

## Pigment Metabolism II.—Determinations of Bilirubin and Carotinoids in the Plasma and of Coproporphyrin in the Urine and Faeces of Cattle Experimentally Infected with *Theileria parva*.

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IN *Theileria parva* infection yellow staining of the fats and the mucous membranes is not conspicuous and, in any case, is much less intense than in certain other protozoal diseases such as anaplasmosis. It had, however, been noted that the urine of cases of *Theileria parva* infection was at times pigmented. Prior to these investigations, however, it was generally held that icterus did not occur in *Theileria parva* infection.

The examination of those cases of the disease in which highly coloured urine did occur revealed that abnormal amounts of urobilin and coproporphyrin were present, that the subcutaneous and peritoneal fat contained both bilirubin and carotinoids and that the yellow colour was due to both these pigments.

On account of the above findings it was decided to carry out a more detailed examination of cases experimentally infected, the object of the experiment being to establish whether or not abnormal pigmentation could be established in the urine, plasma, faeces and fats.

The method employed in this experiment was to examine bovines, experimentally infected with *Theileria parva* by ticks, both before and during the course of the disease as to the presence of bilirubin and carotinoids in the plasma and fat and of coproporphyrin in the urine and faeces.

### LITERATURE.

#### 1. *Bilirubin and carotinoids in plasma and fat.*

A review of the literature on bilirubinaemia and bilirubinuria was made in number 1 of the series of these communications (Roets, 1942). It is known that the plasma of men and animals ingesting large amounts of green plant food contains carotinoids. Thus a "pseudo-icterus" can be produced in man feeding for a few weeks large amounts of green vegetables [v. d. Bergh (1924)]. Rimington (1938) estimated quantitatively the bilirubin and carotinoids in icteric bovine plasma and discussed the respective contribution of the two pigments towards the colouration of such plasma.

The intensity of the yellow colour (icterus index) produced by bilirubin is not always in direct proportion to the amount of bilirubin in the different plasmas [Elton (1931)].

Rimington and Fourie (1938) introduced a method of differentiating carotinoids and bilirubin in fats.

## II. *Coproporphyrin in Faeces and Urine of Cattle.*

Rimington and Roets (1937) found not only coproporphyrin I but also coproporphyrin III in the faeces and the urine of bovine congenital porphyria cases. Quantitative determinations of these coproporphyrin isomers were reported upon by Rimington, Roets and Fourie (1938). The presence of these isomers in the faeces and the urine of bovines infected with *Theileria parva* was first described by Roets (1938). The excretion of coproporphyrin in the faeces and the urine of normal bovines has also been determined [Fourie and Roets (1939); Roets (1941)].

### TECHNIQUE.

#### I. *Bilirubin of Plasma.*

The method employed for the quantitative estimation of plasma bilirubin was that of v. d. Bergh and Grotepass (1934) as described in number I of this series of communications [Roets (1942)].

#### II. *Carotinoids of Plasma.*

The amount of carotinoids in plasma was determined by the method of Rimington (1937) viz.:—

The carotinoids were precipitated with the plasma proteins by mixing 15 c.c. of plasma with 30 c.c. of 96 per cent. alcohol. After centrifugation the protein mass was extracted two to three times with ether, about 20 c.c. of fresh ether being used for each extraction. The combined ethereal extracts were transferred to a separatory funnel, shaken with 0.1 N. NaOH solution to remove any bilirubin present, and then washed with distilled water to remove the alkali. The ethereal solution was evaporated on a water bath down to 15-50 c.c. (depending on the concentration). The colour intensity of this solution was measured in a colorimeter against a dye standard [Guilbert (1934)] and the amount then calculated.

#### III. *Differentiation of the Carotinoids and the Bilirubin in Fat.*

The method employed to differentiate carotinoids from bilirubin in fat was originated by Rimington and Fourie (1938). A 2 gm. sample of fat, as free as possible from blood, was boiled in a strong glass test tube with 5 c.c. of 5 per cent. aqueous sodium hydroxide. The tube was cooled rapidly to about blood temperature and then approximately an equal amount of ether was added. The mixture was well shaken and set aside until it

separated into two layers. The bile pigments remained in the lower greenish yellow aqueous layer, the carotinoids in the upper yellowish ethereal layer.

#### IV. Coproporphyrin in Urine and Faeces.

The coproporphyrin was extracted by acetic acid and ether from urine and faeces as described by Fourie and Roets (1939) for those of normal cattle. The coproporphyrin was transferred from the ethereal solution to 5 per cent. hydrochloric acid solution. The intensity of the spectroscopic absorption bands in this solution was measured against that of a standard coproporphyrin solution and the amount then calculated.

The coproporphyrin I to coproporphyrin III ratios were obtained by separating the methyl esters as described by Rimington, Roets and Fourie (1938).

