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Pigment Metabolism I.—The Examination of the Urine and Blood of Dogs for Bilirubin and Haemoglobin.

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In the past the examination of animal diseases in South Africa in relation to the haemoglobin metabolism and disintegration has not been carried out to any great extent. In dogs, especially, no work of this nature has been undertaken. A number of investigations on and routine examinations of biological material have recently been commenced at this Institute. The object of this article is to report on the various examinations already performed on the bilirubin and haemoglobin content of urine and blood of dogs admitted for the purpose of clinical examination. Most of the cases for examination were selected by means of the v. d. Bergh test on the urine which is a routine clinical examination.

When bilirubin is detected in the urine, the routine procedure is to examine the plasma bilirubin. This latter examination is all the more necessary as a number of cases of bilirubinuria have been shown to be due to hepatic derangement. Full reliance cannot be placed on the microscopic examination of smears or on the symptomatology for the diagnosis of canine piroplasmosis as an occasional case shows negative smears notwithstanding a careful examination and practically no symptoms of the disease.

The value of the examination of the urine and especially of the plasma for the presence of bilirubin and haemoglobin has proved to be such that the continuation and expansion of this method of diagnosis are indicated.

Literature.

In a review on jaundice McNee (1923) refers to the observations of Virchow (1847) who believed that haemotoidin was an isomer of bilirubin and was responsible for haemolytic jaundice and to those of Minkowski and Naunyn (1886) who found that geese whose livers had been surgically removed did not show jaundice when poisoned by arsine. Virchow's claim that there were two types of bilirubin, namely, one present in cases of hepatic disturbances and the other in haemolytic jaundice, was neglected for many years on account of the findings of Minkowski and Naunyn. McNee (1912), however, repeated the experiments of Minkowski and Naunyn and determined that the removal of the liver in birds did not produce the same effect as the removal of that organ in higher animals. In birds the reticulo-endothelial cells (Kupffer cells) are mainly concentrated in the liver, whereas in higher animals, such as dogs, these cells also occur in other parts of the body [Aschoff (1922)]. Prusik and McNee (1924) demonstrated the formation of bilirubin outside the liver.

Blankenhorn (1921) dialyzed icteric plasma and demonstrated dialyzable and non-dialyzable pigments. He expressed the opinion that non-dialyzable pigment was associated with the blood proteins, Andrews (1924) on the other hand presented experiments which indicated that the linkage of bilirubin with protein or with any phospholipoid probably did not occur.

V. d. Bergh (1918) introduced a method of differentiation between the two types of bilirubin. This method is even at present commonly empolyed and is based on the principle of the Ehrlich's diazo-reaction.

Collison and Fowweather (1926) found that free bilirubin isolated from heavily pigmented gallstones, behaved like the bilirubin present in cases of haemolytic jaundice when tested by the v.d. Bergh method, i.e., it gave a positive indirect reaction. The alkaline salt of bilirubin, however, behaved like the bilirubin found in cases of hepatic disturbances, i.e., it gave a positive direct v. .d. Bergh reaction. They expressed the view that bilirubin in serum giving the direct reaction was probably the ammonium salt of the free acid. The ammonium is probably derived from amino-acids in the liver cells during the course of urea production. Subsequently various workers stressed the importance of the pH of solutions on the v. d. Bergh reaction [Davies and Dodds (1927), M'Gowan (1929), Elton (1931)].

The differentiation of the two types of bilirubin found in icteric sera is of great value in establishing whether an icterus is due to hepatic disturbance or to haemolysis [McNee (1924), v. d. Bergh (1924), Willcox 1924), Elton (1931); etc.]. The hepatic and haemolytic types were called by Elton (1931) the crystalloid and suspensoid colloid respectively.

Technique.

1. *Bilirubin.*—A. *Plasma Bilirubin.*

The technique employed for the estimation of plasma bilirubin is the method of v. d. Bergh (1918), improved by v. d. Bergh and Grotepass (1934).

Reagents.

I. *Solution* A.—1 gm. sulphanilic acid, 15 c.c. of 25 per cent. HCl, and distilled water up to 1 litre.

II. *Solution B.*—0·5 per cent. sodium nitrite in aqueous solution.

III. *Reagent.*—The reagent is prepared by adding 10 c.c. of solution A to 0·3 c.c. of solution B. This solution must be freshly prepared before use.

IV. *Buffer Solution.*—27·25 c.c. of 0·1 Mol. citric acid and 72·75 c.c. of 0·2 Mol. secondary sodium phosphate (the pH of such a solution is 6·6).

V. *Buffer Alcohol.*—50 per cent. alcohol containing 10 c.c. of the buffer solution per 100 c.c.

(a) Direct Reaction.

