

## Studies on the Photosensitisation of Animals in South Africa.

### VII The Nature of the Photosensitising Agent in Geeldikkop.

By

CLAUDE RIMINGTON, M.A., Ph.D., B.Sc., A.I.C., Research  
Fellow under the Empire Marketing Board, and

J. I. QUIN, D.V.Sc., Veterinary Research Officer, Onderstepoort.

#### INTRODUCTION.

In the first article of this series it was indicated that outbreaks of "geeldikkop" amongst small stock in the Karroo areas could definitely be ascribed to excessive feeding on *Tribulus* during certain periods of the year. Beyond this, very little is known about the factors rendering the plant toxic or of the toxic principles concerned. This is primarily due to the insidious nature of the disease and the difficulty of carrying out experiments under natural conditions. Thus a pasture which is generally considered excellent for sheep, suddenly becomes extremely toxic. Furthermore, the problem is complicated by the fact that outbreaks of a disease, which appears in all respects to be identical with geeldikkop, may at times be encountered on grass pastures and even in lucerne paddocks where *Tribulus* can be excluded. When, however, any of these suspected plants, including *Tribulus*, are fed to sheep under laboratory and even under field conditions, the results usually obtained are either negative or insignificant. Where watery extracts of *Tribulus* are dosed, as shown in the second article, death may result from methaemoglobinuria. This was found to be due to the large amount of nitrite present in such extracts. Clinically, however, geeldikkop appears with a sudden onset of oedema of the exposed and unpigmented parts of the head and accompanied by definite signs of photosensitisation. This is soon followed by a progressive generalised icterus first evident on the visible mucous membranes, and subsequently on the skin. The urine too is deep yellow. In chronic cases the affected skin of the face and ears undergoes necrosis and subsequent sloughing. The blood serum by this time is deep yellow and shows a strongly positive direct van den Bergh reaction. At post-mortem, the most striking finding is the intense icterus, together with enlargement of the gall bladder, although the common bile duct is always found patent. The liver especially is deeply bile-stained.

The problem therefore resolved itself into an investigation of the nature of the photosensitising and icterogenic factors and the genesis of the symptoms and lesions seen in the disease. Once this was achieved it was hoped that rational means of prophylaxis would present themselves.

On account of the disappointing results obtained with plant material supposed to be toxic, one was forced to approach the problem by various indirect means. Reports of this series of investigations were published in the Onderstepoort Journal of Veterinary Science and Animal Industry, Vol. I, No. 2.

In the 6th article, the photosensitisation following ligation of the bile ducts in sheep was described, the cause of which, however, was unknown at the time. Since then, further work on this phenomenon of photosensitisation has been carried out on the bile, blood and faeces of operated sheep as well as on the same materials collected from naturally occurring cases of geeldikkop. In this article it is intended to report upon the findings which led to the identification of the photosensitising principle.

Photosensitisation, or acute sensitivity of the exposed parts of the body to the light of the sun, is a phenomenon well known to occur after the injection of many fluorescent dyestuffs, including the porphyrins, and also following the ingestion by animals of certain green plants. Of the latter, various species of *Hypericum* (St. John's wort) and of *Fagopyrum* (buckwheat) are best known. In the case of the former, it has been proved experimentally that there is present in the plant a pigment ("hypericin") which is capable of causing direct photosensitivity when injected into the blood stream.

*Tribulus* plants, including specimens taken from a farm where geeldikkop was active, were examined for the presence of fluorescent colouring matters of the hypericin type, but no trace of such could be found. The photosensitising pigment had consequently to be looked for in the animal's body, not in the external plant material.

#### THE PHOTSENSITISING FACTOR IN EXPERIMENTAL BILIARY OBSTRUCTION CASES.

In these investigations sheep were used as experimental animals and the operations carried out as described elsewhere. The animals' diet consisted of fresh green lucerne supplemented by a little dry hay and crushed maize. Blood was withdrawn from animals exhibiting sensitivity and also from control normal sheep in the same camp and an examination made for porphyrin-like substances. In other cases, sensitive and control animals were slaughtered and the chief organs worked up for porphyrin. The methods employed were largely those of Fischer and his collaborators in their post-mortem investigation of the human porphyrinuric Petry, or procedures based upon these. (Fischer, Hilmer, Lindner and Pützer, 1925; Fischer and Zerweck, 1924.)

Typical experiments are the following:—

Comparison of blood of normal and sensitive sheep from the same camp. Three sensitive and three controls used yielding 500 c.c. of blood from each group. Serum and corpuscles worked up separately.

Corpuscles: Normals. Extract in 1 c.c. of ether shows  $\pm$  fluorescence, no spectrum.

Sensitive. Extract in 2 c.c. of ether showed red fluorescence in ultra-violet light: absorption spectrum \* was—

in ether  $\overbrace{635; 600-575; 565-555; 525-520}$  Order III, II, IV, I;  
                   I          II          III          IV

in 25% HCl  $\overbrace{600-595; 570}$  Order II, I.  
                   I          II

\* Absorption spectra measured by a Zeiss direct-vision pocket spectroscope. Liquid layer 2 cm.

Serum: Normals. Extract in 0.5 c.c. ether: no fluorescence, no spectrum.

Sensitive. Ether extract shows deep red fluorescence and following absorption spectrum—

$\overbrace{630; 595; (583); 578; 557; 527-525}$   
                   I          II          III          IV

Order III, II, IV, I.

