

Studies on the Photosensitisation of Animals in South Africa. XII.—An Attempt to Identify the Ictero- genic Factor in Geeldikkop; the Reaction of Nitrous Acid on Chlorophyll.

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INTRODUCTION.

In previous articles of this series the photosensitising agent present in geeldikkop was identified as phylloerythrin, a porphyrin resulting from the normal breakdown of chlorophyll by microbial action during digestion (Rimington and Quin, 1934; Quin, Rimington and Roets, 1935). It was shown that the accumulation of this substance in sufficient quantity to produce photosensitisation was brought about by an obstruction in the bile flow (Quin, 1933; Rimington and Quin, 1934), a condition which was in turn the result of an icterogenic factor derived from ingested plant material. Although an icterogenic substance was isolated from a plant *Lippia rehmanni* (Rimington and Quin, 1937), no substance of like nature was found in *Tribulus* and thus no active principle primarily responsible for geeldikkop could be identified.

Except under certain seasonal conditions and in particular areas the *Tribulus* plants appear to be non-toxic, unless watery extracts are drenched to animals in sufficient quantity to produce nitrite poisoning through the production of a methaemoglobinaemia unassociated with haemolysis (Quin, 1930). This condition arises from the large amount of potassium nitrate normally present in the plant (Rimington and Quin, 1933; Henrici, 1938).

With regard to the formation of a toxic substance giving rise to geeldikkop, two possibilities have to be considered:—

- (a) The toxin may be preformed in the plant itself under certain specific conditions but in a form too unstable for isolation by the standard chemical methods;
- (b) The toxin may be formed from otherwise non-poisonous plant constituents through chemical changes undergone within the digestive tract of the animal, though only under specific conditions. Such interaction might be expected to take place readily under the abnormal conditions ruling in the "geeldikkop season." This second possibility is the one which has been considered in the present work.

Nitrite is produced readily by enzyme action from nitrate and would give the highly reactive nitrous acid on contact with the acid in the digestive system. Moreover, as *Tribulus* is a plant particularly rich in chlorophyll, it was considered rational to study the interaction of these two substances.

Although a large amount of work has been carried out in recent years on the structure of chlorophyll (Steele, 1937; Fischer, 1937), using oxidative, reductive and synthetic methods, no investigation has been undertaken, prior to the present work, of the reaction of nitrous acid on chlorophyll.

In this paper experiments are described in which, under certain conditions, the formation of nitrite from nitrate is shown when the latter is digested with ruminal ingesta *in vitro*. The reaction of nitrous acid on chlorophyll is then studied. This reaction takes place readily in solution, yielding four highly coloured compounds, three of them in considerable yield. The chemical and physiological investigation of these compounds is described in detail.

PRODUCTION OF NITRITE FROM NITRATE WITH RUMINAL INGESTA IN VITRO.

Aqueous solutions of glucose and potassium nitrate were digested at body temperature (39° C.) with ruminal ingesta collected from fistula sheep, tests for nitrate (diphenylamine-sulphuric acid test; Feigl 1935) and nitrite (sulphanilic acid-naphthylamine test, Feigl 1935) being carried out at regular intervals during such digestion.

First experiment.

The ruminal ingesta studied in the first experiment was derived from (a) a sheep fed regularly on poor quality grass hay and (b) a sheep on a diet of good quality lucerne hay. Each tube contained 20 ml. of ingesta. To this was added, in tubes 1, 5 ml. of 0·1 per cent. aqueous potassium nitrate and 1 ml. of 16 per cent. aqueous glucose solution and, in tubes 2, 5 ml. of 0·1 per cent. aqueous potassium nitrate solution. In all cases nitrate was used up rapidly, but whereas in (b) no nitrite could at any time be detected, in (a) there was a definite appearance of nitrite at a stage where the nitrate content had decreased considerably. The results of tests for nitrate and nitrite at measured intervals are shown in Table 1.

Second experiment.

In the second experiment use was made of the ruminal ingesta from two sheep fed on poor quality grass hay and one sheep fed on good lucerne hay. Each set of readings was taken from duplicate tubes (a) and (b). Tubes 1 contained 20 ml. of ruminal ingesta together with 1 ml. of 0·1 per cent. aqueous potassium nitrate and 1 ml. of 16 per cent. aqueous glucose solution. Tubes 2 contained 20 ml. of ruminal ingesta with 1 ml. of 0·1 per cent. potassium nitrate solution. The same result was obtained as in the first experiment, viz. the production of nitrite could be shown where the ingesta was from poorly-fed sheep. The results of tests for nitrate and nitrite are shown in Table 2.

