Onderstepoort Journal of Veterinary Science and Animal Industry, Volume 14, Numbers 1 and 2, January and April, 1940.

Printed in the Union of South Africa by the Government Printer, Pretoria.

Chemical Blood Studies VIII.

A Rapid Spectroscopic Method for (A) The Quantitative Determination of Haemoglobin in Blood and (B) Its Application for the Quantitative Estimation of Haemoglobin in Milk, Urine, Serum or Plasma and Faeces.

By G. C. S. ROETS, Department of Chemical Pathology, Onderstepoort.

In connection with animal pigment metabolism studies the author and his collaborators needed a rapid and efficient method for estimating haemoglobin quantitatively in biological materials. In this communication such a method is recorded, by which the blood haemoglobin and haemoglobin present in milk, urine, serum or plasma and faeces can be quantitatively estimated.

A.—The Quantitative Determination of Haemoglobin in Blood.

Methods considered.

Newcomer, H S. (1919) proposed a haemoglobinometer, with a coloured glass disc as a standard for the quantitative determination of haemoglobin as acid haematin. In 1923 he advocated the use of a modified Duboscq colorimeter. This method, known as the Newcomer disc method, is in common use. Cohen, B and Smith, A. H. (1919) used an Autenrieth-Hellige or Duboscq colorimeter for matching the colour against that of a standard acid haematin solution.

Another technique was developed by Wong, S. Y. (1922) by which the iron present in the blood is determined and from this the total amount of haemoglobin is calculated, the assumption being that iron, apart from that contained in the haemoglobin molecule is present only in traces in blood. In 1928 he described how the iron of the haemoglobin molecule can be detached by the action of sulphuric acid, the decomposition being facilitated with the aid of potassium persulphate. The interfering proteins are completely precipitated by tungstic acid and filtered off. Colorimetric comparisons are made against a standard iron solution. Andes, J. E. and Northup, D. W. (1938) made use of a photoelectric colorimeter for the comparisons. Hanzal, R. F. (1933) determined the iron by digesting blood

with sulphuric acid and hydrogen peroxide over a microburner, and by comparing the characteristic colour obtained with thioglycolic acid and ammonium hydroxide against that of a standard iron solution similarly treated. The last mentioned method was preferred by the author on account of its extreme sensitivity and the elimination of the filtering process. Myers, V. C. and Eddy, H. M. (1939) described slight modifications to permit its use with 0·05 c.c. of blood.

Dénes, A. (1932) established a spectrophotometric method for measuring the 555·8 μ μ and 527·1 μ μ haemochromogen absorption bands ratio. From the extinction coefficients of these two wave-lengths of the spectrum and the absorption ratio, the haemoglobin content of the blood can be calculatetd. The haemin content of liver catalase preparations was determined by Stern, K. G. (1937) by converting the haemin into pyridine haemochromogen and comparing the intensity of the absorption bands against that of a standard prepared from crystalline blood haemin. The same principle had been used by Zeile, K. and Hellström, H. (1930) and also by Keilin, D. and Hartree, E. F. (1936).

The intensity of the 555 absorption band of Pyridine haemochromogen is made use of in my method; a Zeiss pocket spectroscope and the principle of dilution as described by Rimington, C., Roets, G. C. S. and Fourie, P. J. J. (1938) in the quantitative determination of coproporphyrin being used.

Procedure.

Stock Solution.—Dissolve 116·6 mg. pure recrystallised haemin in 40 c.c. pyridine and dilute up to 100 c.c. with ·1N NaOH solution.

Standard.—A dilution of 0·5 c.c. stock solution up to 100 c.c. with ·1N NaOH serves as a standard solution.

Blood.—*Transfer* 0·4 c.c. oxalated blood into a 200 c.c. measuring flask, add ·1N NaOH up to the mark, shake thoroughly and leave at room temperature for 10 minutes.

Comparison.—Take two similar thin glass test tubes 1·5 cm. in diameter, and measure off 5 c.c. of the standard solution into the one and 5 c.c. of the blood solution into the other. To each of the tubes add 1 c.c. pyridine and a few mg. sodium hydrosulphite. Compare the intensity of the 555 absorption bands and dilute the solution showing the more intense band (whether standard or unknown) with a ·1N NaOH solution by means of a calibrated standard pipette until equal intensity is reached in both tubes. After each dilution the solution should be thoroughly mixed by lateral shaking.

Calculations.

M. wt. of haemoglobin = 66,800.

The haemoglobin molecule has four haematin molecules [see Hawk and Bergeim (1931)].

Haemin = $C_{34}H_{32}O_4N_4$ FeCl.

Thus the concentration haemoglobin equivalent to the standard solution $= 116 \cdot 6 \times \frac{66800}{651 \cdot 329 \times 4} \times \frac{\cdot 5}{100}$ mg. per 100 c.c.