On another occasion, 19 c.c. of serum from the same three sensitive sheep was worked up with the following results:—

Extract in 2 c.c. of ether was brownish pink in colour and showed an intense red fluorescence. Spectrum:

$\overbrace{635; (620); 600-590; 565-555; 520}$  Order III, II, IV, I.  
           I          II          III          IV

The pigment was esterified in methyl alcoholic hydrochloric acid solution and crystallised from chloroform-methyl alcohol yielding a small quantity of microscopic prisms.

In chloroform solution, these exhibited the following spectrum:

$\overbrace{632; 595; 577; 560}$  Order III, II, I.  
           I          II          III

The characteristics of this pigment, present only in the blood of sheep which are actually photosensitive at the time of bleeding or slaughter, indicate that it belongs to the class of porphyrins.

It was also demonstrated that in sensitive animals the photo-active pigment is confined almost entirely to the serum, well washed corpuscles yielding only a trace of a porphyrin in all probability identical with protoporphyrin (compare Hymans van den Bergh, Grotepass and Revers, 1932).

The result of the examination of organs taken from sensitive and non-sensitive animals was unfruitful. In no case was an unusual pigment detected.

Some comparative experiments were made with sheep poisoned by the administration of lead acetate. In these cases examination of the blood revealed the presence of considerable quantities of a porphyrin having the properties, absorption spectra, etc., of proto-porphyrin. In no case did the animals exhibit photosensitivity.

Attention was next directed to an examination of the bile. Bile fistulae were introduced into a number of sheep and the secretion collected in bottles strapped to the animal's bodies, a little toluol being added to prevent putrefaction. It was found that even in normal bile, taken from the gall bladder at the time of operation, a small quantity of the same porphyrin-like material was present as had been detected in the blood serum of photosensitive sheep. After the operation in which the fistula was inserted, however, a pronounced rise in the concentration of this pigment was observed in nearly every case. The final concentration, reached in about 4 to 12 days, was anything from 3 to 20 times the normal pre-operative concentration of pigment.

Quantitative comparison was made in the following manner: A representative sample of normal bile was obtained by combining the contents of the gall bladders of healthy laboratory sheep passing through the post-mortem room. In all 450 c.c. was obtained and the porphyrin extracted from this by the usual method. From the hydrochloric acid solution, the pigment was passed back to ether and the volume of the ethereal solution adjusted to 450 c.c. A solution was thus obtained having a pale pinkish-brown colour and exhibiting a well-marked fluorescence in ultra-violet light. When examined in a 2 cm. layer, the absorption band at 560  $m\mu$  was just plainly visible but no trace was seen of the remainder of the absorption spectrum owing to the relative weakness of these bands. Such a solution (found afterwards to contain 12 mg. of the pure pigment per litre) was adopted as the comparison standard of normal bile and other bile samples evaluated by diluting the ethereal solutions of their contained porphyrin until the intensity of the 560  $m\mu$  absorption band matched that of the standard solution. Such a comparison is admittedly rather rough, but in view of the wide fluctuations encountered in porphyrin concentration, was deemed to be of sufficient accuracy. If, for example, the porphyrin from 20 c.c. of bile had to be diluted in ether, to 40 c.c. to match the standard, this concentration was spoken of as "2 bile units", and so on.

The following experiments are selected as illustrating, in a typical manner, the rise in bile porphyrin following the fistula operation. It will be noted that the significant increase occurred within just that space of time that was found requisite, previously, for bile-ligature animals to become sensitive.

*Sheep 32979.*

Gall bladder sample	Vol. 11 c.c.	contained 1.1 units of porphyrin.
15.5 hrs. after operation	105 c.c.	,, 1.1 ,, ,,
39.5	,, ,, 118 c.c.	,, 7.2 ,, ,,
63.5	,, ,, 177 c.c.	,, 9.0 ,, ,,
87.5	,, ,, 224 c.c.	,, 4.3 ,, ,,
		Died.

*Sheep* 28632.

	Gall bladder sample	Vol. 5.5	c.c.	contained	1.6	units of porphyrin.
	18 hours after operation	150	c.c.	„	1.4	„ „
84	„ „	170	c.c.	„	11.0	„ „
108	„ „	180	c.c.	„	11.0	„ „

etc.

*Sheep* 35329.

	Gall bladder sample	contained	...	1.4	units of porphyrin.
	6 hours after operation	contained		1.5	„ „
24	„ „	„ „	„	3.0	„ „
2	days	„ „	„	6.6	„ „
3	„ „	„ „	„	17.3	„ „
4	„ „	„ „	„	33.6	„ „
5	„ „	„ „	„	56.0	„ „
6	„ „	„ „	„	30.5	„ „
7	„ „	„ „	„	15.5	„ „
8	„ „	„ „	„	60.0	„ „
9	„ „	„ „	„	12.5	„ „
10	„ „	„ „	„	12.5	„ „
11	„ „	„ „	„	12.5	„ „

That some external condition such as diet was the factor responsible for the increase in porphyrin concentration after operation appeared most probable, and was at a later stage abundantly proved to be the case. It was our practice to offer the animals a liberal supply of green stuff immediately following the operation since on the ordinary ration they frequently lost appetite. Some incidental observations upon the bile of other animal species may be briefly recorded here. The findings are all in harmony with the thesis that the porphyrin originates from the green feed.

