CLV. THE ESTIMATION OF CYANOGENETIC GLUCOSIDES.

By LAURENCE ROBERT BISHOP.

From the Botany School, Cambridge.

(Received August 30th, 1927.)

As a preliminary to a study of the behaviour of the cyanogenetic glucoside (prulaurasin) in the leaves of cherry laurel (*Prunus laurocerasus*) an attempt was made, at the suggestion of Dr F. F. Blackman, F.R.S., to find a method for the estimation of the glucoside in a single leaf or half leaf. After a trial of various methods the most satisfactory was found to be by decomposition of most of the glucoside by heating the leaf in water and by decomposition of the remainder by added emulsin. During both processes a current of air was passed through the generating vessel into potash. The HCN liberated during the decomposition and absorbed in the potash was titrated in the alkaline solution with silver nitrate (Liebig's method).

Apparatus.

This was composed of six units each connected by equal lengths of fine bore capillary tubing (to equalise the bubbling rates) to a bottle exhausted by a good filter pump. The six generating vessels, 500 cc. filter flasks, were placed in a water-bath which could be heated to boiling and cooled by changing the water.

Fig. 1.

Each generating flask (Fig. 1) had an inlet tube through the stopper with fine openings below the surface of about 100 cc. of water in the bottom of the tilted flask. The side tube of the flask was connected to two test-tubes, in series, with their inlet tubes reaching to the bottom and perforated there with fine holes. The first test-tube was 3 cm. in diameter and contained 15 cc. of 5 % potash, and the second 1.5 cm. in diameter and contained 5 cc. of potash. Each of these test-tubes had a constriction just above the level of the liquid

contained in it, which served as a means of checking frothing and promoting circulation. The outlet tube of the second test-tube was connected to a third with short inlet and outlet tubes. This served to catch any potash which might have frothed over. The last test-tube was connected to the equalising resistance tubing. The contact with rubber tubing was made as small as possible.

Method.

The air current is turned on so as to produce violent bubbling and the first of the weighed leaves is cut up into small narrow pieces as quickly as possible, on a sheet of glazed cardboard, by means of a sharp scalpel. The pieces are then rapidly pushed into the first flask. The other five leaves are treated in a similar manner and put into their respective flasks and the temperature of the bath is raised to 100° during a quarter to half an hour and kept at this temperature for several minutes. The heating is then stopped and the temperature of the bath lowered by running in cold water and running out the hot. About 0.05 g. of emulsin is then added to each flask together with a few drops of amyl alcohol to check frothing.

The time during which the air current should be continued varies with the air rate and other peculiarities of the apparatus and is best determined from a curve of the rate of exhaustion of HCN from amounts of amygdalin similar to those of the glucoside to be estimated. With the apparatus described 12 hours appeared sufficient, so that in practice 14–18 hours' run was given.

Titration of the HCN. The contents of the second and third test-tubes are washed into the first. The resulting volume of liquid (about 25 cc.) is approximately halved by the constriction in the tube. Advantage is taken of this to obtain rapid titration. The tube is placed in a box with three sides and the floor blackened inside and the fourth side open and facing a source of bright diffused light. A standard amount (about five drops) of 5 % potassium iodide solution is added and $M/100$ silver nitrate is run in from a 10 cc. microburette. The silver nitrate is mixed, by means of a stirring rod flattened at the end, with the upper half of the solution only, and this can be titrated quickly to a slight turbidity. A further amount of silver nitrate almost equal to the first is then run in and the whole mixed. By repeating this process of titrating the upper half and then mixing the whole solution, the end-point (a very slight turbidity) can be reached rapidly without fear of over-titrating. The blackened box ensures that the first trace of turbidity will be recognised by means of the Tyndall effect. The potassium iodide must be added since the amounts of HCN are here too small for the precipitation of AgCN to act as an indicator. In the presence of KI the turbidity is due to the precipitation of AgI immediately there is excess of silver nitrate over that required to form the double compound AgCN.KCN .

1 cc. of $M/100$ silver nitrate = 0.0005404 g. of HCN or 0.00590 g. of prulaurasin.

