Onderstepoort Journal of Veterinary Science and Animal Industry, Volume I, Number 2, 1933.

Studies on the Photosensitisation of Animals in South Africa.

II. The presence of a lethal factor in certain members of the plant genus *Tribulus*.

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DURING the investigation of outbreaks of " geel-dikkop ", " yellow-thick-head " or " tribulosis " in sheep due to the ingestion of certain species of the plant genus *Tribulus*, the observation was made by one of us (Quin, 1928) that dosing the expressed juice of these plants to healthy sheep resulted in their death. The most outstanding symptom was a discoloration of the conjunctivae, the blood vessels having a chocolate-brown colour. Post-mortem examination revealed a similar discoloration throughout the body; the blood was dark brown in colour and on spectroscopic examination showed a pronounced absorption band in the red, at about 630 mμ. It was strongly suspected that the abnormal pigment present was methaemoglobin.

No signs of icterus or of photosensitvity, the characteristic outward symptoms of geel-dikkop, were observed following administration of the juice of *Tribulus* plants.

Although, symptomatically, there would appear to be little resemblance between geel-dikkop and poisoning by *Tribulus* juice, it is clear that the plant must contain a lethal factor which under these particular conditions is most effective. The problem therefore on its own merits was worthy of chemical investigation, but the hope was also entertained that its elucidation might help to throw some light upon the naturally occurring disease.

The investigations detailed below have been made with material from three sources, namely, a *Tribulus* species growing in the poison garden at the Laboratory (referred to hereunder as " Onderstepoort *Tribulus* "), a quantity of sun-dried *Tribulus* plants brought from the farm estate " Melton Wold " in the Victoria West District, Cape Province, which was the scene of an outbreak in 1932 (Melton Wold *Tribulus*), and a consignment from a farm in the Northern Transvaal (N. Transvaal *Tribulus*). In view of the uncertainty existing among botanists as to the deliniation of species in the genus, it is

felt advisable to adhere for the present to the designations given rather than to quote botanical names which may shortly require revision. Specimens of the actual plants are preserved in the Onderstepoort Herbarium and also in the Herbarium of the Division of Plant Industry, Pretoria.

Feeding Tests with *Tribulus* Juice and with Aqueous Extracts of the Dried Plants.

A repetition of feeding tests with expressed *Tribulus* juice entirely confirmed previous observations of Quin (1928) made in the field during an outbreak of "geel-dikkop". Quantities of from 4½ to 5 kilos of freshly-gathered Onderstepoort *Tribulus* plants were squeezed in a hand-operated press and the dark green juice collected. The yield averaged 1½ litres from 5 kilos. The juice contained much chlorophyll and protein and was slightly acid in reaction; it was noticed that fermentation, or some type of decomposition accompanied by gas formation, set in very rapidly (1 to 1½ hours) when the liquid was allowed to stand at room temperature. When added to a drop of blood, a drop of the juice caused immediate formation of a pigment similar to methaemoglobin. If dosed as soon as collected to a sheep (by stomach tube) death usually followed within some hours, the blood vessels of the conjunctivae having a chocolate-brown colour and the blood of the animal exhibiting, when examined spectroscopically, a marked absorption band with its centre located at about 630 mμ. Post-mortem examination showed the whole of the blood and organs to be dark brown in colour. The myohaemoglobin was, apparently, not affected, neither were there any other macro-or microscopic changes to be noticed. There was no haemolysis.

Juice which had been desiccated by exposure in shallow trays showed the same effects and had apparently lost little of its potency, since the equivalent of 2¼ kilos of fresh plant when stirred up with water and further treated chemically yet killed a sheep with the typical symptoms in 3½ hours.

That the abnormal pigment produced is actually methaemoglobin was shown by examining the behaviour of the blood when reducing agents were added and by comparison with an authentic specimen of methaemoglobin. Sodium hydrosulphite when added to either sample caused an immediate disappearance of the brown colour and a change of the absorption spectrum to that of reduced haemoglobin which could be re-oxygenated to oxyhaemoglobin by shaking with air. Haematin, with which methaemoglobin might possibly be confused, is transformed by reducing agents to haemochromogen but no sign of the absorption spectrum of this pigment was evident.

Aqueous extracts of about 1 kilo of dried plant were found to produce the same effects as did *Tribulus* juice. Our usual procedure was to allow the plant powder to macerate overnight in 2 to 3 litres of distilled water and then to squeeze off the liquid in the press.

All these specimens of dried *Tribulus* when tested for the presence of alkaloids, by extraction with Prolius' solution, gave negative results.

Thermostability of the Toxic Substance.

Experiments were next made to ascertain whether the toxic substance present in *Tribulus* juice would withstand heating.

By immersion of the flask containing the juice in a water bath heated to 65-70° it was found that complete precipitation of the protein material occurred when the temperature of the liquid reached 61°. When immediately cooled, filtered and dosed to a sheep, such juice was found to be nearly as toxic as the original material causing death with methaemoglobin formation. However, autoclaving the fresh plant for one hour before squeezing destroyed its toxcicity.

Other observations recorded were as follows:—

Treatment of Material Prior to Drenching.	*Result.*
Juice from 2·5 kilos fresh *Tribulus* steamed for 30 minutes..	Negative: No symptoms.
1 kilo dried *Tribulus* in 5 l. water steamed for 30 minutes, then left to macerate overnight. Squeezed off and dosed	Negative: No symptoms.
1 kilo dried *Tribulus* in 5 l. water left macerating overnight, then squeezed off and the extract boiled for 5 minutes before dosing	Positive: Death with typical symptoms.
As above, but boiling continued until extract reduced to 1/3 of its bulk	Transitory methaemoglobinaemia. Animal recovered.
1 kilo dried *Tribulus* in 3 l. of 0·5 aqueous formalin solution left macerating overnight. Squeezed off and dosed	Symptoms appeared 24 hrs. later. Animal listless and staggering about; conjunctivae brown. Died shortly afterwards with typical methaemoglobinaemia.
1 kilo dried *Tribulus* in 3 l. of water left macerating overnight. Squeezed off and mixed with 500 cc. whole sheeps' blood immediately before dosing. Colour of liquid, coffee brown	Negative: No symptoms.

