CXVIII. METHODS FOR THE DETERMINATION
OF THE NITROGENOUS CONSTITUENTS
OF A CYANOPHORIC PLANT:
PRUNUS LAUROCERASUS.

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THE present communication records an investigation of methods applicable to the determination of certain nitrogenous constituents of cyanophoric plants. The technique usually employed in estimation of the nitrogen distribution in plants entails maceration of the tissues with water and some plasmolysing agent, and subsequent separation of the heat-coagulable and water-soluble substances. With rare exceptions, the cyanophoric glucosides present in plants are accompanied by active hydrolytic enzymes, which rapidly bring about liberation of volatile hydrogen cyanide on injury of the plant tissues; thus Treub [1907] has shown that an appreciable hydrolysis of gynocardin takes place if the leaves of *Pangium edule* are put into boiling absolute alcohol; Willaman [1917], moreover, has stated that the hydrolysis of dhurrin in *Sorghum vulgare* may be practically completed during the time taken to raise the temperature of the leaves to boiling-point. A method of extraction involving maceration of the tissues is therefore unsuited to investigations of such plants, as serious losses of cyanide-nitrogen would be thereby unavoidable.

The methods recorded below have been adapted to the use of such quantities of material as can be conveniently utilised in a respiration experiment, namely 10–20 g. of fresh weight of fully grown leaves, quantities considerably smaller than those generally employed in the standard methods for the quantitative determination of the distribution of plant-nitrogen.

The estimation of total nitrogen.

An obvious objection to the use of Kjeldahl's technique for the determination of the total nitrogen content of cyanophoric plants is that incineration with sulphuric acid may cause loss of hydrogen cyanide. In order to test this point, determinations of total nitrogen were carried out on amygdalin. A sample of Merck's amygdalin was recrystallised twice by dissolving in glass-distilled hot water, boiling the solution with charcoal to remove coloured

impurities and immediately filtering the resulting mixture. The amygdalin readily crystallised from the filtrate on cooling, and was air-dried. The compound has the formula $C_{20}H_{27}O_{11}N, 3H_2O$.

Estimation of the total nitrogen of this substance by the method of Kjeldahl gave consistent results within 0.5 % of the theoretical value, if the solutions were incinerated with twice their volume of sulphuric acid. In subsequent determinations of the total nitrogen of the leaves, 10 cc. of concentrated sulphuric acid and a suitable incineration mixture being added to 1.5–2 g. of fresh leaf, no hydrogen cyanide could be detected among the distillation products. It may therefore be assumed that, under these conditions, prussic acid is rapidly decomposed to ammonia.

It is well known that the values obtained by use of Kjeldahl's method do not include the nitrogen present in the form of nitrates, and various modifications have been adopted whereby some reducing agent is added to transform nitrates into ammonium salts. A modification commonly used is known as the Kjeldahl-Gunning process [Moore, 1920], in which incineration is carried out with salicylsulphonic acid, in order that the interaction of inorganic nitrates and salicylic acid may form nitro-compounds, which are reduced by the subsequent addition of sodium thiosulphate. This procedure was found by the present writer to give good results for artificial mixtures of amygdalin and potassium nitrate.

The work of Bordas [1923] and of Margosches and Scheinost [1925] has shown, however, that such a method is unreliable in the presence of tannin. The leaf of *P. laurocerasus* is known to have a high tannin content, and experiments carried out on mixtures of amygdalin, potassium nitrate and tannic acid yielded inconsistent results, thus supporting the conclusions of the above authors. The use of the Kjeldahl-Gunning technique was therefore discontinued, and it was decided to carry out determinations of total nitrogen exclusive of nitrate-nitrogen by the ordinary Kjeldahl method, and to obtain information as to the nitrate from determinations carried out on the non-protein-nitrogen fraction. As recorded below, the concentration of nitric nitrogen in *P. laurocerasus* in all the stages examined was found to be exceedingly low.

The procedure adopted was as follows. The leaves were carefully wiped with absorbent cotton wool to remove dust and traces of moisture, a sample of about 2 g. was weighed as rapidly as possible, then incinerated in a Kjeldahl flask with 10 cc. of sulphuric acid and 2 g. of the incineration mixture described below. The resulting mixture was heated for 2 hours after complete clearing. The digest was then diluted with 10 cc. water and 15 cc. 97 % alcohol and distilled after addition of 40 % sodium hydroxide, to which 5 cc. 25 % sodium sulphide had been added to decompose mercury-ammonium compounds; 5 cc. 2 *N* copper sulphate were added when the mixture started to boil. Distillation was carried out in a slightly modified form of the apparatus described by Cole [1928].

