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Some Aspects of the Toxicology of Hydrocyanic Acid in Ruminants.*

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I. INTRODUCTION.

Although a very large number of poisonous plants occur in the Union of South Africa not all of them can be considered of national importance since a number, mainly on account of localised distribution, are only responsible for isolated cases of poisoning.

Hydrocyanic acid poisoning is, however, of great national importance and is generally known as "Geilsiekte". This form of poisoning has been known for a long time in the Union of South Africa and, although the term "Geilsiekte" is used to signify sudden death from various causes, it is mainly applied to hydrocyanic acid poisoning. Steyn (1934) was the first to demonstrate the causal relationship between hydrocyanic acid and "Geilsiekte".

The importance of hydrocyanic acid poisoning will be readily appreciated if it is pointed out that a large number of cyanogenetic plants, mainly species of *Gramineae* and *Dimorphotheca*, occur in, and are widely distributed throughout, the Union of South Africa. It is well known that various factors influence the toxicity of cyanogenetic plants and that of these, climatic conditions, especially those inducing wilting of plants, are of especial importance, since under conditions of wilting the hydrocyanic acid content of cyanogenetic plants is greatly increased. In the Union of South Africa the climatic conditions are such that wilting often occurs especially in the arid and semi-arid areas. Thus "Geilsiekte" mainly occurs in spring and summer and is mostly encountered in sheep.

In cases of hydrocyanic acid poisoning the history is usually that of sudden death. Since the symptoms and post-mortem appearances are not pathognomonic and since a large number of diseases may be responsible for sudden death great difficulty is often experienced in arriving at a diagnosis. To render the diagnosis easier in such cases, this work was undertaken in an attempt to make it possible to arrive at a diagnosis of hydrocyanic acid poisoning on the results of the analysis of specimens submitted to a laboratory. Such a procedure would also be of great assistance in court cases where a definite diagnosis is so essential.

On account of the frequency with which hydrocyanic acid is ingested by animals in the form of cyanogenetic plants, it was considered necessary also to elucidate the problem of chronic hydrocyanic acid poisoning.

II. THE QUANTITATIVE DETERMINATION OF HYDROCYANIC ACID IN BIOLOGICAL MATERIAL.

A. The Determination of Free Hydrocyanic Acid in Tissues and Body-fluids.

A large number of methods exist for the determination of hydrocyanic acid, different authors employing different methods according to the requirements of the work undertaken. For the determination of hydrocyanic acid in animal tissues it is considered that:—

(i) The method should be specific so as to ensure that only hydrocyanic acid is being determined. In this respect it must be pointed out that the hydrocyanic acid is obtained by aeration, so that other volatile substances may appear in the absorption fluid;

(ii) the method should be sufficiently sensitive and accurate, and

(iii) not be unduly laborious or time-consuming.

The following is a consideration of the quantitative methods for determining hydrocyanic acid and such qualitative methods as may be adapted for quantitative use. It is important to point out that, in the work undertaken by the author, it was necessary to determine quantities of hydrocyanic acid ranging down to 0·001 mg.

(A) For quantities of hydrocyanic acid approaching 0·001 mg. *volumetric and gravimetric methods*, based on the determination of hydrocyanic or thiocyanic acid, not being sufficiently sensitive, do not yield accurate results.

With large quantities of hydrocyanic acid accurate results are obtained, but in view of the foregoing, such methods were not considered for the purpose of determining hydrocyanic acid under the conditions of this investigation.

(B) Colorimetric methods.

(I) *On hydrocyanic acid.*

(*a*) The Guignard test is non-specific since any volatile reducing agent such as sulphuretted hydrogen, aldehydes and sulphur di-oxide will give a positive test. Sulphuretted hydrogen is frequently encountered in ruminal contents. This method is, however, extensively employed by the author as a rapid, preliminary test.

(*b*) In Vortman's test a positive result is indicated by a violet colour changing to blue, green and finally yellow. On account of this colour change no further investigations were made attempting the use of this test for the purpose of quantitative determination.

(*c*) The phenolphthalin method, depending on the oxidation of phenolphthalin to phenolphthalein, will be given by other oxidising agents. According to Childs and Ball (1935) halogens give a positive reaction while sulphides and phenolic compounds interfere with the test.

(*d*) The quaiacum-copper test is non-specific, a positive test also being given by a number of other substances such as bromine, chlorine, hydrogen chloride, ozone, etc.

(*e*) According to Feigl (1937) the benzidine acetate-copper acetate test is specific for hydrocyanic acid only in the absence of oxidising or reducing substances.

(*f*) The iodine-starch method is non-specific since other substances such as sulphuretted hydrogen, sulphur di-oxide, aldehydes and ketones cause a disappearance of the blue colour.

(*g*) The Prussian blue method is specific for hydrocyanic acid but is rendered unsuitable by (i) a green colour is frequently obtained. The use of other acids or the addition of potassium fluoride does not entirely eliminate the green colour (Swanson, 1921), and (ii) the sensitivity of the method is considerably lower than that of other methods such as the thiocyanate method.

(*h*) Since a positive reaction to the ferrous-uranic nitrate test consist of the production of a precipitate no further investigation was made in an attempt to utilise the test for quantitative analysis.

(*i*) In the method of Gales and Pensa (1935) hydrocyanic acid is determined by hydrolysing the hydrocyanic acid to ammonia with concentrated hydrochloric acid and determining the ammonia with the use of Nessler's reagent. Organic cyanides, however, react in the same way.

(II) *On thiocyanic acid.*

(*a*) The method of Lang (1933) for the determination of thiocyanate is based on a reaction described by Spacu (Lang, 1933). According to the literature, available to the author, it is the first time that this reaction has been used for the determination of thiocyanates and no subsequent work has been encountered in which this method has been employed. The method has, therefore, not been widely used and tested under a variety of conditions, so as to prove or disprove the merits claimed, especially that of being totally specific for thiocyanates.

By converting hydrocyanic into thiocyanic acid the method could easily be used for the estimation of hydrocyanic acid. In the course of quite a considerable amount of work on this method the author found that:—

(i) If thiosulphate or a fairly large quantity of chloride is present, a precipitate forms on addition of the copper sulphate and pyridine leading to a decreased yield or even negative results.