Non-extraction of the Toxin by Alcohol.

One kilo of Onderstepoort *Tribulus* (dried and ground) was extracted overnight at room temperature with 4 litres of 96 per cent. alcohol. After squeezing in the press, the residue was re-extracted for some hours with another 2 litres of alcohol and this extract added to the first. The combined extracts were concentrated by distillation *in vacuo* at a temperature not exceeding 50° until much reduced in bulk, when concentration was continued at room temperature in front of an electric fan. The syrup was taken up in 500 c.c. of water filtered from chlorophyll . . . . and drenched to a sheep. The result was *negative*. The plant material remaining after the alcohol extraction was therefore spread out to dry and then extracted overnight by 4 l. of water. This extract when dosed to a sheep caused the typical methaemoglobinaemia within a few hours, the animal dying overnight.

The toxic factor does not, therefore, pass into the 96 per cent. alcoholic extracts.

In a similar manner it was shown that an 80 per cent. alcoholic extract possessed a very slight activity but that the residual plant material was inactive. Attempts to extract the toxic factor by 75 per cent. alcohol and then to precipitate it after various purifications of the extract, by addition of excess of alcohol met with no success.

The apparent disappearance of the "toxin" when concentrations of alcohol less than 96 per cent. were used to extract the plant seemed to us very baffling at the time although in view of our later findings, this behaviour is readily understandable. That an enzyme was the toxic agent, was considered to be definitely excluded by the undiminished toxicity of expressed plant juice which had been heated to 60-65° for as long as one hour.

Non-precipitation by Lead Acetate or Mercuric Chloride.

Following our usual system in the examination of toxic plants, we next endeavoured to precipitate the substance from aqueous extracts, prepared by maceration overnight, by lead acetate, mercuric chloride or phosphotungstic acid. In the former case, clear-cut results were obtained showing that neither with neutral nor with basic lead acetate nor with basic lead acetate and ammonia was an insoluble compound formed; the toxicity was preserved in the filtrate from these substances. It would appear, then, that the toxic substance was not acidic in character nor is its glucosidal nature likely although many glucosides do resist precipitation even by lead acetate and ammonia. Experiments with mercuric chloride were equally convincing, thus casting doubt upon the basic character of the toxin. Complete activity was retained in the filtrate after lead and mercury precipitation. Less satisfactory results followed the use of phosphotungstic acid as, probably owing to the high concentration of acid required when using this reagent, the toxin was invariably destroyed.

Of the substances known to be capable of producing methaemoglobin when fed to animals, by far the largest class comprises organic nitro-compounds such as nitrobenzene, nitroglycerine and picric acid, or amino compounds derived therefrom by reduction of the nitro group. Toluylendiamine may be cited as a typical example of such substances. Inorganic nitrites are, of course, known to produce the transformation of haemoglobin into methaemoglobin *in vitro* as does also the bitter pincipal podophyllotoxin.

Reviewing our results, it appeared that a simple organic substance could be definitely excluded on account of the insolubility of the lethal factor in 96 per cent. alcohol, nevertheless attempts to demonstrate its presence in the inorganic fraction failed. The apparent disappearance of the toxin when alcohols of lower concentration than 96 per cent. were used and its reactions towards heating were also confusing phenomena.

An examination was then made of an extract cleared by means of lead acetate and by mercuric chloride in the hope that after the removal of such quantities of extraneous material, further *in vitro* tests would help to throw light upon the problem. As an index of activity the action upon freshly-drawn sheep's blood was adopted since it appeared to us that the *in vivo* and *in vitro* actions were certainly related.

The following observations were recorded:—

1. When added to diluted blood, immediate formation of methaemoglobin occurs if the pH is about 4·6. Acid haematin was not formed at this acidity, neither was conversion to methaemoglobin appreciable at higher pH values.
2. At a pH of 4·6 the ferrous ion, added as ferrous ammonium sulphate, is immediately oxidized to the ferric state, shown by the blood-red colour with potassium thiocyanate.
3. Acid potassium permanganate is reduced.
4. Acid potassium iodide-starch solution is coloured intensely blue (oxidation).
5. The metaphenylenediamine reagent gives a brown colour.
6. The Griess-Ilosvay reagent (naphthylamine-sulphanilic acid in acid solution) gives an immediate, intense, rose-red colour.
7. The brown ring test with ferrous ammonium sulphate and concentrated sulphuric acid was positive.

From these reactions it appeared that the oxidising agent, the toxic factor converting haemoglobin to methaemoglobin was none other than inorganic nitrite. Reactions 5 and 6 which depend upon the diazotization of an amino body are specific for nitrites. A solution of sodium nitrite gave all the above tests in a manner identical with the plant solution. When reviewed in this light our preliminary experiments were seen to be in perfect harmony with such a conclusion. The only point which at first caused us doubt was that nitrites are only encountered in traces in plants and it seemed highly improbable that a kilogram of dried plant should contain sufficient nitrite to kill a fully grown sheep when given by the mouth.

Feeding tests, which will be considered in more detail below, showed that the lethal dose of sodium nitrite is about 2 gm. *per os* for a sheep weighing approximately 20 kilos.

A consideration of the form in which nitrite could be present in these plant extracts enables the possibilities to be limited to three, namely:—

1. That it is present in solution as free, inorganic nitrite.
2. That it is present in combination with glucose as an extremely unstable glucoside such as the nitrite-glucoside reported to be present in the leaves of *Erythrina* sp.
3. That it is formed from some other precursor.

On account of its reactivity, nitrite is not easily determined in a plant extract rich in proteins, amino acids, etc., however, preliminary experiments showed quite clearly that its amount varied considerably in different extracts and in one and the same extract under different conditions, thus ruling out possibility number 1.

To quote only one example, three extracts prepared on different occasions by macerating 200 gm. lots of dried *Tribulus* overnight with 800 c.c. of water were found to contain quantities of nitrite equivalent to 1·6, 0·15 and 0·31 gms. of $NaNO_2$ per kilogram of plant. Extracts made with boiling water contained no more than traces of nitrite.

That nitrite could be present in the plant in the form of an unstable glucoside was a possibility considered since Weehuizen (1907) reported the occurrence of such a glucoside and of an enzyme decomposing it with liberation of nitrous acid in the leaves of an unidentified *Erythrina* species, an observation subsequently substantiated by Betting (1909).