The incineration mixture was prepared by grinding together potassium sulphate, copper sulphate and mercuric sulphate in the proportions of 10 g. potassium salt to 1 g. copper salt and 0.75 g. mercury salt. The writer is indebted to Dr Lindestrøm-Lang for details of the above technique. The use of the incineration mixture enabled digestions to be carried out in 5 hours, whereas previous experiments had taken more than a day. The practice of using metallic mercury in incinerations is open to many objections, chiefly on account of the uncertainty of the blank determinations; with the above technique, consistent blanks have been obtained throughout the course of the work. It has not been found possible to substitute sodium thiosulphate for sodium sulphide and copper sulphate, added after alkalisation in the distilling process.

Fig. 1.

THE NITROGEN DISTRIBUTION IN THE LEAF.

The usual methods for the separation of soluble and insoluble nitrogen compounds from leaves not being applicable to *P. laurocerasus* because of the liberation and volatilisation of hydrogen cyanide which ensues on maceration of the tissue, the following procedure was adopted.

The leaves were cleaned as described above, a sample was removed for the total nitrogen determination, and the remaining portion was rapidly weighed. The pieces of leaf were quickly cut into thin strips with a safety razor blade and placed in a wide-mouthed conical flask of 450 cc. capacity (A in Fig. 1) which contained about 150 cc. cold glass-distilled water¹, and the apparatus as indicated in the diagram was rapidly assembled. Aeration was immediately started, a strong current of air being drawn through the apparatus. The water and leaf fragments in A were brought to boiling-point by direct heating, and the distillation products were received in flask B, of

¹ The subsequent estimations of ammonia-, amide- and nitrate-nitrogen are on such a small scale, that it is obligatory to use glass-distilled ammonia-free water throughout the manipulations, and to work in a room where ammonia is not used.

250 cc. capacity, which contained 25–30 cc. of 5 % sodium hydroxide. The test-tube *C*, also containing 5 % sodium hydroxide, acted as a guard tube. The flask *B* was surrounded by ice, to inhibit the decomposition by hot alkali of the cyanide obtained in the distillate. The air condenser *D* prevented an excessive volume of water from distilling over. The outlet tube of the flask *A* was bent round in the form of a hook, this being found to be necessary as the strong aeration tended to entrain traces of liquid. The wash-bottle contained *N* sulphuric acid, to remove traces of ammonia from the air.

Boiling of the mixture in the flask *A* was continued for about 20 minutes, provided that this period was allowed by the volume of water present, the source of heat was then removed and aeration was continued for 3 hours. At the end of this period the contents of *C* were added to *B*, and after washing down the inlet tubes the contents of *B* were titrated with 0.01 *N* silver nitrate solution, a crystal of potassium iodide being added to ensure a definite end-point. The amount of cyanide liberated before destruction of the glucosidase by boiling was thus determined.

Three hours' aeration were found to be necessary to remove all traces of cyanide from the leaf extract; furthermore, the aeration must be powerful in order to remove benzaldehyde from the distillate, otherwise this substance reacts with silver nitrate used in the titration.

It has been found in general that adult leaves from a well-grown bush generally yield about half of their cyanide in this preliminary treatment. In the case of very young leaves and seedlings, or small mature leaves from young bushes, the whole of the cyanide contained may be liberated in this process¹.

The contents of the flask *A* were then gradually transferred to a mortar, the leaf fragments were finely cut up with scissors, and then ground into a fine, even pulp. A mechanical pestle and mortar proved to be unsuitable. The pounded tissue and accompanying extract were then transferred to a pyrex beaker of 500 cc. capacity, and again boiled, to obtain as complete a coagulation of albumins and globulins as is possible. The resulting mixture was filtered under pressure through a weighed 50 cc. Jena-glass crucible with a ground-glass filter-plate (2 G. 3/5–7). After thorough washing of the residue with hot water, the crucible was placed in an oven at 103° and dried to a constant weight.

The total insoluble nitrogen.

The total insoluble nitrogen, which is generally regarded as being the protein-nitrogen, was determined by estimations of aliquot portions of the residue by Kjeldahl's method. After the final weight of the crucible had been

¹ When the total cyanide-nitrogen only was to be estimated on a given sample of leaves, the material was placed in the conical flask *A*, the mixture was boiled for about 20 minutes after assembling the apparatus, then, after cooling, 10 cc. of 0.25 % emulsin were introduced by means of a pipette through the inlet tube of *A*. Aeration was then continued for 3 hours, the flask *A* being placed in a water-bath at 40°.

ascertained, the residue was ground to a uniform powder. It was then replaced in the oven to drive off moisture acquired during the process of grinding, cooled in a desiccator, and transferred to a weighing bottle. Amounts of about 0.5 g. were weighed into Kjeldahl flasks, and the nitrogen was estimated as described for total nitrogen.

The non-coagulable nitrogen.

The filtrate obtained as described above was transferred to a 200 cc. Claisen flask and concentrated *in vacuo* at 35°. The contents of the flask were then washed into a graduated flask and made up to 75 cc., the resulting solution being used for analyses of the soluble nitrogenous constituents. It was generally very viscous, apparently on account of the high tannin content, and the accurate measurement of small aliquots entailed the use of finely tipped pipettes.