(ii) The liberation of the thiocyanic acid from the silver precipitate as described by Lang (1933) was found to be difficult, time-consuming and unpleasant and frequently complete liberation was not obtained.

The author attempted to use the reaction of Spacu (Lang, 1933) by converting the hydrocyanic acid into thiocyanic acid by means of a polysulphide. For removal of the excess sulphur the following was attempted:—

(i) acidification followed by aeration;

(ii) precipitation of the sulphur as zinc sulphide, this process permitting the thiocyanate to pass into the filtrate on filtering off the zinc sulphide.

In both cases a quantity of sulphur passed into the filtrate, apparently in the form of thiosulphate, the latter being formed during the preparation of the polysulphide solution. The thiosulphate interferes with the method as stated above.

Finally it was found that this difficulty could be obviated by precipitating the excess sulphur as silver sulphide by an excess of silver nitrate. This heavy silver sulphide precipitate was found to carry down the silver thiocyanate quantitatively. By centrifuging the combined precipitates are easily isolated. However, as previously stated, the liberation of the thiocyanic acid from the silver precipitate, according to the method of Lang (1933), was found to be unsatisfactory and no satisfactory method could be found to accomplish this.

At this stage no satisfactory method had been evolved to use the reaction of Spacu (Lang, 1933) for the determination of hydrocyanic acid. It had also been demonstrated that the reaction was influenced by a number of compounds. In the course of the above work control tests were carried out with the ferric thiocyanate method which on the whole yielded much better results than the above method.

(*b*) The ferric thiocyanate test is a well-known and much used qualitative test for hydrocyanic acid and regarded to be specific. The test is carried out by converting hydrocyanic into thiocyanic acid.

The test, as basis for the quantitative estimation of hydrocyanic acid, has been used by Francis and Connell (1913) and subsequently by Johnson (1916) and Gettler and Baine (1938). The method of Francis and Connell (1913) was rejected by Greene and Brazeale (1937) on the grounds that low yields were obtained probably due to the incomplete conversion of hydrocyanic into thiocyanic acid and furthermore due to the difficulty of removing the excess free sulphur and the fact that the long manipulation gave many chances of errors. Coleman and Gardner (1939) compared the method of Johnson (1916), which is a modification of the method of Francis and Connell, with the modified alkaline titration method on cyanogenetic plants and aqueous solutions of potassium cyanide. Of the two methods the modified alkaline titration method gave the most reliable results. With the larger quantities of hydrocyanic acid the thiocyanate method gave low yields. Both methods, however, proved more satisfactory than the Prussian blue method.

Brodie and Friedman (1937) use the ferric thiocyanate test for the determination of thiocyanate in tissue. They state that although the thiocyanate test is very sensitive the method, as described by them, is not applicable to small amounts of thiocyanate in tissues since some unknown substance, originally present in tissues or formed during the analysis, reacts with the ferric ion in acid solution to give a yellow colour, which, superimposed on the colour of ferric thiocyanate, makes readings difficult if tissues contain less than 250 micrograms of thiocyanate per gm. of wet tissue.

The colour of even the smallest quantities of ferric thiocyanate have a brownish tinge and should not be confused with the above interfering colour. Consequently, under the above conditions, the ferric thiocyanate test cannot be regarded as non-specific. It appears quite possible to the author that the yellow colour may be due to the presence of chloride derived from the tissue.

Lang (1933) working on the determination of thiocyanate in biological material states, without giving any definite proof, that the ferric thiocyanate test is non-specific and furthermore that the colour intensity is influenced by many substances such as oxyacids, phosphates and trichloracetic acid. He, furthermore, states that Becher (Lang, 1933) has also shown that results by this method are unreliable.

There is an important difference between the determination of thiocyanate and hydrocyanic acid. The determination of thiocyanate is done directly on the material analysed whereas hydrocyanic acid is obtained by aeration. It is, therefore, obvious that interfering substances are less likely to be encountered in the determination of hydrocyanic acid than in the determination of thiocyanate.

Gettler and Baine (1938), whose work entailed a large number of determinations of hydrocyanic acid in biological material do not in any way comment unfavourably on the ferric thiocyanate method.

The author, in the course of his work, never encountered any evidence indicating that the method is non-specific. The only substance encountered, with a colour similar to that of thiocyanate, is ferric acetate, but since the test is carried out in the presence of nitric acid this is of no importance since ferric acetate does not exist in the presence of an excess of nitric acid. It is

perfectly true that the colour intensity of ferric thiocyanate is influenced by many factors, as is explained later, but it will be shown that this can be controlled by preparing the standard in the same way as the test solution.

Since the ferric thiocyanate method is very sensitive, not very laborious or time-consuming and since it can be regarded as specific, it was decided to adopt this method. The main objections to the method are given by Greene and Brazeale (1937), namely:—

(1) Low yields are obtained probably due to the incomplete conversion of hydrocyanic to thiocyanic acid.

(2) Difficulty is experienced in the removal of the excess sulphur.

In the ensuing detailed study of the method it will be shown how these difficulties are overcome and, furthermore, that the method, as modified by the author, is an accurate and sensitive method for the determination of hydrocyanic acid.

The method can be briefly sketched as follows:—The hydrocyanic acid liberated by aeration of the acidified specimen is converted into thiocyanic acid by the use of a polysulphide and the excess polysulphide removed. The next step is the concentration of the filtrate to a suitably small volume, the addition of a ferric salt and the test solution is finally read against a standard.

It is now proposed to discuss the method step by step beginning with the conversion of hydrocyanic into thiocyanic acid.

Conversion of Hydrocyanic into Thiocyanic Acid.

For the above process the sulphides as well as the polysulphides of sodium, potassium and ammonium can be used. Using sulphides, the author found the conversion to be very much slower than when the polysulphides are used and for this reason it was decided to use the latter. Ammonium polysulphide may not, however, be used since during the subsequent evaporation in the presence of sodium hydroxide ammonia is displaced and escapes. When doing a number of analyses the rate of evaporation varies so that varying quantities of ammonia escape. All the solutions, will, therefore, not have the same degree of alkalinity so that on acidification the acidity of the solutions will vary. This will lead to erroneous results since the colour intensity of ferric thiocyanate is influenced by the degree of acidity.