For several reasons, however, the occurrence of a nitrite-yielding glucoside in our *Tribulus* samples appeared to us unlikely in view of our experimental findings. Although nitrite is absent from the dry, powdered plant material, it is rapidly formed, and in considerable quantity, when such preparations are soaked in water in a closed vessel. In one instance 100 gm. plant powder (Onderstepoort *Tribulus*), and 1 litre of water to which had been added 1 c.c. of cloroform was left in a closed jar at room temperature for 60 hours. When opened the smell of nitrous oxide was apparent and a strip of starch-iodide paper held in the vapour was immediately coloured deep blue. An attempt was made to determine the quantity of nitrite (or nitrous acid) still in solution by filtering, decolorising the filtrate by shaking with animal charcoal (proved to contain no nitrite) and carrying out a colorimetric determination upon the filtrate by means of the Griess-Ilosvay reagent. Reckoned as sodium nitrite, 0·8 gm. was found to be present.

The most likely source of this nitrite formed in extracts of *Tribulus* or in the press juice of the plant appeared to be inorganic nitrates, which are known to be frequently, although not invariably, present in plant tissues. The only possible objection to such an hypothesis was the relatively large quantity demanded by the formation of so much nitrite. A reducing enzyme or enzyme system capable of bringing about the reduction of added nitrate has been shown to be present in quite a number of plant tissues (Anderson 1924).

It became imperative to carry out determinations of the nitrate present in our *Tribulus* samples, and for this purpose the method proposed by Strowd (1930) was finally adopted, the values being checked later by the highly specific phenolsulphonic acid colorimetric method.

Determination of Nitrate in Dried *Tribulus*.

All the preliminary work was done using samples taken from a large stock of ground, dried *Tribulus* (from all three localities in order to check the reproducability of the results).

Preparation of the extract. Since it was important that all enzymic changes should be eliminated, extracts were made by adding a weighed sample of the plant powder to a known volume of boiling water, maintaining ebullition for 2 or 3 minutes and then allowing the mixture to stand, plus a few drops of toluol, in a stoppered flask overnight. A satisfactory proportion was to take 20 gm. of plant and 200 c.c. of water.

The extract was strained off through fine muslin and the residue squeezed in a press, after which it was quantitatively transferred to another portion of 200 c.c. of boiling water and the extraction process repeated. The extracts were combined and the resulting volume measured. Aliquots of 50 c.c. were found to be satisfactory for the determination of nitrate by the Devarda alloy method.

Two 500 c.c. pyrex flasks were fitted with kjeldahl splash-bulb distillation traps and these, in turn, connected to two vertical water-cooled condensers. The delivery tubes dipped beneath the surface of 30 c.c. of decinormal sulphuric acid contained in 250 c.c. flasks provided with a mark at 180 c.c. volume. In one of the pyrex reaction flasks, which served as the control, was placed 50 c.c. of the extract, water to 240 c.c. and a few pieces of porcelain to promote smooth boiling. 10 c.c. of 25 per cent. sodium hydroxide solution was then added and the stopper carrying the splash-bulb inserted. The other flask had similar contents, but in addition 1 gm. of Devarda's alloy was added. The flasks were warmed by very low gas flames, actual boiling or distillation being avoided until the reaction between the alloy and the alkali had subsided. Heating was then continued more vigorously but always in such a way that both control and determination were distilling at the same rate, and 150 c.c. of distillate collected. The condensers were disconnected and rinsed out and the excess of acid in the two flasks determined by back titration with decinormal sodium hydroxide using methylred as indicator. Duplicate determinations were found to agree excellently and, as already pointed out by Strowd, the method gives results in good agreement with the gasometric method of Schultz. In making the final calculations, the volume of acid neutralized by the control was subtracted from that in the determination since sodium hydroxide alone is capable of liberating ammonia or volatile amines from some of the organic constituents of the plant, e.g. bases like choline, etc.

As an example of the method, the figures obtained in one experiment on Onderstepoort *Tribulus* are reproduced below:—

25 gm. dry plant extracted by 500 c.c. boiling water in two lots of 250 c.c. each. Nitrate determination upon 50 c.c.

	c.c.
(i) Acid neutralized in blank determination	1·8
Acid neutralized in nitrate determination	10·7
Therefore due to nitrate	8·9

Since the acid was 0·09523 normal this is equivalent to $8{\cdot}9 \times 0{\cdot}09523 \times 101$ mgm. KNO_3 or 3·42 gm. KNO_3 per 100 gm. plant.

(ii) Acid neutralized in blank determination	1·4
Acid neutralized in nitrate determination	10·1
Therefore due to nitrate	8·7

i.e. 3·35 gm. KNO_3 per 100 gm. plant.
Mean of determinations 3·39 gm. per cent.

The above figure is typical of that found by this method in many analyses of dried Onderstepoort *Tribulus*. The average figures for the other two varieties are given below for comparison.

Nitrate content of *Tribulus* samples (dried material):—

Onderstepoort *Tribulus*: 3·39 gm. KNO_3 per 100 gm.
Northern Transvaal: 2·29 gm. KNO_3 per 100 gm.
Melton Wold *Tribulus*: 1·20 gm. KNO_3 per 100 gm.

It is clear that these plants are considerably richer in nitrate than are most dicotyledons. It is also apparent that the nitrate content is more than sufficient to account for the quantities of nitrite found experimentally and demanded by the minimal quantity of the plant juice or extract known to be toxic when fed to sheep.

Although the Devarda alloy method has been shown by Strowd to be reliable it was felt that confirmation of these figures by some other method would be desirable in view of the importance to be attached to them in the elucidation of the whole problem of the toxicity of this plant.

At the time of conducting the experiments the method of Pucher, Vickery and Wakeman (1932) had not yet reached us, however, the very specific colorimetric phenol-sulphonate method used in the determination of nitrates in water analysis was adopted and found to give results in excellent agreement with those yielded by the reduction method.