Conclusions.

From the above tables there is evidence to indicate that, in cases where the animal is on poor diet, the nitrite—probably a normal intermediate in the synthesis of protein in the forestomachs of ruminant animals by specific bacterial activity—tends to be used up comparatively slowly. Consequently such an animal on a poor diet would be more predisposed towards systemic derangement due to the presence of nitrous acid than a similar animal kept on a diet of lucerne hay only.*

*It has since been shown, in work which will be published later, that nitrite is produced in the same way when larger amounts of nitrate are digested *in vitro* in ruminal ingesta from lucerne-hay fed sheep. In this case, however, the nitrite formed is found to disappear more rapidly than is the case when ingesta from grass-hay fed sheep is used.

TABLE 1.

Time in Minutes.	(a) INGESTA FROM GRASS-HAY FED SHEEP.				(b) INGESTA FROM LUCERN-HAY FED SHEEP.			
	Tube 1 : Nitrate + Glucose.		Tube 2 : Nitrate Alone.		Tube 1 : Nitrate + Glucose.		Tube 2 : Nitrate Alone.	
	Nitrate.	Nitrite.	Nitrate.	Nitrite.	Nitrate.	Nitrite.	Nitrate.	Nitrite.
20.....	+	—	+	—	+	—	+	—
40.....	+	—	+	—	+	—	+	—
60.....	+	—	+	—	+	—	+	—
80.....	+	—	+	—	+	—	+	—
100.....	+	—	+	—	+	—	+	—
120.....	+	—	+	—	+	—	+	—
140.....	+	—	+	—	+	—	+	—
160.....	+	—	+	—	+	—	+	—
180.....	+	—	+	—	+	—	+	—
210.....	+	—	+	—	+	—	+	—
285.....	Slight	—	+	Slight	+	—	+	—
340.....	Slight	Slight	+	Stronger	+	—	Slight	—
375.....	Slight	—	Slight	Good	+	—	Slight	—

TABLE 2.

	Time in Minutes.	INGESTA FROM GRASS-HAY FED SHEEP A.				INGESTA FROM GRASS-HAY FED SHEEP B.				INGESTA FROM LUCERNE-HAY FED SHEEP.			
		Nitrate.		Nitrite.		Nitrate.		Nitrite.		Nitrate.		Nitrite.	
		a.	b.	a.	b.	a.	b.	a.	b.	a.	b.	a.	b.
Tubes 1: Nitrate and Glucose.....	30	+	+	-	-	+	+	-	-	-	-	-	-
	60	+	+	-	-	+	+	-	-	-	-	-	-
	90	+	+	-	-	+	+	-	-	-	-	-	-
	120	Slight +	Slight +	-	-	Slight +	+	-	-	-	-	-	-
	150	Very Slight +	Very Slight +	-	-	-	-	Slight +	-	-	-	-	-
Tubes 2: Nitrate only...	30	+	+	-	-	+	+	-	-	-	-	-	-
	60	+	+	-	-	+	+	-	-	-	-	-	-
	90	+	+	-	-	+	+	-	-	-	-	-	-
	120	+	+	-	-	+	+	-	-	-	-	-	-
	150	Slight +	Slight +	-	Slight +	Slight +	Slight +	Slight +	Slight +	Slight +	Slight +	Slight +	Slight +

THE REACTION OF NITROUS ACID ON CHLOROPHYLL.

A. The Reaction.

The chlorophyll used in the experimental work was a commercial powdered product supplied by Messrs. Hodgkinson, Prestons and King, London. Although this sample is still to be analysed, it could normally be expected to contain a small proportion of impurities (xanthophyll, fatty substances, etc.) and chlorophyll a and b roughly in the proportion of 3 to 1.