The total non-coagulable nitrogen.

This value was obtained by the addition of the amount of nitrogen estimated as hydrogen cyanide in the preliminary aeration to that determined by Kjeldahl's procedure. As stated above, the nitric nitrogen was not estimated by the ordinary Kjeldahl technique, and the Kjeldahl-Gunning modification was unsatisfactory. The present worker has furthermore been unable to obtain consistent results in small scale experiments by the use of the method suggested by Bordas [1923] and by Gallagher [1923]. The residual soluble nitrogen was therefore estimated as described above for total nitrogen, 5 cc. of the solution being used for each determination, and the values for cyanide- and nitric-nitrogen were added to the figure obtained.

The residual cyanide-nitrogen.

The apparatus used for this estimation was a smaller and slightly modified form of that used in the preliminary cyanide determination. The conical flask and air condenser were substituted by a 100 cc. Kjeldahl flask with a three-holed stopper fitted with inlet and outlet tubes and capillary tap funnel. A flask of 100 cc. capacity containing 15 cc. of 5 % sodium hydroxide was used as a receiver. 5 cc. of the plant extract were placed in the Kjeldahl flask, a few drops of caprylic alcohol were added, and the apparatus was assembled. After aeration had been started, 20 cc. of 0.25 % emulsin were added by means of the funnel. The Kjeldahl flask was immersed in a water-bath at 40° and aeration was continued for 3 hours, further amounts of caprylic alcohol being added if necessary. If any traces of caprylic alcohol remained in the receivers at the close of the experiment, they were removed by continuation of the air current after the apparatus had been disconnected. The cyanide was titrated with *N*/200 silver nitrate, a small crystal of potassium iodide being added as an indicator. The value of cyanide-nitrogen obtained in this way, added to that determined by the preliminary aeration, gave the total cyanide-nitrogen.

The use of a long-necked flask fitted with a hook-shaped outlet tube was found to be necessary on account of the tendency of traces of the extract to be carried over by the air current. If even very small traces were thus carried over, the soda in the receiver became bright yellow on account of the presence of flavone in the plant extract, whereupon the titration with silver nitrate became impossible. The necessity of a rapid removal of the products of hydrolysis has been discussed by Alsberg and Black [1916]; moreover a slow aeration must be exceedingly prolonged in order to obtain the maximum amount of cyanide. The present writer has been unable to use successfully the colorimetric method of estimation proposed by Viehover and Johns [1915].

Control experiments which were carried out with amygdalin and emulsin gave results indicating a 96 % yield of hydrogen cyanide.

The methods which have been employed for the determination of cyanide-nitrogen are founded on that originally described by Roe [1924], and modified by Bishop [1927].

The proteose-nitrogen.

Attempts to precipitate a proteose fraction with saturated zinc sulphate solution [Chibnall, 1922] or with sodium sulphate at 33°, and to estimate the nitrogen of the resulting precipitate were unsuccessful owing to the high tannin content of the plant extract, the precipitates obtained being too sticky for manipulation.

The method finally adopted was that suggested by Wasteneys and Borsook [1924]. 5 cc. of the extract were placed in a 20 cc. beaker and 2.5 g. anhydrous sodium sulphate were added. The beaker was kept at 33° with occasional shaking, until the supernatant fluid was clear. The mixture was filtered into a Kjeldahl flask, the beaker being washed out with small successive quantities of sodium sulphate solution saturated at 33°. The operations of filtration and washing were carried out at a temperature of 37°. It was found important to restrict the amount of sodium sulphate solution used for washing as much as possible, as an excessive quantity caused bumping in the subsequent distillation. The Kjeldahl flask was then immersed in a boiling water-bath, and the solution evaporated to dryness. The total nitrogen of the residue was determined by Kjeldahl's method as described above, the value for proteose-nitrogen being the difference between the figure thus obtained and that for the non-coagulable nitrogen found by the previous Kjeldahl estimation.

It is necessary to state that the partition of protein-nitrogen into coagulable and proteose-nitrogen may not give absolute values in the analyses of plants, the conditions of coagulation of plant proteins being imperfectly known; moreover, it is probable that, when the plant has a high tannin content, the proteoses may be partially precipitated with the albumins and globulins, and appear in the insoluble nitrogen fraction.

The amino-nitrogen.