$= 14 \cdot 95$ mg. or $0 \cdot 01495$ g. per 100 c.c.

In the unknown, the amount of haemoglobin expressed as grams haemoglobin per 100 c.c. blood

$$= \frac{x}{6} \times \frac{200}{0 \cdot 4} \times 0 \cdot 01495$$

$x =$ Volume in c.c. to which the unknown is diluted

$$\text{or } = \frac{6}{y} \times \frac{200}{0 \cdot 4} \times 0 \cdot 01495$$

where $y =$ Volume in c.c. to which the standard is diluted.

Precautions.—The addition of too much sodium hydrosulphite produces a slight turbidity, which may result in too high values. With experience the exact quantity necessary can easily be judged.

Fresh stock solutions should be made up monthly and kept in the dark since decomposition takes place. Standard solutions should be made up daily.

Discussion.

As a check on this method simultaneous determinations were made by the iron method as described by Hanzal and by the Newcomer disc method. The results obtained by the former compared quite satisfactorily whilst those of the last mentioned method did not. Andes and Northup (1938) had the same experience when comparing the results obtained by Wong's method and the Newcomer disc method.

The following table shows comparative results obtained by these three methods.

TABLE I.

Comparison of the Haemoglobin Values by the Three Methods.

Animal Species.	Iron Method. g. Hb. per 100 c.c. Blood.	Spectroscopic Method. g. Hb. per 100 c.c. Blood.	Newcomer Disc Method. g. Hb. per 100 c.c. Blood.
Sheep normal	12·61	12·57	14·31
Bovine normal	11·47	11·42	12·61
Bovine normal	11·78	11·42	13·06
Bovine normal	11·48	11·82	14·04
Bovine normal	11·48	11·82	13·78
Bovine normal	12·10	12·37	15·18
Bovine normal	15·71	14·93	16·87
Bovine normal	12·10	12·46	12·61
Bovine normal	10·92	10·59	10·87
Bovine anaplasmosis	4·14	4·08	6·11
Bovine anaplasmosis	6·22	6·85	5·08

With the necessary practice and sufficient equipment 70 to 100 determinations can easily be made during an eight hour working day.

The accuracy and simplicity will undoubtedly be increased with a more efficient spectroscopic apparatus. The author attached a Zeiss microspectroscope ocular with a comparison prism to a microscope and transferred the standard solution to a tube in front of the comparison prism which was illuminated with a mirror in a fixed position. The stratum of the standard in a little beaker on the microscope stage was adjusted by addition until equal intensity in both spectra was reached. The unknown was now diluted with known quantities and replaced in the tube in front of the comparative prism until equal intensity was once more reached. Better results were not obtained. Similar apparatuses for comparing spectra intensities were designed by Stern, K. G. (1937) and Keilin, D. and Hartree, E. F. (1936) where the strata of liquids could be altered by movable plungers similar to the principle employed in ordinary colorimeters.

(B) The Application of this Pyridine Haemochromogen Method to the Quantitative Estimation of Haemoglobin in Milk, Urine, Serum or Plasma and Faeces.

Methods Considered.

Numerous methods for the qualitative detection of blood in biological materials can be traced in physiological and biochemical text books like those of Hawk, P. B. and Bergeim, O. (1931), Plimmer, R. H. A. (1918) and White, D. S., and Fischer, P. (1920). These methods can be classified into four groups:—

(1) *Peroxidase Reactions.*—Haemoglobin reacts upon peroxide giving off oxygen. Oxygen thus liberated gives marked colour reactions with substances of the following type:—

(*a*) Guaiacum—blue.
(*b*) Aloin—purple-red.
(*c*) Benzidine—blue-green.
(*d*) Malachite green—green.
(*e*) p-Phenylenediamine hydrochloride—brown-red.
(*f*) Phenolphthalein—red.
(*g*) Ortho-toluidin—bluish.

These tests, with all their modifications, are based on the same property of haemoglobin. Thus any substance which liberates oxygen in the same way as haemoglobin, will give a positive reaction. In practice, therefore, pus, milk, faeces, etc., very often give a positive result in the absence of haemoglobin.

(2) *The microscopic identification of erythrocytes.*—This method is one of the best when non-haemolysed blood is present, for then liquid materials such as urine and milk can be centrifuged and the sediment examined microscopically for erythrocytes.

(3) *The conversion of haemoglobin into haematin.*—(*a*) In the potassium hydroxide test (Heller) the red colour of haematin is made use of to detect the presence of blood. This test is very unsatisfactory since many other substances like porphyrins, for example, may contribute to a red colour of the solution.

