manger of a bull which was kept confined to the barn. The bull had not eaten the cornstalks clean, but what he had eaten had no ill effects on him. The remaining portion, estimated at about 4 armfuls, was fed to the cattle, which seemed to relish the stalks and ate them readily. Within eight hours 7 out of the 12 animals were dead. A farmer in Iowa, reported in Bulletin No. 10 of the Bureau of Animal Industry, states that he had 14 acres of corn on high land. The corn being good, he turned 24 cattle, mostly calves, 4 to 7 months old, into the stalk field on November 15. For several weeks prior to this they had been on clover pasture. For the first three days the cattle were allowed to remain in the stalk field for about three hours daily. They were allowed to run at will in a clover meadow adjoining the cornstalk field and appeared to do well for several days, when they began to die. Another case has been reported from Iowa (Bulletin No. 10, Bureau of Animal Industry), where 25 cattle, mostly yearlings, were turned into a 15-acre cornstalk field. For the first few days they were in the cornstalk field for a few hours only each day, but later they were left in the field continually. Rye had been sown in the cornfield, which gave a considerable amount of green food. In four days the animals began to die. These are only a few examples of numerous similar cases where animals had access to some pasture other than the cornstalks, while in all cases the stalk field contained a number of plants that the cattle ate besides the cornstalks.

The death of a portion of the animals goes to show that these probably ate some material which the others did not, or that they got the poisonous material in a larger quantity, as is illustrated by the case of sorghum poisoning, which occurs only in a portion of the animals

eating the sorghum; or it may be that some of the animals were more resistant to the action of the poison.

The discovery of the presence in cornstalks of an enzyme which has the property of forming prussic acid when acting on a proper medium, such as the glucoside amygdalin, was made so late in the fall that no work could be taken up leading to the detection of a glucoside in other plants to which the cattle had access. In order to approach this question as nearly as possible, however, some grains and plants used for cattle foods were tested for a glucoside which could be broken up into prussic acid, and also for the presence of a glucoside-splitting enzyme, with the following result:

| Food. | Glucoside-splitting enzyme. | Glucoside. |
|---|---|---|
| Alfalfa (green) | − | − |
| Timothy (green) | − | − |
| Wheat (grain) | − | − |
| Corn (grain) | + | − |
| Linseed (grain) | + | + |
| Cotton seed: | | |
| Pride of Georgia | + | − |
| King | + | − |
| Excelsior | + | − |
| Doughty | + | − |
| Culpepper | + | − |
| Sea-island | + | − |
| Sunflower | + | − |
| Staple | + | − |
| Truitt | + | − |
| Russell | + | − |

Linseed was the only food material examined that contained both a glucoside and a glucoside-splitting enzyme. The amount of prussic acid formed after 25 grams of the ground linseed had stood in contact with 200 c. c. of water containing chloroform at 38.5° C. for twenty hours was determined by filtering the incubated extract and distilling off about 50 c. c., the distillate being collected in 20 c. c. of $\frac{N}{10}$ silver nitrate solution. The precipitate formed was found to be silver cyanide, which weighed 0.0055 gram, equivalent to 0.0011 gram of prussic acid, or 0.0196 gram of prussic acid for every pound of linseed. A sample of ground linseed-cake meal free of the oil was treated in the same manner and formed only one-half the amount of prussic acid as the whole seed, showing that probably some of the glucoside was broken up by the heat when the linseed was "cooked" before the oil was taken from it. The amount of prussic acid formed in linseed meal is too small to prove fatal in ordinary feeding, but

might prove so if the animal were allowed to obtain the meal in too large quantities.

These investigations, while not conclusive in regard to the relation prussic-acid poisoning bears to the so-called "cornstalk disease," have at least established that there is present in cornstalks an enzyme which has the property of decomposing amygdalin, and thereby producing prussic acid. In view of this fact, and also because the symptoms seen in many outbreaks of cornstalk disease resemble those which would be expected to follow prussic-acid poisoning, it seems not unlikely that at least some of the instances of so-called cornstalk disease may be due directly to prussic acid formed by the action of an enzyme upon a glucoside. As has been stated previously, no glucoside capable of furnishing prussic acid as a result of enzyme action was found by the writer in the comparatively small number of examinations made. When the great variety of plants found in ordinary fields, and also when the possible influence of physical conditions of the soil and atmosphere upon the physiological processes of the corn itself are considered, however, the failure to find a glucoside in a few samples of cornstalks does not prove that the glucoside may not exist in cornstalks under different conditions, or in plants other than corn. It is hoped that in the near future we shall be able to investigate more fully the question of the existence of an amygdalin-like glucoside in corn and other plants.

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