For this determination the ordinary Van Slyke technique has been followed, the micro-form of the apparatus being used. Estimations of amino-nitrogen carried out on the concentrated aqueous plant extract gave exceedingly irregular results, probably owing to the high tannin content of the solution; tannins were therefore removed by precipitation with colloidal ferric hydroxide, or with alkaline lead acetate. In general, precipitations were carried out according to the instructions given by Burrell and Phillips [1925], namely, to 25 cc. of the plant extract were added 7.5 cc. *N* sodium hydroxide and a slight excess of 25 % lead acetate. The resulting mixture was stirred and filtered immediately through a Jena-glass funnel with a glass filter-plate (3 g. 4) the residue being washed with boiling water. Excess of lead was then precipitated from the filtrate by sulphuric acid, and the acidified mixture was left to stand overnight to ensure complete separation of lead sulphate: it was then filtered into a 100 cc. Claisen flask and distilled to dryness *in vacuo*. The readily soluble residue was dissolved in water and made up to 15 cc., two volumes of 3 cc. being used for the amino-nitrogen determination. The readings were often very small, but concordant results were obtainable. The remainder of the solution was utilised in the determination of nitric nitrogen, to be described below.

Vickery and Vinson [1925] and Hiller and Van Slyke [1922] have criticised the use of such precipitants as lead acetate and colloidal ferric hydroxide if the filtrate is to be examined for soluble protein-degradation products, as the latter may themselves be thrown down with the precipitate. It is, however, obligatory to remove tannin from extracts of *P. laurocerasus* before use of Van Slyke's technique, and these methods appeared to be justified, as identical results were obtained whether the precipitant were lead acetate or colloidal ferric hydroxide; moreover, in the case of seedlings of *Sorghum vulgare*, which do not appear to contain tannin, identical values for amino-nitrogen were obtained before and after precipitation with lead acetate.

The ammonia-nitrogen.

It is well known that the ammonia content of the normal green leaf is exceedingly small; a microtechnique for the estimation of ammonia in relatively small quantities of material is therefore essential.

The first method tested in this work was that due to Nash and Benedict [1921] which entails powerful aeration without the application of heat. The present writer was, however, unable to obtain more than a 90 % yield of ammonia in estimations of standard solutions, although the time of aeration advised was quadrupled.

The method and apparatus described by Stanford [1923] were subsequently adopted with the modifications suggested by Watchorn and Holmes [1927]. By this method a 96 % yield of ammonia was obtained in control experiments carried out on standard solutions. 5 cc. of the extract were used for each

estimation, a saturated solution of borax being used to liberate ammonia, which was estimated colorimetrically, after nesslerisation, in a Bausch and Lomb colorimeter.

The amide-nitrogen.

The usual method of estimation of amide-nitrogen is that described by Sachsse [1873], whereby the solution under examination is subjected to mild hydrolysis with a mineral acid for 2 or 3 hours, and the ammonia thus produced is estimated after distillation with magnesia. Concentrations of acid of 3-10 % have been used by different workers to bring about hydrolysis.

It is obvious that this procedure cannot be utilised for extracts of a cyanogenetic plant, on account of the partial decomposition of cyanide to ammonia under such conditions.

Preliminary studies were carried out on the hydrolysis conditions of amygdalin, the strength of the solution used being such as might be found in the plant extract. If hydrolysis were carried out with 0.5 *N* hydrochloric acid, volatile products being removed by a current of air and the volume of the hydrolysate being kept constant, about half the hydrogen cyanide present was recovered, whereas the remainder was converted to ammonia. Hydrolysis with dilute sulphuric acid yielded similar results, although it had been anticipated from the work of Walker and Krieble [1909] that a fairly complete decomposition of cyanide to ammonia might have been expected in such circumstances, in which case the amount of such ammonia could easily have been calculated from cyanide estimations.

As the total nitrogen of amygdalin estimated by such means, namely, that obtained by addition of the value for ammonia-nitrogen and that for cyanide-nitrogen, was practically theoretical, it was at first considered that if the amount of cyanide produced during hydrolysis were determined, the additional ammonia might have been computed from the separate estimations of residual hydrogen cyanide-nitrogen. Unfortunately, however, as was perhaps to have been expected from the work of Alsberg and Black [1916], potassium cyanide or amygdalin added to plant extracts treated in this way could never be quantitatively recovered. Hydrolysis with mineral acids was therefore abandoned.

Attempts to dispose of the cyanide present by the addition of emulsin, and then to remove completely the nitrogen added with the enzyme by means of precipitation with colloidal ferric hydroxide or tannic acid, were unsuccessful, additional ammonia always being present after the precipitation.

Willaman has stated that the hydrolysis of dhuririn with production of hydrogen cyanide could not be brought about by 5 % tartaric acid, though his experiments did not preclude a decomposition by such hydrolysis with production of ammonia. The hydrolysis of amygdalin, asparagine, and mixtures of these substances by tartaric acid was therefore studied. The concentrations of amygdalin and asparagine were such as might occur in the plant

extract, the asparagine value being taken from Chibnall's data [1922]. It was found that whereas 5 % tartaric acid did not bring about complete hydrolysis of the amide group, this was practically complete in 5 hours if normal acid were used, whereas only negligible traces of ammonia were liberated from amygdalin by this treatment. Hydrolysis of the leaf extracts was then carried out with normal tartaric acid, control experiments with aliquot amounts of extract to which a known amount of asparagine had been added being estimated concurrently. In these circumstances the hydrolysis of asparagine was apparently incomplete, possibly on account of the presence of buffering substances in the extract, but quantitative recovery of amide-nitrogen was obtained if the strength of the acid used was increased by one half.