In preliminary experiments chlorophyll was suspended in accurately measured quantities of very dilute hydrochloric acid, cooled in a freezing mixture and measured quantities of sodium nitrite added gradually with the application of mechanical stirring. The process was conducted using from 1 to 10 mols. of nitrous acid per mol. of chlorophyll, but no appreciable reaction took place. Since, however, any interaction between nitrous acid and chlorophyll in the digestive tract of an animal would take place in the presence of possible solvents (organic acids) and catalysts (enzymes, etc.), it was decided to continue the experiments using a solvent for the chlorophyll. Chlorophyll, when dissolved in chloroform, was found to react very readily with nitrous acid. The procedure adopted was as follows:—

Ten grams of chlorophyll were dissolved in 300 ml. of chloroform and 200 ml. of 3 per cent. hydrochloric acid were added; then 12 grams of sodium nitrite in 50 ml. of water were added, with vigorous shaking, over a period of about half a minute. The chloroform layer, which underwent a striking colour change from green to dark red over this period, was separated immediately, washed with two portions of water, filtered to remove water and evaporated. There remained 10 grams of solid, consisting of a mass of needle-like crystals. This material appeared yellow-brown in transmitted light and greenish in reflected light and was found to consist of a certain amount of unchanged chlorophyll together with at least four definite compounds, which for convenience, are referred to below as "A", "B", "C" and "D".

B. Separation of the Reaction Products.

A and D were separated from the mixture by a chromatographic method. When the solution of the reaction products in chloroform was passed through a column of activated alumina, the chromatogram, developed with chloroform, consisted of the following:—

- (i) Various blue, green and yellowish-green bands, apparently due to residual plant colouring matters;
- (ii) A deep red band, shown later to consist of an adsorbed aluminium salt of A; and
- (iii) A somewhat scattered yellow area, consisting of an adsorption of D or its aluminium salt. B and C were not adsorbed and were the main constituents of the solution after it had passed through the column. The bands containing A and D were separated and the separate substances recovered from them by boiling with water containing a little sodium carbonate, filtering and acidifying the coloured filtrate. The finely divided substances thus precipitated were extracted with chloroform.

The chloroform solution containing B and C, after the chromatographic separation, was evaporated and B extracted from the residual solid by boiling with several batches of 50 per cent. aqueous alcohol, in which solvent C was found to be practically insoluble.

The quantities of the compounds obtained from 10 grams of chlorophyll in several experiments were:—

A:	1—1·5 grams.
B:	3—5 grams.
C:	1·5—2 grams.
D:	0·03 grams.

C. Purification and Properties of the Compounds.

Compound A.

Purification.

The compound was dissolved in a small amount of chloroform, absolute alcohol added and the solution boiled to remove most of the chloroform. The pure material separated on cooling.

Physical Properties.

The pure compound consists of odourless, Indian-red prisms, appearing light yellow-green in transmitted light. Its melting point is 151-153° C. (corrected). It is very soluble in pyridine, benzol and chloroform (red or brown-red solutions) and soluble in alcohol (brown solution) and in ether (yellow solution). It is soluble also in 50 per cent. aqueous alcohol. It dissolves in molten camphor, giving a red solution and in concentrated sulphuric acid, giving an orange-red solution. It is insoluble in either dilute or concentrated hydrochloric acid but dissolves in a mixture of concentrated hydrochloric acid and alcohol. Compound A cannot be either steam-distilled or sublimed in a vacuum.

Tautomerism and Salt Formation.

Compound A dissolves in dilute alkalis to give red solutions, from which it is precipitated by hydrochloric acid in the form of orange rhombs of melting point 147—151° C. (corrected). Evaporation of a solution of A in dilute caustic soda yields dark, purple-red prisms of melting point 146-150° C. (corrected.) This substance was shown to contain no sodium and to have all the other properties of the original compound; it would appear to be simply an acid form of the compound A. From a dilute potassium hydroxide solution, however, a very dark red potassium salt, decomposing explosively at 268-270° C., is obtained by evaporation. It is concluded from these results that compound A has two forms, probably exhibiting a nitroso-isonitroso tautomerism. Neither of the two forms could, however, be obtained quite free from the other.

That compound A is adsorbed on alumina in the form of an aluminium salt was shown by elution of the adsorbed material with water. Evaporation of the eluate yielded a dark red solid, without melting point. On the addition of dilute hydrochloric acid to the aqueous eluate and removal of the precipitated compound A by filtration, the filtrate was found to contain aluminium chloride.

Addition of alcoholic silver nitrate solution to an alcoholic solution of A produces a dark red silver salt which, however, on washing with water, seems to become largely hydrolysed.

No definite platinumchloride of A could be obtained.

Indications of structure.