DISCUSSION OF VARIOUS METHODS.

Estimation of the glucoside by means of the benzaldehyde liberated was rejected owing to the liability of this substance to oxidation [Armstrong and Horton, 1908].

Estimation of the glucoside sugars by the amount of change in optical rotation or copper-reducing power after the addition of enzyme involves the extraction of the glucoside before estimation. Complete extraction is difficult and it will be shown later that dropping the leaves into boiling absolute alcohol, to kill them before the estimation, involves a loss of about 6 % of the glucoside, judged by the liberation of HCN which takes place at this stage.

Distillation method.

Evidence has been produced by several workers [Alsberg and Black, 1916; Willaman, 1917] that the distillation of plant tissue with acid results in incomplete recovery of the HCN from the cyanogenetic glucosides.

This was confirmed by comparison with the aeration method, using cherry laurel leaves. Direct distillation with acid gave a recovery of 27 % and distillation after maceration 14 %. Distillation without acid gave 49 % and subsequent distillation with acid a further 3 %. These recoveries are calculated by taking as 100 % the HCN content, determined by the aeration method, of the corresponding halves of the leaves.

It can be seen from this that the distillation method is not a reliable quantitative method. Distillation without acid gives higher yields than with it.

The distillation of small amounts of amygdalin with acid gave similar incomplete yields (20–30 % recoveries with 10 % acid).

When KCN solution was distilled with acid there was a 95–97 % recovery of the HCN. When benzaldehyde was added as well only 50–70 % was recovered.

The apparent reaction between benzaldehyde and HCN at the boiling point may explain the similar losses which occur during the distillation of the prulaurasin of cherry laurel and of amygdalin.

Aeration method.

The other method investigated was based on that of Roe [1924] for the estimation of amygdalin, where the glucoside is decomposed by means of emulsin and the HCN carried by a rapid current of air from the generating liquid into potash.

The method was tested with small amounts of amygdalin.

With 0.0400 g. of amygdalin in each experiment	With 0.00348 g. of amygdalin in each experiment
Recoveries	Recoveries
98.9 %	99.4 %
102 %	99.6 %
100.2 %	96.9 %
100.0 %	98.2 %

In applying the aeration method to leaves, steam was first used to inactivate the glucoside-hydrolysing enzyme, but it was found that nearly all the glucoside had been decomposed, as shown by the HCN liberated, before the inactivation occurred. Treub [1907] also found that he could not prevent the decomposition of less than $\frac{1}{2}$ to $\frac{3}{4}$ of the glucoside when suddenly heating leaves in steam.

The volatile product, HCN, here gives evidence of a great deal of enzyme action before the enzyme is inactivated. It suggests that in other cases, where the enzyme is supposedly inactivated instantaneously by plunging into boiling water or by steaming, a great deal of decomposition may occur before the inactivation takes place.

Plunging the leaf into boiling absolute alcohol was then tried, and even with this a part of the HCN is liberated (about 6 %). The cut leaves were dropped into the boiling alcohol in a flask, through which a current of air was passed into potash. The alcohol was distilled over into the potash, water and a small amount of emulsin were then added to the leaves, and the liberated HCN carried over into the potash in an air current. It was found that the alcohol had only a very slight effect on the end-point of the titration. This loss of HCN when leaves are dropped into boiling alcohol has also been shown by Treub [1907].

This "alcohol" method, although complicated, ensures that the action of other enzymes shall not interfere. The liberation of HCN at high temperatures and at the time of opening the flask is avoided. Hence the results were regarded as probably showing the whole of the HCN present as glucoside in the leaf.

After the alcohol method had been elaborated a simpler one for class experiments was devised in collaboration with the Hon. Mrs Onslow. In this the leaves were placed in cold water and gradually heated to 100° in a current of air. After cooling to 35° emulsin was added and the aeration carried on so that traces of glucoside remaining were decomposed.

Most of the glucoside is hydrolysed by the enzyme of the leaf while the temperature is being raised to 100°, but some still remains and is split by the added emulsin.

