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ESTIMATION OF HAEMOGLOBIN BY THE ALKALINE HAEMATIN METHOD

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Haemoglobinometry has at present two chief functions—first, the detection of diseases characterized by deficiency or excess of haemoglobin; secondly, the study of changes in haemoglobin concentration caused by loss or gain of plasma. For both purposes it is desirable that the method employed should estimate the concentration of all the forms of haem-pigment circulating in the blood. Ammundsen (1939, 1941) has shown that under modern conditions a normal adult may have from 2 to 12% of his total haemoglobin circulating in an inactive form. The inactive fraction is chiefly composed of carboxyhaemoglobin, methaemoglobin, and sulphaemoglobin. Drugs of the sulphonamide class often increase the proportion of inactive haemoglobin in patients receiving this form of treatment. Most methods of estimating haemoglobin are standardized by reference to determinations of the oxygen-carrying power of blood. These yield valuable information concerning one important function of the blood, but they may provide a false picture of the pigment metabolism, since they take no account of inactive haemoglobin which may be capable of regeneration to the active form. They may imply the presence of anaemia where none exists. Several methods of estimation are in general use, and it is pertinent to inquire not only into the accuracy of each but also if total pigment or merely active haemoglobin is being measured.

The Haldane method enjoys a wide popularity, yet, being based on the gas (CO)-carrying power of blood, it may not measure total pigment. Samples of blood are frequently encountered in which the colour is not comparable with the standard; this is probably due to the presence of a large amount of inactive haemoglobin which is not convertible to carboxyhaemoglobin. The standards available commercially are often unreliable and may quickly fade. In addition the method requires a source of carbon monoxide.

There are several acid haematin methods in use, of which the most familiar are the Sahli and Newcomer. Several disadvantages are common to them all. The colour developed by the test does not quickly reach a maximum; while most of it develops in the first 30 or 40 minutes, the greatest optical density is not reached for from 24 to 36 hours, and the velocity of the colour reaction is not constant. Some observers claim the colours are not easily matched and that there is a high degree of personal error: according to Heilmeyer and Von Mutius (1938) this may be as high as ± 10%. Wu (1922) has shown that the colour developed depends upon the concentration of protein and lipoid in the test; and, since acid haematin is insoluble and exists as a colloid suspension protected by the protein present, there is often turbidity or actual precipitation.

The pyridine haemochromagen method of Roets (1940) [cf. Rimington, 1942] depends on the following principle. Haemoglobin in the sample is converted by sodium hydroxide, pyridine, and sodium hydrosulphite into pyridine haemochromagen. The standard is crystalline haemin, which is converted to the haemochromagen with the same reagents. The two solutions are compared colorimetrically and the concentration of haemoglobin in the test is calculated on the assumption that each milligramme of iron in the haemin standard is exactly the equivalent of a milligramme of haemoglobin iron in the test. This method has the advantage of a standard which is readily reproducible, and in addition it measures total pigment; but most pathologists would probably agree that pyridine, because of its extremely objectionable odour, is an unsuitable reagent for use in a routine clinical laboratory.

Recently we have been searching for some method of haemoglobin estimation which fulfils the following criteria. It must measure total pigment with a reasonable degree of accuracy and yet be simple enough to be employed in a laboratory where many determinations are performed daily. Measurement must be in terms of some simple easily reproducible standard so that the results at one laboratory may be directly comparable with those obtained in another. This standard must be either haemoglobin or some other substance with constant properties which may be referred to haemoglobin. From a review of the literature we decided that the most likely procedure was the alkaline haematin method of Wu (1922), which is recommended by Peters and van Slyke (1932). Wu found that the acid haematin method of Cohen and Smith (1919), the CO method of Palmer (1918), the methaemoglobin method of McEllroy (1920), and the cyanhaemoglobin method of Stadie (1920) gave divergent results with sheep's blood, but that if the test solutions were all rendered alkaline before the colorimetric readings were made the differences largely disappeared. The acid haematin method applied to solutions of pure haemoglobin gave lower results than those obtained when plasma was added. The errors increased with increasing amounts of plasma, but were not proportional to the plasma present. With solutions of washed red cells the reverse was the case: added plasma decreased the apparent observed concentration of haemoglobin. These extraordinary effects, probably due to the colloidal nature of haematin in the acid medium and of proteins and fats from the plasma, were abolished by the