The procedure usually adopted to bring about the conversion of hydrocyanic acid into thiocyanic acid is to evaporate the solution of sodium hydroxide containing the former, after addition of the sulphide or polysulphide, to dryness in a crucible on a waterbath. There are two objections to the above procedure:—

(1) There is a danger of some of the hydrocyanic acid escaping, especially if sulphides are used, on account of the very slow conversion. If, however, the solution being evaporated is sufficiently alkaline such escape may be prevented.

In the method used by the author the hydrocyanic acid cannot escape as the conversion is conducted in stoppered vessels of suitable capacity immersed in a water bath.

(2) Thiosulphate results from the solution of sulphur in the alkaline medium especially when heated. The above procedure will, therefore, lead to the formation of large quantities of thiosulphate. As will be shown later the excess sulphur is removed by acidification and filtration to remove the precipitated sulphur. Should thiosulphate be present the above process would not remove it and no satisfactory procedure could be found to completely eliminate any thiosulphate present.

Thiosulphate interferes with the method as follows:—

(1) Thiosulphate, if present in more than minute quantities, reduces the colour intensity of ferric thiocyanate. If the standard solution is prepared in the same way as the test solution, according to the procedure of the author which is subsequently outlined, both the standard and test solutions will contain an equal concentration of thiosulphate so that the reduction of the colour intensity of ferric thiocyanate will be equal in both solutions. Besides thiocyanic acid the test solution, as will be seen later, contains, apart from the accidental presence of thiosulphate, only sodium nitrate and free nitric acid. The standard solution may, therefore, also be prepared by adding besides thiocyanic acid, equivalent quantities of sodium nitrate and free nitric acid. Standard solutions prepared in such a way will, on account of the absence of thiosulphate have a higher colour intensity than test solutions containing an equal quantity of thiocyanic acid unless the concentration of thiosulphate in the test solution is so small as not to interfere. With the procedure usually adopted, which is outlined above, it is obvious that the rate of evaporation on a water bath will not be the same for all the solutions, so that, during evaporation, varying quantities of thiosulphate will be formed in the different solutions and it will be impossible to obtain the same concentration of thiosulphate in the standard and various test solutions.

(2) Thiosulphate, being slowly decomposed by acids, gives rise to a slow continuous precipitation of sulphur. Thiosulphate, to interfere in this way, must be present in a greater concentration than in the above. Therefore, if the standard and test solutions are prepared so that they contain an equal concentration of thiosulphate, a greater concentration of thiosulphate is permissible than when the solutions are not prepared in this way.

It was found impossible to prepare a solution of polysulphide without the formation of some thiosulphate. Since no satisfactory method could be found to eliminate thiosulphate, it is essential to prevent the formation of thiosulphate in quantities that will interfere. To limit its formation as much as possible it is essential to observe the following:—

(1) The polysulphide solution must be as dilute as possible.

(2) The period during which the polysulphide is allowed to react must be as short as possible, after which the polysulphide must be immediately destroyed.

(3) The temperature of the water bath must be as low as possible. The procedure usually adopted, i.e., evaporation of the test solution with sulphide or polysulphide, does not observe these points and can, therefore, not be used.

The following procedure is adopted by the author:—

(1) *Preparation of Sodium Polysulphide*.—Sodium sulphide is freshly prepared since samples of crystalline sodium sulphide were found to contain thiosulphate. A 20 per cent. solution of sodium hydroxide is saturated with sulphuretted hydrogen and an excess of finely powdered sulphur added. The mixture is shaken regularly for ten minutes and filtered. The filtrate is then diluted with distilled water until its colour equals that of a 3·5 per cent. aqueous solution of potassium dichromate. One c.c. of the sodium polysulphide solution is used per 50·0 c.c. of the solution containing the hydrocyanic acid.

The above sodium polysulphide solution will contain extremely little thiosulphate but, on standing, thiosulphate forms. The solution must, therefore, be freshly prepared before use.

(2) As stated before, the conversion of hydrocyanic to thiocyanic acid is carried out in stoppered vessels using 1·0 c.c. of the sodium polysulphide solution per 50·0 c.c. of the solution containing the hydrocyanic acid. With this procedure 20 minutes in a water bath at 40° C. proved sufficient for complete conversion. At the same time the thiosulphate formed is limited to a quantity smaller than that which will interfere provided that with an original volume of 50·0 c.c. the final volume of the test solution does not comprise less than 30·0 c.c. Should the original volume not comprise 50·0 c.c. proportionately more or less sodium polysulphide solution should be added and the final volume should also be proportionately increased or reduced. If the final volume, with an original volume of 50·0 c.c., should be decreased below 30·0 c.c. the thiosulphate concentration may become sufficiently high to interfere. If the above procedure is very carefully followed the formation of thiosulphate, in concentrations that interfere, will be prevented.

If quantities of potassium cyanide corresponding to quantities of hydrochloric acid up to 5·0 mg. in 25·0 c.c. of water and 0·5 c.c. sodium polysulphide solution were used complete conversion was obtained. The procedure described by Winkler (1941) is considered more laborious and gave less satisfactory results than the above method.

Removal of the Excess Sulphur.

All the difficulties which had been experienced in the removal of the excess sulphur were found to be due to thiosulphate. Polysulphide is readily decomposed by acids and the procedure adopted is as follows:—

After conversion of the hydrocyanic into thiocyanic acid a slight excess of dilute nitric acid is added and the solution aerated for one hour in order to remove the sulphuretted hydrogen and render the precipitated sulphur filterable. The latter is very important since if

any sulphur passes the filter it may dissolve on subsequent evaporation of the filtrate in the presence of sodium hydroxide to form thiosulphate. Sintered glass filters No. 4 were used. Aeration of the acidified solution does not lead to a loss of thiocyanic acid or nitric acid. If the solution stands overnight no loss of thiocyanic acid will occur provided not more than 3·0 c.c. of dilute nitric acid (20 per cent. by volume) per 10·0 c.c. of solution is present.

The procedures of Francis and Connell (1914) and of Johnson (1916) are considered more laborious than the above.

Concentration of the Filtrate.

This is done by evaporation of the filtrate, after the addition of a slight excess of sodium hydroxide, on a boiling water bath. The solution is then rendered acid with a slight excess of dilute nitric acid. The acidified solution is then brought to 29·0 c.c. with distilled water (if original volume before evaporation was 50·0 c.c.) before 1·0 c.c. of the ferric reagent is added (see later). If the ferric reagent is added before diluting to 29·0 c.c. the concentration of thiosulphate may be so high as to interfere. Evaporation in acid solution leads to a loss of thiocyanic acid. In alkaline solution, even when strongly alkaline, such loss does not occur.

