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Chemical Blood Studies.*

I. Comparative Studies on Blood, "Laked" and "Unlaked" Blood Filtrates of Animals in Health and Disease, with particular reference to methods and technique employed.

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CONTENTS.

INTRODUCTION.

The present paper is intended to be the first publication of a series of researches into animal diseases occurring in South Africa. Further studies have been planned with a view to concentrating thereafter on correlating the chemical data with the pathology and pathological physiology of each particular disease in an attempt to get a rational explanation of any changes in the composition of the blood which

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may have been observed. The blood data are not to be restricted to the constituents enumerated in this paper, but will include in addition data on other constituents such as cholesterol, pigments, ammonia, lactic acid, mineral constituents and physico-chemical data such as viscosity, hydrogen ion concentration and sedimentation rate of cellular elements, etc. It would have been preferable to get all this information from one and the same experimental subject, but the available facilities did not permit of this.

No serious attempt has, therefore, been made at present to offer explanations of the changes in blood composition which have been recorded, this aspect being reserved for such a time as the data referred to have been gathered. The same extensive " normal " data are being obtained for various domestic animals over a period of 12 months, those for sheep will be published by Hamersma of this Division in this Journal at a later date. Since a comparison with data obtained by other workers on normal animals is being discussed there, this aspect has been omitted. Publications on comparative data for " laked " and " unlaked " filtrates of domestic animals have not been found, in spite of a wide search through all available literature.

THE PLAN OF RESEARCH.

The present series of investigations have been primarily undertaken with a view to determining the actual changes, if any, occurring in the composition of the blood during the course of a number of different protozoan and virus diseases. The conditions investigated up to the present are: (1) Heartwater of sheep (*Rickettsia ruminantium* infection), (2) Horsesickness (*Pestis equorum*), (3) Anaplasmosis of cattle (*A. marginale* infection)), (4) Piroplasmosis of cattle (*P. bigeminum* infection), (5) Bluetongue of sheep, and (6) Anaplasmosis in the Blesbok (*Damaliscus albifrons*). For the results of these investigations up to the present see " Chemical Blood Studies III-V " in this Journal. Such data in respect of the diseases studied, have up to now, been completely wanting in South Africa, and in spite of an exhaustive search in the available literature no chemical data on the conditions detailed here could be found. It was felt that such data may materially contribute towards a deeper understanding of the pathology of these diseases, also enabling a clearer conception to be formed of the processes taking place in the body as a result of such specific infections. It was also anticipated that these researches, apart from increasing our knowledge as to the actual changes in the composition of the blood during infectious conditions, may become of value from a diagnostic, prognostic or prophylactic point of view.

No search was made for constituents not normally occurring in the blood, but which may possibly be present as a result of the abnormal metabolism of the body under the stimulus of the causal agent or its excretory products, or secondary stimuli associated with the symptoms of the disease such as hyperexia, anaemia, anorexia, etc.; or which may constitute the toxins themselves or the by-products of the metabolism of the causal agent. The aim was rather to note the changes in the relative proportions of certain normal constituents during any particular infection.

Determinations in respect of the total nitrogen (T.N.) and haemoglobin content (Hb) of the whole blood, and non-protein nitrogen (N.P.N.), urea nitrogen (U.N.), amino-acid nitrogen (A.A.N.), uric acid nitrogen (U.A.N.), total creatinine nitrogen (T.C.N.), and sugar (S) were, therefore, made in each case on both "laked" and "unlaked" blood filtrates, which were prepared according to the method of Folin and Wu (laked) and Folin (unlaked). "Laked" and "unlaked" filtrates were studied in order to obtain figures comparable with those obtained elsewhere during similar investigations into animal diseases, whether done on either filtrate. In view of the unequal distribution of most constituents over the plasma and the cellular elements, a study involving both types of filtrates was an additional inducement to undertake the large amount of extra labour involved in analysing two filtrates of one and the same blood.

Owing to the absence of normal figures for ovines, bovines and equines for the above constituents under South African conditions, several blood analyses were made prior to infecting the experimental subject. In a few cases where this was not found possible, analyses were made on the day of infection, and in some rare cases even a day or two later.

DIET OF EXPERIMENTAL ANIMALS.