The phenol-sulphonic acid reagent was made up according to Charnot, Pratt and Redfield (1911) by dissolving 25 gm. phenol in 150 c.c. pure concentrated sulphuric acid and adding 75 c.c. of fuming sulphuric acid (13 per cent. SO_3) mixing and heating in a boiling water bath for 2 hours.

Since the method is capable of determining 10 parts of nitrate nitrogen per million, an extensive dilution of the plant extract could be carried out thereby very considerably diminishing the quantities of chloride and organic matter present. The following example will serve to illustrate the procedure and to demonstrate the agreement between the two methods.

An extract was made in the usual way of 20 gm. of *Tribulus* by 200 c.c. of boiling water. Nitrate determination by the Devarda alloy method gave values corresponding to 2·74 and 2·86 mgm. per c.c. or 2·74 per cent. and 2·86 per cent. KNO_3 respectively in the original plant powder. Mean 2·80 per cent.

1 c.c. of the filtered extract was diluted to 100 c.c. and of this solution duplicate samples of 10 c.c. each were mixed in centrifuge tubes with 5 c.c. of a suspension of freshly precipitated aluminium hydroxide. After centrifugation, 10 c.c. of each of the supernatant liquids plus 2 drops of decinormal sodium hydroxide were pipetted into small evaporating basins. A range of standards were similarly prepared by taking suitable quantities of a standard potassium nitrate solution. All solutions were evaporated to dryness upon the water bath, 2 c.c. of the phenol-sulphonic acid reagent was then added and after thorough mixing of this with the residue, 20 c.c. of water was carefully added to each (it was found convenient to float the dishes in a large basin of water in order to avoid great development of heat in this and the next stage). After stirring, 10 c.c. of a 10 N solution of potassium hydroxide was then added, which quantity of alkali is slightly more than sufficient to neutralize the acid present, and the resultant yellow solutions compared in vessels of similar size and shape. The dilution of the standard most nearly matching the unknown solutions was picked out and from this the original

nitrate content of the plant extract calculated. In the above case it was found to be 2·70 mgm. KNO_3 per c.c. corresponding to 2·70 per cent. of KNO_3 in the plant powder.

In a later experiment using freshly gathered green *Tribulus* values of 0·577 per cent. and 0·570 per cent. KNO_3 were found by the Devarda method and 0·58 per cent. by the colorimetric method (percentages calculated upon fresh wt.). There is no doubt therefore that in the present case the results of the Devarda method can be relied upon to give the true nitrate contents.

The presence of such a quantity of nitrate in the plant suggested that it would be possible to isolate the salt in the pure crystalline form. This was accomplished by making a water extract, clearing with a considerable quantity of decolorizing charcoal (free from nitrate) and evaporating to a syrup. 96 per cent. alcohol was then added and this mixture filtered. The residue was well washed with 96 per cent. alcohol and then subjected to fractional crystallization. Potassium nitrate was obtained in fair yield.

It would thus appear that the greater part of the nitrate is present in the plant as the potassium salt.

That the production of nitrite from nitrate is brought about by an enzymic reducing system present in the plant is fully capable of explaining the salient characteristics summarised below of nitrate formation in *Tribulus* juice or extracts. What substance acts as the hydrogen donator in the system it is impossible to state:—

1. Absence of nitrite in fresh plant but slow liberation when macerated with water.
2. Thermolability of the system.
3. Abundance of nitrate in the original material.
4. Capability of thoroughly washed plant powder to bring about the reduction of added nitrates.
5. The persistence of the reaction under conditions excluding the possibility of bacterial action.

With regard to findings 4 and 5, the evidence may be presented as follows.

In order to ascertain whether aqueous extracts of the plant powder possessed the reducing activity and could thus be utilized for closer study of the system, 10 gm. of dried Onderstepoort *Tribulus* was allowed to stand overnight in 50 c.c. of 5 per cent. disodium-hydrogen phosphate solution (pH 8·4) to which a few drops of chloroform were added. The liquid was expressed, centrifuged, saturated with ammonium sulphate and the resulting precipitate centrifuged off and washed by saturated ammonium sulphate solution. It was then redissolved in 5 c.c. of water.

This solution was tested for activity by addition of potassium nitrate (25 c.c. of a 0·2 per cent. solution), 3 mgm. of xanthine freshly dissolved in 3 c.c. of N/10 sodium hydroxide and 0·1 c.c. of chloroform. After 4 days the mixture was examined quantitatively for nitrite by means of the Griess-Ilosvay reagent and 0·21 mg. found to be present, i.e. less than 1 per cent. of the amount theoretically derivable from the nitrate added.

5 gm. of the plant residue was freed from nitrate and nitrite by steeping once more in water overnight, squeezing off and then thoroughly washing the residue in repeated changes of distilled water. One quarter of the material so obtained, 2·5 gm. dry wt., was suspended in 50 c.c. of water plus 0·1 c.c. chloroform. An equal quantity was added to 20 c.c. of 0·2 per cent. potassium nitrate solution, 30 c.c. of water and 0·1 c.c. of chloroform added and the two mixtures allowed to stand in stoppered flasks for 4 days at the temperature of the laboratory after which time they were tested for nitrite with the following result, from which it is evident that the nitrate reducing system is retained in the plant material and cannot be washed out by water.

1. Washed residue plus water; Nitrite nil.
2. Washed residue plus KNO_3 solution; 25·36 mgm. i.e. 63·4 per cent. of that theoretically possible.

The remaining 5 gm. of washed residue was used in attempts to reconstitute the system, replacing nitrate by methylene blue (0·5 c.c. of 1 in 5,000), and working at temperatures ranging from 20° to 65° in vacuum tubes of the usual Thunberg type. In some cases xanthine or acetaldehyde was also added but in no instance was any appreciable rate of discoloration of the methylene blue recorded although the pH was also varied over a wide range.

