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Studies on the Photosensitisation of Animals in South Africa.

VIII. The Biological Formation of Phylloerythrin in the Digestive Tracts of various Domesticated Animals.

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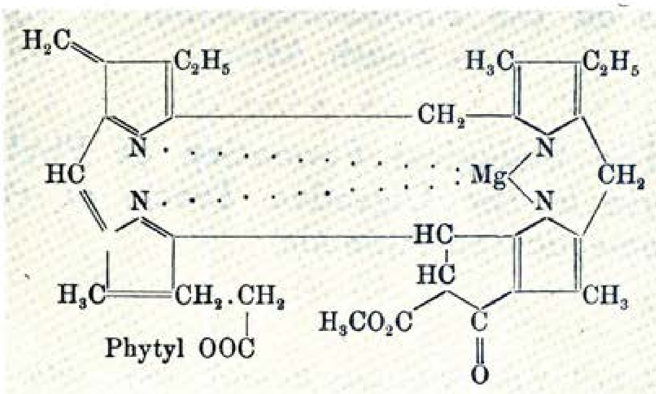
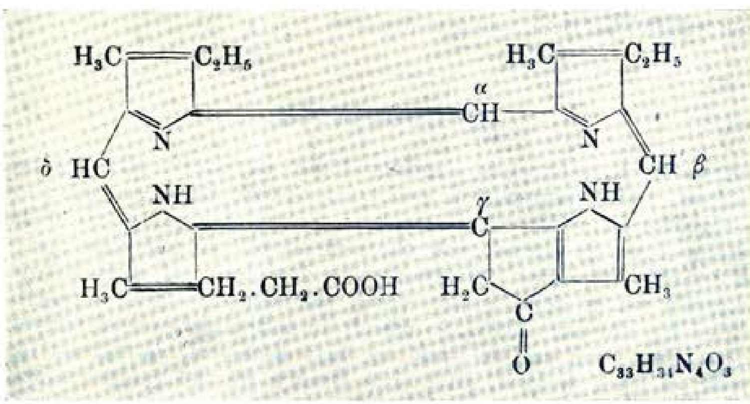
INTRODUCTION.

In the various researches conducted by us previously (Quin and Rimington, 1933; Rimington and Quin, 1933, 1934) on the problem of geeldikkop amongst small stock in South Africa, great difficulty was experienced in elucidating the genesis of the peculiar symptoms shown in this disease. Although the aetiology of geeldikkop had been known for a good many years through the field experiments conducted by Theiler (1918), in which he definitely proved that wilted *Tribulus* vegetation could, under certain seasonal conditions lead to outbreaks of this disease, little was known of the factors operating in bringing about the condition and especially those responsible for the acute photosensitisation and the subsequent grave jaundice. Numerous attempts at isolation or even of demonstration of such active principles as would lead to the above symptoms in experimental animals have failed. Thus the cause of the marked photosensitisation remained obscure as no photo-active substance could be demonstrated in the plant, as for example, has been found possible in various species of *Hypericum* and other plants. However in examining the bile and faeces of naturally occurring cases of geeldikkop as well as the same materials from experimentally produced cases of obstructive jaundice in sheep, we were able to show in a previous communication that there were present relatively large amounts of a fluorescent pigment, identified as phylloerythrin, which could be isolated in pure form from all these materials. Furthermore, minute amounts (40 mgms) of the same pigment, when injected into Merino sheep, produced typical photosensitisation and swellings of the head similar to those seen in geeldikkop. It therefore seemed reasonable to accept phylloerythrin, a product arising from the disintegration of chlorophyll a (the chemical relationship between these two substances will be apparent from the subjoined structural formulae) as the real cause of the photosensitisation.



Chlorophyll a.



Phylloerythrin.

Since no trace of the pigment as such could be found in the fresh green plant, its formation somewhere in the animal body had to be assumed. Several workers (Marchlewski, 1905; Rothemund and Inman, 1932) had demonstrated the presence of small amounts of phylloerythrin in the digestive tracts of herbivorous animals although no systematic work on its formation and distribution in the animal body had been carried out previous to our investigations. In preliminary experiments we were able to show, in the Merino sheep, that besides being present in the bile and faeces, fair amounts of the pigment could also be isolated from the rumenal contents but only when such animals were maintained on a ration containing chlorophyll. When dry, bleached hay, practically devoid of chlorophyll, was fed exclusively, phylloerythrin gradually diminished until after a period of 7-10 days it could no longer be detected in the digestive tract.