#### EXPERIMENTAL DETAILS.

All the cases of *Theileria parva* infection were produced by tick infestation.

Bilirubin was first determined in the plasma of two bovines, 7076 and 6371, suffering from the disease. The plasma was examined for bilirubin and carotinoids, fats collected at post mortem for bilirubin and carotinoids and the urine for coproporphyrin.

Two 2 year old bovines (6722, 7031) were infected and the examination was carried out on the plasma for bilirubin and carotinoids and on the faeces and urine for coproporphyrin before the reaction commenced and up to the time of death. The ratio of coproporphyrin I to coproporphyrin III was determined in the faeces of each of these two animals two days before death. The coproporphyrin I to coproporphyrin III ratio of the urine was also determined in a sample accumulated over two days subsequent to the period of incubation.

A similar examination to the previous one was carried out on bovines 6725 and 6902 except that the faeces of these were examined as combined samples for the reason that they shared a box.

The blood for the determinations on the plasma was drawn from the jugular. The anticoagulant was 0.75 c.c. of a 20 per cent. potassium oxalate solution per 50 c.c. blood.

The animals were not housed in metabolism boxes as they had to be kept in quarantine on account of the danger of the disease spreading to other animals. The faeces were collected from each animal before the stables were cleaned. The total faeces for each day were thoroughly mixed and samples for analysis were then taken.

The urine was collected in glass jars during the act of urination and was immediately examined.

RESULTS.

(a) Quantitative estimations of bilirubin and carotinoids were made from time to time in the plasma of bovines 7076 and 6371 infected with *Theileria parva* on the 3.11.37. Bovine 6371 was killed *in extremis* on 28.11.37 and 7076 died on the 7.12.37. The data obtained are presented in Table 1.

TABLE 1.

No. of Animal.	Date.	Mg. Carotinoids per Litre Plasma.	Plasma Bilirubin in v. d. Bergh Units.
7076.....	19/11/37	2.9	1.97
7076.....	22/11/37	3.7	2.02
7076.....	24/11/37	3.4	1.0
7076.....	29/11/37	2.04	1.57
6371.....	19/11/37	3.7	2.02
6371.....	22/11/37	3.0	4.33
6371.....	24/11/37	—	4.25
6371.....	27/11/37	—	5.12

(b) Bovines 6722 and 7031 were infected on the 25.4.38. They reacted on the 6.5.38 and the 5.5.38 respectively. The results obtained on their plasma bilirubin and carotinoids and faeces coproporphyrin are presented in Table 2. Plasma bilirubin less than 1 v. d. Bergh unit is expressed as "Positive".

TABLE 2.

Date.	BOVINE 6722.			BOVINE 7031.		
	Faeces.	Plasma.		Faeces.	Plasma.	
	Mg. Copro-p. per 100 g.	v. d. Bergh Units Bilirubin.	Mg. Carotinoids per Litre.	Mg. Copro-p. per 100 g.	v. d. Bergh Units Bilirubin.	Mg. Carotinoids per Litre.
26/4/38	0.018	Trace only	3.6	0.014	Negative	4.6
28/4/38	0.025	Negative	4.1	0.022	Negative	5.0
3/5/38	0.014	Negative	4.5	0.025	Negative	3.7
5/5/38	0.016	—	—	0.038	—	—
7/5/38	0.024	Positive	4.8	0.093	Positive	3.8
9/5/38	0.039	Positive	3.3	0.104	Positive	2.6
10/5/38	0.031	Positive	3.1	0.082	Positive	—
11/5/38	0.094	2.0	—	0.082	Positive	—
12/5/38	—	5	1.5	—	Positive	1.7
13/5/38	0.128	—	—	0.065	—	—
14/5/38	—	1	—	—	Positive	—
16/5/38	0.082	Positive	—	0.091	—	—
17/5/38	0.144	—	—	0.146	—	—
18/5/38	0.102	8.3	—	0.108	7.8	—
20/5/38	—	10.5	0.9	0.271	9	0.4

TABLE 3.

Date.	BOVINE 6725.	BOVINE 6902.	Mg. Copro-p. per 100 g. in the combined Faeces of 6725 and 6902.
	Plasma Bilirubin in v. d. Bergh Units.	Plasma Bilirubin in v. d. Bergh Units.	
21/10/38.....	Negative	Negative	—
24/10/38.....	—	—	0·04
25/10/38.....	Negative	Negative	0·055
28/10/38.....	Negative	Negative	—
31/10/38.....	—	—	0·06
1/11/38.....	Positive	Positive	—
2/11/38.....	—	—	0·075
4/11/38.....	5·1	3·9	0·12
7/11/38.....	11·25	3·7	0·182
8/11/38.....	9·375	5·0	No faeces passed.
9/11/38.....	9·2	7·03	0·5
10/11/38.....	11·1	7·7	0·151
11/11/38.....	10·0	6·8	0·322
14/11/38.....	12·0	7·3	—

The concentration of coproporphyrin in the urine of 6722 on the 18th and 19th of May was 0·338 and 0·202 mg. per 2000 c.c. respectively. In the urine of 7031 on the 18th of April the coproporphyrin concentration was 0·395 mg. per 2000 c.c. These coproporphyrin fractions of the two animals were combined and the ratio of coproporphyrin I to coproporphyrin III in the combined sample was found to be 1:0·31.