No special diet was given, the rations being in all cases those supplied to the stock at this Institute. Sheep received 1 lb. of mealies, veld hay and green feed (when available) *ad lib.*, plus $\frac{1}{8}$ ounce of salt per day; cattle received 2 lb. mealie meal, 2 lb. mealie bran, veld hay and green feed (when available) *ad lib.*, plus 1 ounce of salt per day. Horses received 5 lb. of mealies, veld hay and green feed (when available) *ad lib.*, plus $\frac{1}{2}$ ounce of salt per day. Sick horses received 6 lb. of mealie bran and 2 lb. of crushed oats instead of the 5lb. of mealies. The green feed consisted of either lucerne, green barley or oats. The food was given at 7 a.m. and 4.30 p.m.; water three times a day. As the nature and amount of the diet influences to some extent the composition of the blood, the system of analysing the blood repeatedly before and after infection of one and the same animal on a "fixed" diet, eliminates largely the complication of the influence of the diet on composition. In this connection it should also be borne in mind that during severe hyperrhexias, especially during the critical period, animals not uncommonly refuse food—this anorexia *per se* influencing the composition of the blood.

As, however, such an anorexia constitutes a part of the symptom complex, its influence on the composition must be regarded as abnormal and as part and parcel of any pathological changes which may be observed.

It was not considered of sufficient value to determine accurately the intake of food by each individual animal, since the present work is not so much concerned with how the food influences the blood composition but rather a study of the influence on the body of various infections as reflected in the blood. This system also furthermore permits of seasonal variations in the normal composition of the blood to be taken into consideration, whether due to diet, environmental tempera-

ture, humidity, etc., or not. It, therefore, allows for an accurate comparison of the figures obtained for the various constituents in health and during any particular infection.

The subjects were in all cases placed on temperature for long periods prior to infection and clinical examinations made whenever it was deemed necessary for the purposes of these investigations.

TECHNIQUE AND METHODS.

Full reference to the origin of the methods here used are given under the various sub-sections and only where modifications of any given method were introduced, or special points of interest emerged, have these been detailed here. In all cases such changes of the prescribed procedure have been thoroughly checked before being adopted for the analyses of blood filtrates.

(1) Method of taking Blood Samples (Neser, 1923).

The blood was in all cases drawn from the jugular vein with sterilised trocar and canula or hollow bleeding needles and collected in 30-35 c.c. vaccine bottles containing 0·25 c.c. of a 20 per cent. potassium oxalate solution. These bottles were filled with blood so that less than one c.c. of oxalate per 100 c.c. blood was used. This amount of anticoagulant proved sufficient, except in the case of sheep blood, with which occasional clotting took place, such bloods having then to be discarded. The bleeding took place, in the majority of cases, between the periods 8.30 a.m.–9 a.m., except where otherwise stated. This enabled me to collect as nearly as possible comparative data as far as the period between the morning feeding of the stock and the withdrawal of the blood was concerned, and further permitted the analyses being completed the same day (except the amino-acid nitrogen determinations, since with these the colorimetric readings had to be delayed for 24 hours). Serial analyses could thus be made without difficulty.

In order not to introduce a complicating factor through the production of an anaemia as a sequel to too frequent bleedings, bleedings were undertaken with as long intervals as the main objects of this research permitted. The analyses were begun immediately, precipitation of the proteins being started within a few minutes of bleeding.

(2) Preparation of Blood Filtrates.

As previously stated, determinations were made on so-called " laked " and " unlaked " protein-free filtrates, the method followed being in both cases those advocated by Folin and Wu (1919), and Folin (1930), i.e. using tungstic acid as the protein precipitant.

(*a*) " *Laked* " *Blood Filtrates*.—The addition of 10 c.c. of blood to 70 c.c. of distilled water in a 100 c.c. container was followed by this mixture being well shaken to permit of thorough laking. 10 c.c. of 11 per cent. sodium tungstate solution ($Na_2WO_4 \cdot 2H_2O$) were now added, followed by 10 c.c. ·725 per cent. normal sulphuric acid, the mixture being again well shaken. With all samples having an

approximately normal haemoglobin content, the colour changes in the mixture are those described by Folin and Wu, the higher the haemoglobin content, the darker is the brown colour of the precipitate, but with anaemic bloods the colour is of varying depths of dirty pink, the more anaemic the lighter the colour remains.