In order to gain further insight into the mechanism of its formation, studies were made of the distribution of phylloerythrin in the various parts of the digestive system in the different species of domesticated animals, all values being reduced to dry weight basis and the phylloerythrin expressed in terms of " rumen units " per gram of alimentary contents.

Extraction of the pigment was carried out by the acid-ether method as described by Rimington and Quin (1934). The same standard was used for comparison but, for convenience, the results were divided by ten (10) and called " rumen units " so that " 1 rumen unit " = 10 " bile units " as previously defined.

For the collection of materials, healthy animals were kept on a known diet, usually for seven days, then killed and the whole of the digestive tract carefully removed immediately after death. The stomachs, together with the intestines detached from the mesentery were spread out so that representative samples of ingesta could be taken from the various levels of the tract. Extraction of the materials was usually commenced immediately after collection, in this way minimising the risk of any change in phylloerythrin content. A smear of the moist material was also examined microscopically in order to ascertain whether infusoria and bacteria were present.

EXPERIMENTAL FINDINGS.

In order to obtain significant information concerning the phylloerythrin contents of the digestive tracts of the various species of animals, these were divided into the following groups according to the type of food generally consumed by them:—

A. *Herbivora.*

1. Ruminants—sheep and cattle.
2. Those with a single, simple stomach—horses, rabbits and guinea pigs.

B. *Omnivora*—Pigs and rats.

C. *Carnivora*—Dogs.

D. *Birds*—Fowls.

A. 1. Ruminants.

Sheep.

As most of the preliminary experiments were conducted on sheep, further studies were carried out on this species. For this purpose healthy half-grown and adult Merino sheep kept under stabled conditions were utilised. That the phylloerythrin formed in the digestive tract was subject to absorption into the blood stream was proved by the fact that this pigment could be recovered from fistula bile in amounts proportional to the chlorophyll-content of the food. As already mentioned, fair quantities of phylloerythrin could be detected in the rumen and other fore-stomachs, i.e. before the ingesta had been subject to true peptic or intestinal digestion. In the first place it therefore seemed desirable to ascertain how this phylloerythrin in the fore-stomachs was formed, and the following possibilities had to be considered:—

(*a*) Action of digestive enzymes possibly present in the saliva swallowed with the food.

(*b*) Action of plant enzymes in the rumen.

(*c*) Action of the bacterial flora in the rumen.

(*d*) Action of infusoria in the rumen.

(*a*) In order to test out the first possibility, viz. the action of the saliva, quantities of this secretion were collected from the mouths of several sheep, and after mixing with freshly expressed green plant juice were incubated at 37° for several days. Attempts to demonstrate the presence of phylloerythrin in these cultures failed. In this connection it may be added that the saliva from both sheep and cattle is generally considered to be free from any diastatic enzymes, the main feature of this fluid in these species being the fairly high concentration of alkaline salts which it contains.

It would thus seem that the saliva by itself could not be held responsible for the breakdown of the chlorophyll and its subsequent change into phylloerythrin.

(*b*) *Plant enzymes.*—To investigate the possible rôle that these might play in the production of phylloerythrin, two sheep were fed exclusively for 4 weeks on green lucerne which had been thoroughly autoclaved each day before feeding. In spite of this procedure it was found that the phylloerythrin level in the faeces was not changed from the normal thus indicating that the destruction of possible enzymes by autoclaving did not inhibit the formation of phylloerythrin.

(*c*) *Action of rumenal bacteria.*—It is a well known fact that a very rich and varied bacterial flora is maintained in the fore-stomachs of the ruminant animal. Although it has not been found possible to isolate and test out every single species of organism found in the rumen, pure cultures were made in which *B. coli*, present in large numbers in the fore-stomachs, was incubated with fresh, green plant juice, and also with sterile broth to which small quantities of commercial chloryphyll had been added. In both instances it was found possible to demonstrate the presence of small amounts of phylloerythrin. Although other organisms from the rumen were not tested out systematically it would be reasonable to assume that several species of bacteria may cause the disintegration of chlorophyll with the formation of phylloerythrin under suitable conditions.