addition of excess sodium hydroxide to the test solutions. Haematin exists in true solution in alkaline solutions of *p*H 10 and over, and plasma proteins and fat are likewise much better dissolved by alkali than by acid. The presence of variable amounts of the abnormal forms of haemoglobin (CO-, met-, cyan-), moreover, exerted a pronounced effect on the acid haematin method, but no effect when the solutions were made alkaline, the same amount of alkaline haematin being produced from any form of haemoglobin or from mixtures of them. The alkaline haematin method of Wu, then, appeared to satisfy our criteria of reasonable accuracy, of simplicity, and of measuring all forms of haemoglobin.

The majority of the standards in use for the different methods of haemoglobinometry are prepared from blood. This is a grave disadvantage, as the blood needs careful standardization by the oxygen capacity method, a procedure which requires elaborate apparatus and a skilled technique and which is difficult for those who are not experienced in gas analyses. The standards prepared from blood are, moreover, not easily preserved, and frequent restandardization is necessary. Because of these difficulties there has been a wide-spread adoption of coloured glass standards, but these have proved unsatisfactory because of lack of uniformity and of the failure to give an equally good match of the haemoglobin colours in light from different sources. The alkaline haematin method carries the great advantage that a simple reproducible standard can be prepared from crystalline haemin, a substance which is easy to prepare and which can be weighed out in equal amount and dissolved in soda by any worker to give standards of identical colour value.

Haematin Standards

Thirteen samples of crystalline haemin have been prepared by slightly different methods from ox and human bloods.[1] The iron content of each sample was determined and the results are shown in Table I. Solutions of alkaline haematin containing

TABLE I.—*Specific Extinction Coefficients of Various Preparations of Haemin**

No.	Blood Used	% Fe	Analyst	Method of Analysis	Es† (for 1 mg. Fe/100 c.cm.)	Es‡ (for 1 mg. Fe/100 c.cm.)
1	Ox	8·55	Weiler and Strauss	"Micro Fe_2O_3"	0·764	0·768
2	Ox	8·29 8·28	Geochem. Labs. Delory	$TiCl_3$	0·782	0·786
3	Human	8·53	Weiler and Strauss	"Micro Fe_2O_3"	0·752	0·768
4	,,	8·45	Delory	$TiCl_3$	0·760	0·752
5	,,	8·21	,,	,,	0·779	0·766
6	,,	7·81	,,	,,	0·793	0·800
7	Human	8·29	,,	,,	0·769	0·757
8	,,	8·26	,,	,,	0·786	0·771
9	,,	8·18	,,	,,	0·752	0·758
10	,,	8·50	,,	,,	0·756	0·748
11	,,	8·45	,,	,,	0·766	0·759
12	,,	8·32	,,	,,	0·762	0·753
13	Washed cells	8·40	,,	,,	0·775	0·764
				Mean =	0·769	0·766
				Standard deviation (corrected) =	0·009	0·014
				Coefficient of variation =	1·18%	1·83%

* 10 mg. (approx.) per 100 c.cm. solution in N/10 NaOH. Two-stage Leitz compensating colorimeter; mercury-green illumination; 1·01 D Ilford neutral grey screen, average depth of solution 15 mm.
† Average of 3 separate determinations by 3 observers.
‡ Determined in photo-electric colorimeter (King, 1942), Chance green filter. The extinction coefficients re-determined on these same solutions 6 months after their preparation were identical with those shown.

approximately 10 mg. of haemin per 100 c.cm. were prepared and the specific extinction coefficients of the solutions measured. The mean of the 13 samples is 0.769, with a standard deviation of 0.009 and a coefficient of variation of 1.18%. Three other samples, one prepared in bulk by B.D.H. and two kindly supplied by Dr. R. G. Macfarlane of Oxford, agree closely with the results in the table. These solutions, which have remained stable for over six months, obey the Beer-Lambert law. Clearly when alkaline haematin is prepared from crystalline haemin the intensity of the colour developed is proportional to the concentration of iron in the sample.