The following procedure was therefore adopted. 4 cc. of the leaf extract were hydrolysed for 5 hours with 4 cc. 3 *N* tartaric acid, the mixture was then cooled and filtered into a graduated 25 cc. flask, filtration being necessary to remove phlobaphens which were precipitated during the hydrolysis; the filtrate was then brought to about p_H 7 by the addition of a previously determined volume of sodium hydroxide and the volume made up to 25 cc. 10 cc. of the resulting solution were then transferred to Stanford's apparatus, and the ammonia present was determined colorimetrically after distillation with 5 cc. of saturated borax solution. This amount of ammonia, after subtraction of the quantity estimated before hydrolysis, gave the value for amide-nitrogen.

The nitrate-nitrogen.

Preliminary efforts to obtain this value were carried out according to the colorimetric technique suggested by Burrell and Phillips [1925], who have pointed out that nitrate determinations by the usual means of reduction to ammonia should not be carried out in the presence of amides. Colourless extracts of *P. laurocerasus* were, however, rarely obtained; a colorimetric method was, therefore, not feasible. Dr Burrell, in a personal communication, stated that he had encountered similar difficulties in experiments with ever-green plants, and was good enough to make valuable suggestions as to possible alternative methods. Attempts were then made to utilise the well-known method of reduction of nitrate to ammonia by means of Devarda alloy in the presence of alkali. The experience of the present worker confirmed the statement of Burrell and Phillips, namely, that the comparison method of Strowd [1920] was unsatisfactory even if amide- and ammonia-nitrogen had previously been removed. It has been found, however, that if a complete alkaline hydrolysis is carried out before addition of the alloy, concordant results are obtainable, and added nitrate can be quantitatively recovered.

The procedure adopted was as follows. 4 cc. of the solution prepared for the analysis of amino-nitrogen were put into a round-bottomed resistance flask of 50 cc. capacity, and an equal volume of 40 % sodium hydroxide was added. The flasks were then plugged and autoclaved for 1.5 hours at 15 lbs. pressure, then filtered into the Stanford apparatus, any residual ammonia

being distilled off. A second distillation generally yielded a perfectly ammonia-free distillate. The volume of the mixture in the distilling-flask was then made up to about 10 cc. and 40 mg. of powdered Devarda alloy were added. Slow aeration was then carried out for about 14–16 hours (overnight), the air having previously passed through a wash-bottle containing sulphuric acid; the mixture was then distilled and the ammonia in the distillate was estimated colorimetrically. After treatment with Devarda alloy, extracts of *P. lauro-cerasus* showed a great tendency to foam; caprylic alcohol was therefore added, and removed by evaporation from the distillate before nesslerisation, or else the distillation was carried out with very cautious heating. This difficulty was not encountered in experiments with *Sorghum vulgare* or *Sambucus nigra*.

EXPERIMENTS ON *PRUNUS LAUROCERASUS* VAR. *ROTUNDIFOLIA*.

The experimental values recorded below are intended in no wise to present a basis for a theory of the course of nitrogen metabolism of a cyanophoric plant. In most of the experiments performed the chief aim was to study the method rather than to gain physiological information; nevertheless, the available data on the nitrogen content of plants are so scanty that the results are not entirely without interest.

Table I.

		Leaves under one year old	Weight of leaf g.	Total N as % of fresh weight	
Dec. 9th, 1925	Five leaves from the same stem. South side of tree. Leaves numbered from apical leaf	1st leaf	1.998	0.6223	
		2nd „	1.644	0.6619	
		3rd „	1.8810	0.6352	
		4th „	1.736	0.5935	
		5th „	1.467	0.6204	
Dec. 15th, 1925	Leaves from same stem. North side of tree	1st „	1.146	0.7954	
		2nd „	1.479	0.5914	
		3rd „	0.7848	0.5722	
		4th „	1.050	0.5559	
		6th „	0.9075	0.5398	
Dec. 16th, 1925	Leaves from same stem. South side of tree	1st „	1.411	0.6891	
		2nd „	2.175	0.6616	
		3rd „	2.134	0.6579	
		5th „	1.975	0.6106	
July 4th, 1926	Leaves from same stem. South side of tree	1st „	1.146	0.4899	
		2nd „	1.703	0.5090	
		4th „	1.161	0.4863	
		5th „	1.1125	0.4670	
May 4th, 1927	Leaves from same stem. South side of tree. Tree in flower	2nd „	1.675	0.6160	
		4th „	1.749	0.5804	
		5th „	2.142	0.5600	
May 18th, 1927	Leaves from two neighbouring stems	7 leaves	2.223 (av.)	0.8899	
		4 „	1.1639 (av.)	0.8970	
		Weight of leaf g.	Total N as % of fresh weight	Weight of leaf g.	Total N as % of fresh weight
Nov. 7th, 1927	Half leaves	(i) 0.758	0.5838	(ii) 0.686	0.5685
		(i) 0.512	0.5808	*(ii) 0.529	0.5689
		(i) 0.738	0.5817	*(ii) 0.775	0.5754

* The second half was kept at -10° for 5 hours after halving before incineration.