These observations are in agreement with the findings of others who have worked upon plant oxide-reductase systems. Thus Abelous and Alloy (1904) found that, in the presence of salicylaldehyde, potato juice reduced nitrates to nitrites. Bach (1913) interprets this action as being due to a water-splitting oxido-reductase and capable therefore of carrying out the same reaction in the absence of oxygen. The presence of an aldehyde was shown to be essential to complete the system with freshly prepared extracts, although in extracts which have been allowed to stand, its place can be taken by some substance formed as the result of autolysis. Michlin (1928) in a very thorough investigation of the nitrate-reducing system of the potato, showed that this differs in certain fundamental respects from the nitrate-reducing xanthine-oxidase system of milk, the so-called ' Schardinger enzyme.' In the first place, the milk system is destroyed at reactions greater than pH 9·5 whilst that of potato juice is unaffected. In the former, methylene blue can be substituted for aldehyde without diminution of the activity of the system which moreover possesses a wide pH range extending from pH 3·0 to 8·6, whilst in the latter the reductase is practically inactive with methylene blue, even this feeble action, which Bernheim (1928) has shown to be limited to a narrow range between pH 7·3 and 7·8 and to require strict anaerobiosis, being inhibited by addition of m/300 potassium cyanide. The milk enzyme is unaffected in presence of m/50 potassium cyanide. Michlin found that the potato extracts with which he worked had no effect whatever upon the purine bases and that the feeble anaerobic activity with methlylene blue is only shown by extracts which have been cleared with charcoal. He suggests that the difference between the plant and animal systems may be due to the absence of some co-enzyme or carrier from the former.

In the present work the best antiseptics for use with the *Tribulus* system were found to be toluol, chloroform (0·2 per cent.) or ether (0·4 per cent). Formaldehyde (0·2 per cent.) exerted a very pronounced inhibiting action which is in accordance with the experience of Bach (1911) who found both the Shardinger enzyme and the oxido-reductase of liver to be markedly inhibited by formaldehyde or even by acetaldehyde.

Determination of Nitrate Content in Fresh Plant Material.

Since it appeared likely that the existence in *Tribulus* of an energetic reducing system would lead to decomposition of nitrate during the drying process, efforts were made to determine the quantity of nitrate present in the freshly gathered green plants.

For this purpose, two sources of supply were used: plants raised from the seed of local *Tribulus* and growing in the Onderstepoort poison garden, and *Tribulus* which was found growing next to one of the sheep camps in the laboratory yard and which had evidently seeded itself from material used in feeding experiments.

Determination of nitrate content was carried out as follows. A 50 gm. to 100 gm. sample was dropped into ten times the volume of boiling water, ebullition maintained for two or three minutes, a few c.c. of toluol added and the flask closed and allowed to stand until next day. After straining off the liquid and squeezing, the residue was put through a small mincing machine and returned to the flask with one half or an equal quantity of water to that first used. All parts of the mincer and all vessels used were rinsed in this operation.

The contents of the flask was brought to boiling and extraction allowed to take place as before. The two extracts were combined and their volume measured, aliquot portions of about 200 c.c. being taken for nitrate determination by the Devarda alloy method, or after suitable dilution, for the phenol-sulphonic acid colorimetric procedure. Results were checked by duplicate determinations. A representative sample of the fresh material was always taken for moisture determination, drying being continued at 95° to 100° until the weight remained constant.

Most surprising differences in nitrate content were found both between plants gathered on different days (after rain, etc.), and even between specimens taken at the same time from the same small plot and differing only in their outward appearance and condition of growth—luxuriant or stunted. That the technique was not at fault, nor errors of sampling too large, was proved by the agreement of duplicates. In the table immediately following, some of the more striking results are reproduced. Nitrites were never found to be present in quantities exceeding the merest traces in any of the specimens tested.

TABLE

Variation in Nitrate Content of Freshly Gathered Tribulus.

Source of Material.	Date of gathering.	Description.	Moisture content	KNO_3 content per cent. of wet weight.	KNO_3 content per cent. of dry weight.
Laboratory poison garden	9.30 a.m. of 28/10/32	Well grown	76·6	50 gm. sample 0·58 (colorimetric 0·58) 30 gm. sample 0·57	2·44
,, ,,	2 p.m. of 2/11/32*	Well grown	70·6	100 gm. sample 0·39	1·32
,, ,,	10 a.m. of 7/11/32	Luxuriant, Ditto, after drying	74·0	50 gm. sample 0·40	1·53 1·33
,, ,,	,,	Stunted, poorly grown	74·0	50 gm. sample 0·03	0·12
Laboratory yard, sheep camp 47	Noon, 7/11/32	Well grown, Ditto, after drying	74·4	50 gm. sample 1·04	4·06 2·96
Laboratory poison garden	10 a.m., 5/1/33	Single large plant in plot, very well grown	82·17	50 gm. sample 1·27	7·12
Bethulie, O.F.S.....	10/12/32	Small plants	—	20 gm. sample (dry)	0·65
Middelburg, C.P.....	8/2/33	Stunted: Gathered at scene of outbreak of dikkop	—	10 gm. sample (dry)	1·42

* Fairly heavy rain fell between 28/10/32 and 2/11/32.

The very great variability is apparent of the nitrate content found although in all of the above cases only one species of *Tribulus* was being considered. The very high nitrate content of the plants growing near to the sheep camp is probably to be explained by the rich manuring of such soil. With regard to the other variations, especially the difference between the luxurious and the stunted plants from the same plot little can be said by way of attempted explanation.

That the amount of nitrate present in a plant may vary very considerably was pointed out so long ago as 1884 by Berthelot (1884). Anderson (1924) has recently reported that *Mercurialis perennis* also shows a seasonal variation, the test for nitrate being positive in October but negative in June. The leaves of *Lupinus sp.* follow a similar cycle, whilst in the case of *Solanum Dulcamara L.* the nitrate content was much higher in the early morning than in the evening. Locality also influences the nitrate content very markedly as is shown by the examples of *Suaeda fruticosa*, free from nitrate when growing on highly insolated shingle, but giving a strongly positive test when grown in shaded garden soil, and *Sambucus nigra* which was very rich in nitrate when growing in shaded valleys.

Klein (1913) has shown that the distribution of nitrate in the different parts of the plant varies largely.

The activity of the nitrate-reducing mechanism according to Anderson (1924) also fluctuates considerably. *Solanum Dulcamara*, for example, exhibits a very high activity in June, the season at which the nitrate content of this plant was at its lowest, but evinces such a feeble activity in October as to be considered practically negative.

How the activity of the enzyme system may affect the nitrate content of *Tribulus* plants, as also the effect of growth, locality, etc., it is at present impossible to state with certainty. Such variations may conceivably bear some relationship to the periodic and fluctuating character of geeldikkop.