Alkaline Haematin Method Applied to Blood

When adult human blood is added to decinormal sodium hydroxide a brown colour is quickly developed which on spectroscopic examination contains the specific absorption band of alkaline haematin (607 mμ). Blood taken from infants develops this colour much more slowly owing to the fact that the foetal type of haemoglobin is more resistant to alkaline denaturation. Brinkman and Jonxis (1935) have shown that the foetal type of haemoglobin steadily diminishes after birth and is absent at 7 months; at about 3 years another type of alkali-resistant haemoglobin appears, and persists throughout life. This adult resistant haemoglobin occurs only in small concentrations and the main bulk of adult haemoglobin has little resistance to alkali. The rate of alkali denaturation of all forms is markedly accelerated by increasing the *p*H and the temperature. We have investigated the rate at which alkali denaturation becomes complete in samples containing varying proportions of resistant and inactive haemoglobin.

If a 1 in 100 dilution of umbilical cord blood in N/10 soda is made, the solution remains bright pink, with the absorption bands of oxyhaemoglobin clearly visible for some hours at room temperature. With brief heating the colour change is dramatic, and the oxyhaemoglobin bands are replaced by those of alkaline haematin. The effect of heat was studied by making a 1 in 100 dilution of umbilical cord blood and placing it in a boiling-water bath. Aliquots were removed at various intervals and promptly cooled under the tap before being compared in a colorimeter with a standard alkaline haematin solution. It was found that the colour reaction, often complete after 2 minutes in the bath, was invariably so after 5 minutes. For a further short and variable time the colour remained constant, but turbidity developed later, giving an apparent increase in colour. No significant turbidity was detected after only 5 minutes in the bath (Table II).

TABLE II.—*Effect of Heat on Alkali Denaturation of Foetal Type Haemoglobin*

Time of Heating	Blood A	Blood B	Blood C	Blood D
Before heating ..	0·50*	0·43*	0·88*	0·60*
2 minutes	0·318†	0·285†	0·670†	0·455†
5 ,, ...	0·308†	0·285†	0·662†	0·440†
10 ,, ...	0·310†	0·285†	0·680‡	0·440†
15 ,, ...	—	0·330‡	0·710‡	0·463‡

* Still pink. † Clear brown. ‡ Turbidity.
The figures represent the extinctions E read in the King (1942) photo-electric colorimeter, Chance green filter.

Many observations were made on hospital patients to determine if any appreciable concentration of resistant haemoglobin might be encountered. 0.05 c.cm. of blood was added to 5 c.cm. of N/10 soda. After this unheated solution had been compared with a standard solution it was allowed to stand overnight, when the comparison was repeated. In many cases there was no significant difference between the two observations, but in some adults and all infants the overnight reading

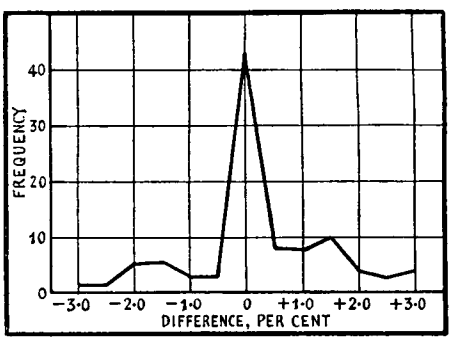

FIG. 1.—Difference in colour of blood in N/10 NaOH produced by heating for 5 minutes and by standing overnight at room temperature.

was significantly lower than the initial reading—i.e., the change from pink to brown was not complete in the few minutes before the first reading.

The effect of heating all specimens was then studied, 0.1 c.cm. of blood being added to 10 c.cm. of N/10 soda and the specimen divided. One-half of each test was placed in the boiling-water bath for exactly 5 minutes, cooled under the

[1] The methods of preparation and of iron analysis are published elsewhere (Delory, 1942).

tap, and compared with the standard haematin solution. The other half was allowed to stand overnight and also compared with the standard. Fig. 1 is a distribution curve of 100 consecutive observations on 96 patients and 4 specimens of umbilical cord blood. No observation falls outside the limits of 3% of the same reading, 91% of cases are within 2%, and in 43% of cases the observations are identical. The mean overnight reading is 100.285 of the 5-minute reading, and the standard deviation is 1.72.

