THE ESTIMATION OF HYDROCYANIC ACID AND THE PROBABLE FORM IN WHICH IT OCCURS IN SORGHUM VULGARE.

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INTRODUCTION.

The biochemistry of cyanogen compounds has received a considerable amount of attention, not only because of their practical importance in such substances as bitter almonds, linseed cake, fodder grasses, etc., but also because of the theoretical interest which attaches to their probable function in plants. From either viewpoint, facts of first importance to be ascertained are the nature of the cyanogenetic compounds, means of estimating them quantitatively, their situation in the plant, and their reaction to mechanical and chemical treatment of the plant. Although the first two points have received more attention than the others, they are still far from being answered satisfactorily. It is the object of the present paper to describe some experiments on Sorghum vulgare bearing on these questions.

Concerning the form in which prussic acid occurs in plants, it is generally believed that it exists free in but few cases, being usually combined as glucosides. About eight such glucosides have been isolated and identified. The nature of the cyanide body, however, is still unknown in the great majority of the plants which are known to contain the cyanide group. Only a few cases are on record where the evidence pointed definitely to the existence of uncombined hydrocyanic acid. Treub1 found it "free or in a very loosely combined state" in Pangium edule, a tropical


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tree containing as high as 0.3 per cent of the dry weight of the leaves as hydrocyanic acid. Greshoff, with the same plant, came to similar conclusions. Later Peche, in working out his microchemical test for cyanide, found it free or "in a very labile form" in both Pangium edule and in Prunus laurocerasus. Moore believed that he had free hydrocyanic acid in cassava. The writer, however, questions this interpretation of his results, as will be discussed later.

Concerning the quantitative estimation of cyanogenetic compounds, it has usually been accomplished by the estimation of the cyanide itself, since it is impossible to extract and crystallize out the glucosides quantitatively. And since in most instances the hydrocyanic acid itself, and not the glucoside, is the critical element under observation, the investigator is content to determine the total cyanide present and disregard any other bodies which may be combined with it. A glucoside linkage is usually assumed, and the plant tissues treated with a view to hydrolyzing this glucoside and setting free the hydrocyanic acid. This is done (1) by autolysis (maceration with water), (2) by added enzyme (emulsin), or (3) by boiling with acids. Autolysis has been used frequently; in fact, the first discovery of hydrocyanic acid in the plant world by Böhm in 1803 was in water which had been in contact with crushed bitter almonds. The writer used it in his first studies on the dhurrin in sorghum. Autolysis depends upon the coincident existence of glucoside and glucosidase in the same tissues, but not in contact with each other until the tissues are crushed. Since in a given tissue the enzyme must be perfectly specific for its accompanying glucoside, this method of hydrolysis, where applicable, is very satisfactory. Emulsin seems to act on all cyanogenetic glucosides; and, although its action is less rapid

\[\text{Peche, K., Mikrochemischer Nachweis der Cyanwasserstoffsaure in Prunus laurocerasus L., Sitzungsb. k. Akad. Wien, 1912, cxxi, 33.}\]
\[\text{Moore, C. C., Cassava: its content of hydrocyanic acid and starch and other properties, U. S. Dept. Agric., Bureau of Chemistry, Bull. 106, 1907, 1.}\]
\[\text{Böhm, Neues allgemein. J. Chem., 1803 (original not consulted).}\]
\[\text{Willaman, J. J., and West, R. M., Notes on the hydrocyanic-acid content of sorghum, J. Agric. Research, 1915, iv, 179.}\]
than that of the autolyzing enzymes, it is nevertheless a highly
effective agent in bringing about the hydrolysis of various glu-
cosides. Acid hydrolysis in most cases gives the same final pro-
ducts as does enzyme hydrolysis, but probably does not go to
completion or follows a different course. Caldwell and Court-
auld, using a 10 per cent solution of amygdalin and normal hy-
drochloric acid, found only 85 per cent hydrolysis in 200 hours at
60°C., and 92 per cent in 16 hours at 80°C. Walker and Kriebles
obtained slow and incomplete hydrolysis with hydrochloric and
sulfuric, and none with oxalic or trichloroacetic acid. Recently
several investigators have used 5 per cent sulfuric or 5 per
cent tartaric acid. They judged the completeness of their hydrol-
ysis only by the failure of further hydrocyanic acid to distill
over. That this may be a false assumption, at least in the case
of certain plants, will be brought out in the following experiments.
The writer and West, in the 1915 work on sorghum at the Minne-
sota Experiment Station, following the directions of Viehoever
and Johns, ground the leaves in a food chopper and then distilled
from 5 per cent tartaric acid. No further cyanide was obtained
after an hour's distillation. That cyanide obtained in this way
is not the product of acid hydrolysis, but of enzyme hydrolysis
taking place previous to the addition of the acid, is in part the
object of the present paper to show.