(*b*) The crystals of haematin can easily be prepared by the acetic acid method and microscopically identified. This is a specific test but the very small quantities to be dealt with in practice makes this method often impracticable.

(4) *Spectroscopic examination.*—The spectroscopic examination of translucent materials like lymph, serum, plasma or urine is one of the most satisfactory ways by which haemoglobin can be detected in such materials.

The method described in this article makes it possible to detect haemoglobin in concentrations as low as 4 mg. haemoglobin per 100 c.c. liquid, on account of the extreme intensity of the 555 band of pyridine haemochromogen.

Procedure.

(1) *Milk.*—Shake the milk sample thoroughly to obtain an even distribution of erythrocytes, which might have gravitated towards the bottom of the container on standing. Transfer 30 c.c. to a calibrated centrifuge tube, add a few mg. saponin, stir well with a glass rod and leave at room temperature for 30 minutes. Add 0·3 c.c. of strong hydrochloric, stir well and centrifuge for 15 minutes. Note the volume of the liquid. Measure off 10 c.c. of this liquid into a centrifuge tube, add 2 c.c. of pyridine, mix well and centrifuge for 10 minutes. To 6 c.c. of the supernatant liquid is added sodium hydrosulphite. The comparison is now made.

Calculation.

$$\frac{x}{6} \times \frac{y}{30} \times 14{\cdot}95 \text{ mg. haemoglobin per 100 c.c.}$$

x = Volume in c.c. to which the unknown is diluted.

y = Volume in c.c. of the liquid phase.

or

$$\frac{6}{z} \times \frac{y}{30} \times 14{\cdot}95 \text{ mg. haemoglobin per 100 c.c.}$$

where z is the volume in c.c. to which the unknown is diluted.

Thus 30 c.c. of a milk sample containing blood equivalent to 27·41 mg. haemoglobin per 100 c.c. yielded 25 c.c. of liquid after being centrifuged. 6 c.c. of the final liquid after being treated with pyridine and centrifuged had to be diluted up to 13 c.c. to match the standard.

$$\text{Haemoglobin found} = 14{\cdot}95 \times \frac{13}{6} \times \frac{25}{30} \text{ mg. per 100 c.c.}$$

$$= 26{\cdot}99 \text{ mg. per 100 c.c.}$$

The haemoglobin of the erythrocytes in fresh milk can be determined as follows: Centrifuge a known volume of the sample for 30 minutes, decant off the liquid carefully, lake the sediment with a 0·05 per cent. saponin solution (1 to 5 c.c. or more) and perform the test as above.

It was observed that the haemoglobin concentrations decreased in the course of time. The following experiment was therefore carried out:—

2·5 c.c. of a solution containing 4·96 g. haemoglobin per 100 c.c. was made up to 500 c.c. with milk. This mixture was thoroughly shaken. 250 c.c. was exposed in an open flask at room temperature and 250 c.c. was stored in an open flask in the ice chest. Tests were carried out on these two samples and it was found that the haemoglobin concentrations decreased equally in both flasks. Table II shows the percentage decrease at different periods.

TABLE II.

Time in hours.	*Percentage decrease.*
2	3·5
5	24·5
24	62
72	74·5
96	80·4

It is therefore necessary to examine milk within two hours after withdrawal from the udder to avoid too large an error. Furthermore the rate of haemoglobin destruction may vary considerably in different milks, whether from normal or diseased udders, and this point merits further investigation.

(2) *Urine and serum (or plasma).*—Measure off 10 c.c. into a centrifuge tube, add 2 c.c. of pyridine, mix well and centrifuge for 10 minutes. The test is performed on 6 c.c. of the clear supernatant fluid as previously described.

Calculations.

$$14{\cdot}95 \times \frac{x}{6} \text{ mg. per 100 c.c.}$$

Where x = Volume in c.c. to which the unknown is diluted.

$$\text{or } 14{\cdot}95 \times \frac{y}{6} \text{ mg. per 100 c.c.}$$

where y = Volume in c.c. to which the standard is diluted.

The haemoglobin concentration in a normal human urine to which blood had been added, remained constant within the limits of experimental error, for at least 5 hours, but decreased by 34 per cent. and 66·9 per cent. after 2 and 7 days respectively. The haemoglobin concentration in urine should therefore be estimated as soon as possible after collection.

(3) *Faeces*.—Grind in a mortar 5 grams fresh faeces with 30 c.c. of a 0·05 per cent. saponin solution, leave at room temperature for 15 minutes and centrifuge for 15 minutes. Pipette off 10 c.c. of the liquid, add 2 c.c. of pyridine and a pinch of sodium chloride, stir well and centrifuge for 15 minutes. The test is performed on 6 c.c. of this solution.