Final Volume of the Test Solution.

On the final volume of the test solution will greatly depend the sensitivity of the method. As already explained the test solution should not be concentrated to a volume smaller than three-fifths of its original volume. This concentration of the test solution is only necessary when working with small quantities of hydrocyanic acid so as to increase the sensitivity and thus the accuracy of the method.

Working with different quantities of ammonium thiocyanate dissolved in 20·0 c.c. of distilled water it was found that a quantity, corresponding to 0·01 mg. of hydrochloric acid, is still distinctly visible. A quantity corresponding to 0·001 mg. of hydrocyanic acid produced a colour just visible, whereas a quantity, corresponding to 0·0005 mg. of hydrocyanic acid, produced no visible colour. The colour intensity of the quantities of thiocyanate representing 0·01 to 0·001 mg. of hydrocyanic acid is, however, very slight.

The Ferric Reagent.

This consists of a 10·0 per cent. solution of ferric-ammonium sulphate made up as follows:—

> Dissolve 50·0 gm. of ferric-ammonium sulphate in 250·0 c.c. of distilled water and add 250·0 c.c. concentrated nitric acid. The solution is boiled to expel any oxides of nitrogen, and finally filtered, if necessary.

Using quantities of thiocyanate, corresponding to 1·0 mg. of hydrocyanic acid dissolved in 30·0 c.c. of distilled water it was found that 0·5 c.c. of the ferric reagent produced a much intenser red colour than 0·2 c.c., whereas 1·0 c.c. produced only a slightly intenser red colour than 0·5 c.c. and again 2·0 c.c. produced only a slightly intenser red colour than 1·0 c.c.

Therefore with 0·5 c.c. of the ferric reagent the maximum colour intensity had been obtained. In 30·0 c.c. of distilled water 1·0 c.c. of the ferric reagent produces only a slight colouration, whereas 2·0 c.c. produces a fairly distinct colouration. It was, therefore, decided to use 1·0 c.c. of the ferric reagent per 30·0 c.c. of solution.

The use of ferric chloride in equivalent quantities would produce a distinct yellow colour which, being constant, would interfere with the proportionality of the colour intensity produced by various quantities of thiocyanic acid. The standard would, therefore, have to be near the test solution so that for a series of determinations a large number of standards would have to be prepared, which is obviously undesirable. If ferric-ammonium sulphate is used, but if chloride is present, the same yellow colour will be produced. For this reason nitric acid is used throughout, avoiding the use of hydrochloric acid.

Colour Intensity of Ferric Thiocyanate.

The colour intensity of ferric thiocyanate is influenced by many factors. In alcohol it is greater than in water and sulphates decrease the intensity, whereas chlorides increase it. The larger the quantity of ferric reagent added, the greater the colour intensity. This finding corresponds with that of Gettler and Blaine (1938) who state that $Fe+++$ ions tend to increase the colour intensity of ferric thiocyanate. Lang (1933) has already been quoted as stating that substances like phosphates, oxy-acids and trichloracetic acid influence the colour intensity of ferric thiocyanate.

In the method of the author the only adventitious substances are free nitric acid and sodium nitrate. To various quantities of a 30 per cent. solution of sodium nitrate thiocyanate corresponding to 1·0 mg. of hydrocyanic acid was added and the volume made up to 30·0 c.c. with distilled water. These were read against a standard containing thiocyanate corresponding to 1·0 mg. of hydrocyanic acid in 30·0 c.c. of distilled water.

TABLE 1.

Influence of Sodium Nitrate on the Colour Intensity of Ferric Thiocyanate.

No.	C.c. $NaNO_3$ Solution.	Percentage of Standard.
1	3	100
2	5	100
3	10	100
4	15	100

Sodium nitrate, therefore, does not influence the colour intensity of ferric thiocyanate.

Nitric acid was found to increase the colour intensity of ferric thiocyanate. To quantities of thiocyanate, corresponding to 1·0 mg. of hydrocyanic acid, various quantities of dilute nitric acid (40 per cent. by volume) were added and the volume made up to 30·0 c.c. These were read against a standard prepared in the same way but not containing any nitric acid.

TABLE 2.

Influence of Nitric Acid on the Colour Intensity of Ferric Thiocyanate.

No.	C.c. Dilute HNO_3.	Percentage of Standard.
1	0·5	103
2	1·0	109
3	3·0	112
4	5·0	112

These results correspond with the findings of Johnson (1916) who states that small quantities of hydrochloric acid increase the colour intensity of ferric thiocyanate.

Using the prescribed quantity of ferric reagent the colour intensities of various quantities of thiocyanate dissolved in distilled water is exactly proportionate. In view of the influence of nitric acid on the colour intensity of ferric thiocyanate it was necessary to determine whether the proportionality would be retained on the addition of this substance. Varying quantities of thiocyanate, representing varying quantities of hydrocyanic acid, were dissolved in 30·0 c.c. of water containing 1·0 c.c. of a 20·0 per cent. solution of sodium hydroxide and 7·0 c.c. dilute nitric acid. The solutions were prepared in this way so that their composition would approach that of the test solutions obtained according to the author's method. The solution containing a quantity of thiocyanate corresponding to 0·5 mg. of hydrocyanic acid was used as a standard and adjusted at 20 in a colorimeter.

TABLE 3.

Influence of Nitric Acid and Sodium Nitrate on the Proportionality of the Colour Intensity of various Quantities of Thiocyanate.

Mg. HCN.	Colorimeter Reading.	Colorimeter Reading if Colour Intensities are Proportionate.
1·0	10·0	10·0
0·9	11·2	11·1
0·8	12·5	12·5
0·7	14·4	14·3
0·6	16·8	16·7
0·5	20·0	20·0
0·4	24·9	25·0
0·3	33·4	33·3
0·2	49·6	50·0

From the above it is thus evident that the colour intensities of various quantities of thiocyanate remain proportionate in the presence of sodium nitrate and nitric acid. This means that for quantities of thiocyanate, representing quantities of hydrocyanic acid ranging from 1·0 mg. to 0·02 mg. only two standards are required, namely a standard, representing 0·5 mg. of hydrocyanic acid, for the range 1·0 mg. to 0·2 mg. of hydrocyanic

acid and a standard, representing 0·05 mg. of hydrocyanic acid, for the range 0·1 mg. to 0·02 mg. of hydrocyanic acid. A Leitz colorimeter is used, employing filters No. 6 (green) for larger quantities of hydrocyanic acid and No. 3 (blue) for smaller quantities.