Estimations of the total nitrogen of individual leaves.

The values given in Table I show that appreciable differences in total nitrogen content exist between individual neighbouring leaves, so that in comparative experiments, made on small quantities of material, precautions must be taken to eliminate a sampling error. The difference in total nitrogen between halves of the same leaf is not so considerable.

The highest value obtained in total nitrogen was that recorded for Exp. xi (Table II) for young first year leaves of an average weight of 0.1308 g., namely 1.306 % of the fresh weight. The value for second year leaves (cf. Table II, Exp. x b) was below 0.5 % and that of the third year leaves about 0.35 % of the fresh weight.

The cyanide content.

In *Prunus laurocerasus* the cyanide content undergoes conspicuous seasonal variations. The highest value was obtained from an experiment on young leaves of *P. laurocerasus* var. *longifolia*, of an average weight of 0.359 g., which contained 0.2312 % of the fresh weight and 62.3 % of their non-coagulable nitrogen in the form of cyanide. It is not known whether the cyanide content of this variety is invariably higher than that of var. *rotundifolia*, where leaves of an average weight of 0.1308 g. (cf. Table II, Exp. xi) contained 0.1979 % of cyanide-N, and those of an average weight of 0.3815 g. 0.123 % of cyanide-N (Table II, Exp. ii). This value decreased slowly during the growth of the leaves, until about 0.065 % of the fresh weight and about 40 % of the non-coagulable nitrogen was present as cyanide-nitrogen. During the winter months an increase was again noted, about 0.08 % of the fresh weight and 50 % of the total soluble nitrogen consisting of cyanide-nitrogen.

The second year leaves, examined in the summer, contained about 0.0454 % of the fresh weight and 30 % of the total soluble nitrogen as cyanide-nitrogen, third year leaves about 0.03 % of the fresh weight. No experiments were carried out on leaves just about to fall, but it may be assumed, from the work of Godwin and Bishop [1927] that in such cases the amount of cyanide present will be very small.

The most striking change is obtained in leaves which have been exposed to frost, when the cyanide content may increase by about 70 % of its former value in the course of one night. This fact has been previously noted, in the case of *Sorghum vulgare*, by Willaman [1917] who observed the same effect after artificial refrigeration of gathered leaves. The present writer has been unable to obtain an increase of cyanide in leaves or half leaves of *P. laurocerasus* which were placed in a cold chamber at -10° , but it is possible that such freezing was too rapid to be comparable with a moderate night frost. In support of this supposition, two samples of leaves from different trees, examined in February 1929, after a sudden spell of extreme cold, gave a value of 0.07 % of fresh weight as cyanide-nitrogen, instead of 0.12–0.13 %, the amounts previously obtained earlier in the winter after mild frosts.