Calculations.

$$14 \cdot 95 \times \frac{x}{6} \times \frac{30}{5} \text{ mg. per 100 g. faeces.}$$

Where x = Volume in c.c. to which the unknown is diluted.

$$\text{or } 14 \cdot 95 \times \frac{6}{y} \times \frac{30}{5} \text{ mg. per 100 g. faeces,}$$

where y = Volume in c.c. to which the standard is diluted.

It should be noted that a possible haemoglobin destruction may also take place in faeces, and to investigate this possibility the following experiment was conducted:—

0·3 c.c. quantities of a solution of blood in distilled water (4·485 g. haemoglobin per 100 c.c.) were added to 5 g. samples of fresh haemoglobin-free bovine faeces; thus the theoretical amount in each sample should be 269 mg. per 100 g. faeces.

Concentration found = 284 mg. per 100 g.

After the samples had been left at room temperature for 2 hours it was 268 mg. per 100 g. A further decrease of 30 per cent. was found after 24 hours. To obtain the most reliable concentration, estimations should therefore be made as soon as possible after sampling, preferably within 2 hours after defaecation.

In applying this pyridine haemochromogen method for the quantitative estimation of haemoglobin in milk, urine, serum or plasma and faeces a slight opalescence or other pigments apart from haemoglobin may interfere with the penetration of light to a certain extent. The pyridine haemochromogen, however, has such an intense absorption band that this defect is not a serious one. It should also be kept in mind that methaemoglobin, which is frequently encountered is also converted into the pyridine haemochromogen.

Summary.

A spectroscopic method for the quantitative determination of the haemoglobin content of blood is described. The haematin is converted into pyridine haemochromogen and a comparison of the intensity of the 555 absorption band made against a known standard. Haemoglobin can be quantitatively estimated in milk, urine, serum, plasma or faeces.

Acknowledgment.

I wish to thank Dr. H. Graf, Head of this Department, for his interest and encouragement in this work.

LITERATURE

ANDES, J. E., AND NORTHUP, D. W. (1938). Photoelectric colorimetry. 1. Determination of blood iron and haemoglobin. *J. Lab. and Clin. Med.*, Vol. 24, pp. 197-206.

COHEN, B., AND SMITH, A. H. (1919). The colorimetric determination of iron and haemoglobin in blood. *J. Biol. Chem.*, Vol 39, pp. 439-496.

DENES, A. (1932). Über die Lichtabsorption des Globinhämochromogens und über seine Verwendbarkeit zur Bestimmung des Farbstoffgehaltes des Blutes. *Biochem. Zeitschr.*, Vol. 255, pp. 379-386.

HANZAL, R. F. (1933). Determination of iron in boilogical material. *Proc. Soc. Expt. Biol. and Med.*, Vol. 30, p. 846.

HAWK, P. B., AND BERGEIM, O. B. (1931). *Practical Physiological Chemistry.*

KEILIN, D., AND HARTREE, E. F. (1936). On some properties of Catalase Haematin. *Proc. Roy. Soc. of London*, Vol. 121, pp. 173-191.

MYERS, V. C., AND EDDY, H. M. (1939). The haemoglobin content of human blood. *J. Lab. and Clin. Med.*, Vol. 24, pp. 507-508.

NEWCOMER, H. S. (1919). Absorption spectra of acid haematin, oxyhaemoglobin and carbon monoxide haemoglobin. A new haemoglobinometer. *J. Biol. Chem.*, Vol. 37, pp. 465, 496.

NEWCOMER, H. S. (1923). A new optical instrument for the determination of haemoglobin. *J. Biol. Chem.*, Vol. 55, pp. 569-574.

PLIMMER, R. H. A. (1918). *Practical organic and bi-chemistry.*

RIMINGTON, C., ROETS, G. C. S., AND FOURIE, P. J. J. (1938). Quantitative studies upon porphyrin excretion in bovine congenital porphyrinuria (pink tooth). No. 1. *Onderstepoort Jnl. of Vet. Sci. and Animal Industry*, Vol. 10, No. 2, pp. 421-429.

STERN, K. G. (1937). On the absorption spectrum of catalase. *J. Biol. Chem.* Vol. 121, pp. 561-572.

WHITE, D. S., AND FISCHER, P. (1920). *Malkmus outlines of clinical diagnostics.*

WONG, S. Y. (1922). Colorimetric determination of iron and haemoglobin in blood. *J. Biol. Chem.*, Vol. 55, pp. 421-425.

WONG, S. Y. (1928). Colorimetric determination of iron and haemoglobin in blood II. *J. Biol. Chem.*, Vol 77, pp. 409-412.

ZEILE, K., AND HELLSTRÖM, H. (1930). Über die aktiewe Gruppe der Leberkatalase. *Z. Physical. Chem.*, Vol. 192, pp. 171-192.