(*d*) *The possible rôle played by infusoria.*—That infusoria form a considerable proportion of the bulk of the rumenal mass is obvious from the large number, consisting of different species, to be seen microscopically in a single drop of the expressed fluid. Moreover, they are capable of ingesting various kinds of materials including starch granules, chlorophyll particles and bacteria. Since it has not been found possible to cultivate any of these organisms *in vitro* it is obviouslly difficult to decide whether they are capable of transforming chlorophyll into phylloerythrin. In a preparation consisting of several grams of thoroughly washed infusoria from the rumen of a sheep, phylloerythrin was definitely demonstrated, although again it would be difficult to state whether this was taken in with ingested bacterial bodies or whether natural chlorophyll was disintegrated by the infusoria themselves. Particles of green plant materials could be seen inside many infusoria.

An experiment was devised in which, by indirect means, it was hoped to gain further information as to the action of the infusoria on chlorophyll. By regular systematic dosing of various toxic substances to sheep, it was expected that the sensitive infusoria in the fore-stomachs could either be completely killed off, or at least, their numbers greatly reduced. Should they normally have been responsible for phylloerythrin formation, this experiment should have led to decreased values of the pigment both in the rumenal contents and in the faeces, which could be examined regularly throughout the experiment. Twelve (12) full-grown Merino sheep were placed for a week on a diet containing liberal amounts of fresh green lucerne. By means of a stomach tube inserted into the rumen of each sheep daily, a small quantity of the rumenal fluid was withdrawn and immediately afterwards examined microscopically to ascertain its richness in infusoria. At the same time faeces were collected and their phylloerythrin content determined.

In all animals, infusoria were constantly present in large numbers while the faeces showed relatively large amounts of phylloerythrin. Daily dosing of the sheep was then commenced as follows, the food remaining unchanged:—

(*a*) Two sheep each dosed 2 gm. potassium permanganate in 1 litre water daily for 11 days. Dose thereafter increased to 3.5 gm. daily for 9 days.

(*b*) Two sheep each dosed 0·2 gm. calomel in 1 litre water daily for 11 days. Dose thereafter increased to 0·3 gm. daily for 3 days.

(*c*) Two sheep each dosed 0·125 gm. potassium cyanide in 1 litre water daily for 11 days. Thereafter dose increased to 0·2 gm. daily for 11 days.

(*d*) Two sheep each dosed 1 gm. quinine sulphate in 1 litre water daily for 10 days. Dose thereafter increased to 2 gm. daily for 11 days.

(*e*) Two sheep each dosed 0·1 gm. Government Wireworm Remedy (sodium arsenite and copper sulphate) in 1 litre water daily for 7 days. Thereafter dose increased to 0·3 gm. daily for 10 days.

(*f*) Two sheep kept as controls were not dosed at all.

During the course of the experiment, regular examinations of rumenal fluid samples revealed the fact that the infusoria were flourishing in these sheep equally well as in the controls. Furthermore, the phylloerythrin content of the faeces only showed minor fluctuations similar to those shown in the controls.

From this it may be concluded that the various substances (ranging from simple oxidising to definitely toxic agents) dosed in daily sublethal amounts to sheep were also withstood with impunity by the infusoria. Hence through inability to destroy the infusoria these experiments failed to reveal the rôle these organisms played in the breakdown of chlorophyll and the formation of phylloerythrin. Apart from the definite action exerted by bacteria in this direction, the circumstantial evidence to be revealed later makes it highly probable that the infusoria also, are concerned in the process of chlorophyll disintegration in the fore-stomachs of the sheep.

With regard to the phylloerythrin content in the rest of the digestive tract, the following findings are recorded (see Figs. 1 to 3):—

(*a*) Abomasal contents showed approximately the same amounts as found in the rumen indicating that this pigment was simply passing through with the ingesta, unaccompanied by either further formation in the abomasum or destruction of the phylloerythrin by the acid pepsin.

(*b*) The contents of the small intestines showed a slight progressive decrease in the phylloerythrin content towards the ileum due, probably, to its absorption.

Where no precaution had been taken to exclude the influx of bile from the common bile duct, a relatively large increase in phylloerythrin concentration occurred on passing to the lower region of the small intestine. In the case of a sheep, operated upon prior to the experiment, and in which a permanent bile fistula had been introduced, this was not apparent. A slight rise is probably to be accounted for by the preferential absorption of sugars, amino-acids, etc., thus causing phylloerythrin to assume a relatively greater concentration in the intestinal contents.