Table II. *Nitrogen distribution in first year leaves.*

Exp. No.	Date	Details of leaf	Values given as percentages for fresh weight of leaf.										Nitrate-N
			Fresh wt. g.	Total insol. N	Protease-N	Cyanide-N	Ammonia-N	Amide-N	Amino-N				
i a	(1928)												
i b	June 4	46 leaves	8.197	0.637	0.3941	0.056	0.1916	0.00957	0.0064	Trace			
ii	"	57 leaves from same stems as i a, kept for 7 days in semi-darkness at room temp.	10.639	0.6028	0.4205	0.0596	0.0701	0.0461	0.0320				
iii a	June 18	28 leaves, about 7.5 cm. long	10.683	0.5305	0.2521	—	0.123	0.00182	0.00492	—			
iii b	June 20	17 leaves, about 9 cm. long	11.076	0.4338	0.1793	0.059	0.0863	0.00158	0.00348	Trace			
iv a	"	Opposite leaves, kept for 7 days at room temp.	11.38	0.401	0.2023	0.0365	0.0832	0.0019	0.0124	0.0158			
iv b	July 1	7 leaves, about full size	10.67	0.3718	0.1771	0.049	0.0716	0.00133	0.0049	—			
v a	"	Opposite leaves, kept for 13 days at room temp.	9.97	—	0.188	0.027	0.0307	0.0021	0.0178	—			
v b	Oct. 16	6 leaves	10.85	0.5345	0.1864	0.0423	0.1027	0.0039	0.006	—			
v b	"	Opposite leaves, kept for 10 days at room temp.	11.21	0.4997	0.1936	—	0.0906	0.0043	0.0104	—			
vi a	Nov. 16	Branches bearing leaves had been kept in greenhouse for a few days on account of low outside temp. Leaves halved, midribs removed. Half leaves used	14.861	0.6068	0.2083	0.0511	0.1101	0.00189	0.0045	0.0006			
vi b	"	Other halves of leaves of vi a, kept for 3 days at 0°	14.739	0.5974	0.2185	0.054	0.1014	0.00167	0.0053	0.0006			
vii a	Nov. 16	Leaves as in vi a	14.626	0.5516	0.2244	0.0628	0.0945	0.00178	0.0069	Trace			
vii b	"	Other halves from vii a, kept for 12 days at 0°	15.385	—	0.2228	0.0730	0.1134	0.00161	0.0056	—			
viii a	(1929) Jan. 16	Leaves halved, midribs removed. Half leaves used	13.44	0.607	0.2235	0.0391	0.1182	0.0013	0.0048	0.0091			
viii b	"	Other halves kept for 13 days at room temp.	13.00	—	0.2467	—	0.1015	0.0023	0.0109	0.03414			
ix a	Feb. 7	Leaves halved, midribs removed. Half leaves used	15.52	0.5971	0.2135	—	0.1136	0.00152	0.00548	0.00479			
ix b	"	Other halves kept for 4 days in lab.	15.67	0.5762	0.2267	—	0.07215	0.00157	0.00865	0.00889			
x a	Feb. 14	15 leaves picked after sudden spell of acute cold, midribs removed, both halves used	35.28	0.5807	0.1404	0.0304	0.0719	0.00123	0.00368	0.0089 Trace			
x b	July 30	Opposite leaves, left on tree till date of exp., i.e. leaves more than a year old	21.32	0.3093	0.148	0.025	0.0454	0.00182	0.0074	0.0153			
xi	May 14	36 young leaves, length 2.8-3.9 cm.	4.71	0.8108	0.4959	0.2879	0.1979	0.0032	0.0137	0.0163 Trace			

In "starvation" experiments, for which comparative samples of leaves were kept for some days in the laboratory in semi-darkness, with their petioles in water, before analysis, the cyanide content was generally, but not invariably, considerably reduced, and could not be restored to the original level by exposure to temperatures below zero.

The figures in Table III illustrate the comparative values for cyanide-nitrogen obtained firstly, when the determination is carried out directly in the presence of emulsin (see footnote, p. 1102) and secondly, when it involves two processes, as described above, in the technique for nitrogen distribution.

Table III.

Date 1926	Details of leaves	Weight of leaves g.	Cyanide-N estimated directly as % of fresh weight	Cyanide-N estimated by two processes
Feb. 17th	2nd and 5th leaves from two separate stems on north side of tree	3.20	0.09266	—
	Six leaves from the same two stems	8.13	—	0.1018
Feb. 23rd	Three leaves. South side of tree from three separate stems	5.48	0.07424	—
	Eight leaves from same three stems	15.575	—	0.07421
March 15th	Tree in flower, samples from eleven leaves	3.07	0.0802	—
	Residual part of leaves	10.18	—	0.0790
July 17th	Samples from thirteen leaves	3.707	0.074	—
	Residual parts of leaves	10.852	—	0.0706
August 24th	Tree in fruit, samples from twelve leaves	13.339	0.0644	—
	Residual parts of leaves	11.612	—	0.0659

The nitrogen distribution in first-year leaves.

In Table II values given are typical of those which have been obtained throughout the course of the work. It will be noted that in starvation experiments the increases of amino-, ammonia- and amide-nitrogen show irregularities, similar to that of the decrease in cyanide. Dr Godwin has suggested that the practice of keeping the leaves under bell-jars in the laboratory or the cold chamber with their petioles in water may not provide a sufficient control of the water content, variations in which might cause these anomalies.

EXPERIMENTS ON *SORGHUM VULGARE*.

A small number of experiments, using the methods described above, have been carried out on *S. vulgare*. The fluctuations in the cyanide content of this plant were first investigated by Dunstan and Henry [1902], who isolated and analysed the cyanogenetic glucoside, dhurrin, which is probably characteristic of the Gramineae. The above authors found that there was no cyanophoric substance in the seed, whereas two samples of bright green plants, 12 in. high, contained 0.1044 % and 0.1093 % of their dry weight as cyanide-nitrogen. Yellowish-green ripe plants, about 3 ft. high, yielded, on the other hand, only small traces of hydrogen cyanide.

The following values for cyanide-nitrogen were obtained from the shoots of plants grown in soil in a temperate pit in the Cambridge Botanic Gardens,

the seeds having been kindly supplied by the Director of the Royal Botanic Gardens, Kew.