*Horse bile.* Sample taken post-mortem volume 5 c.c.; golden-brown in colour, mixed with a little blood. Extract in 2 c.c. of ether

showed  $\overline{598; 560; 525}$  Order II, I, III

I    II    III

in 25% HCl 610; 565.

Porphyrin obtained in crystalline condition from chloroform.

*Ox bile* (No. 4225) sample taken post-mortem volume 120 c.c.; contained 3 units of porphyrin. Spectrum in ether

$\overline{598-580; 560; 525}$  Order II, I, III.

I    II    III

*Milk-fed calf.* Sample taken from gall bladder at operation (ligature of bile duct). Porphyrin nil. Animal did not become sensitive.

*Dog's bile.* White bull terrier. Sample taken at operation (ligature of bile duct); spectrum in ether (greenish-blue).

$\overline{660-630; 595-580; 535; (500)}$ . Animal did not become sensitive.

*Cat's bile.*—Sample taken post-mortem, combined from 4 young cats, volume 5 c.c. Porphyrin nil.



The characteristics of the porphyrin present in normal and fistula sheeps' bile were as follows:—

Normal sheeps' bile. Spectrum in ether  $\overbrace{635; 593; 578; 560; 525}$

I            II            III    IV

Order III, II, IV, I.

In 25% HCl  $\overbrace{605; 570}$  Order II, I.

I            II

Fistula bile. Spectrum in ether  $\overbrace{630; 595; 578; 558; 525}$ .

I            II            III    IV

Order III, II, IV, I.

In 25% HCl  $\overbrace{620-600; 580-555}$  (525) Order II, I.

610            570  
I                II

The pigment passed completely into chloroform from hydrochloric acid solution on repeated shaking with the solvent. The chloroform solution was a deep purplish-crimson in colour and left the pigment in a crystalline state on evaporating spontaneously at room temperature. Washing of the residue with ether removed a small amount of accompanying pigment showing a pronounced absorption band in the region of 650  $m\mu$ . The porphyrin itself was insoluble in ether, sodium carbonate, water or alcohol, very sparingly soluble in chloroform but readily soluble in glacial acetic acid or in pyridine.

That this pigment was actually responsible for the photosensitisation noticed in the experimental animals was proved by tying off the fistula tube and so causing an obstruction icterus as exemplified in the following experiment:—

Sheep 35326 was operated upon and a fistula tube inserted into the ductus choledochus. The secretion of bile was collected in a bottle and analysed daily. After some days the porphyrin concentration was 9 units and remained at this level without sign of altera-

tion. The spectrum in ether was  $\overbrace{630; 595; 578; 558; 525}$ . The fistula tube was then closed externally by ligature. Within 24 hours the animal had become markedly photosensitive, the blood plasma was yellow in colour and gave a direct van den Bergh reaction, whilst signs of clinical icterus were also visible. 7 c.c. of serum was worked up for porphyrin and sufficient pigment obtained to show the following spectrum in about 0.5 c.c. ether:—

$\overbrace{596; 578; 560; 525-520}$ .

It fluoresced strongly in ultra-violet light.

### THE PHOTOSENSITISING PIGMENT IN GEELDIKKOP.

At about this stage in the work, the opportunity arose of investigating a fairly severe outbreak of geeldikkop on the farm Dassiespoort, in the Fauresmith area of the Karroo. Although the disease had nearly subsided by the time the affected area was reached, we were able to select several cases typical of the chronic, advanced

stage with sloughing lips and ears, also some animals still in the earlier stages. Specimens were slaughtered and samples brought back to the laboratory for chemical examination. Examination of the *Tribulus* growing in the affected paddocks, showed that the plants were small and stunted, moderately parasitised by a weevil-like grub but otherwise in no way peculiar. Chemical tests for alkaloïds and nitrites were negative. It was not possible to carry out extensive feeding trials.

The chemical post-mortem findings on the animals may be summarised as follows (examination of the organs were negative; description therefore omitted):—

Blood serum: In every case examined, deep yellow. Positive direct van den Bergh reaction.

Case 1 (of approximately 48 hours standing). Serum from 660 c.c. of blood worked up for porphyrin. Extract in ether had a brownish colour and exhibited well marked fluorescence in ultra-violet light. Spectrum  $\overbrace{630; 595; 577; 560; 525}$  Order III, II, IV, I.

I            II            III    IV

Case 2 (chronic). Serum from 660 c.c. of blood worked up for porphyrin. Ether extract pinkish-brown; strong fluorescence.

Spectrum  $\overbrace{633; 595; 578; 560; 523}$  Order III, II, IV, I.

I            II            III    IV

The combined pigment from cases 1 and 2 was esterified in methyl alcohol-hydrochloric acid and passed into chloroform. Upon evaporation, the chloroform solution deposited a small quantity of microscopic prisms but the amount was too small to permit of analysis.

Corpuscles: The red-cell volume in all cases was found to be normal. There was no indication of any extensive haemolysis having taken place.

Oedema fluid: 30 c.c. of yellow fluid obtained from the intra-mandibular space was examined for porphyrin but the result was negative.

Bile: The chemical examination of the bile afforded very striking results. From the gall bladder of case 2 was obtained 25 c.c. of dark, brownish-green, rather viscid bile. This was worked up for porphyrin in the usual way. The final ether solution (100 c.c.) was of an intense rose-red colour and exhibited the following spectrum:—

$\overbrace{635; 593; 578; 565-555; 530-515}$  Order III, II, IV, I.