The success of any method for the determination of prussic acid
depends not only on the complete hydrolysis of the glucoside, but
also on the complete removal of the cyanide from the hydroly-
sate. A thorough discussion of this latter phase of the question
was given by Alsberg and Black. In their experiments with

7 Caldwell, R. J., and Courtauld, S. L., The hydrolysis of amygdalin
8 Walker, J. W., and Krieble, V. K., The hydrolysis of amygdalin by
9 Viehoever, A., and Johns, C. O., On the determination of small quan-
10 Alsberg, C. L., and Black, O. F., The separation of autogenous and
added hydrocyanic acid from certain plant tissues and its disappearance
during maceration, J. Biol. Chem., 1916, xxv, 133.
11 Viehoever, A., Johns, C. O., and Alsberg, C. L., Cyanogenesis
in plants. Studies on Tridens flavus (tall red top), J. Biol. Chem., 1916,
xxv, 141.
12 Willaman and West, Effect of climatic factors on the hydrocyanic
several cyanogenetic plants they noticed a loss of hydrocyanic acid when the macerated tissue was allowed to stand some hours before distilling from acid. Also, added cyanide was lost in the same way. A non-cyanogenetic plant, *Sambucus canadensis*, did not have this power. They proved that the phenomenon was not due to enzymes, and suggested that there was a chemical reaction between the hydrocyanic acid and some other substances in the tissues, probably aldehydes. In another paper by Viehovver, Johns, and Alsberg, retention of hydrocyanic acid on maceration was reported in *Tridens flavus*; maceration in presence of tartaric acid prevented this retention. Henry and Auld devised a scheme which was designed to prevent retention of cyanide by the macerated tissue. They extracted the material with alcohol, evaporated the alcohol, then took up with water, and distilled with 2 per cent HCl. The cyanogenetic compounds were no doubt removed in this way, but whether they were all hydrolyzed by the acid is doubtful.

It is evident that there is considerable cause for questioning our knowledge of the actual amount of hydrocyanic acid present in various plants and the conditions in which it exists there. Most workers in this field have not taken into account (1) the possible incomplete liberation of hydrocyanic acid from its compounds, (2) the possible incomplete removal of the acid by distillation, and (3) the existence of various states of combination between the hydrocyanic acid and other constituents of the plants. The following experiments deal with these three points in the cyanogenesis of sorghum.

**EXPERIMENTAL.**

**Material and Method.**

The sorghum used in these experiments was of the Early Amber variety grown on the plots of the Minnesota Agricultural Experiment Station. Most of the samples were taken between the stages of full bloom and maturity, when the plants were 6 feet high or over. The leaves only were used.

The distilled hydrocyanic acid was collected in an excess of sodium hydroxide, and determined colorimetrically by the Prussian blue method of Viehovver and Johns.

The procedure with some samples furnished illustrations for more than one factor under observation. Hence in the tables considerable duplication of laboratory numbers will be noticed. Instead of describing and discussing the procedure followed for each sample of leaves in chronological order and noting the various points observed, each factor (as acid hydrolysis, autolysis, etc.) will be taken up by itself, and in the tables reference will be made to the experiments from which the data were obtained.