When an alcoholic solution of compound A is treated with an alcoholic solution of mercuric chloride, no precipitate is formed. Addition to the mixture of one drop of N/10 potassium hydroxide solution gives a voluminous orange precipitate. This reaction possibly indicates that A is a tetra-substituted pyrrole derivative (Küster, 1922), which is in agreement with the likelihood that part of the porphyrin structure of chlorophyll would be preserved in it.

Molecular Formula.

From several micro-analyses, A has tentatively been given the molecular formula $C_{14}H_{13}N_3O_3$. Analytical figures are given below:—

	Carbon Per Cent.	Hydrogen Per Cent.	Nitrogen Per Cent.	Molecular Weight (Rast).
Found:—				
1.....	61.91	4.93	16.2	252
2.....	62.18	4.75	16.2	261
3.....	62.50	4.80	15.4	255
4.....	62.26	4.66	15.4	282
Calculated for $C_{14}H_{13}N_3O_3$	61.98	4.80	15.5	271

Acetyl Derivative.

0.2 gram of A was dissolved in 5 ml. of pyridine, 2 ml. of acetic anhydride added and the mixture refluxed gently for one-half hour. The liquids were then removed by fanning and the residual solid recrystallised from absolute alcohol. This gave 0.155 gram of orange-coloured prisms, with a sharp melting point of 113.5-115° C. (corrected), soluble in benzol, pyridine, chloroform, alcohol and in molten camphor. Micro-analyses indicated the addition of one acetyl group:—

	Carbon Per Cent.	Hydrogen Per Cent.	Nitrogen Per Cent.	Molecular Weight (Rast).
Found:—				
1.....	61.69	5.17	12.9	298
2.....	61.42	5.01	13.2	272
Calculated for $C_{16}H_{15}N_3O_4$	61.35	4.79	13.4	313

Hydrolysis of the acetyl derivative with alcoholic potash rapidly regenerated the original compound A (Melting point and mixed melt, appearance, etc.).

Quantitative hydrolysis with standard alcoholic potash also indicated the presence of one acetyl group. For this determination a weighed quantity of the acetyl derivative was refluxed with a measured excess of decinormal alcoholic potash for two hours. After cooling, the solution was diluted with water, a

measured excess of decinormal sulphuric acid added and the precipitated compound A filtered off. The filtrate and washings were then titrated with decinormal alcoholic potash, using phenolphthalein as indicator.

Weight of acetyl derivative used: 0.0916 gram.

	N/10 Alcoholic Potash, ml.	COCH ₃ Group, Per Cent.
Found.....	2.99	14.0
Calculated for C ₁₄ H ₁₂ N ₃ O ₃ (COCH ₃).....	2.93	13.7

Compound B.

Purification.

B could be purified by dissolving in boiling N/5 caustic soda solution and acidifying with dilute hydrochloric acid. A better method of purification, however, was found to be by sublimation *in vacuo*. Temperatures of 100 to 130° C. were applied in conjunction with a pressure of 20 to 30 mm.

Physical Properties.

The sublimed product consists of large crimson needles, of melting point 103.5-105° C. (corrected). It is readily soluble in pyridine and chloroform (red solutions), benzol and ether (reddish-brown solutions) and is soluble in alcohol as well as in hot 50 per cent. aqueous alcohol (yellow or brownish-yellow solutions). It dissolves in molten camphor to give an orange-red solution. It gives a yellowish solution in concentrated hydrochloric acid but is almost insoluble in dilute hydrochloric acid. The solution in concentrated sulphuric acid is deep red. Compound B has an odour reminiscent of that of o-nitrophenol. It sublimes readily near its melting point and is volatile in steam.

Tautomerism and Salt Formation.

Like compound A, compound B seems to exist in both an acid and an alkaline form. When the material is sublimed, the first trace of sublimate is light yellow and this darkens to red as more material collects. The yellow form, precipitated from solutions of B in dilute alkali by the addition of dilute hydrochloric acid, melts at 100-104° C. (corrected) with the partial formation of the red form. When a hot solution of B in dilute alkali is cooled, the red form is deposited, having a sharp melting point of 103-104° C. (corrected). It would appear from these results that the yellow (alkaline) form is the lower melting and the red (acid) form the more heat-stable. Neither form could, however, be obtained free from the other. Since B is formed by a reaction with nitrous acid, it is probable that its tautomerism represents a change between nitroso and isonitroso structure.

B dissolves slightly in cold dilute sodium and potassium hydroxides and more readily on warming. The solutions are brown-red in colour and on evaporation they give no salts but only the original compound in its red form. No silver salt or platinichloride could be obtained from B by the ordinary methods.