I            II            III            IV

It was found that a concentration of 9 per cent. of hydrochloric acid was necessary to remove most of the pigment from the ether (i.e. its acid number was in the region of 9).

In 25 per cent. HCl the spectrum was:—

610-600; 595; 580-555 Order III, I, II.  
           I      II      III

On evaporating the acetic acid-ether solution of the pigment at room temperature, a crystalline deposit of fine prisms was obtained (see Fig. I). These, together with a similar crop from bile of case 1, were converted into the methyl ester, freed from fatty material by washing with petroleum ether, and taken up in chloroform.

Spectrum of ester in chloroform 630; 595; 560; 530-525 Order III, II, IV, I.  
   I    II    III    IV



Fig. 1.—Porphyrin from dikkop bile (Dassiespoort) crystallised from acetic acid ether. Magn. 175 $\times$ .

From the gall bladder of case 1 was obtained 20 c.c. of bile similar in appearance to that of case 2. It was very rich in porphyrin, the ether solution exhibiting the following spectrum:

635; 598; 580; 560; 530-520; (492) Order III, II, IV, I.  
           I      II      III    IV



The crude pigment could be extracted from 25% HCl by shaking with chloroform and was deposited from this solvent in clusters of fine needle-like crystals very similar in appearance to those similarly obtained from fistula bile crude porphyrin (see Fig. 4).

Urine: The urine from all three cases appeared to be free from porphyrin, although containing much bile pigment.

The above results with the bile from these two geeldikkop cases were amply confirmed when an examination was made of specimens secured during a subsequent outbreak at Middelburg, C.P. The quantities of porphyrin were evaluated in terms of the arbitrary "normal" standard and were found to range from 4 to 13 units, as follows:—

<i>Case No.</i>	<i>Volume of Bile, c.c.</i>	<i>Porphyrin Concentration in "Units".</i>
1	66	11.0
2	56	8.3
3	63	8.4
4	127	8.0
5	52	8.0
6	34	10.4
7	20	8.5
8	100 ("yellow bile")	3.8
9	97	10.0
10	33	6.0
11	59	12.5
12	60	10.0
13	5 (no clinical icterus: "yellow bile").	4.2

It was concluded from these experiments that the pigment causing photosensitivity in geeldikkop is identical with that found to be responsible for this symptom in the experimental, biliary-obstruction cases produceable at the laboratory. In addition, in both instances, the porphyrin content of the bile was found to be high, considerably higher than in sheep subsisting upon the usual diet supplied to the available pen animals.

### ISOLATION OF THE PHOTOSENSITISING PIGMENT.

Clearly, the most readily available source of the pigment for isolation purposes was fistula bile, consequently the isolation and chemical identification of this pigment was approached as the next step in the logical elucidation of the geeldikkop problem. Quantities of bile amounting to 12.5 litres in all were obtained from fistula sheep. The porphyrin concentration was such that this quantity was equivalent to 67 litres of normal bile. It was evaporated to dryness by fanning thin layers exposed in large shallow trays. The residue was extracted repeatedly with pyridine until this solvent was no longer coloured pink, some glacial acetic acid was then added to the pyridine, followed by a considerable quantity of ether and sufficient water to cause the separation of two phases. Great difficulty

was experienced in washing out the pyridine and water-soluble materials from the ether owing to the tendency to form emulsions; however, by cautious shaking with consecutive quantities of water this was achieved. The entire pigment was then transferred to 10% hydrochloric acid and ether and lipoidal impurities removed from this solution by aeration. After again transferring to ether, the crude porphyrin was fractionated by shaking with different concentrations of hydrochloric acid. The 8-10% fraction was purified by frequent transferences, finally taken into chloroform and this solution evaporated. The crystalline residue was taken up in a little warm pyridine and, after filtration, about 4 volumes of boiling methyl alcohol added and a drop of glacial acetic acid. Upon cool-



Fig. 2.—Bile porphyrin crystallised from pyridine-methyl alcohol.  $\times 270$ .

ing, a fine crop of crystals was deposited. These were centrifuged off, washed well with ether and alcohol and finally recrystallised from hot pyridine-methyl alcohol. The pigment was thus obtained (in a yield of 0.3 gm.) in fine, large obliquely-ended prisms having a metallic lustre when seen in quantity (see Fig. 2). Other crystalline forms were obtained by crystallising from neutral ether (an acetic-ether solution washed repeatedly with water until freed from acid), which yielded tufts of very fine needles or needle-like prisms (see Fig. 3), and from acetic acid-ether solution on evaporation when the pigment was deposited in the form of slender, obliquely-ended prisms (see Fig. 4).



Fig. 3.—Porphyrin from fistula bile crystallised from neutral ether.  $\times 170$ .

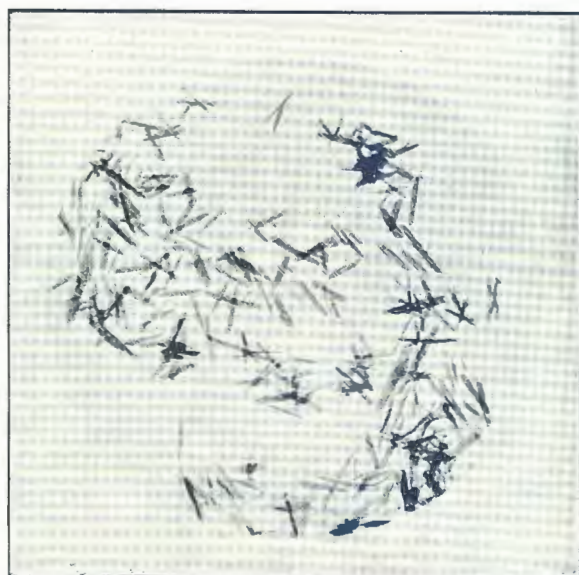


Fig. 4.—Porphyrin from fistula bile crystallised from acetic acid ether.  $\times 270$ .