Feeding Experiments and Animal Tests.

Experiment 1.—*Feeding Green Tribulus plants to Sheep at Onderstepoort.*

As pointed out previously (15th Report, D.V.S. p. 765), cases of geeldikkop were produced in sheep in the Burghersdorp district in January, 1929, when animals were fed exclusively on green *Tribulus* plants.

On account of rain falling while the experiment was in progress, the disease disappeared abruptly, so that the work had to be discontinued. However, in February, 1932, a similar experiment was started at Onderstepoort, where a small patch of the locally found *Tribulus* (*Tribulus murex*?) was established. When in the flowering stage, and growing luxuriantly, the plants were allowed to be grazed for one hour twice daily by a merino lamb about 6 months old, not receiving any other food besides the *Tribulus*. The animal was exposed to sunlight for several hours each day. After seven days feeding on *Tribulus* the sheep started losing condition, and after three weeks it was very weak and emaciated, although throughout the whole period *Tribulus* was readily being eaten. At no time did the animal become photosensitive, or did any swellings or icterus develop. Blood samples were drawn each day and the van den Bergh test carried out. This remained negative.

After three weeks of grazing on the plot, it was decided to dose the juice from 2 kilos of the same fresh plant. Within three hours after dosing the animal died of acute methaemoglobinaemia. Except for the intense chocolate brown colour of the blood no other lesions were to be found.

Although from the above single experiment no conclusions can be drawn, as to the ability of this plot of *Tribulus* to produce true cases of geeldikkop, seeing that similar experiments carried out in an affected area may fail, it nevertheless clearly shows that the juice even from a comparatively small amount of the green plant can be very toxic, although the immediate cause of death in this case is different (methaemoglobinaemia) from that encountered in sheep dying from geeldikkop under natural conditions (icterus, oedema and skin necrosis).

Experiment 2.—*Repeated drenching with dried Melton Wold* Tribulus

This *Tribulus* was collected on the Melton Wold Estate in the Victoria West district during an outbreak of geeldikkop on the estate. The material was air-dried, finely powdered up and dosed to sheep at Onderstepoort.

One sheep was dosed daily for 10 days with 500 grams of the powder freshly mixed in 3 litres water, and the animal kept in the sun. No signs of illness were noted during this time.

Unless specifically stated, all extractions were made by allowing the dried plant material to macerate in water for several hours, usually overnight, under conditions which permitted any enzymic changes to take place.

One sheep dosed with the watery extract from 1 kilo of the same dried Melton Wold *Tribulus*, died from acute methaemoglobinaemia within three hours.

It is thus clear that dry Melton Wold *Tribulus*, as is the case with Onderstepoort *Tribulus* does not produce true geeldikkop when dosed repeatedly, although acute methaemoglobinaemia again is the constant cause of death, when the fermented watery extract is dosed.

Experiment 3.—*Drenching of* Tribulus *Extracts.*

In this experiment 31 young merino sheep were used. The routine procedure was to dose all sheep through a stomach tube. All animals were closely shorn and kept exposed to sunlight between hurdles. Any shade was avoided as far as possible. The object of the experiment was to ascertain whether regular dosing of different extract of *Tribulus* plants would ultimately lead to the typical disease. In addition to the dose a little roughage and green lucerne were also allowed, unless otherwise stated.

One sheep was dosed with freshly expressed juice from 1·5 kilos green Onderstepoort *Tribulus*. Although the animal became dull and listless within a few hours after dosing, it was apparently completely recovered the next morning, when the same amount of juice was again dosed. The animal died in the afternoon from intense methaemoglobinaemia.

From this experiment it may be concluded that death was due either to the cumulative effect of the toxin in the two doses, or only that the body had been rendered more susceptible by the first dose, which in itself produced only very slight symptoms.

It may be pointed out that in previous experiments with *Tribulus* juice the minimal lethal dose was found to vary within fairly wide limits, depending apparently not only on the toxicity of the juice, but also on the age and condition of the animal.

Experiment 4.—*Drenching with Dried Juice Expressed from Onderstepoort* Tribulus.

Ninety grams of dried juice obtained from 2 kilos fresh plant were dissolved in 2 litres water and dosed to a sheep. Death from methaemoglobinaemia followed within 8 hours.

This indicates that desiccation did not in any way effect the toxicity of the juice.

Experiment 5.—*Drenching of Watery Extract of Dry Onderstepoort* Tribulus.

Some of the green *Tribulus* was air-dried and subsequently finely powdered. Of this 1 kilo amounts were used in these experiments. Four litres of tap water were added to each one kilo of powder and the material allowed to extract overnight. Fermentative changes were generally visible the next morning and a peculiar musty odour was found to have set in. The watery fraction was thoroughly removed by means of a strong, hand-operated press. It was turbid and greenish-brown in colour. As a rule two-thirds to three-quarters of the water added was again recovered in the extract. On dosing the extract so prepared from 1 kilo amounts, sheep were regularly found to die from methaemoglobinaemia in 2 to 6 hours.

Experiment 6.—*Drenching of Watery Extract of Onderstepoort* Tribulus *in Acid Medium.*

The object of this experiment was to ascertain whether the pH of the extract, or of the rumen of the animal, would effect the toxicity. For this purpose a sheep was dosed on two consecutive days with 500 c.c. of $^{N}/_{10}$ hydrochloric acid. On the third day the animal received the watery extract from 1 kilo of dried Onderstepoort *Tribulus*, to which 40 c.c. concentrated HCl was added just before dosing. No symptoms were noted during the day, but the aimal died during the night, with the blood still somewhat brown the next morning, i.e. death was apparently again due to methaemoglobinaemia.

Another sheep, which was dosed with the watery extract from 1 kilo *tribulus* to which 15 c.c. concentrated HCl was added before dosing, showed respiratory distress within 5 hours, and died shortly afterwards from methaemoglobinaemia.

A third sheep was dosed with the acidified watery extract from 1 kilo dried *Tribulus*. In this case 20 c.c. concentrated HCl was added to 3 litres of water and the *Tribulus* soaked in this overnight. The animal died from typical methaemoglobinaemia within 3 hours.