**Comparison of Acid and Enzyme Hydrolysis.**

In order to determine the efficiency of various acids as hydrolyzing agents for cyanogenetic glucosides, the trials described in Table I were carried out, using amygdalin as the glucoside. Hydrolysis by emulsin was also performed for comparison.

TABLE I.

<table>
<thead>
<tr>
<th>No.</th>
<th>Method of hydrolysis.</th>
<th>HCN Present</th>
<th>Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>53</td>
<td>34.4 mg. amygdalin distilled 1½ hrs. with 5 per cent tartaric...</td>
<td>2.00</td>
<td>0.00</td>
</tr>
<tr>
<td>54</td>
<td>34.4 &quot; &quot; distilled 1½ hrs. with 5 per cent H₂SO₄...</td>
<td>2.00</td>
<td>0.00</td>
</tr>
<tr>
<td>55</td>
<td>200 &quot; &quot; in 40 cc. water, 100 mg. emulsin, 24 hrs. at 45°C...</td>
<td>10.56</td>
<td>10.40</td>
</tr>
<tr>
<td>56a</td>
<td>200 &quot; &quot; distilled 2½ hrs., 200 cc. 5 per cent H₂SO₄...</td>
<td>10.56</td>
<td>0.40</td>
</tr>
<tr>
<td>56b</td>
<td>200 &quot; &quot; distilled 2½ hrs., 200 cc. 5 per cent HNO₃...</td>
<td>10.56</td>
<td>0.00</td>
</tr>
<tr>
<td>56c</td>
<td>200 &quot; &quot; distilled 2½ hrs., 200 cc. 5 per cent tartaric...</td>
<td>10.56</td>
<td>0.00</td>
</tr>
<tr>
<td>57</td>
<td>200 &quot; &quot; boiled 24 hrs. with 200 cc. 5 per cent H₂SO₄*...</td>
<td>10.56</td>
<td>0.70</td>
</tr>
<tr>
<td>58a</td>
<td>50 &quot; &quot; 10 mg. emulsin, 47°C. 4 hrs...</td>
<td>2.64</td>
<td>2.20</td>
</tr>
<tr>
<td>58b</td>
<td>50 &quot; &quot; 5 mg. emulsin, 47°C. 4 hrs...</td>
<td>2.64</td>
<td>1.40</td>
</tr>
<tr>
<td>73</td>
<td>200 &quot; &quot; 200 cc. 2 per cent HCl, 72 hrs. boiling...</td>
<td>10.56</td>
<td>2.80</td>
</tr>
</tbody>
</table>

*The material was boiled under a reflux connected with bulbs of NaOH. After the cyanide had been determined, the ammonia was distilled off; 1.0 mg. of N as NH₃ was obtained, corresponding to 1.9 mg. of HCN.
The results show that no acid is at all efficient for the hydrolysis of amygdalin. As evidenced in No. 57, considerable of the hydrocyanic acid in the glucoside is set free as ammonia by strong acids, according to the well known mandelonitrite decomposition. Emulsin proved a very efficient hydrolyzing agent. How far we are safe in assuming that what is true for amygdalin is also true for dhurrin, we cannot say at present. However, the succeeding experiments will show that dhurrin is probably not hydrolyzed by acids to any greater extent than is amygdalin.

Effect of Autolysis.

In Table II are collected the data obtained in the various experiments dealing with the autolysis of the macerated sorghum leaves. The procedure in general was to grind the leaves in a food chopper, and then put them into 300 cc. of water for autolysis, or into 300 cc. of 5 per cent tartaric acid for distillation. It was not expected that the acid would hydrolyze any dhurrin; it merely furnished an acid medium from which to distill the hydrocyanic acid present in the tissues from any source whatsoever, and prevented enzyme action. In order to preclude entirely any chance for enzyme action on the dhurrin, in some cases the leaves were moistened with the tartaric acid before grinding, and then were ground directly into more of the acid. In the tables this method is called "grinding with tartaric," and is used as a check determination in most cases to judge the effect of autolysis.