These three crystalline forms were shown to be interconvertable and to yield similar analytical figures.

The acid number of the pigment was shown to be 9. The absorption spectra were:—

In ether	<u>635; 595; 578; 560; 523</u>	Order III, II, IV, I.
	I            II            III    IV	
in 25% HCl	<u>620; 607; 568</u>	Order II, I.
	I            II	

Its methyl ester was prepared by suspending some of the crystalline pigment in absolute methyl alcohol and saturating the ice-cooled mixture with gaseous hydrochloric acid. Chloroform was then added and a considerable quantity of ice-water. After shaking, the chloroform layer was removed, washed repeatedly with dilute alkali and water to remove the excess of acid and finally concentrated to dryness. The residue was taken up in a little anhydrous chloroform and filtered; five volumes of hot methyl alcohol were then added and the ester allowed to crystallise. It was obtained in fine prisms M.P. 259° (see Fig. 5). Combustion micro-analyses were performed upon the preparations of free pigment and of the methyl ester. From the collected results the pigment was identified with phylloerythrin. This was confirmed by preparing a sample of the methyl ester of the latter and demonstrating that the melting point was not lowered by admixture of the methyl ester of the bile pigment. The analytical figures for carbon content are somewhat low, but it is well known that phylloerythrin is very difficult to combust quantitatively and always tends to give low figures (in this connection compare Noack and Kiessling, 1930). The pertinent data are recorded below:—

	C	H	N	CH <sub>3</sub> O
Micro-analysis: Bile pigment from pyridine ... ..	72·26	7·02	9·69	
Bile pigment from ether	72·16	6·52	10·07	
Phylloerythrin				
C <sub>33</sub> H <sub>34</sub> N <sub>4</sub> O <sub>3</sub> requires	74·15	6·37	10·49	
Bile pigment methyl ester (M.P. 259°) ...	73·08	6·59	11·08	6·06
C <sub>34</sub> H <sub>36</sub> N <sub>4</sub> O <sub>3</sub> (M.P. 262°) requires ... ..	74·45	6·57	10·22	5·65

The ethyl ester of the bile pigment melted at 245°, whereas that of phylloerythrin (from faeces) is given as 248°.

The absorption spectra of phylloerythrin are as follows:—

In ether (Hellström 1931)	<u>635·2; 595; 589·8; 558; 526·6-512·3</u>
	I            II            III            IV
Order III, II, IV, I.	
In 25% HCl	<u>619; 607; 577</u>
	I            II

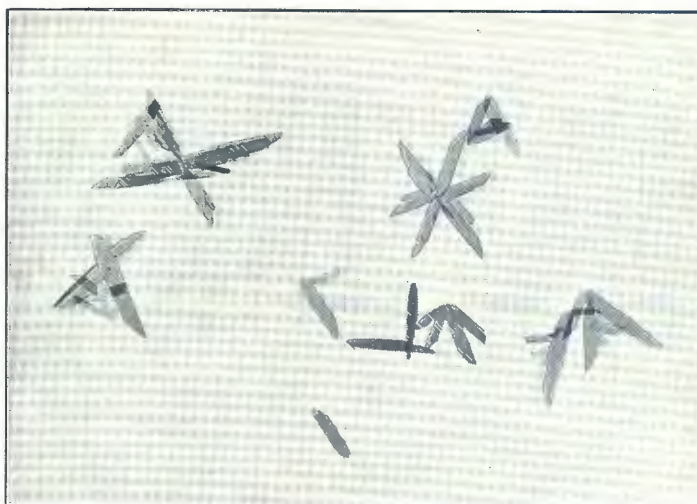


Fig. 5.—Methyl ester of porphyrin from fistula bile, crystallised from chloroform-methyl alcohol. M.P. 259°. 180 $\times$ .

The relative intensity of the band at 558  $m\mu$  is well brought out in the figure which Hellström gives comparing the spectra of phylloerythrin, mesoporphyrin and phaeoporphyrin  $a_5$ , and from *ou*, photographs taken with a Zeiss grating instrument and achromatic plates. Figs. 6 and 7.

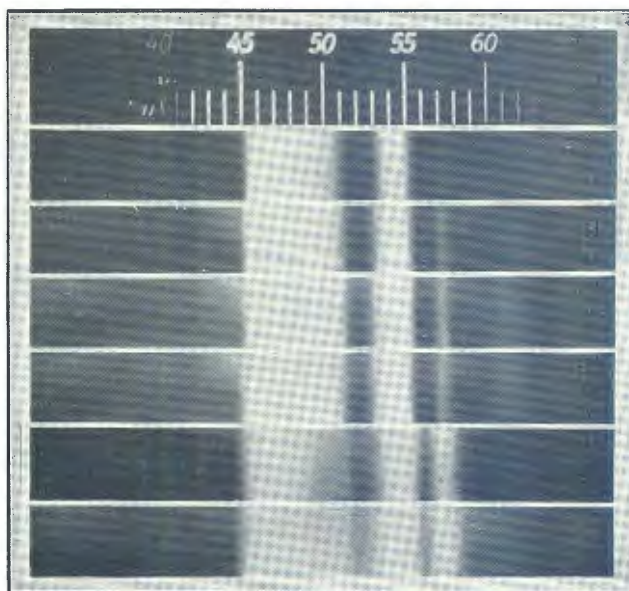


Fig. 6.—Absorption spectrum of phylloerythrin in ether solution.