Indications of structure.

An alcoholic solution of B treated with alcoholic mercuric chloride solution and then one drop of N/10 potassium hydroxide gives a similar precipitate to that obtained from compound A, indicating the possibility of a tetra-substituted pyrrole structure.

Molecular Formula.

A molecular formula of $C_{14}H_{16}N_2O$ is tentatively proposed for compound B, based on several micro-analyses, as shown below:—

	Carbon Per Cent.	Hydrogen Per Cent.	Nitrogen Per Cent.	Molecular Weight (Rast).
Found:—				
1.....	73·89	6·41	13·1	225
2.....	73·81	6·39	12·8	213
3.....	73·89	6·28	12·6	210
4.....	73·55	6·00	12·4	200
5.....	73·57	6·11	12·7	202
6.....	73·82	6·09	12·5	194
Calculated for $C_{14}H_{16}N_2O$	73·69	7·02	12·3	228

Acetyl Derivative.

0·2 Gram of B in 5 ml. of pyridine was refluxed with 2 ml. of acetic anhydride for half-an-hour. After fanning off the liquids, the gummy product was induced to crystallise by seeding from a small trial sample which had taken about four months to crystallise. The product was twice recrystallised from small amounts of absolute alcohol, in which it was appreciably soluble in the cold, seeding being again necessary to induce crystallisation. 0·108 Gram of deep orange, capped prisms was obtained, with a melting point of 76-79° C. (Corrected).

Determinations of nitrogen and molecular weight and quantitative hydrolysis indicated the presence of one acetyl group. The hydrolysis was conducted exactly as for the acetyl derivative of compound A, using 0·2292 gram of the acetyl derivative:—

	Nitrogen Per Cent.	Molecular Weight.	N/10 Alc. KOH, ml.	$COCH_3$ group Per Cent.
Found.....	10·4 10·5 10·3	271 253	8·45	15·9
Calculated for $C_{14}H_{15}N_2O(COCH_3)$	10·4	270	8·49	15·9

The hydrolysis product was shown to be identical with the original compound B (melting point, mixed melt, appearance, etc.).

*Compound C.**Purification.*

Compound C was crystallised from glacial acetic acid, which freed it from any unchanged plant colouring matters which had escaped adsorption. It was then further purified by recrystallisation from chloroform-absolute alcohol.

Physical Properties.

Compound C consists of indigo-coloured needles, with a melting point of 219-222° C. (corrected). It has no smell and is non-volatile. It dissolves readily in pyridine, benzol and chloroform to give deep greenish-blue solutions and is soluble in ether and slightly soluble in cold alcohol to give clear blue solutions. The solution in molten camphor is deep blue and even in concentrations of 1:20 is too dark for observation of melting point depression in the Rast method for determining molecular weight. With concentrated sulphuric acid compound C gives a deep purple solution which turns blue on careful dilution with water. C is insoluble in hot 50 per cent. aqueous alcohol and is quite insoluble in hydrochloric acid of any strength.

Indications of structure.

Compound C is insoluble in alkalis and does not exhibit either tautomerism or change of form, as A and B do. It yields no acetyl derivative with acetic anhydride.

C gives a positive result in the test with alcoholic mercuric chloride and N/10 potassium hydroxide, the precipitate resembling those obtained with A and B thus it is probable that C is a tetra-substituted pyrrole derivative. This is the only deduction made up to the present with regard to the structure of compound C.

Empirical Formula.

The molecular weight of compound C has not yet been determined. As a result of several micro-analyses, the empirical formula of the compound has been tentatively fixed as $C_{13}H_{10}NO$ or $C_{13}H_{11}NO$:—

	Carbon. Per Cent.	Hydrogen Per Cent.	Nitrogen Per Cent.
Found :—			
1.....	79.45	5.20	7.20
2.....	79.07	5.27	6.78
3.....	79.36	5.03	7.12
4.....	79.02	4.87	7.23
Calculated for $C_{13}H_{10}NO$	79.58	5.10	7.14
Calculated for $C_{13}H_{11}NO$	79.18	5.58	7.11

Comparison of the Properties of A, B, C, and D.