In Nos. 60 and 62 there is a marked increase in hydrocyanic acid after hydrolysis; there is no increase in Nos. 70 and 65, where no tartaric acid was added before distilling. This is the reverse of what was suggested by Viehoever, Johns, and Alsberg. They did not offer an explanation of why the addition of acid after the period of autolysis should affect the retention of cyanide. In Nos. 87 and 84, the check was ground with tartaric; the other sample had a chance to autolyze from the moment the tissues were ruptured till the macerated mass was heated to the killing temperature of the enzyme, probably 10 minutes. This was evidently sufficient time for considerable hydrolysis to take place,

but insufficient for much "retaining" action of the tissues on the hydrocyanic acid. In comparison to this, Nos. 87 and 85 show complete retention in 16 hours' autolysis. Two examples of 4 hour autolysis are given in Nos. 88a and 88b, and Nos. 91a and 91b. There is a marked increase in both cases.

It is apparent from these data that the hydrolysis of dhurrin in sorghum leaves by the action of the contained glucosidase takes place rapidly; it is probably completed in a few hours, perhaps in a few minutes. Auld\textsuperscript{15} found that when linseed cake was auto-

\begin{table}[ht]
\centering
\caption{The Effect of Autolysis of Sorghum Leaves for Various Lengths of Time, on the Yield of Hydrocyanic Acid.}
\begin{tabular}{|c|c|c|c|}
\hline
Nos. & Conditions of autolysis. & Method of determining HCN. & HCN per 100 gm. of dry matter. \\
\hline
& & & Before autolysis. & After autolysis. \\
\hline
60 and 62 & 24 hrs., 47°, 300 cc. & Distilled from 5 per cent tartaric. & 7.12 & 12.25 \\
70 & 65 & 24 " 47°, 300 " & Distilled from water. & 12.40 & 12.40 \\
87 & 84 & 10 min., previous to reaching the boiling point. & " " & 1.74 & 10.45 \\
87 & 85 & 16 hrs., 47°, 300 cc. & Cheek ground with 5 per cent tartaric; autolyzed sample distilled from water. & 0.00 & 5.51 \\
88a & 88b & 4 " 23°, 300 " & & 0.00 & 7.16 \\
91a & 91b & 4 " 25°, 300 " & & & \\
\hline
\end{tabular}
\end{table}

lyzed in water at 37°C., most of the hydrocyanic acid was liberated in 15 minutes, and all of it in 6 hours.

In Moore's investigation of cassava\textsuperscript{4} he ground the tissues to a pulp, placed it in a retort, and slowly distilled for 2 hours. Most of the cyanide passed over in the early stages. He says: "From this fact and the nature of the results obtained there is no reason to believe that the figures given represent other than the HCN existing in the free state." He got no increase in cyanide by the

\textsuperscript{15} Auld, S. J. M., Formation of prussic acid from linseed cake and other feedingstuffs, \textit{J. Southeast Agric. College Wye}, 1911, no. 20, 289.
use of sulfuric acid. Now, if autolysis in cassava proceeds at anything like the rate it does in sorghum, there was time in Moore’s process for considerable liberation of cyanide before the mass of tissue reached a temperature fatal to the enzymes. There may be free hydrocyanic acid in cassava; but it is the writer’s conviction that the above process gives considerably more than the non-glucosidic hydrocyanic acid.

In the same way, when Peche concluded that he had free hydrocyanic acid when a section of the tissue gave a reduction of mercurous nitrate, it is possible that the act of sectioning brought glucoside and enzyme in contact for a time sufficient to set free enough hydrocyanic acid to give a test. By the time these facts on sorghum were ascertained, material was not available for making a more thorough study of this rate of autolysis. The writer believes, however, that for each plant species an optimum time for autolysis can be found during which a maximum yield of hydrocyanic acid can be obtained. This may not represent all the cyanide present; it probably is the difference between the total amount set free and the amount retained by the macerated tissue. What may be the nature of this retention is not known; it is suggested that, if aldehydes are responsible, some substance may be found which, added to the macerated tissue, will combine with the aldehydes and prevent their action on the cyanide. De Jong, in working with the decomposition of gynoecardin in the leaves of *Pangium edule*, found that a diketone and HCN were produced, which combined in part, thus holding back some of the prussic acid from distillation.