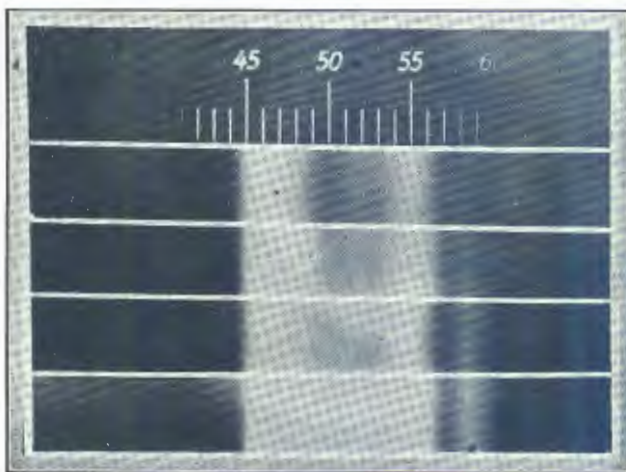


Fig. 7.—Absorption spectrum of phylloerythrin in 25 per cent. HCl.

A 0.1 gm. sample of the crystalline material from the dried bile was injected, dissolved in a little pyridine, intravenously into a sheep. Well marked photosensitisation resulted, the animal flinching, crouching, and eventually lying down in a most contorted position (see Figs. 8 and 9). Next day the head and ears were markedly swollen and a pouch of oedematous fluid distended the loose skin in the region of the intermandibular space. No sign of icterus was observed (Fig. 10).



Fig. 8.—Sheep showing photosensitisation after injection of bile porphyrin.



Fig. 9.—Sheep showing photosensitisation after injection of bile porphyrin.



Fig. 10.—The same sheep 24 hours later.

Although the chemical identification seemed quite conclusive, a biological test was also carried out upon an authentic sample of phylloerythrin. This was prepared from chlorophyll by refluxing for 18 hours with 20% hydrochloric acid, decanting the dark blue-green liquid from tarry impurities, transferring the pigment to ether and purifying in the usual way. 2% and 5% hydrochloric acid extracted considerable quantities of pigment, but the bulk passed into 9% acid. In spite of frequent transferences, crystallisation of

this material was very poor. The spectrum was identical, however, with that of phylloerythrin and the methyl esters were similar. 41 mgm. of the free porphyrin were injected intravenously into a sheep. Well marked photosensitisation with the usual symptoms and sequelae was obtained, but there was no indication of any icterus.

*The conclusion could thus safely be drawn that the photosensitising agent in geeldikkop is the pigment phylloerythrin. The significance of this discovery lies in the fact that phylloerythrin is a porphyrin of plant origin derived from chlorophyll. From the colouring matter of the Tribulus plant, therefore, the photosensitising factor takes its origin.*

According to H. Fisher, the constitution of chlorophyll a and of phylloerythrin are to be expressed as follows:—

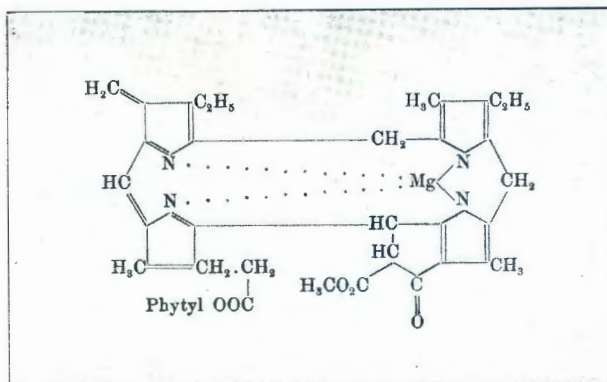


Fig. 11.—Chlorophyll.

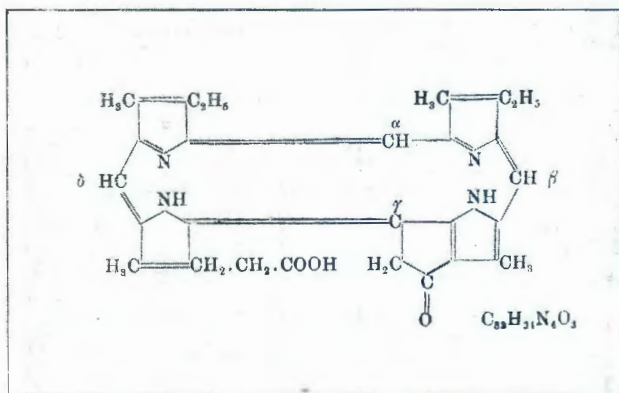


Fig. 12.—Phylloerythrin.

It will be seen that the production of the porphyrin necessitates only the removal of magnesium, saponification of the phytol and methyl ester linkages and a simple decarboxylation of the chlorophyll molecule.

Phylloerythrin was first isolated from the faeces of herbivorous animals by Marchlewski (1904) who demonstrated that its excretion was related to the quantity of chlorophyll in the diet. This we have verified by our own experiments.