Sorghum cernuum. Sown Feb. 6th. Gathered Feb. 26th. Length of shoot 6 in. Cyanide-N in terms of fresh wt. 0.0247 %.

S. cernuum. Sown Feb. 6th. Gathered March 20th. Length of shoot 10 in. Cyanide-N in terms of fresh wt. 0.00705 %.

S. caffrorum var. *Roma*. Sown Feb. 6th. Gathered March 27th. Length of shoot 10 in. Cyanide-N in terms of fresh wt. 0.00755 %.

S. caffrorum var. *Roma*. Sown Feb. 6th. Gathered April 22nd. Length of shoot 16 in. Cyanide-N in terms of fresh wt. 0.00497 %.

S. caffrorum var. *Tegalegenung*. Sown Feb. 6th. Gathered March 27th. Length of shoot 9 in. Cyanide-N in terms of fresh wt. 0.00774 %.

S. caffrorum var. *Tegalegenung*. Sown Feb. 6th. Gathered April 22nd. Length of shoot 16 in. Cyanide-N in terms of fresh wt. 0.00656 %.

For the experiments recorded in Table IV on the nitrogen distribution, seeds of *S. vulgare*, obtained from Messrs Sutton, were germinated between large porcelain plates, lined with moist filter-paper, then planted out on cotton netting which had been tightly stretched over porcelain rings of about 8 in. diameter, the rings being placed over appropriately sized earthenware vessels, filled with tap water or a nutrient medium. By this means, the water or nutrient medium could be changed daily, without disturbing the seedlings. After planting out, the seedlings were kept in a greenhouse.

Table IV.

	No. of seedlings	Length of shoot cm.	Length of rootlet cm.	Total N for 7 seedlings %	Insol. N %	Sol. N %	Cyanide-N %	NH ₃ -N %	Amide-N %	Pro-teose-N %	Amino-N %	Nitric-N %
I	115	3-5	9-11	0.374 0.393	0.2524 —	0.1302 —	0.00654 —	0.00516 —	0.0104 —	0.0858 —	0.0183 —	— —
II	115	11 av.	17 av.	0.373 0.3707 0.3771	0.2080 — —	0.1422 — —	0.00344 — —	0.0064 — —	0.0086 — —	Trace — —	0.01505 — —	0.0292 — —
	115	11 av.	17 av.	—	0.2018	0.1521	0.00337	0.0067	0.0084	Trace	0.0166	0.0400
III	115	13-15	22-23	0.3761 0.3684	0.2365 —	0.134 —	0.00339 —	0.00317 —	0.0057 —	0.0228 —	0.00922 —	0.00643 —
	115	13-15	22-23	—	0.2301	0.1266	0.00274	0.0032	0.0065	0.0273	0.00907	—

One additional complication was encountered in the experiments on *S. vulgare*, namely, after the filtered water extract of the non-coagulable nitrogen fraction was concentrated *in vacuo*, an insoluble, nitrogen-containing residue was formed, part of which adhered to the walls of the Claisen flask. Such of this insoluble matter as was suspended in the water-soluble fraction was therefore removed by centrifuging, the supernatant fluid and washings were made up to 75 cc. and the residue returned to the Claisen flask and there incinerated, its nitrogen content being estimated by Kjeldahl's method, and the value obtained added to the figure for insoluble nitrogen.

The first batch of seedlings examined had been grown for 7 days on tap water; the second batch had been grown for 6 days on tap water and for 5 subsequent days on a medium containing 20 mg. KH₂PO₄ and 10 mg. MgSO₄ per litre of tap water, the latter, which contained a considerable

quantity of calcium salts, being adjusted to a p_H of 5 with nitric acid. The third batch had been grown for 6 days on tap water and then for 11 days on the above medium.

These figures do not necessarily give a correct picture of events of the normal nitrogen metabolism of *Sorghum*, as the given conditions of growth may not have been wholly suitable for the plants, although the latter appeared to be healthy and normal.

The technique described above has been applied furthermore to leaflets of *Sambucus nigra*. This plant would seem to be an ideal subject for the study of cyanogenesis, as the glucoside sambunigrin is apparently unaccompanied by a hydrolytic cyanide-liberating enzyme. The low concentration of cyanide present in young leaves (0.0042 % of the fresh weight in early spring), however, involves the use of larger quantities of material in order that a reasonable figure may be obtained in the determination of cyanide-nitrogen on aliquot portions of the plant extract.

SUMMARY.

Methods have been described for the estimation of certain of the nitrogenous constituents of cyanophoric plants, namely, total, insoluble, non-coagulable, proteose-, cyanide-, ammonia-, amide- and nitrate-nitrogen, and examples of their application to leaves of *Prunus laurocerasus* and seedlings of *Sorghum vulgare* have been given. These methods are applicable to small quantities (10–20 g.) of material.

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