The coproporphyrin samples from the faeces of 6722 on the 11th and 13th of April, on which the quantitative estimations of coproporphyrin had been made, were combined. The ratio of coproporphyrin I to coproporphyrin III in this combined sample was 1:1·5. In a similar composite sample of coproporphyrin from the faeces of 7031 collected on the 9th, 10th and 11th of April, only a trace of coproporphyrin I was detected.

(c) Bovines 6725 and 6902 were infected on 21.8.38. The results obtained on their plasma bilirubin and on the coproporphyrin concentrations of their combined faeces are presented in Table 3.

Bovine 6725 was killed *in extremis* on the 14th November and 6902 on the 16th November.

Samples \* of peritoneal and subcutaneous fats from all the animals examined were collected at post mortem. Carotinoids and bilirubin were found to be present in all the cases.

\* A sample of urine collected during the post mortem examination of a typical case (bovine 8593) was sent to my laboratory for examination on the 22nd of January, 1943. This sample contained 0·4 mg. Coproporphyrin per 2000 c.c. urine. This sample of urine was also examined for haemoglobin by the pyridine haemochromogen method [Roets (1940)]. A haemoglobin concentration of 210 mg. per 100 c.c. urine could be determined.

## DISCUSSION.

The following points should be borne in mind when considering the significance of the data presented:—

I. The concentration of the carotinoids in the plasma depends on the type of food fed to the animals and is not associated with haemopoietic activities.

II. The concentration of the bilirubin in the plasma in a protozoal disease, such as *Theileria parva* infection, is indicative of haemoglobin disintegration.

III. Coproporphyrin is of endogenous origin and it is excreted via the bile and urine. Thus the relative quantities appearing in the faeces and the urine depend largely upon the total excretion of faeces and of urine.

In the advanced stages of the disease the animals ate little or no food. The intake of food gradually decreased and ultimately ceased and the amount of faeces passed decreased. For example, bovines 6725 and 6902 (Table 3) showed decrease of food intake and passed no faeces on the 18.11.38. The concentration of coproporphyrin in the faeces passed the following day was the highest during the whole period of examination. In all cases examined the amounts of faeces passed during the advanced stages of the disease were very small, whereas the concentrations of coproporphyrin increased considerably in such faeces. It is, however, doubtful whether the total amount of coproporphyrin excreted increased.

The presence of both coproporphyrin I and coproporphyrin III in the faeces and the urine of *Theileria parva* infected is of special interest. Haematin and the bile pigments are type III pigments. The type III pigments are sometimes called the "physiological" types. Theories, such as those of Rimington (1937b) and Turner (1940), were put forward to explain the relationship of natural porphyrins and their dualism to normal and pathological haemopoiesis. These theories are based on the presence of a porphyrin stage in haemoglobin metabolism. The type I porphyrins are considered to be "byproducts" in the process of haemopoiesis or "products" which result from the acceleration of the control processes in the direction of the type I products.

It is known that in normal bovines both coproporphyrin I and coproporphyrin III are excreted in the faeces and urine [Fourie and Roets (1939); Roets (1941)]. The effect of *Theileria parva* infection on the blood forming tissues, therefore, does not prevent the formation of both types. On account of the very small amounts encountered in such investigations it is extremely difficult to determine the influence of the disease on the quantitative relationship of the two types during the course of the disease.

The level of the bilirubin in the plasma of the same animal fluctuated considerably from day to day, especially during the early stages of the disease (Tables 1, 2 and 3), but ultimately in all the animals, shortly before death, it reached a high level. The highest levels obtained in the different animals also differed considerably.

## SUMMARY.

The investigations on *Theileria parva* infection of cattle revealed:—

(1) That bilirubinaemia did occur, the highest figure for plasma bilirubin being 12 v. d. Bergh units.

(2) That the yellow staining of the fat is due to the combined effect of carotinoids and bilirubin.

(3) That the intensity of the yellow colour of the plasma is in part due to carotinoids, a normal constituent of the blood of cattle, especially if they be fed on food containing carotinoids.

(4) That there is a definite rise in the concentration of coproporphyrin in the faeces and urine. Such a rise, however, does not necessarily mean that there is an increase in the total amount excreted as the rise may be due to the decrease in the amount of faeces passed as a result of the disease.

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