For the quantitative estimation of bilirubin in plasma giving the direct reaction, a mixture of 2 c.c. reagent, 1 c.c. plasma and 2 c.c. water is diluted up to 10 c.c. (or more in cases of high concentrations of bilirubin) with the buffer alcohol solution.

(*b*) *Indirect Reaction.*

For the quantitative estimation of bilirubin in plasma giving the indirect reaction, the plasma proteins are precipitated by mixing one part of plasma with two parts of 96 per cent. alcohol. The mixture is centrifuged and to 8 c.c. of the supernatant fluid 2 c.c. of the reagent is added.

Comparisons.—The comparisons are made in a colorimeter against a known standard. V. d. Bergh and Grotepass used smoked glass or painted wire gauze as standards for comparison. Because these standards were not available a number of solutions used by various workers were investigated as possible substitutes.

The various solutions tested were:—

I. 2·16 per cent. cobalt sulphate [McNee (1925)].

II. The ethereal rhodate iron solution [Hawk and Bergeim (1931)].

III. Standard methyl red solution, pH 4·63 [Beaumont and Dodds (1939)].

IV. 10 per cent. cobalt nitrate [Wester (1935)].

V. A bilirubin standard containing 5 mg. bilirubin per litre [v. d. Bergh and Grotepass (1934)].

These standards were compared against one another in a colorimeter but no two were found to match. The following experiments were, therefore, made:—A solution of bilirubin, containing 5 gm. bilirubin per 800 c.c. was made as described by v. d. Bergh and Grotepass (1934). A mixture of 8 c.c. of this solution and 2 c.c. of the reagent was used in each experiment. Immediately after the reagent had been added the solution was transferred to a colorimeter cup and observed in comparison with the 10 per cent. cobalt nitrate standard (fixed at a depth of 30 mm.). There occurred a gradual change in the colour of the reagent mixture. It was only after two minutes that a good match of the colours was obtained at 38 mm. After 3 to 4 minutes the best match was obtained at 32-30 mm. After 4 minutes it was no longer possible to match the colours. In an attempt to find a stage at which the colour of such a reacting mixture would remain constant, the colours of a series of such mixtures which had been allowed to react in a dark cool place for 15 minutes and for 4, 6, 24 and 48 hours were examined. No constant stage was, however, obtained. The best match was also obtained after 3 to 4 minutes when plasma or urinary bilirubin was used instead of the pure bilirubin solutions. Consequently the amount of bilirubin in materials under examination could be calculated from the results obtained in comparison with a standard of 10 per cent. cobalt nitrate solution equivalent to a mixture containing 5 mg. bilirubin per 1000 c.c. (equivalent to 1 v. d. Bergh unit).

Differentiation of the Direct and the Indirect Types of Bilirubin.

Lepehne's technique [McNee and Keefer (1925)] was employed to determine whether the plasma bilirubin gave the direct, indirect or delayed v. d. Bergh reaction. In three tubes were placed 2·0 plasma containing the bilirubin. To tube 1 was added 0·2 c.c. of water. This was the negative control. To tube 2 was added a small amount of caffeine-sodium salicylate. When this had dissolved, 0·2 c.c. of reagent was added. The reaction commenced immediately, but time should be allowed for it to reach its maximum. To

tube 3 the reagent (0·2 c.c.) is now added. The response can then be compared with that obtained in tube 2, i.e., the direct or delayed reaction could be judged by the intensity of the colour and its rapidity of development.

B. *Bilirubin in Urine.*

Methods Considered.

Marsh (1940) dissolved dried egg albumen up to 10 per cent. in strongly icteric urines. He then applied the indirect v. d. Bergh method for estimating the bilirubin quantitatively. Hunter (1930) absorbed the urinary bilirubin with barium chloride and then recovered it from the absorbent, which was filtered off, by dissolving it in 96 per cent. alcohol.

The bilirubin present in urines of cases examined at this laboratory always was the direct type. Because bilirubin of the direct type is only sparingly soluble in alcohol concentrations of 60 per cent. and higher, there occurred appreciable losses of bilirubin, when the above-mentioned methods were used. It was also observed that all the bilirubin present in urine was not absorbed by the barium chloride.

The method of Siede and Zink (1931), based on the number of drops of a 2 per cent. aqueous solution of methylblue necessary to produce a green colour in a known volume of urine is even less satisfactory, especially as the colour of icteric urines makes it impossible to judge the endpoint.

Method Employed.