Folin and Wu advocate the use of a 10 per cent. sodium tungstate solution and ·66 N sulphuric acid, but the author has encountered blood specimens where, when using these concentrations, the filtrate either did not come through water clear or came through clear but later became slightly turbid, especially was this the case with equine blood. When using the slightly higher concentrations, clear filtrates were always obtained.

On making check determinations with filtrates obtained with 10 per cent. and 11 per cent. sodium tungstate solutions for all the constituents, no differences in the results—within the experimental error—could be noted. During filtration—through Schleicher and Schüll's folded filter paper No. 588, 15 cm. diameter—the first few c.c. of filtrate were always returned. The filtrate is only very slightly on the acid side, except with anaemic blood, in which case it is distinctly more acid.

Ten c.c. blood yields sufficient filtrate for duplicate determinations of all the above-mentioned constituents.

In view of the number of different constituents which are determinable in the filtrate, this method of precipitation offers undoubted advantages and must be regarded as a notable advance in the technique for the study of such a complex tissue as blood.

(*b*) "*Unlaked*" *Blood Filtrates.*—For these Folin's (1930) method was employed, except that filtration, instead of centrifugation was used, no trouble being experienced in getting sufficient filtrate before disintregration and haemolysis set in. The filtration was carried on only sufficiently long to obtain sufficient filtrate for the determinations—generally about 30 minutes—the darkening of the blood during this period being only slight.

With the majority of blood samples visible disintregration, except darkening of the precipitate which takes place earlier, occurs only about after an hour, except in the case of bovine blood, which haemolyses more rapidly. In very rare cases disintegration occurred within 20-25 minutes with some pathological samples. Anaemic blood samples can immediately be spotted by their paler pink colour after the addition of the $\frac{1}{3}$ N sulphuric acid. In the case of the laked filtrate there is undoubtedly the objection raised by Wu (1922), that constituents of the disintegrated blood corpuscles are included, but where both filtrates are used concurrently, this very fact is of some interest in indicating in which fraction, i.e. whether in the cellular or plasma fraction, the constituents are concentrated, and whether any changes in the normal relative proportions can be noted under pathological conditions. This aspect will again be referred to in the final discussion of the data obtained.

(3) Amino-acid Determinations.

Folin's (1922) method was used, utilising 10 c.c. of each filtrate. The colorimeter was set generally at 10 mm. and no trouble in reading experienced except where the amino-acid content was found to be relatively low. In such cases the colour tint of the unknown tended more towards a yellow, and exact matching became virtually impossible. No efforts were made to attempt to determine why the colour in such cases differed from the standard. The cause may be the "dilution phenomenon" as suggested by Folin (1922) or be associated with a change in the relative proportions of the various amino-acids composing the "amino-acid nitrogen" fraction of the blood, when the total "amino-acid N", as given by this method, is low. Folin draws attention, during the discussion of the method, to the fact that only a part of the nitrogen, i.e. the nitrogen in the $-NH_2$ grouping reacts with the quinone reagent and that, therefore, certain amino-acids give readings which are in reality too low. He instances, amongst others, histidine which only reacts with one-third of its nitrogen, and tryptophane reacting with one-half. It is, therefore, theoretically at least, possible to obtain a low reading, even although the total amino-acids actually present may be relatively high. Under normal conditions the actual proportions and number of different amino-acids circulating in the blood is probably fairly constant, subject to the influence of the diet, but during pathological conditions especially septicaemias, selective destruction by the micro-organisms of one or more amino-acids would tend to disturb the normal proportions.

Unfortunately our knowledge of the metabolism of the micro-organisms in biological fluids or tissues intravitam during any specific infection, what they live on, what products are excreted by them, and what changes, if any, are brought about by these excreted substances in the surrounding medium, is extremely limited at present.

(4) Uric Acid Determinations.

The "uric acid N" in both "laked" and "unlaked" filtrates was determined by Folin's 1930 method. It was, however, found to be more satisfactory in the majority of cases, especially with sheep blood, where the uric acid concentration is low, to make the volume up to 15 c.c. instead of 25 c.c. for the colorimetric readings.