The main properties of A, B, C and D are listed hereunder in tabular form:—

Property.	A.	B.	C.	D.
Colour.....	Indian-red by reflected light; pale yellow-green in transmitted light.	Crimson	Indigo	Amber.
Crystalline.....	Prisms	Needles	Needles.	Plates.
Odour.....	Odourless.	Smells like o-nitrophenol.	Odourless.	Smells like o-nitrophenol.
Volatility.....	Non-volatile	Sublimes and steam-distils.	Non-volatile.	—
Melting point.....	151–153° C.	103.5–105° C.	219–222° C.	(18–28° C.)
Solubility in:—				
Pyridine.....	Very soluble.	Very soluble.	Very soluble.	Soluble.
Benzol.....	Very soluble.	Very soluble.	Very soluble.	—
Chloroform.....	Very soluble.	Very soluble.	Very soluble.	Soluble.
Ether.....	Soluble.	Very soluble.	Soluble.	Soluble.
Alcohol.....	Soluble.	Soluble.	Soluble.	Insoluble.
50 per cent. Alcohol.....	Soluble.	Soluble hot.	Insoluble even hot.	—
Conc. HCl.....	Insoluble.	Soluble.	Insoluble.	—
Dil. HCl.....	Insoluble.	Insoluble.	Insoluble.	—
Conc. H ₂ SO ₄	Soluble.	Soluble.	Soluble.	Soluble.
Tautomerism.....	Soluble.	Soluble.	Soluble.	Soluble.
Salt formation.....	Potassium salt, no sodium or stable silver salt, no platinumchloride.	Acid-alkali forms.	No tautomerism observed.	—
Reaction with Acetic Anhydride.....	Mono-acetyl derivative, orange prisms, M. Pt. 118.5–115° C.	Mono-acetyl derivative, deep orange prisms, M. Pt. 76–79° C.	No acetyl derivative.	—
Formula Assigned.....	C ₁₄ H ₁₃ N ₃ O ₃ .	C ₁₄ H ₁₆ N ₂ O.	C ₁₃ H ₁₀ NO or C ₁₃ H ₁₁ NO (Empirical formula).	—
Structure.....	Probably tetra-substituted pyrrole derivative.	Probably tetra-substituted pyrrole derivative.	Probably tetra-substituted pyrrole derivative.	—

Compound D.

Up to the present, too small an amount of this compound has been obtained for detailed investigation. The properties of the compound, as obtained by the elution of its adsorption band, are as follows:—

D is an amber-coloured liquid, with a strong odour resembling that of 0-nitrophenol. It solidifies on cooling with ice to yellow plates which melt from about 18 to 28° C. It is soluble in pyridine and in molten camphor to give yellow solutions. It crystallises from alcohol on cooling with ice, but with no improvement of the melting point. It is apparently insoluble in 50 per cent. aqueous alcohol. D is found to contain nitrogen.

Colour Illustration.

The colour plate gives a comparison of the colours and crystalline forms of most of the compounds described above. In the circles are shown the substances as seen under the microscope with ocular No. 2 and objective a₁ (magnification approximately 15x), whilst in the lower illustration the compounds are shown in tubes as seen with the naked eye. The compounds represented are:—

- 1: Compound A.
- 2: Compound B.
- 3: Compound C.
- 4: A, acetyl derivative.
- 5: B, acetyl derivative.
- 6: A, potassium salt.

Tests on the compounds A, B, C and D for Icterogenic and Photosensitising Properties.

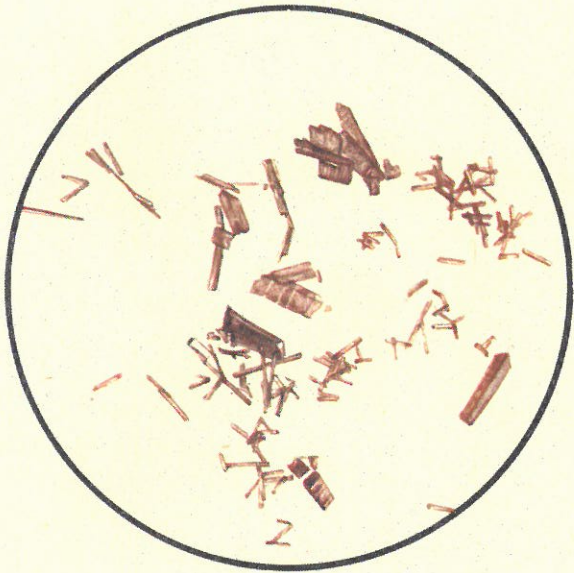
1. Quantities of 0.05 to 0.1 gram of A, B and C, dissolved in each case in 2 ml. of pyridine, were administered separately to sheep by subcutaneous injection. For five days after the injections the sheep were kept in bright sunlight. In no case was photosensitisation induced nor was there any visible effect on the blood serum to indicate the development of jaundice.