It is also evident from Table II that grinding with tartaric acid completely inhibits enzyme action, as in two cases, Nos. 88a and 91a, no cyanide was obtained in this way, whereas by normal hydrolysis from 5 to 7 mg. were obtained. In No. 87, where cyanide was obtained even with the acid treatment, this cyanide no doubt existed in some non-glucosidic form. This will be discussed under another heading.

Effect of Vacuum Distillation on the Yield of Cyanide.

In order to ascertain whether distillation under reduced pressure, and hence lower temperature, would decrease the retention of cyanide, the experiments in Table III were carried out. After removing the cyanide under reduced pressure, redistillation was found to be necessary because foaming had discolored the distillate. It is unlikely that this redistillation had any effect on the cyanide. In two of the cases a slight increase in hydrocyanic acid in favor of vacuum distillation is seen, but it is inappreciable.

TABLE III.
The Effect of Distilling Macerated Sample 1½ Hrs. at 50-55°, 15 Mm. Pressure, Then Redistilling at Ordinary Pressure.

<table>
<thead>
<tr>
<th>Nos.</th>
<th>Description of samples</th>
<th>HCN obtained.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>By ordinary</td>
</tr>
<tr>
<td></td>
<td></td>
<td>distillation.</td>
</tr>
<tr>
<td>72a and 72b</td>
<td>Ground with 5 per cent tartaric.</td>
<td>0.40</td>
</tr>
<tr>
<td>76a &quot; 76b</td>
<td>&quot; &quot; 5 &quot; &quot; &quot;</td>
<td>0.00</td>
</tr>
<tr>
<td>82 &quot; 83</td>
<td>and 1.0 mg. HCN (as KCN) added.</td>
<td>0.60</td>
</tr>
</tbody>
</table>

Form of the Occurrence of Hydrocyanic Acid in Sorghum.

In the discussion of the data in Table II, it was pointed out that grinding in the presence of tartaric acid completely prevented enzyme action on glucoside. Nevertheless, cyanide was obtained in this way in some cases. Assuming that the acid cannot appreciably affect dhurrin, this cyanide must have existed in the plant in some non-glucosidic form. The only reference the writer could find on the nature of the hydrocyanic acid compounds of sorghum was one by Dunstan and Henry,17 who, in discussing their newly found sorghum glucoside dhurrin, said that the hydrocyanic acid apparently does not occur in the free state, but only as the glucoside. They did not state their reasons for this conclusion. In Table IV is brought together the evidence which

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points towards the existence in *Sorghum vulgare* of non-glucosidic hydrocyanic acid. It all hinges upon two assumptions: first, that the method of grinding with 5 per cent tartaric acid prevents any

enzyme decomposition of glucoside; and second, that the acid itself does not accomplish any hydrolysis even during boiling. The first is based upon the fact that a given sample of sorghum