The direct v. d. Bergh technique was employed to get an idea of the concentration of bilirubin in urine. Quantitative estimations could not be made in urines coloured by pigments such as haemoglobin. The quantity of haemoglobin in the urine of a dog suffering from piroplasmosis was estimated at 498 mg. per 100 c.c. urine. The haemoglobin was precipitated by alcohol using 1 part of urine and 4 parts of alcohol, and centrifuged. The supernatant liquid was fairly clear. The bilirubin in this solution was found to be 20 v. d. Bergh units. It was, however, felt that such a figure was not justifiable because the direct type of bilirubin, which was the one present, is only very sparingly soluble in alcohol of such a high concentration.

C. *Haemoglobin.*

The haemoglobin determinations were made by the method developed by Roets (1940). The haemoglobin was converted into pyridine haemochromogen and the intensity of the 555 spectroscopic absorption band measured against that of a standard prepared from pure haemin.

Details of Materials Examined.

Blood.—The blood for the examination was in every case drawn from the jugular vein, the anticoagulent used being 0·5 c.c. of a 20 per cent. potassium oxalate solution per 25 c.c. of blood.

Urine.—The urine was collected in a beaker during the act of urination.

Both urine and blood samples were examined immediately after collections were made.

Results.

In the tabulated data, quantities of bilirubin less than one v. d. Bergh unit are expressed as " positive ". If haemoglobin be also present the bilirubin is expressed as " positive + ".

The plasma bilirubin and haemoglobin concentrations in bloods of various cases examined are presented in Table 1.

Table 1.—*Bilirubin and Haemoglobin in Blood.*

Date.	No. of Dog.	Disease.	Haemoglobin in gm. per 100 c.c.	Bilirubin.	
				Type.	v. d. Bergh Units.
5/ 8/39	253/39	Hepatic disturbance	10·66	Direct	5
16/ 8/39	261/39	Piroplasmosis	6·99	Indirect	1·25
13/10/39	321/39	Piroplasmosis	3·0	Indirect	Positive.
17/ 1/40	17/40	Normal	16·2	Negative	Negative.
8/ 2/40	37/40	Piroplasmosis	9·34	Indirect	Positive.
14/ 2/40	41/40	Piroplasmosis	11·9	Indirect	Positive.
28/ 3/40	99/40	Piroplasmosis	3·7	Indirect	53
13/ 4/40	118/40	Rheumatism	13·7	Negative	Negative.
18/ 7/40	182/40	Piroplasmosis	9·0	Indirect	3
5/ 9/40	238/40	Enteritis	14·4	Indirect	Positive.
14/ 2/41	71/41	Hepatic disturbance	17·8	Direct	3
14/ 3/41	71/41	Hepatic disturbance	14·0	Direct	2·5
27/ 3/41	71/41	Hepatic disturbance	17·9	Direct	3·2
19/ 4/41	205/41	Piroplasmosis (Chr.)	14·4	Indirect	Positive.
20/11/41	441/41	Piroplasmosis	7·8	Direct delayed	2·2

Results obtained on bilirubinuria and haemoglobinuria are summarized in Table 2.

Table 2.—*Bilirubin and Haemoglobin in Urine.*

Date.	No. of Dog.	Disease.	Bilirubin in v. d. Bergh Units.	Haemoglobin in mg. per 100 c.c.
5/ 8/39	253/39	Hepatic disturbance	3	Negative.
16/ 8/39	261/39	Piroplasmosis	10	4
11/10/39	304/39	Normal	Negative	Negative.
4/11/39	329/39	Piroplasmosis	Positive	Negative.
5/ 1/40	4/40	Piroplasmosis	1·9	Negative.
13/ 1/40	7/40	Piroplasmosis	Positive +	12·83
17/ 1/40	17/40	Normal	Negative	Negative.
14/ 2/40	41/40	Piroplasmosis	Positive	Negative.
22/ 2/40	48/40	Piroplasmosis	Positive +	99·7
27/ 3/40	97/40	Piroplasmosis	Positive +	799
28/ 3/40	99/40	Piroplasmosis	Positive +	498
13/ 4/40	118/40	Rheumatism	Negative	Negative.
18/ 7/40	182/40	Piroplasmosis	5·6	Negative.
14/ 3/41	71/41	Hepatic disturbance	15	Negative.
27/ 3/41	71/41	Hepatic disturbance	7·5	Negative.
20/11/41	441/41	Piroplasmosis	Positive	Negative.

In some cases simultaneous examinations of both urine and blood were made for haemoglobin and bilirubin. These results are presented in Table 3.

TABLE 3.