Marchlewski (1904-5) showed the identity of phylloerythrin with the pigment "bilipurpurin" detected in bile by Löbisch and Fischler (1904). Kemerl (1924) described a similar pigment in human faeces. Fischer and his collaborators (1931; 1931; 1932) were the first to show that phylloerythrin is a true porphyrin and to elucidate its chemical constitution.

With regard to the mechanism responsible for the biological formation of phylloerythrin from chlorophyll and the site of these reactions our knowledge is, at present, very meagre. The entire question of the metabolism of ingested chlorophyll by various animal species is, in fact, in a most unsatisfactory condition. Fischer and Hendschel (1932) have shown that caterpillars break down chlorophyll for the main part to a substance with the following constitution which they have named *phyllobomycin*.

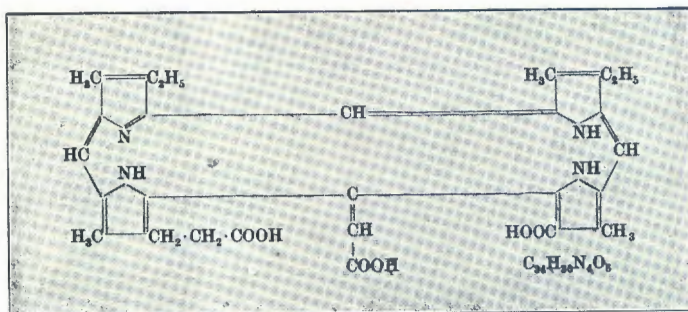


Fig. 13.—Phyllobomycin.

Phylloerythrin is apparently not formed by caterpillars. In sheep's faeces, they were able to detect the presence of three closely similar pigments, the probophorbids  $\alpha$ ,  $\beta$  and  $j$ , in addition to phylloerythrin.

During the course of our own work, which was more particularly concerned with the mechanism of phylloerythrin formation in the alimentary canal of the sheep, a communication appeared by Insman and Rothmund (1932), in which they stated that traces of this pigment were to be found even in the rumen. Their experiments were qualitative and did not go far enough to permit of conclusions being drawn as to the agencies concerned in the formation of this pigment from chlorophyll.

We propose in a subsequent paper of this series to go more fully into the questions of chlorophyll metabolism in different animals, reproducing here only such data as is a necessary adjunct to the experimental findings in the main enquiry as recorded above.



**RELATION OF PHYLLOERYTHRIN EXCRETION IN FAECES  
AND BILE TO THE QUANTITY OF CHLOROPHYLL IN  
THE DIET.**

As stated previously, an increase in the concentration of phylloerythrin in fistula bile was invariably noticed subsequent to the operation when the animals were liberally supplied with green stuff. In order to study this point more closely, a number of sheep were transferred to a diet consisting of coarse, yellow packing straw shown to be practically free from chlorophyll. The excretion of phylloerythrin in the faeces was followed by examining samples daily by the acetic acid-ether method. Within about a week, phylloerythrin and other chlorophyll derivatives were present only in traces. A fistula was then inserted into the gall bladder and after examining the bile for a few days, the animals were transferred to a chlorophyll-rich diet consisting of fresh, green lucerne or barley supplied *ad libitum*. Records were kept of the volume of bile secreted daily but these data along with other pertinent matter will be presented in a subsequent communication. Typical experiments are the following:—

Sheep 35287 previously maintained upon a chlorophyll-free diet was operated and a biliary fistula introduced.

<i>Days after Operation.</i>	<i>Units of Phylloerythrin in Bile.</i>
0	Nil
1	Nil
3	Nil
4)	
5)	
6)	Nil
7	0.2
8	6.5
9	12.4
10	13.0
11	9.5
12	9.5
13	13.2
14	14.2
15	16.2
17	21.6
18	25.0
19	32.0
22	32.4
23	—



Sheep 35290, companion to above, treated similarly:—

<i>Days after Operation.</i>	<i>Units of Phylloerythrin in Bile.</i>
0	Nil
4 } 5 } 6 }	worked up together Nil
7	put on to fresh green lucerne 0·8
8	5·5
9	9·2
10	13·9
11	8·6
12	5·5
13	17·8
14	tube partially blocked by <i>Stilesia</i> worms: animal photosensitive.

As was to be expected, it was found that sheep in which the bile duct had been ligated did not show any signs of photosensitivity so long as they were maintained on a chlorophyll-free diet, but did so within a short space of time when green lucerne was allowed.

The following two examples may be quoted:—

Sheep 35430 and 35422 maintained on chlorophyll-free diet, then operated and the bile duct ligatured. The bile taken from the gall bladders at the time of operation contained a trace only of phylloerythrin. They were exposed daily to the sun but evinced no sensitivity. After one week green lucerne was fed. Two days later sheep 35430 was markedly photosensitive and on the third day 35422 also became sensitive. Samples of faeces showed the presence of phylloerythrin and other chlorophyll derivatives.

Attention was next paid to the distribution of phylloerythrin in the various parts of the alimentary canal of sheep with a view to disclosing the mechanism of its formation. The methods employed were based upon the acetic acid-ether procedure, all values being related to the dry weight basis, since moisture content is a very important factor in quantitative data of this kind.

Individual sheep that had been maintained for some time upon a chlorophyll-rich diet were slaughtered and representative samples taken post-mortem from the contents of the rumen, omasum, abomasum, duodenum, jejunum, ileum, caecum, proximal, middle and distal portions of the large intestine.