In view of the influence of sodium nitrate and nitric acid on the colour intensity of ferric thiocyanate it is obvious that the standard should be prepared in the same way as the test solution. This is best accomplished by treating an equal volume of distilled water, after the addition of the same quantity of sodium hydroxide as used to absorb the hydrocyanic acid, in the same way as the solution containing the hydrocyanic acid.

Brodie and Friedman (1937) state that dilute solutions of thiocyanates, such as are necessary for the preparation of standards, do not keep well and that more concentrated solutions should be used for stock solutions. 2·817 mg. of ammonium thiocyanate correspond to 1·0 mg. of hydrocyanic acid. A stock solution containing 14·085 gm. of ammonium thiocyanate per litre is used, 1·0 c.c. corresponding to 5·0 mg. of hydrocyanic acid.

The solution was found to keep well.

The Liberation of Hydrocyanic Acid from Biological Material.

This can be effected by direct distillation, steam distillation or aeration. Distillation either direct or with steam, is the method most frequently employed but some authors like Harker (1936), Gettler and Baine (1938), Roe (1924) and others have discarded it for the method of aeration.

Steam distillation is usually employed to obtain hydrocyanic acid from plant material. It is used by Greene (1936), Finnemore and Williams (1935), Briese and Couch (1938) and by other authors. Doak (1933), using this method, states that collecting the distillate in sodium hydroxide and titrating with silver nitrate is unsatisfactory as in many instances the presence of reducing substances in the distillate precluded the possibility of obtaining, even approximately, an end point. Askew (1933) states that it is desirable to use a good volume of water in the distilling flask otherwise decomposition compounds appear in the distillate which interfere with the titration with silver nitrate. Elsdon and Stubbs (1937) used steam distillation to obtain hydrocyanic acid from blood.

Chelle (Harker, 1936) employed direct distillation to obtain hydrocyanic acid from organs. Childs and Ball (1935) employed the same method. They distilled 500·0 c.c. quantities of water adding 0·5 gm. of tartaric acid and state that 95 per cent. of the hydrocyanic acid is present in the first 25·0 c.c. of distillate, whereas if 50·0 c.c. distillate are collected not more than 1·0 per cent. of the hydrocyanic acid will remain in the distilling flask. They, furthermore, state that chlorides, bromides, iodides, chromates, ferricyanides, ferrocyanides, cyanates, thiocyanates, and other non-volatile substances will not pass over into the distillate but if ferrocyanides are present ferrous hydrogen cyanide will be precipitated and an equivalent quantity of hydrocyanic acid liberated.

They also state that oxidising agents will oxidise hydrocyanic acid to cyanate, reducing the yield and that if sulphuretted hydrogen be present it can be precipitated by lead nitrate and the lead sulphide filtered off. Furlong

(1914) working on hydrocyanic acid in feeding-stuffs does not use the silver nitrate or iodine titration methods since hydrolytic and distillation products sometimes interfere. Klein (1932) points out that traces of hydrocyanic acid may develop by direct heating of aqueous extracts of plants containing acids, sugars and nitrites or nitrates and thus advises removing the hydrocyanic acid by aeration when traces are being determined. Direct heating can be avoided by using an oil bath. Morris and Lilly (1933) demonstrated that rubber connections as well as rubber and cork stoppers caused a decreased yield by adsorption of hydrocyanic acid. Harker (1936), controlling the method of Chelle (Harker, 1936), gives the following information:—

> The method of Chelle consists of the direct distillation of the tissue with 5·0 c.c. phosphoric acid and the subsequent removal of the hydrocyanic acid from the distillate by aeration into 1·0 c.c. of $\frac{N}{10}$ potassium hydroxide solution.

With this method Harker (1936) obtained an average hydrocyanic acid yield of 55 per cent. Reducing the acid to 0·5 c.c., phosphoric acid yielded only slightly higher results. Using aqueous solutions of cyanide instead of tissues, similar results were obtained. The loss of hydrocyanic acid was found to be caused by the distillation. Aeration, on the other hand, did not lead to a similar loss if the air was first freed from carbon di-oxide. Harker (1936), therefore, removes the hydrocyanic acid from tissues by aeration obviating the second part of Chelle's method and obtains far better results.

From the above it is evident that the following are the objections against steam distillation and direct distillation:—

(1) From the method of the author it is obvious that, since the test solution should not be concentrated to a smaller volume than three-fifths of the original, the volume of the distillate should be as small as possible. With steam distillation the volume of the distillate would be by far too great and although direct distillation reduces the volume of the distillate, the reduction is not sufficient.

(2) Distillation, direct or with steam, may cause a serious loss of hydrocyanic acid.

(3) Unless care is exercised direct distillation may lead to the formation of traces of hydrocyanic acid.

(4) Volatile interfering agents may appear in the distillate.

Gettler and Baine (1938) state that of the three methods, namely steam distillation, direct distillation and aeration they chose the last-named method since it reduces the volume of the distillate and markedly reduces the quantity of foreign organic volatile substances in the distillate, which interfere with the method. The author selected the aeration method for the same reasons as Gettler and Baine (1938) and also because (1) there is no danger of formation of traces of hydrocyanic acid, and (2) no significant loss of hydrocyanic acid was experienced.

The material to be analysed is placed in the distilling flask B which is immersed in a boiling water bath H (see Figure I). The distilling flask is connected to a condenser C. A splash-bulb L is inserted to prevent any material from passing over into the absorption tubes. The tube of the

condenser is connected to the test tube E. The test tubes E and F, containing a 0·5 per cent. sodium hydroxide solution, serve to absorb the hydrocyanic acid and are placed in a water bath K, which is kept cold by the addition of ice. Tube G is connected to a suction pump. Flask A contains a 30·0 per cent. solution of sodium hydroxide and serves to free the air of carbon dioxide before being drawn through the apparatus. The air is drawn through the apparatus at a rate sufficient to agitate the contents of the distilling flask. In view of the findings of Morris and Lilly (1933), already referred to, care is taken to have a minimum of exposed rubber surfaces in order to minimise the loss of hydrocyanic acid by adsorption by rubber.