(*c*) The large intestines, especially the latter portion of the caecum and the beginning of the floating colon, again contained relatively large amounts, due in all probability to the rich bacterial flora usually found in these parts and acting on chlorophyll which had escaped disintegration in the rumen, as also to the aforementioned possibility that other nutrients are more readily absorbed before this level is reached. The faeces too showed high phylloerythrin values.

Cattle.

Only a few phylloerythrin analyses were carried out on cattle, as the distribution and values closely approximated those obtained for sheep. Moreover, infusoria were present in large numbers in the fore-stomachs.

A. 2. Herbivora.

Horses.

In this species, although herbivorous, the arrangement of the stomach is far more simple than in the ruminant. Consequently, as could be surmised, the phylloerythrin distribution showed a variation. Samples of ingesta collected from the cardiac as well as from the pyloric divisions of the stomach revealed no phylloerythrin at all. Similarly the small intestines were devoid of all but a trace of this pigment. The caecum and large colon, however, showed appreciable quantities of phylloerythrin, while several large species of infusoria were present in millions. Thus it would again seem that those localities of the gut in which bacteria and infusoria flourish best are the actual sites where phylloerythrin is formed.

Rabbits.

These animals were kept upon a diet of lucerne, then killed and examined in the usual way. Phylloerythrin was present in the stomach where infusoria and bacteria were also seen. The faeces were in most cases relatively rich in phylloerythrin. Typical distribution curves are reproduced in Figs. 4 and 5.

Guinea Pigs.

The diet in this case was green barley. A fairly abundant formation of phylloerythrin was observed as is evident from the distribution curves, Figs. 6, 7 and 8.

B. Omnivora.

Pig.

As typical omnivorous animals, the pig and rat were selected. The pig was drenched each morning for several days with freshly expressed lucerne juice. Only a very small quantity of phylloerythrin could be detected in the faeces.

Rat.

Albino rats were kept upon a diet of lucerne and killed in pairs for each experiment. Unfortunately only very small quantities of material were present in the stomach and small intestine which rendered the detection of phylloerythrin less certain. In the caecum and large intestine appreciable quantities of pigment were found and numerous spirochaetes were also detected microscopically.

C. Carnivora.

Dog.

A dog was drenched repeatedly with lucerne juice but no trace of phylloerythrin could be found in the faeces.

D. Birds.

Fowl.

A fowl, as typical of an avian, was fed on fresh lucerne. When slaughtered, no phylloerythrin was detected anywhere except in the large intestine and then it was only present in traces.

The accompanying table serves to summarise these results and correlates the presence of phylloerythrin with that of infusoria and bacteria. When the chemical change is considered, which is required to transform chlorophyll into phylloerythrin, it is seen that only reactions are necessary which have familiar counterparts in microbiological chemistry. Thus, phaeophytin—the magnesium-free pigment derived from chlorophyll is converted into phylloerythrin by (1) saponification of the phytol and methyl alcoholic groups, and (2) elimination of CO_2 from the carboxyl group attached to the isocyclic ring. Both types of reaction, saponification and decarboxylation are known to occur commonly in bacterial cultures.

Previous systematic studies upon the fate of chlorophyll in the animal organism have been surprisingly few. Machlewski and his co-workers (1905, 1913, 1933) devoted some attention to the subject and more recently Fischer and Hendschel (1931) have investigated the pigments present in the excrement of the silk-worm caterpillar. They (1932) were able to isolate a crystalline pigment phyllobombycin which proves to be a tricarboxylic acid and no longer contains the isocyclic ring characteristic of phylloerythrin and chlorophyll. It was also shown (1931) that *Aspergillus oryzae* and *Penicillium glaucum* are both capable of forming phylloerythrin from chlorophyll.

Another paper of interest is that of Von Linden (1903) who followed the fate of chlorophyll in the digestive tract of caterpillars by means of a microscopical fluorescence method.

Rothemund and Inman (1932), as previously stated, carried out some qualitative work with ruminants and detected phylloerythrin in the rumen.

Conclusions.