<table>
<thead>
<tr>
<th>Nos.</th>
<th>HCN per 100 gm. of dry weight of leaves.</th>
</tr>
</thead>
<tbody>
<tr>
<td>60, 61, 62</td>
<td>I. Leaves ground, then distilled from 5 per cent tartaric, allowing about 5 min. autolysis. 7.12 mg.</td>
</tr>
<tr>
<td>69, 70, 65</td>
<td>I. Ground with tartaric, all enzyme action prohibited. 5.13 mg.</td>
</tr>
<tr>
<td>76a, 87, 84</td>
<td>I. Ground with tartaric, all enzyme action prohibited. 0.00 mg.</td>
</tr>
<tr>
<td>89a1 and 82a2</td>
<td>I. Ground with tartaric, all enzyme action prohibited. 0.00 mg.</td>
</tr>
<tr>
<td>90a1 and 90a2</td>
<td>I. Ground with tartaric, all enzyme action prohibited. 4.50 mg.</td>
</tr>
</tbody>
</table>
leaves will yield cyanide after autolysis, but will not yield any if ground in presence of tartaric acid; and that still other samples give some cyanide when ground with the acid, but will give more if not ground with it. The second assumption is based on the following facts: (1) Amygdalin cannot be hydrolyzed with 5 per cent tartaric acid (see Table I) and amygdalin and dhurrin are very similar in their composition and properties. (2) Samples of leaves which yield hydrocyanic acid by autolysis, yield none on boiling with tartaric acid. Therefore it is believed that sorghum does not contain all of its hydrocyanic acid in the form of dhurrin; that at times part or all of it may exist in some more unstable compound which can be decomposed by boiling 5 per cent tartaric acid. Whether the prussic acid actually exists free in certain of the cells, as found by Treub in *Pangium edule*, or even as the salt of metals, the data at present do not show. Peche suggests that in *Prunus laurocerasus* it may be linked with a ketone. Treub, in demonstrating the presence of free hydrocyanic acid in *Pangium edule*, treated the leaves with boiling alcohol to prevent enzyme action. De Jong modified this by using large volumes of absolute alcohol at $-10^\circ$C.

This discovery may explain the apparent discrepancy between Auld's results from the study of the possibility of cyanide poisoning in stock, and what actually happens in practice. He concluded, after a study of the conditions favorable for the liberation of cyanide in feedingstuffs by enzymes, that such hydrolysis is unlikely except under unusual circumstances, since it is prevented by the reaction of the alimentary pouches, and by the presence of large amounts of fiber, salt, glucose, etc. Cyanide poisoning, however, is very common in districts growing sorghum, and it is probable that it is caused in large part by the non-glucosidic hydrocyanic acid.

The demonstration of the inefficiency of tartaric acid as a hydrolyzing agent for dhurrin, of the rapidity of autolysis on grinding the tissues, and of the existence of non-glucosidic hydrocyanic acid in sorghum, necessitates a revision of the conclusions arrived

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at by Willaman and West in regard to the effect of climatic factors on the prussic acid content of sorghum. The method employed there throughout the season was to grind the tissues, and then put them into 5 per cent tartaric acid. From the evidence given above, this allowed from 4 to 7 minutes for autolysis; a time sufficient for considerable, if not for complete, hydrolysis. If it was complete, or if the degree of hydrolysis was the same in all cases, the comparative value of the data would still obtain. If there was considerable variation in the relative amount of the total cyanide in the non-glucosidic condition, there would also be a corresponding variation in the amount of hydrolysis of the glucoside, and the data would carry but little significance. Which of these two cases is correct remains for future work to determine.

It would be of interest to study the form of occurrence of the prussic acid in the hosts of cyanogenetic plants known to science. No doubt more exact information would be obtained as to the nature of this non-glucosidic union of cyanide, as well as to the variation in the proportion of glucosidic to non-glucosidic cyanide present under various conditions.

**SUMMARY.**

1. The methods in use for the determination of the hydrocyanic acid content of plant tissues are of questionable accuracy, because of the difficulty in getting complete hydrolysis of the glucosides by means of acids, and because of the retention of the cyanide from distillation by the tissues involved.

2. Hydrolysis of the dhurrin in sorghum is best accomplished by means of the glucosidase found in the same tissues (autolysis). It takes place very rapidly at 45°C.

3. Retention of hydrocyanic acid by the tissues during distillation cannot be prevented by the presence of tartaric acid, nor can it appreciably be lessened by distilling under reduced pressure.

4. *Sorghum vulgare* contains hydrocyanic acid in two forms: a glucosidic, as dhurrin, and a non-glucosidic, the nature of which is as yet unknown. It is the latter portion of the cyanide of this plant which is probably responsible for the poisoning of stock.

5. The non-glucosidic cyanide can be distinguished from the glucosidic by grinding the leaves in the presence of 5 per cent tartaric acid to prevent any enzyme action, and then distilling.
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