Date.	No. of Dog.	Disease.	Blood.			Urine.	
			Haemoglobin in g. per 100 c.c.	Plasma Bilirubin.		Haemoglobin in mg. per 100 c.c.	Bilirubin in v. d. Bergh Units.
				Type.	Amount in v. d. Bergh Units.		
5/ 8/39	253/39	Hepatic disturbance	10·66	Direct...	5	Negative	3
16/ 8/39	261/39	Piroplasmosis.....	6·99	Indirect.	1·25	4	10
11/10/39	304/39	Normal.....	10·4	Negative	Negative	Negative	Negative.
14/ 2/40	41/40	Piroplasmosis.....	11·9	Indirect.	Positive.	Negative	Positive.
28/ 3/40	99/40	Piroplasmosis.....	3·7	Indirect.	53	498	Positive+
18/ 7/40	182/40	Hepatic disturbance	9	Indirect.	3	Negative	5·6
14/ 3/41	71/41	Hepatic disturbance	14	Direct...	2·5	Negative	15
27/ 3/41	71/41	Hepatic disturbance	17·9	Direct...	3·2	Negative	7·5
16/ 6/41	205/41	Piroplasmosis (Chr.)	14·4	Indirect.	Positive.	4	Positive.
20/11/41	441/41	Piroplasmosis.....	7·8	Direct delayed	2·2	Negative	Positive.

DISCUSSION.

To interpret the results in cases of bilirubinaemia and bilirubinuria, considerations should be given to the origin and the fate of bilirubin. Watson (1938) deals with this problem. After haemoglobin is transformed via haematin into bilirubin by the reticulo-endothelial system [Aschoff (1922)], it passes to the liver and, under normal conditions, is excreted via the bile duct into the intestinal tract, where it is broken down into products such as urobilinogen and stercobilinogen. A portion is passed out with the faeces and a portion is reabsorbed and excreted in the urine.

In diseases such as piroplasmosis the abnormal destruction of erythrocytes and the resultant production of bilirubin produce a rise in plasma bilirubin. Bilirubin under such conditions is partly absorbed by tissues such as fats or mucous membranes and partly excreted. By such excretions the bilirubin concentrations of urines, which is normally very low [v. d. Bergh (1924)], are considerably increased.

In the case of hepatic disturbances the bilirubin generated in normal or abnormal amounts, passes from the liver into the circulation and thus the level of plasma bilirubin is raised. This eventually gives rise to absorption by various tissues and an increased bilirubin excretion in the urine.

When the results summarised in Table 1 are considered, the cases examined can be grouped according to whether the plasma bilirubin gives the indirect or direct v. d. Bergh reaction, into haemolytic and hepatic disturbances cases respectively, with the one exception of case No. 441/41, suffering from piroplasmosis. In this particular case the examination was made after all the parasites had disappeared from the blood. The bilirubinaemia, however, was still present. Finally liver damage probably due to protozoal intoxication [Willcox (1924)] occurred.

Haemoglobinuria frequently occurs in canine piroplasmosis. In such cases the haemoglobin disintegration in the body is not sufficiently rapid to cope with all the haemoglobin liberated by the destruction of the erythrocytes, consequently the unchanged haemoglobin is excreted in the urine. This is especially evident in acute cases, such as Nos. 99/40 and 97/40, in which bilirubin as well as free haemoglobin are excreted in the urine. The process of haemolysis was definitely interferred with in the case of No. 441/41 by the treatment, which was probably the cause of the absence of haemoglobin in the urine. The absence of haemoglobin in the urine of cases such as Nos. 182/40, 41/40, 4/40 and 329/39, may be due to various factors, such as mild infections or different stages of the course of the disease. Such factors will receive due consideration during the course of investigations to be made in the near future.

The haemoglobin concentrations in the blood of the piroplasmosis cases (Tables 1 and 3), as would be expected, are low except in the chronic case 205/41. In this case the haemoglobin destruction was probably at a very low rate. In the case of hepatic disturbances, however, the figures for the haemoglobin are similar to those of normal dogs.

Summary.

1. In all the cases of bilirubinaemia examined, whether the animals suffered from canine piroplasmosis or hepatic disturbances abnormal amounts of bilirubin were excreted in the urines.

2. Haemoglobin was frequently present in the urine of dogs suffering from piroplasmosis. The highest concentration of haemoglobin found was 799 mg. per 100 c.c. urine. No haemoglobin could be detected in the urines of dogs suffering from hepatic disturbances.

3. The plasma bilirubin of the piroplasmosis cases gave an indirect v. d. Bergh reaction whereas that of the hepatic disturbance cases gave the direct reaction with the v. d. Bergh reagent. The highest plasma bilirubin figure obtained was 53 v. d. Bergh units in a case of canine piroplasmosis.

4. In the piroplasmosis cases an increase in bilirubin was accompanied by a decrease in haemoglobin in the blood, whereas in the hepatic disturbance cases there was no decrease of the blood haemoglobin, the bilirubin in the plasma being due to retention and not to haemolysis.

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