It appears from the facts which we are able to present that there is a considerable weight of circumstantial evidence in favour of the hypothesis that the agencies responsible for the formation of phylloerythrin from chlorophyll in the digestive tract of animals are the symbiotic infusoria and bacteria present in these localities. Only when an abundant microbial flora was present, could phylloerythrin be demonstrated.

In the elucidation of the geeldikkop problem, a further significant fact is thus established. The photo-sensitising pigment is not present in the plant, but arises as a result of microbial activity during the normal course of digestion. It is produced from any chlorophyll-containing plant.

In this connection it seemed of interest to compare the phylloerythrin excretion in the faeces of sheep maintained upon *Tribulus* only with that of animals on a lucerne diet. *Tribulus* is particularly rich in chlorophyll (see a forthcoming contribution to this series by M. Henrici) and the quantity of phylloerythrin formed in the alimentary canal is correspondingly great. The two curves reproduced in Fig. 9 illustrate the daily fluctuations in the phylloerythrin-content of the faeces of two sheep receiving only

Tribulus. The mean level for an animal on lucerne is also shown. It will be remarked that the two curves run closely parallel which must indicate that some factor was affecting each sheep equally. This could conceivably be due to variations in the quality of the feed from day to day. It is also apparent that sheep may differ individually to quite a large extent in the quantity of phylloerythrin which they excrete even when on the same diet. Possibly this difference may be due to the presence of a richer and more active rumenal flora in the one than in the other.

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Distribution of Phylloerythrin in relation to Micro-organisms in different animals.

Animal.	Diet.	Stomach.		Small intestine.	Caecum.	Large intestine.	Faeces.
		Fore stomach. (*Rumen*).	True stomach. (*Abomasum*).				Bile units/ gm.
Sheep......	One week on fresh lucerne	xxx inf. and bact. numerous	xxxx	xxxx	xxxx inf. and bact. numerous	xxxx inf. few., bact. numerous	1076
,,	,,	xxx inf. and bact. numerous	xxx	xxx	xxxx inf. and bact. numerous	xxxx inf. few, bact. numerous	1098
Sheep......	Permanent biliary fistula	xx inf. and bact. numerous	xxx	xxx	xxxx inf. and bact. numerous	xxxx inf. few, bact numerous	690
Bovine (calf)	Mixed diet......	—	—	—	—	—	351
Horse......	Mixed diet......	Nil. inf. absent, bact. present	—	Trace.	Trace. large inf. numerous present	x inf. and bact. present	72
Rabbit.....	Lucerne........	xx inf. absent, bact. present	—	x inf. absent, bact. present	xx inf. and bact. present	xxx inf. and bact. present	420
Guinea-pig..	Green Barley...	xx small inf. present bact. few	—	xxx small inf. and bact. present	xxxx small and large inf. present	xxxx inf. and bact absent	826
Pig........	Drenched lucerne juice	—	—	—	—	—	57
Rat........	Lucerne........	Nil.	Nil.	Nil.	xx Bact. and spirochaetes present	xx Bact. and spirochaetes present	169
Dog........	Drenched lucerne juice	—	—	—	—	—	Nil.
Fowl.......	Lucerne........	Crop, gizzard and prov inf., bact. and spiroch	entriculurs nil. aetes present.	Nil.	—	Trace.	Trace.

Key:

x	0– 100	bile units phylloerythrin per gm. dry weight.
xx	100– 250	,, ,, ,, ,,
xxx	250– 500	,, ,, ,, ,,
xxxx	500–1,000	,, ,, ,, ,,