(5) The Determination of "Urea N."

For this Folin and Svedberg's urease (1930) method [see also Folin and Denis (1916)] was employed and once the technique had been acquired was found to be simple and convenient. At the beginning, however, difficulty was experienced in obtaining uniform results when making a series of duplicate determinations on solutions of urea and blood filtrates to which known amounts of urea had been added. Jorden and Graf (1933) of this Institute, investigated the method, and after a thorough check of all the reagents and the technique employed, suggested that possibly the fault lay with the amount of buffer solution added to the unknowns. Further detailed work in this connection confirmed this suspicion. It was found that constant results were obtainable through the addition of more buffer

solution, the optimum being determined to be 1·5 c.c. instead of the two drops recommended by the authors of the original method. Full details of this work are given in this (1933) Journal (see pages 279-283). In my determinations I have used 1·5 c.c. of the buffer solution and have obtained excellent results. For the preparation of the urease paper extracts made from locally grown soya beans and from " Merck's Soya bean meal ", gave equally satisfactory results. After the addition of the urease paper, the tubes were allowed to stand for 1-1½ hours, with occasional shaking. The small anti-bumping tubes not being available, a small glass bead was substituted and no trouble experienced with bumping once the technique of the urea distillation had been mastered. In most cases a standard corresponding to 10 mgm. urea nitrogen per cent. was employed owing to the low blood urea often encountered, although the 20 mgm. per cent. standard was also always made up. With high urea-containing filtrates, dilutions were made in such a way as to give colours after nesslerisation, closely approximating the colour of the standard. Repeated blanks were made throughout the course of these investigations, and in consequence an average figure of 1 mgm. urea N per cent. was subtracted from the " urea N " figures obtained.

(6) Non-Protein Nitrogen.

Folin and Wu's (1919) method [see also Folin and Svedberg (1930)] was used and all determinations made in duplicate, two standards in each case being made up, containing ·3 and ·15 mgm. N respectively. Where the N.P.N. was high, the determination was repeated with smaller amounts of filtrate, so that the colour obtained would approximate the standard solution. It is of great importance to continue the micro-digestion sufficiently long to ensure complete digestion, otherwise the readings will be too low. I have found the best results are obtained if the digestion is carried so far that when 15-20 c.c. of water are added, the solution is very slightly turbid. This turbidity disappears on making up to the final volume of 50 c.c. If the digestion is carried further, even for only a few seconds, a marked turbidity frequently results, which then interferes with accurate colorimetric readings, results which are too high being obtained. With experience the correct degree of digestion can be readily acquired.

(7) " Total " Creatinine Determinations.

Folin and Wu's (1919) method was used, the picric acid being purified with the method of Benedict (1929) from glacial acetic acid. In connection with the method as described on page 100 of the Journal of Biological Chemistry, Vol. 38, 1919, an error in the calculation has crept in. Instead of multiplying by 6 it is necessary to multiply by 12, since 20 c.c. of the standard solution recommended contain ·12 mgm. of creatinine. With 10 c.c. of the standard solution (Hawk, 1931), the calculation as given, would be correct.

When accidental overheating in the autoclave occurred a turbidity due to the presence of a white precipitate necessitated a repeat determination.

(8) Total Nitrogen (on Whole Blood).

For this 1 c.c. of oxalated blood was digested in a Kjeldhal flask with 15 c.c. of sulphuric acid-copper sulphate (15 c.c. acid and 1 c.c. 6 per cent. copper sulphate) digestion mixture until clear, the ammonia being distilled into a known volume (25-35 c.c.) of ·1 N sulphuric acid. A blank of 20 mgm. was allowed for.

(9) Haemoglobin.

One c.c. of whole blood was diluted to 200 c.c. with ·1 N hydrochloric acid to convert the haemoglobin into acid haematin. Full development of the colour is not obtained at once, all readings being taken with day-light illumination four hours after dilution. The standard employed was a Newcomer (1919) disc which had been standardised in this laboratory against the van Slyke (1921, 1924, and 1927) gasometric haemoglobin determination method.