The quantities of phylloerythrin found are expressed for convenience in "rumen units", one rumen unit being equal to 10 of the bile units as previously defined.

A typical result is shown in Fig. 14, the phylloerythrin contents of the different parts of the alimentary canal being recorded graphically. It is evident that the primary seat of formation of this pigment is the rumen; some secondary formation may occur in the caecum and colon. It appears probable, as will be demonstrated in a subsequent communication, that the symbiotic micro-organisms inhabiting the alimentary canal are the agencies responsible for the formation of phylloerythrin.

**SUMMARY.**

The pigment responsible for the photosensitivity in geeldikkop and also that developing after operative ligation of the bile duct in sheep (experimental icterus) has been isolated and identified as phylloerythrin, a porphyrin derived from chlorophyll.

In the absence of chlorophyll from the diet, experimental animals neither became photosensitive nor could phylloerythrin be isolated from the bile, serum or faeces.

The agencies responsible for the biological transformation of chlorophyll into phylloerythrin in the sheep are under investigation. Preliminary results show that the phylloerythrin is formed in the fore-stomachs and is probably a product of protozoal or bacterial activity.

The icterogenic factor in geeldikkop is still under investigation.

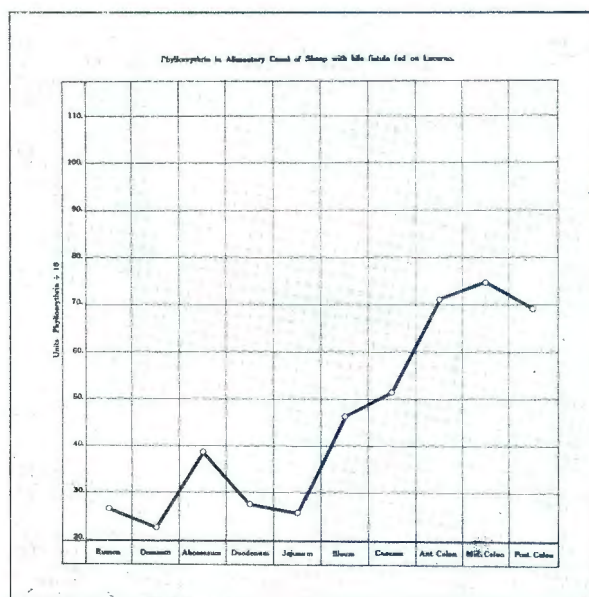


Fig. 14.—Phylloerythrin in alimentary canal of sheep with bile fistula fed on lucerne.

**REFERENCES.**

- FISCHER, H., AND HENDSCHEL, A. (1932). Phyllobombycin and the Probophorbids. *Zeit. f. physiol. Chem.*, Vol. 206, pp. 255-278.
- FISCHER, H., HILMER, H., LINDNER, F., AND PUTZER, B. (1925). Studies on the natural porphyrins. XVIII. Chemical findings in a case of Porphyrinuria (Petry). *Zelt. f. physiol. Chem.*, Vol. 150, pp. 44-101.
- FISCHER, H., MOLDENHAUER, O., AND SUSS, O. (1931). Phyllo- and Pseudo-Phylloerythrin. *Liebig's Ann.* Vol. 485, pp. 1-25.
- FISCHER, H., and RIEDMAIR, J. (1931). Synthesis of Desoxo-phylloerythrin, the basic substance of Chlorophyll. *Liebig's Ann.* Vol. 490, pp. 91-9.
- FISCHER, H., AND RIEDMAIR, J. (1932). Synthesis of Phylloerythrin. Change of Phaeoporphyrin A<sub>2</sub> into A<sub>1</sub>. *Liebig's Ann.*, Vol. 497, pp. 181-93.

- FISCHER, H., AND ZERWECK, W. (1924). Studies on the natural porphyrins. v. Koproporphyrin in the urine and serum in normal and pathological conditions. *Zeit. f. physiol. Chem.*, Vol. 132, pp. 12-33.
- HELLSTRÖM, H. (1931). The Relation between Constitution and Spectra of Porphyrins. *Zeit. physikal Chem.*, Vol. 14 B, pp. 9-17.
- KEMERI, D. (1924). A New Porphyrin Constituent of Human Faeces. *Biochem. Zeitsch.*, Vol. 151, pp. 438.
- LÖBISCH, W., AND FISCHLER, M. (1904). A New Pigment of Ox-bile. *Monatshfte f. Chemie*, Vol. 24, pp. 335.
- MARCHLEWSKI, L. (1904). *Bull. intern. de l'Acad. des Sciences de Cracovie*, Vol. 1904, pp. 276.
- MARCHLEWSKI, L. (1904-05). The identity of Cholehaematin, Bilepurpurin and Phylloerythrin. *Zeit. f. physiol Chem.* Vol. 43, pp. 464-468.
- NOACK, K., AND KIESSLING, W. (1930). The formation of Chlorophyll and its relation to Blood Pigment. II. *Zeit. f. physiol Chem.*, Vol. 193, pp. 97-137.
- ROTHEMUND, P., AND INMAN, O. (1932). Occurrence of Decomposition Products of Chlorophyll. I. *J. Amer. Chem. Soc.*, Vol. 54, pp. 4702-6.
- VAN DEN BERGH, H.; GROTEPASS, W.; AND REVERS, F. (1932). *Klin. Wochenschr.*, Vol. 11, No. 37, p. 1534.