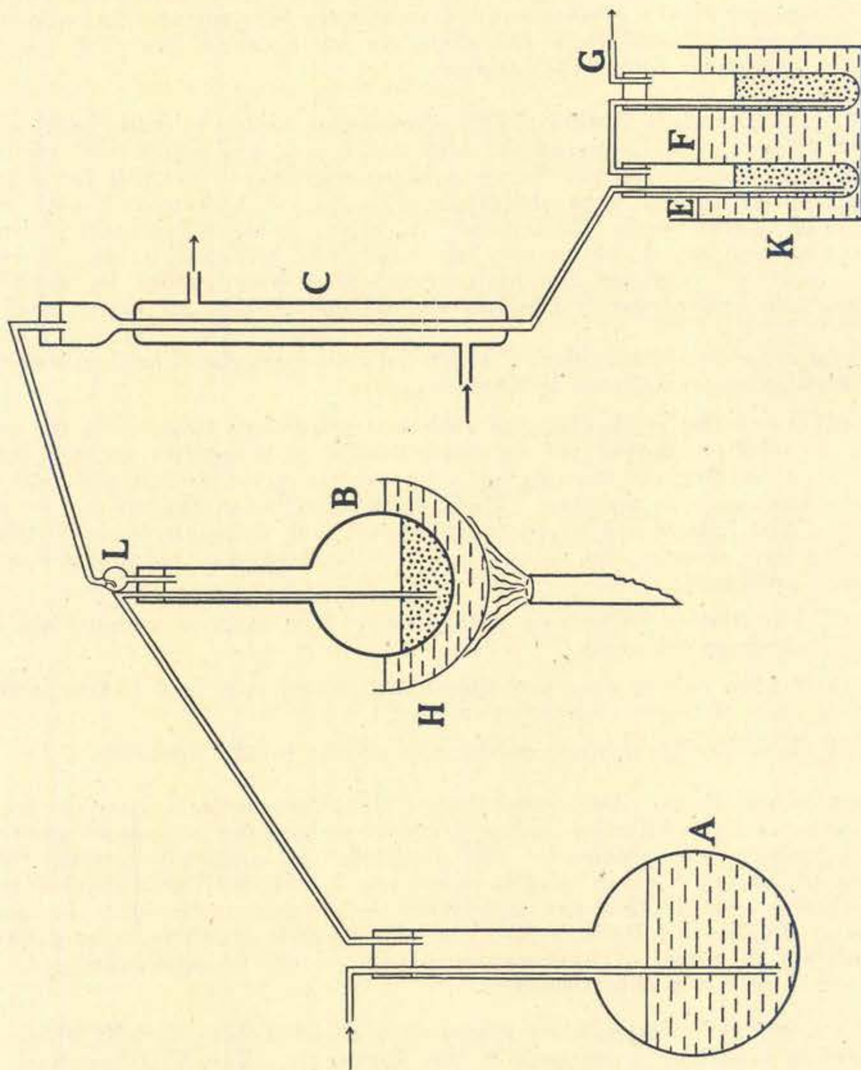

Figure 1.

The apparatus, as constructed by the author, does not allow any water to distil over on account of the length and slope of the tube connecting the distilling flask to the condenser. This fact is important, for should water distil over into the absorption tubes it would result in an increase in the final volume of the test solution and so lower the sensitivity of the method.

The material to be analysed is, except in the case of fluids, minced or ground. Of such material as liver, brain, etc., which contain relatively little hydrocyanic acid 50·0 gm. are taken since higher yields were obtained from 50·0 gm. than from 100·0 gm. Of material containing larger quantities of hydrocyanic acid relatively smaller quantities are taken, *viz.*, of ruminal contents 25·0 gm. are usually taken. To minimise the loss of hydrocyanic acid during the process of mincing or grinding, the material, if suitable, is first rapidly cooled to a low temperature in a refrigerator.

To liberate hydrocyanic acid the material in the distilling flask must be acidified. Tartaric acid is used for this purpose. Working with various quantities of potassium cyanide dissolved in 200·0 c.c. of water, quantities of tartaric acid up to 4·0 gm. had no effect on the yield of hydrocyanic acid. It was, therefore, decided to use 1·0 gm. of tartaric acid per 100·0 gm. material. It is, however, essential to test the reaction of the contents of the distilling flask to ensure that it is acid, otherwise more acid must be added. Sufficient water should be added to the specimen, but an excess is to be avoided since it will retard the liberation of the hydrocyanic acid. To 50·0 gm. specimens of liver and 25 gm. specimens of ruminal contents the author adds 150·0 c.c. of water. Working with quantities of potassium cyanide, corresponding to quantities of hydrocyanic acid up to 20·0 gm., dissolved in 200·0 c.c. of water, all the hydrocyanic acid was found to pass over within an hour. In Table 4 will be found the time required to remove the hydrocyanic acid from specimens of liver and ruminal contents of sheep that were dosed with potassium cyanide.

A control test was made as follows: To 25 gm. of ruminal contents, containing no hydrocyanic acid, in 150 c.c. of water, a quantity of potassium cyanide, corresponding to 0·87 mg. of hydrocyanic acid, was added. Aeration was continued for 3 hours. At the end of this period 0·82 mg. of hydrocyanic acid had been recovered. This finding confirms the results recorded in Table 4.

TABLE 4.

Time required for the Liberation of HCN from Specimens of Liver and Ruminal Contents.

Material.	Weight.	Mg. HCN per 100 m. Obtained during First Two Hours.	Mg. HCN per 100 m. Obtained during Third Hour.
Ruminal contents............	25 gm.	0·76	Less than 0·01.
Ruminal contents............	25 gm.	0·38	Less than 0·01.
Liver........................	50 gm.	0·09	Very faint trace.
Liver........................	50 gm.	0·07	Very faint trace.
Liver........................	50 gm.	0·1	Less than 0·01.
Liver........................	50 gm.	0·13	Less than 0·01.
Liver........................	50 gm.	0·15	Less than 0·01.