The colorimetric readings were then converted by means of tables supplied with the disc into " haemoglobin per cent. Williamson's Standard " and this reconverted into " Grams Haemoglobin per 100 c.c. blood ". No correction was made for the small amount of anti-coagulant used—less than 1 per cent. All precautions such as detailed in Fourie's (1931) paper, viz. thorough shaking of the acid haematin solution, absence of gas bubbles in the pipette, etc., were taken into account.

(10) Sugar Determinations by Folin's (1929) Method.

In connection with this method the special Folin-Wu sugar tubes were not available in time and in their stead 18 mm. diameter test tubes 21 cm. long had to be utilised, only this type of test tube being used throughout. With them more constant results were obtained when the heating was continued for 20 minutes instead of the 14-15 minutes recommended. No time should be lost in the cooling after the heating and the addition of the acid molybdate reagent. Thorough mixing is essential. Owing to the relatively low blood sugar content of some animals, particularly sheep, only 1 c.c. standard glucose solution had frequently to be used, the final volumes being made up to 15 c.c. instead of 25 c.c.

(11) The Use of the Colorimeter.

For all the colorimetric work a " Holri " 50 mm. E. Leitz colorimeter was utilised. Particular attention was paid to noting zero points, setting the two fields evenly, interchangeability of cups, etc., in order to obtain accurate readings. As a general rule I preferred setting the standard at 10 mm., except for abnormally dilute solutions. With the lighter tints of the fields obtained in this way it appeared easier to get more constant readings, the difference in tints being more readily noticeable. 2-3 Careful readings were taken for each unknown, more readings throwing an undue strain on the eyes and tending to inaccuracy rather than accuracy. The source of illumination was always daylight against a white background.

(12) Temperature Charts.

In order to indicate the type of temperature reaction, and more particularly to demonstrate at what periods of the reaction blood examinations have been made, charts have been incorporated. The periods at which blood has been drawn has been indicated on the curves. The temperatures were taken twice daily (once only on Sundays) at 6.30 a.m. and 3.30-4 p.m. respectively. Only the actual reactions are recorded, the normal temperature records being omitted for the sake of economy of space, no useful purpose being served by the incorporation of several weeks of such normal records.

(13) Tables of Analytical Data.

These are mostly self explanatory, all constituents being expressed in " mgm. per 100 c.c. of blood " except haemoglobin and total nitrogen, both of which are expressed as " grams per 100 c.c. ". The " coaguable nitrogen " has been obtained by calculation (Total N – N.P.N.). The " Rest nitrogen " represents the nitrogen fraction unaccounted for in any specific form after the " urea N ", " Total creatinine N ", " uric acid N ", and " Amino acid N " had been subtracted from the " Non-protein N " figure. The urea (46·66 per cent. N), total creatinine (37 per cent. N) and uric acid (33·33 per cent. N) have for the sake of convenience been expressed both as such and as " Nitrogen ". In the column " plasma " the symbols " n.u." (nothing unusual) refer to the physical appearance of the plasma, more particularly to its colour. In anaplasmosis, redwater and horse-sickness, icteric plasmas were quite frequently encountered. The " plasma " column has been omitted in the case of heartwater, since the plasma at no time showed any haemolytic or icteric discolouration.

In the column " Temperature Reaction " the following symbols have been adopted: —

N (Normal) = meaning that no abnormal temperature reaction is going on at the time of bleeding.

P.I.N. (Post infectionem, normal) = indicating that the animal has been injected with virus, but that as yet no temperature reaction has set in.

R (Reaction) = indicating that blood was withdrawn during the course of a reaction.

" Time of bleeding " = where no symbol is given it means that the blood was drawn between 8.30 a.m. and 9.30 a.m.

In other cases the time of bleeding is inserted. The above system has been applied throughout these publications.

SUMMARY.

A scheme has been outlined of a series of researches into various animal diseases and the technique and the chemical methods utilised have been described. The present paper is to be regarded as the first of a series of publications to be issued under the general title of " Chemical Blood Studies " and is to serve as a general introduction for the series. The chemical determinations include total nitrogen (T.N.), haemoglobin (Hb), " Total " creatinine nitrogen (T.C.N.), urea nitrogen (U.N.), uric acid nitrogen (U.A.N.), amino-acid nitrogen (A.A.N.), sugar (S.), and non-protein nitrogen (N.P.N.).

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