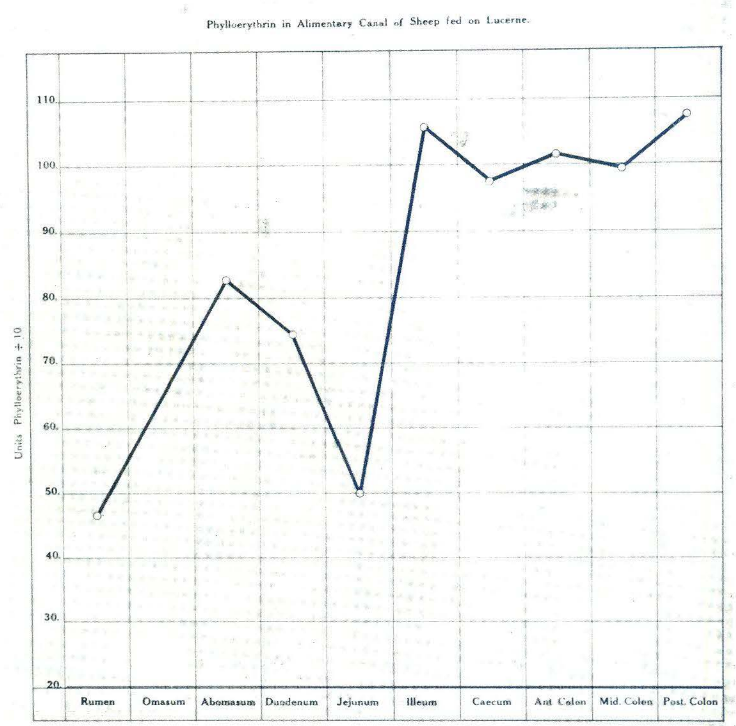
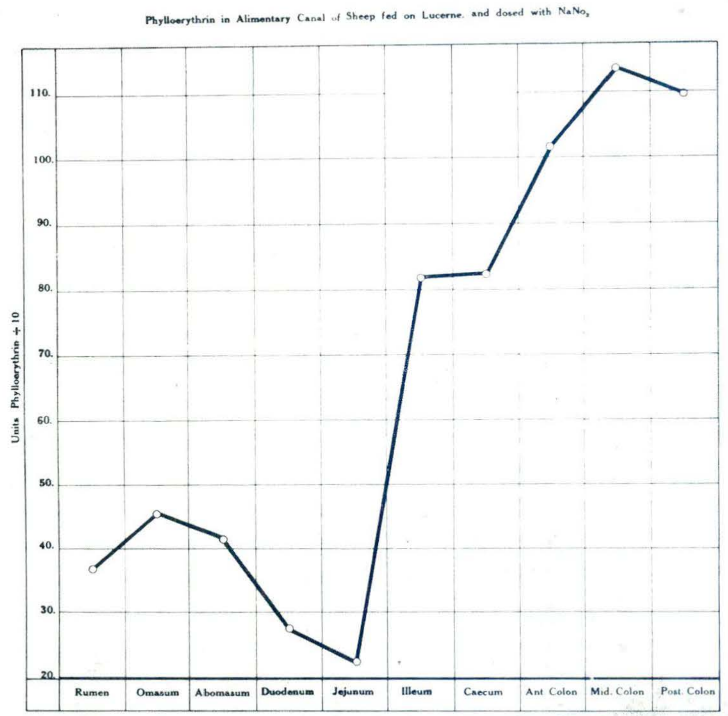

Phylloerythrin in Alimentary Canal of Sheep fed on Lucerne.
Units Phylloerythrin ÷ 10
110.
100.
90.
80.
70.
60.
50.
40.
30.
20.
Rumen
Omasum
Abomasum
Duodenum
Jejunum
Ileum
Caecum
Ant. Colon
Mid. Colon
Post. Colon

Fig. 1.



Phylloerythrin in Alimentary Canal of Sheep fed on Lucerne. and dosed with NaNo₂
Units Phylloerythrin ÷ 10
110.
100.
90.
80.
70.
60.
50.
40.
30.
20.
Rumen
Omasum
Abomasum
Duodenum
Jejunum
Illeum
Caecum
Ant Colon
Mid. Colon
Post. Colon

Fig. 2.