From Table 4 it is evident that aeration for 3 hours is sufficient for the satisfactory liberation of the hydrocyanic acid from such specimens as liver and ruminal contents. Whenever one is not sure of the time required for the satisfactory liberation of the hydrocyanic acid from a specimen, fresh absorption tubes should be substituted every hour in order to ensure that complete liberation of the hydrocyanic acid from the specimen is effected.

The absorption of hydrocyanic acid by sodium hydroxide is very efficient so that, unless excessive quantities of hydrocyanic acid are present, all the hydrocyanic acid will be absorbed in the first test tube and only the contents of this tube need further consideration.

Sulphuretted hydrogen, if present in the material analysed, will pass over and be absorbed by the sodium hydroxide solution. Should excessive quantities be present, the hydrocyanic acid will be displaced from the absorption tubes and lead to erroneous results. For the same reason the air is first freed from carbon di-oxide (Harker, 1936). This difficulty can be surmounted by adding a solution of mercuric chloride to the specimen to be analysed when mercuric cyanide is formed and mercuric sulphide precipitated. The mercuric cyanide is decomposed by the addition of stannous chloride and, after acidifying with tartaric acid, aeration is carried out. With this procedure very little sulphuretted hydrogen will pass over, insufficient to interfere with the method. The yield of hydrocyanic acid is not affected.

The author's method can now be summarised as follows:—The specimen to be analysed is minced or ground if necessary and a suitable quantity placed in the distilling flask together with sufficient water. Where the approximate hydrocyanic acid content is not known from previous experience it can be determined by the use of a rapid qualitative method such as the sodium picrate test. If the presence of an excess of sulphuretted hydrogen is suspected a 5·0 per cent. solution of mercuric chloride is added to the contents of the flask and the flask well shaken.

Sufficient stannous chloride to decompose the mercuric chloride is then added to the contents of the flask and the flask again shaken. Finally add tartaric acid and commence aeration, bringing the water bath to boiling. Aeration is continued for a sufficiently long period.

Ten or twenty c.c. of a 0·5 per cent. solution of sodium hydroxide in each absorption tube is employed to absorb the hydrocyanic acid depending on the hydrocyanic acid content of the specimen.

The two absorption tubes are then treated separately in case sufficient hydrocyanic acid was present to enter the second tube. To each absorption tube add the correct quantity of freshly prepared sodium polysulphide solution, stopper the tube and immerse it in a water bath at 40° C. for twenty minutes, then add a slight excess of nitric acid to each tube and aerate the contents of the tube for one hour to remove the sulphuretted hydrogen and to render the sulphur filterable. Filter off the sulphur. Should the specimen have contained a fair amount of hydrocyanic acid, concentration of the filtrate is unnecessary, so that the ferric reagent can be added directly to the filtrate. If the specimen contained a smaller quantity of hydrocyanic acid it is advisable to render the filtrate alkaline by the addition of a slight excess of sodium hydroxide and to evaporate it in a crucible on a boiling water bath to a suitably small volume. Acidify with dilute nitric acid and filter if

necessary. The volume is then correctly adjusted with distilled water and the ferric reagent added. The standards are prepared in exactly the same way as the test solutions.

Using potassium cyanide in aqueous solution the following results were obtained by the aeration procedure.

The hydrocyanic acid was determined in the absorption tubes by means of the alkaline titration method.

TABLE 5.

Recovery of Hydrocyanic Acid from Aqueous Solutions of Potassium Cyanide by the Aeration Procedure.

Mg. HCN Added.	Mg. HCN in Absorption Tube I.	Mg. HCN in Absorption Tube II.	Per Cent Recovery of HCN.
20·0	19·8	Nil	99·0
15·0	14·8	Nil	98·0
10·0	10·0	Nil	100·0
5·0	4·9	Nil	98·0
1·0	0·99	Nil	99·0
0·5	0·5	Nil	100·0

By adding varying quantities of potassium cyanide to 20 c.c. of a 0·5 per cent. solution of sodium hydroxide and determining the hydrocyanic acid according to the thiocyanate method, the following results were obtained:—

RECOVERY OF HCN BY THE FERRIC THIOCYANATE METHOD.

TABLE 6.

Mg. HCN Added.	*Per Cent HCN Recovered.*
5·0	96·0
1·0	97·0
0·8	100·0
0·6	98·0
0·4	99·0
0·2	97·0
0·08	96·0
0·05	97·0

TABLE 7.

Mg. HCN Added.	*Per Cent. HCN Recovered.*
20·0	60·0
10·0	90·0
5·0	96·0
1·0	98·0
0·5	97·0

From the results in Table 7 it is evident that if the absorption tube contains more than 5·0 mg. of hydrocyanic acid erroneous results will be obtained, probably due to the incomplete conversion of hydrocyanic to thiocyanic acid. In such cases the contents of the absorption tube should be diluted with a 0·5 per cent. solution of sodium hydroxide and a suitable aliquot used.

If the approximate hydrocyanic acid content is not known from previous experience it should be determined by a rapid qualitative method such as the sodium picrate test.

B. Determination of Hydrocyanic Acid in Plants.

In determining the hydrocyanic acid content of cyanogenetic plants it must be remembered that part of the hydrocyanic acid is present in the free state and that part of the hydrocyanic acid is bound in the form of a glucoside from which it is liberated by the action of an enzyme. It is possible that further hydrocyanic acid may be present in the plant bound in other forms, such as cyanhydrins, isothiocyanates, etc. The occurrence of these latter combinations of hydrocyanic acid have not been investigated by the author. Hydrocyanic acid poisoning under field conditions (" geilsiekte ") is a very rapid and fatal form of hydrocyanic acid poisoning. Consequently it is the free hydrocyanic acid in the plant which is of importance as well as that which is bound in the form of a glucoside from which it can readily be liberated by the action of the enzyme.

The hydrocyanic acid determined by the author, therefore, comprises the hydrocyanic acid present in the free state in the plant and that bound in the form of a glucoside. For the determination of the former it is essential to destroy the enzyme so as to prevent any liberation of hydrocyanic acid from the glucoside during the determination. According to Couch and Briese (1939) the enzyme is best destroyed by adding boiling water, containing mercuric chloride in solution, to the plant material. The author's work, the results of which are recorded in Table 8, confirms this. After destruction of the enzyme the analysis is continued as usual after stannous chloride has been added to liberate the hydrocyanic acid from the mercuric cyanide.