It may thus be concluded that an acid medium whether of the plant extract, or in the rumen of the animal does not in any way effect the toxicity.

Experiment 7.—*Drenching of Watery Extract of Onderstepoort* Tribulus *in Alkaline Medium.*

One sheep was dosed with the watery extract from 1 kilo dried Onderstepoort *Tribulus* to which 35 grams sodium carbonate was added just before dosing. The animal died during the night from methaemoglobinaemia.

Another sheep was dosed with an alkaline extract prepared by soaking 1 kilo *Tribulus* in 3 litres water containing 40 grms. sodium carbonate. The animal died within 3 hours, the blood again showing the typical brown discoloration.

From this experiment it is clear that an alkaline medium does not effect the toxicity of the plant extracts.

Experiment 8.—*Administration of Sodium Nitrite to Sheep.*

Once it was known that a nitrite present in the *Tribulus* extracts was causing the death of the sheep, it was decided to obtain more information about the action of inorganic nitrites when administered in different ways, and it was hoped that by these means light would be thrown on the cause of the symptoms in true geeldikkop.

For this experiment 18 young merino sheep were used. The animals were again closely shorn and kept out in the sunlight each day as in the previous experiments.

(*a*) *Drenching of Sodium Nitrite.*

Eight young sheep were drenched with amounts of sodium nitrite ranging from 1·5 to 2·5 grams per dose. The material was dissolved in a few c.cs. of water and dosed through the stomach tube.

As a rule no symptoms were shown within the first two hours after dosing although a progressive brown discoloration of the conjunctivae could frequently be noted. Soon thereafter a varying degree of respiratory distress was shown. In single doses of 2 grams and more, death usually supervened after the third or fourth hour, the main symptoms being those of acute respiratory distress and asphyxia. In doses of less than 2 grams, most of the animals after a short transitory period of distress, rapidly recovered to apparently normal health. In several cases it was observed that recovery could rapidly take place even after the animal had passed into a state of coma, the brown colour of the blood and mucous membranes soon changing back to the normal red. Where death supervened, the only post-mortem finding was that of an intense methaemoglobinaemia. No signs of haemolysis were ever seen, the serum always remaining perfectly clear and colourless. In these sheep it could therefore be taken that a single dose of 2 grams sodium nitrite represented the minimal lethal dose.

After this, it was decided to ascertain the effects of repeated dosing of sublethal amounts of sodium nitrite. In the case of one sheep, daily dosing was commenced with 1 gram sodium nitrite. On the 9th day this was increased to 1·5 grams, on the 12th day to 2 grams, from the 18th to 2·5 grams and from the 24th day to 3 grams. On the 29th day the animal was discharged after having received 57·5 grams sodium nitrite. Although slight browning of the conjunctivae was noted at different times, no outward symptoms of illness were ever shown. Another sheep was dosed over a period of 41 days with 82 grams sodium nitrite (maximum dose 3 grams), without showing any signs of illness. By the end of this period the red cell volume had increased to 40 per cent. from an initial 26 per cent.

From these experiments it was evident that sodium nitrite alone did not produce any symptoms of true geeldikkop. On the contrary, it became clear that the animals developed a definite tolerance to nitrite and that the volume of red cells was markedly increased. This may have been due to an attempt on the part of the body to compensate for the chronic anoxaemia caused by repeatly dosing with nitrite.

(*b*) *Drenching of Sodium Nitrite and Potassium Permanganate.*

It was hoped that potassium permanganate when dosed simultaneously with the nitrite would inhibit the toxic effects of the latter. Doses of 0·25 grams potassium permanganate were given daily immediately after the nitrite. In this way one sheep was dosed with 71·8 grams sodium nitrite and 6·5 grams potassium permanganate over a period of 30 days (maximum dose of nitrite 3·5 grams). Although browning of the conjunctivae was noted off and on, no signs of illness were shown until on the 30th day, when the animal developed marked respiratory distress and died of typical acute methaemoglobinaemia, which also was the only post-mortem finding.

(*c*) *Drenching of Sodium Nitrite with Sodium Bicarbonate.*

One sheep was dosed with 53·5 grams sodium nitrite (maximum amount 3 grams) and 650 grams sodium bicarbonate (maximum amount 50 grams) over a period of 47 days. The animal remained in good health and was discharged on the 50th day, indicating that the sodium bicarbonate had in no way influenced the course of events.

(*d*) *Intravenous Injections of Sodium Nitrite.*

Injected intravenously 0·75 to 1 gram sodium nitrite was regularly found to cause death of sheep from methaemoglobinaemia within a few hours. Repeated daily injections starting with 0·1 gram and gradually increasing to 0·8 gram usually produced no symptoms. With doses above 1 gram animals may, however succumb very rapidly. In the case of a dog, 0·5 grams intravenously caused death from methaemoglobinaemia within 2 hours, while another dog of the same size withstood 11·5 grams sodium nitrite injected over a period of 23 days. One sheep injected intravenously with a total of 10·8 grams (maximum dose 1 gram) died after the 23rd day with acute brown discoloration of the blood.

(*e*) *Influence of Starving on Sodium Nitrite Poisoning.*

One sheep was starved for 48 hours and then dosed 2 grams sodium nitrite. Death took place within 2 hours, the blood being intensely brown.

Another sheep similiarly starved was dosed 5 oz. meatmeal together with 2 grams sodium nitrite. Death followed within 6 hours, the blood again being intensely brown.

(*f*) *Drenching of Sodium Nitrite followed by Injection of Haematoporphyrin.*

One sheep was dosed 2 grams sodium nitrite on two consecutive days, after which 0·2 gram haematoporphyrin was injected intravenously. Photosensitisation became very marked soon afterwards. A subsequent dose of 2·5 grams sodium nitrite rapidly caused death from methaemoglobinaemia.

Discussion.—From the above experiments it is clear that sodium nitrite is very poisonous to sheep, although as a rule repeated sub-lethal doses provoke a well-marked tolerance. Death, when it takes place, always results from an intense methaemoglobinaemia. In no case does sodium nitrite poisoning lead to symptoms simulating those seen in true geeldikkop, the clinical picture and post-mortem findings resembling those reported in the literature for other animals. No essential difference was found between sodium nitrite and potassium nitrite poisoning. Higher doses of the latter (about 3·5 to 4 gm.) were required to produce death.