2. 1 Gram of B was administered to a sheep *per os*. The sheep was thereafter kept in bright sunlight for several days, but no photosensitisation resulted and no bilirubinaemia was noted in observation on the blood serum.

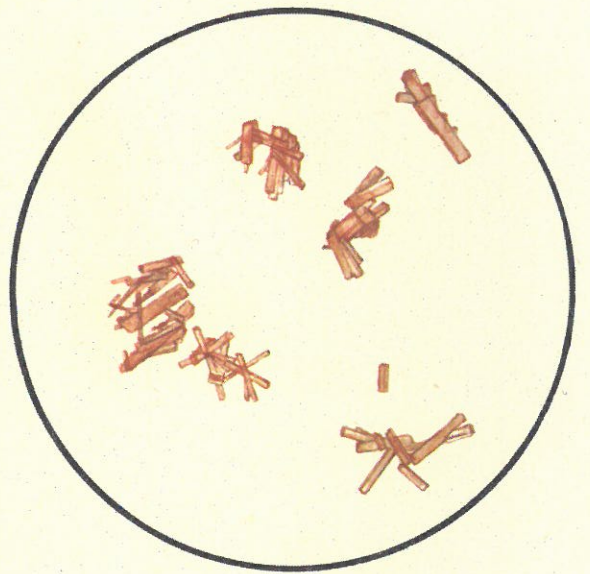
3. The reaction product from 5 grams of chlorophyll treated with nitrous acid was administered, without separation of the constituents, to a sheep *per os*. This mixture would contain:—

- A: 0.5 to 0.75 gram.
- B: 1.5 to 2.5 grams.
- C: 0.75 to 1 gram.
- D: about 15 milligrams.

The sheep was thereafter kept in bright sunlight for several days, without, however, any photosensitisation or bilirubinaemia appearing.



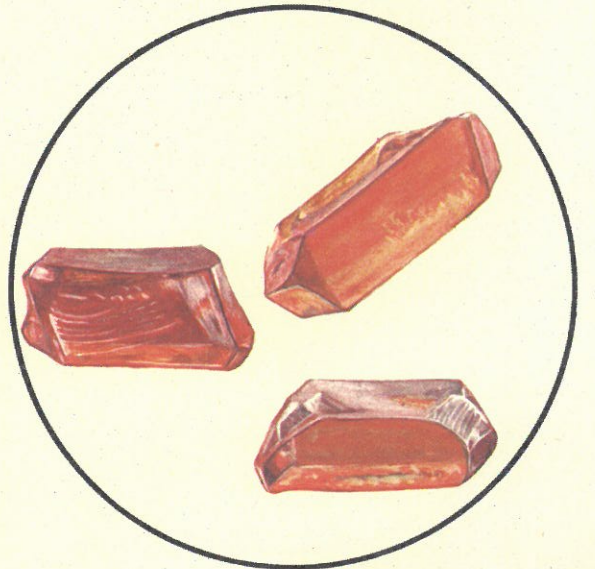
1



4



2



5



3



6



1



2



3



4



5



6

SUMMARY.

1. The reaction of nitrous acid on chlorophyll yielded four compounds, denoted for convenience as A, B, C and D.
2. The compounds are all distinctive in appearance, are highly coloured and three of them have sharp melting points.
3. A convenient process of separation of the compounds and easy methods for the purification of A, B and C have been found.
4. A, B, and C all appear to be tetra-substituted pyrrole derivatives. A and B each exhibit tautomerism, probably of the nitroso-isonitroso type, whereas C appears to have only one form.
5. A and B form mono-acetyl derivatives, but C yields no derivatives of this type.
6. Formulae have been tentatively proposed as follows:—
 - A (Molecular formula): $C_{14} H_{13} N_3 O_3$.
 - B (Molecular formula): $C_{14} H_{16} N_2 O$.
 - C (Empirical formula): $C_{13} H_{10} N O$ or $C_{13} H_{11} N O$.
7. None of the compounds exhibited icterogenic or photosensitising properties when administered to sheep, either by subcutaneous injection or by oral administration.

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