	(1)	(2)
HCN given off at the end of heating	95.5 %	91.3 %
HCN given off after emulsin addition	4.5 %	8.7 %

Owing to the slower heating the enzyme is inactivated at a temperature of 60–80° and the apparent loss of HCN which takes place in the distillation method does not take place here, since the results by this "water" method agree with those by the "alcohol" method, where nearly all (about 94 %) of the decomposition is by the added emulsin at 35°. The agreement between the two methods is shown by the following experiments with the corresponding halves of leaves.

In one experiment the results were 100 : 100.4 (HCN by "water" method : HCN by "alcohol" method), and in another 100 : 104.6. Since this was near

the limit of experimental error and within the variations between the corresponding halves of leaves, this was taken as showing that the simpler water method was about as accurate as the "alcohol" method. Hence the arguments on the non-interference of enzymes, etc. in the alcohol method must hold for the "water" method also. The simpler method was adopted in the final apparatus.

Estimation of the HCN. Since in the aeration method it is necessary to absorb the HCN in potash, titration in the alkaline solution by silver nitrate was the most convenient method. When the necessary precautions were taken this was found to be quite accurate enough for the present biological requirements. Results by this method were found to agree with those by the iodine method [Dunstan and Henry, 1904].

Using the blackened box and small volumes of titrated fluid the addition of one drop from the microburette (0.01 cc.) is sufficient to give a distinct end-point in clear solutions. Hence readings may be obtained accurate to 0.02 cc. of $M/100$ AgNO_3 , which corresponds to 0.00001 g. of HCN. Thus the quantities of HCN in a single leaf, 0.001–0.005 g., can be estimated with sufficient accuracy.

It is difficult to obtain an idea of the error of the whole process when used with leaves, but with amygdalin in amounts corresponding to those of prunellin in a cherry laurel leaf, the error is 1–3 %. The HCN contents per 100 g. of fresh weight of the corresponding halves of leaves also agree within these limits.

It is of course open to question whether the HCN as measured in this way is representative of the cyanogenetic glucoside in the plant. This however seems probable, since in the "alcohol" method it is only the β -glucosidic HCN which can be liberated, as nearly all the decomposition is by added emulsin. Further, by the kindness of Mrs Onslow, I am able to state that the average of some of her unpublished determinations of the glucoside sugar of young cherry laurel leaves is in substantial agreement with the aeration results for corresponding leaves; moreover, data obtained during starvation by the two methods fall on closely parallel curves. The values for glucoside sugar give a somewhat lower amount of equivalent HCN. This is what would be expected since a certain amount of decomposition must have taken place, previous to the estimation, during the killing of the leaves in boiling absolute alcohol.

SUMMARY.

Various methods of estimating the cyanogenetic glucosides in plant tissues are discussed and most of them considered unsatisfactory. In particular the distillation method has been found to give untrustworthy results.

Two new methods are described in which the HCN of the glucoside is liberated by enzyme action and carried over into potash in a current of air. The HCN absorbed is titrated in the alkaline liquid by silver nitrate. This method is considered satisfactory if certain details of procedure are adopted.

The "alcohol" method is considered to be a sound method for the estimation of cyanogenetic glucosides in leaves.

The results by this method agree with those by a simpler "water" method which was adopted for use in plant physiological investigation. The details of apparatus and procedure used in this are described.

It is hoped to publish elsewhere data obtained by this method on cherry laurel leaves.

This research was carried out during the tenure of a grant from the Department of Scientific and Industrial Research and later of the Frank Smart Studentship in Botany. I should like to tender my very grateful thanks to Dr F. F. Blackman, F.R.S., and to the Hon. Mrs Onslow for help and advice throughout the investigation.

REFERENCES.

- Alsberg and Black (1916). *J. Biol. Chem.* **25**, 133.
Armstrong and Horton (1908). *Proc. Roy. Soc. Lond. B*, **80**, 321.
Dunstan and Henry (1904). *Proc. Roy. Soc. Lond.* **72**, 285.
Roe (1924). *J. Biol. Chem.* **58**, 667.
Treub (1907). *Ann. Jard. Bot. Buitenzorg*, **21**, 101.
Willaman (1917). *J. Biol. Chem.* **29**, 25.