Experiment 9.—*Drenching of Potassium Nitrate.*

Since it had been shown that potassium nitrate as such was contained in the fresh *Tribulus* plant, it was thought advisable to dose some of the pure salt to sheep. One sheep dosed with 10 grams potassium nitrate on two consecutive days died within 6 hours after the second dose from typical methaemoglobinaemia. Another animal only died after receiving 80 grams potassium nitrate (maximum dose 20 grams) over a period of 5 days. One sheep dosed with 75 grams of potassium nitrate over a period of 14 days developed no symptoms.

It was thus shown that large doses of potassium nitrate may cause death in the same way as does the nitrite.

Experiment 10.—*Administration of Hydroxylamine Hydrochloride.*

The object of this experiment was to ascertain whether a product such as hydroxylamine, which has been detected as an intermediate in the biological reduction of nitrates to ammonia (see Blom 1928), showed any toxic effect on sheep. One sheep was dosed with 16·25 grams (maximum dose 1·5 grams) over a period of 21 days without any ill effect. Another sheep injected with 0·5 grams intravenously showed marked shock-like symptoms, but soon recovered. Three subsequent injections of 0·3 gram produced haemolysis which resulted in the death of the animal on the 6th day.

Experiment 11.—*Administration of Hydrazin Sulphate.*

One sheep was injected intravenously with 0·3 grams hydrazin sulphate daily for 11 days without showing symptoms. Thereafter it received two injections of 0·6 grams each. This produced a transitory weakness lasting two or three days. The animal was then dosed with 1 gram hydrazin sulphate daily for 8 days. The only symptom noticeable was a progressive weakness in the legs, which soon passed off after dosing had been stopped.

Experiment 12.—*Drenching of Ammonium Carbonate.*

The object here was to note whether the NH_4 ion would produce symptoms in a sheep. One sheep was dosed with 244 grams (maximum dose 24 grams) of Ammonium carbonate over 24 days. No symptoms whatever were shown, until on the last day the dose was increased from 20 grams to 24 grams. Three hours after receiving the last dose, the animal suddenly became very weak, the whole body trembling markedly, lips were parted, respirations very fast, there was stiffness in the hind limbs, and frothing from the mouth and nostrils. The animal died within 4 hours after dosing. No signs of methaemoglobinaemia were noticeable on post mortem.

Experiment 13.—*Drenching of Potassium Nitrite, Potassium Nitrate, Ammonium Carbonate, Hydrazin Sulphate, and Hydroxylamin Hydrochloride.*

One sheep was dosed with 252 grams ammonium carbonate, 81 grams potassium nitrate, 20·25 grams potassium nitrite, 20·25 grams hydroxylamin hydrochloride, and 15·75 grams hydrazin sulphate over a period of 43 days. The animal became progressively weaker, without showing any other symptoms. Death occurred on the 43rd day, the only post mortem finding being that of cachexia and general atrophy. It was hoped that by simultaneously dosing of these different nitrogenous compounds, characteristic symptoms might possibly be shown by the animal. This however is not the case.

Experiment 14.—*Drenching of Nitrobenzol.*

One sheep was dosed 0·5 c.c. nitrobenzol on two consecutive days without any ill effect. On the third day it received 2 c.c. Within 1 hour the animal was found to be *in extremis*. It died half an hour later from acute methaemoglobinaemia. Another sheep was dosed 0·5 c.c. on five consecutive days without ill effect. On the 6th day 1 c.c. was given. This caused death within one hour. Nitrobenzol was thus found to be extremely toxic to sheep, death being due to very rapid methaemoglobin formation.

Experiment 15.—*Drenching of Nitrobenzaldehyde.*

One sheep was dosed with 29 grams nitrobenzaldehyde (Maximum dose 4 grams) over a period of 20 days without showing any symptoms, except a slight haemolysis, noticed in the blood serum.

Experiment 16.—*Drenching of Amyl Nitrite.*

One sheep was dosed with 49 c.c. amyl nitrite over a period of 24 days without any symptoms. Another sheep, which received 1 c.c. amyl nitrite intravenously died from methaemoglobinaemia within a few hours.

Summary.

1. It has been demonstrated that a lethal factor is present in the fresh juice expressed from green *Tribulus* plants and in the watery extracts prepared by soaking the dried ground plant material in water for several hours.

2. Death, in each instance, is due to acute asphyxial conditions resulting from the rapid intra-corpuscular change of haemoglobin into methaemoglobin.

3. The agent responsible for this change has been identified as inorganic nitrite—chiefly potassium nitrite.

4. Nitrite as such is absent (except in traces) from the tissues of *Tribulus* plants, but is formed from pre-existing nitrate (isolated in crystalline form) under the influence of an enzymic oxidation-reduction system.

5. The properties of this system have been examined and are found to conform in general to those of other plant oxido-reductases, e.g. that of the potato.

6. The production of nitrite by the plant from added potassium nitrate has been demonstrated.

7. Methods are described for the determination of nitrate in fresh green *Tribulus* plants and in the dried ground material.

8. It has been shown that not only does the nitrate content vary widely in different species of *Tribulus* but also in different plants of the same species and growing in the same locality.

9. Prolonged feeding of fresh green or dried *Tribulus* under the Laboratory conditions failed to produce any ill-effect whereas under field conditions, in the Karroo area, as is well known, grazing of this plant may in certain circumstances give rise to outbreaks of geeldikkop, a disease characterised by an intense photosensitisation and accompanying generalized icterus.

10. Administration of sodium and potassium nitrites, nitrobenzol and amylnitrite all resulted in death from simple methaemoglobinaemia.

11. Prolonged administration of graduadly increasing doses of sodium nitrite provoked a well marked tolerance in sheep.

12. Repeated dosing over an extended period of sodium nitrite, hydroxylamine hydrochloride, hydrazine sulphate, and ammonium carbonate, substances regarded as possible intermediates in the biological formation of ammonia from nitrates failed to produce either photosensitivity or icterus.

Work is being continued in an endeavour to throw further light on the problem of geeldikkop.

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