The plant material used was minced and thoroughly mixed so as to ensure that all specimens were equal in their hydrocyanic acid content.

Table 8.

Results Obtained by Various Methods for the Inactivation of the Enzyme.

Treatment of Specimens—	*Mg. HCN/ 100 gm.*
Plant material added to 200·0 c.c. boiling water	19·8
Plant material added to 200·0 c.c. boiling water containing 1·0 gram $HgCl_2$	18·7
Plant material in 100·0 c.c. cold water added to 200·0 c.c. boiling water containing 1·0 gm. $HgCl_2$ and very rapidly brought to boiling with a powerful flame	18·5
Plant material added to 200·0 c.c. cold water containing 1·0 gram $HgCl_2$	22·4
Plant material added to 200·0 c.c. cold water containing 2·0 c.c. of a 20 per cent. solution of sodium hydroxide. After a few minutes neutralised with nitric acid	23·2

If, besides the hydrocyanic acid present in the free state in the plant, that, bound in the form of a glucoside, is also determined, care should be taken that all the hydrocyanic acid is liberated from the glucoside. The customary procedure to bring this about is to store a number of specimens of the plant in sealed containers and to analyse them at certain intervals. The highest yield of hydrocyanic acid so obtained is taken as the hydrocyanic acid content of the plant. The period required to store the specimen in order to obtain this yield of hydrocyanic acid is taken as the time required for the complete decomposition of the cyanogenetic glucoside by the enzyme. There is, however, the following objection to the above procedure. Specimens, stored for longer periods than the above, yield ever decreasing quantities of hydrocyanic acid the longer the period of storage becomes. It is, therefore, evident that hydrocyanic acid is destroyed in the specimen during storage so that the highest yield of hydrocyanic acid is somewhat lower than the actual hydrocyanic acid content of the plant.

Briese and Couch (1938), have shown that this destruction of hydrocyanic acid in specimens of cyanogenetic plants during storage can be entirely prevented by the addition of mercuric chloride to the specimen. The author has, therefore, adopted the procedure of Briese and Couch (1938) according to which the plant specimens are stored in a solution of mercuric chloride so as to permit the liberation of the hydrocyanic acid from the glucoside by the enzyme. Sufficient time should be allowed to ensure the complete decomposition of the glucoside. It is, therefore, essential to analyse a number of specimens at progressively longer intervals.

That the procedure of Briese and Couch (1938) yields excellent results was proved by the following experiments. The same plant material was used for these experiments. The material consisted of the leaves of *Dimorphotheca Ecklonis* which were minced and thoroughly mixed so as to ensure a uniform hydrocyanic acid content. The free hydrocyanic acid content of this material was determined according to the procedure outlined above and is given in Table 9.

TABLE 9.

The Free Hydrocyanic Acid Content of the Leaves of Dimorphotheca Ecklonis.

Specimen No.	*mg. HCN/100 gm.*
1	39
2	38
3	25
AVERAGE	34

The total hydrocyanic acid content of the material was then determined by placing 10 gm. specimens of the material with a minimum quantity of water in test tubes and removing the hydrocyanic acid by means of a rapid current of air. Aeration was continued until all the hydrocyanic acid was removed from the material. The results are given in Table 10.

TABLE 10.

The Total Hydrocyanic Acid Content of the Leaves of Dimorphotheca Ecklonis.

Specimen No.	*mg. HCN/100 gm.*
1	96
2	83
3	88
AVERAGE	89

The total hydrocyanic acid content of the material was also determined as follows:—10 gm. specimens of the material were stored in 200 c.c. of water, containing 10 c.c. of a 5 per cent. solution of mercuric chloride, in closed containers for 7 days. The hydrocyanic acid content of the specimens was then determined. Table 11 contains the results.

TABLE 11.

The Total Hydrocyanic Acid Content of the Leaves of Dimorphotheca Ecklonis.

Specimen No.	*mg. HCN/100 gm.*
1	91
2	89
3	89
AVERAGE	90

From the above three experiments it is evident—

(1) that 34 mg. of hydrocyanic acid per 100 gm. of plant material was present in the free state whereas 56 mg. of hydrocyanic acid per 100 gm. of plant material was bound in the form of a glucoside;

(2) that the glucoside present was completely decomposed in the presence of mercuric chloride; and

(3) that the procedure of Briese and Couch (1938) yields excellent results.

Briese and Couch (1938) have shown that mercuric chloride inhibits, although it does not inactivate, the enzyme splitting the cyanogenetic glucoside. The inhibiting action of mercuric chloride will obviously depend on its concentration. Furthermore it is obvious that it is the concentration per volume of water and not by weight of plant material which determines the inhibiting action of mercuric chloride. The procedure adopted by the author differs somewhat from that of Briese and Couch (1938). These authors generally use 100 gm. of fresh plant material and 400 c.c. of water, adding 1-2 per cent. mercuric chloride by weight of the plant material. This would equal a 0·25-0·5 per cent. aqueous solution of mercuric chloride. The author uses 200 c.c. of a 0·25-0·5 per cent. solution of mercuric chloride and varies the weight of the plant material added thereto according to its hydrocyanic acid content so as to ensure that the 200 c.c. of mercuric chloride solution is sufficient to combine with all the hydrocyanic acid present in the plant material.

For the determination of hydrocyanic acid in plants numerous methods are employed. In considering the determination of hydrocyanic acid in tissues and body-fluids all the known methods of determining hydrocyanic acid were considered and the remarks made there also apply here. The picrate test has been extensively employed as a qualitative method for the determination of hydrocyanic acid in plants. Of the volumetric methods the best known are the iodine titration method (Finnemore and Williams, 1935) and the alkaline titration method (Green, 1936). Objections have, however, been raised against these volumetric methods.

Doak (1933), titrating with silver nitrate, states that in many instances the presence of reducing substances in the distillate preclude the possibility of obtaining, even approximately, an end-point. The same author [Doak (1938)], distilling macerated clover with steam into sodium bicarbonate and titrating with iodine or into silver nitrate and determining the hydrocyanic acid according to Volhard, obtained abnormally high results. Volhard's method gave higher results than the iodine titration method, whilst the alkaline titration method gave by far the highest results. Usually, according to Doak (1938), the iodine method gave higher results than Volhard's method.