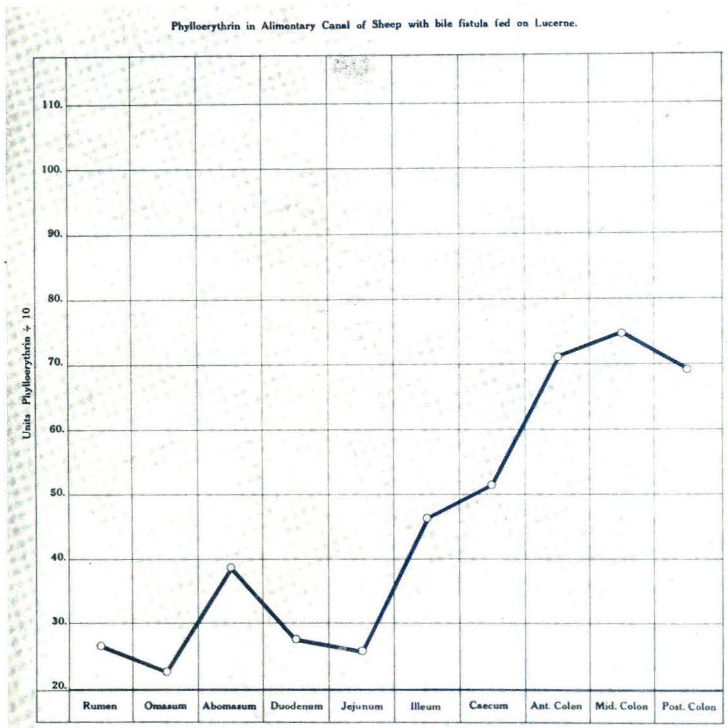

Phylloerythrin in Alimentary Canal of Sheep with bile fistula fed on Lucerne.
Units Phylloerythrin ÷ 10
110.
100.
90.
80.
70.
60.
50.
40.
30.
20.
Rumen
Omasum
Abomasum
Duodenum
Jejunum
Illeum
Caecum
Ant. Colon
Mid. Colon
Post. Colon

Fig. 3.

FIG. 4.

Phylloerythrin in alimentary canal of rabbit fed on Lucerne.

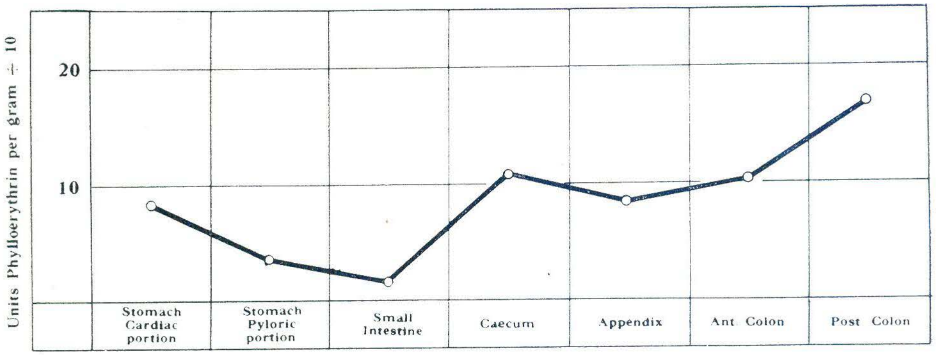


FIG. 5.

Phylloerythrin in alimentary canal of rabbit fed on Lucerne.

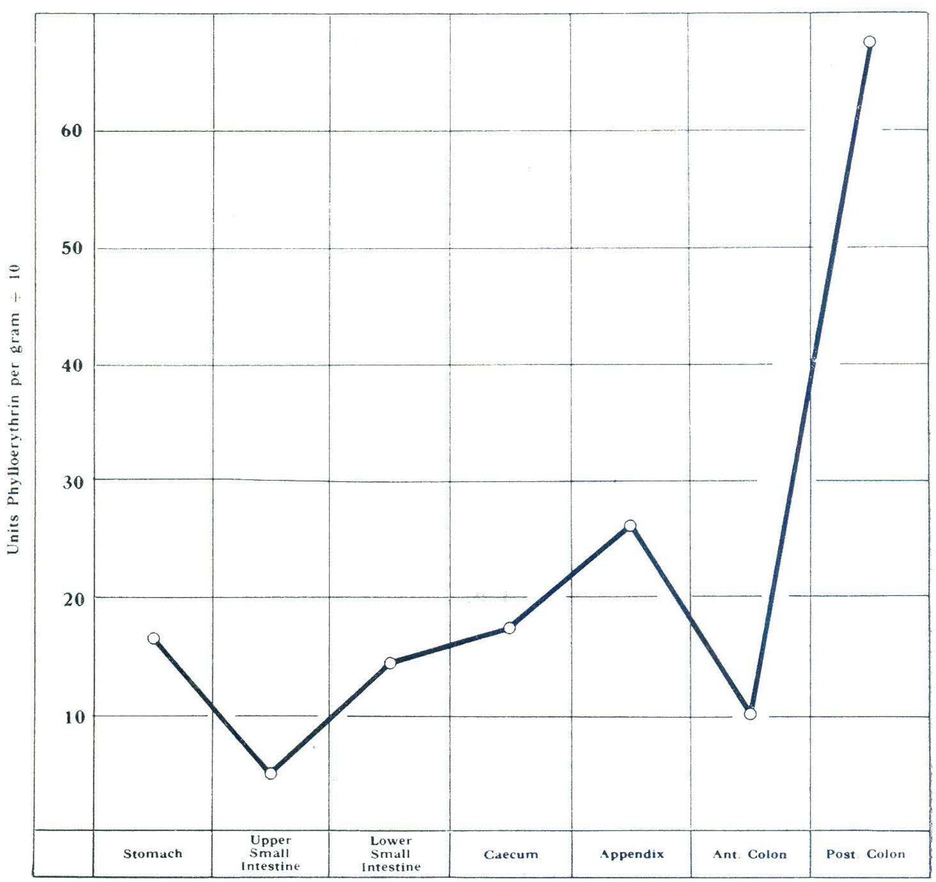


FIG. 6.

Philloerythrin in alimentary canal of Guinea pig fed on Green Barley.

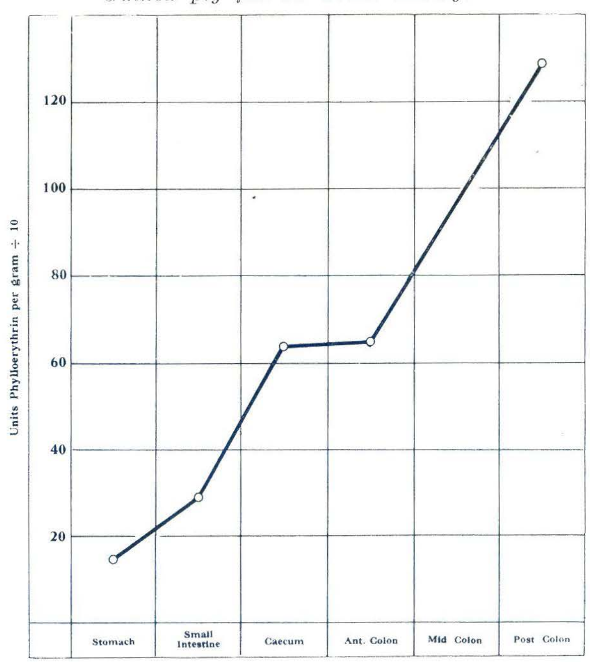


FIG. 7.

Phylloerythrin in alimentary canal of Guinea pig fed on Green Barley.

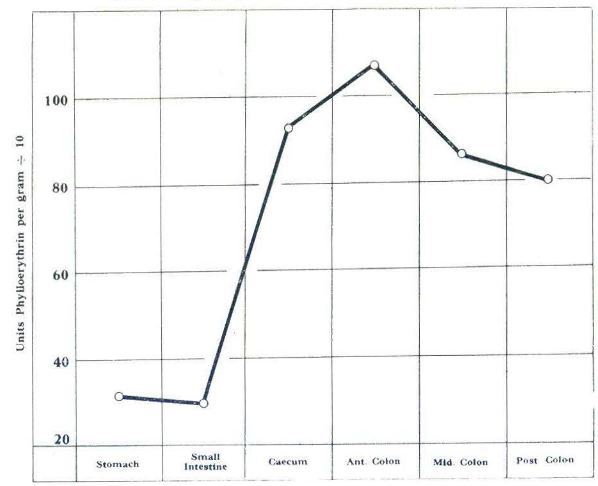


FIG. 8.

Phylloerythrin in alimentary canal of Guinea pig fed on Green Barley.

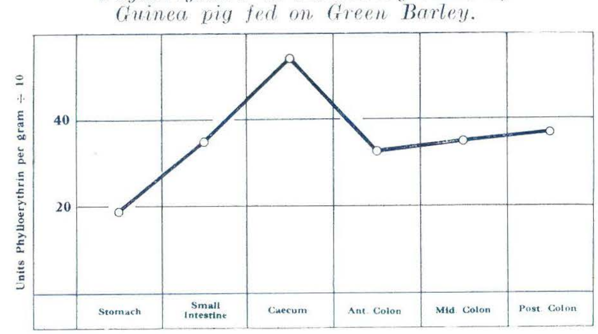


FIG. 9.

Phylloerythrin excretion of 2 sheep fed on Tribulus only.