A comparison was next made of a series of alkaline haematin values produced by 4–5-minute heating in sodium hydroxide with those developed by Wu's procedure—i.e., a 40-minute treatment of the blood with N/10 hydrochloric acid at room temperature followed by the addition of excess sodium hydroxide. Table III shows the close agreement of the two

TABLE III.—*Comparison of Haemoglobin Results by Different Haematin Methods. (Figures represent % of Haden Normal; Consecutive Routine Examinations.)*

Alkaline Haematin Methods		Acid Haematin Method	Alkaline Haematin Methods		Acid Haematin Method
Present Method*	Wu's Method†		Present Method*	Wu's Method†	
%	%	%	%	%	%
42	41	46	36	36	40
69	70	67	40	39	45
84	84	80	53	53	61
83	84	87	74	73	87
88	87	81	84	83	89
91	89	89	80	80	81
83	86	81	83	83	85
86	86	84	37	37	41
56	55	57	88	88	92
76	76	78	76	76	80
25	23	36	51	51	58
43	43	45	77	77	80
55	56	57	59	59	64
57	57	61	76	76	78
48	48	54	94	95	95
80	81	82	55	55	54
83	83	85	72	73	76
70	70	68	84	84	87
47	46	52	72	73	76
66	65	81	90	90	92
73	72	71	55	56	58
74	74	80	29	30	34
79	78	82	98	98	96
32	31	31			

* 0·05 c.cm. blood in 5 c.cm. N/10 NaOH, heated 5 minutes.
† 0·05 c.cm. blood in 4 c.cm. N/10 HCl, let stand 40 minutes, made alkaline with 1 c.cm. N/1 NaOH.

alkaline haematin methods, and the discrepancies between them and the acid haematin procedure.

From these experiments it seems clear that the same final alkaline haematin colour is developed equally well either by treating the blood directly with soda and heating or allowing to stand at room temperature for many hours, or by an initial treatment to convert the haemoglobin into acid haematin followed by the addition of soda to render it alkaline.

To study the effect of inactive haemoglobin it is necessary to subject the sample to considerable manipulation with increased opportunity for introducing sampling errors. To reduce these as far as possible normal blood was lysed with water and filtered, the resulting solutions being much easier to handle than whole blood. Table IV indicates the steps

TABLE IV.—*Colour Produced by Different Forms of Haemoglobin when Estimated by the Alkaline Haematin Method*

Solution	Untreated	As COHb (gassed for 30 min.)	As Sulph-Hb (H_2S passed for 10 min)	As Met-Hb (Treated with Pot. Ferricyanide)
1	0·168	0·162	0·171	0·162
2	0·199	0·204	0·205	0·199
3	0·544	0·548	0·536	0·536
4	0·376	0·376	0·374	0·388
5	0·346	0·336	0·347	0·339

(Figures represent extinctions E, photo-electric colorimeter, Chance green light filter.)

taken to convert the haemoglobin into inactive forms, and also shows that they are quantitatively converted to alkaline haematin by heating for 5 minutes in the water-bath. This experiment confirms the finding of Wu, that the different forms of haemoglobin are quantitatively determined when estimated by the alkaline haematin method.

Likewise, the addition of plasma either to the haemin standard or to tests prepared from blood had little or no effect. Plasma added in amount equal to the volume of blood taken—i.e., 0.05 c.cm. in 5 c.cm. of soda—caused no measurable change in many cases, and in others the change was of the order of 1% increase. The most pronounced effect observed was a 2% increase in optical density due to adding a turbid specimen of plasma.

Relation of Colours Produced from Haemin and from Haemoglobin in Sodium Hydroxide

From theoretical considerations it now seemed possible to equate solutions of alkaline haematin derived from haemin and from blood on the assumption that one milligramme of iron in the haemin standard would be equivalent to one milligramme of iron in the haemoglobin test. Attempts to apply this assumption to the clinical estimation of haemoglobin showed that bloods examined in this way yielded results by the alkaline haematin method which were higher than could be justified either by the clinical condition of the patient or by examination of the stained blood film. In a small series of hospital cases venous blood samples were analysed for haemoglobin by the alkaline haematin procedure and for oxygen capacity with the van Slyke manometric apparatus by Dr. J. McMichael. The results obtained by the haematin method were uniformly about 30% higher than the oxygen

TABLE V.—*Haemin Iron Equivalent of Oxygen Capacity*

Subject	Oxygen Capacity (c.cm./100 c.cm. Blood)	Fe eqv. (mg.)	Fe found by Comparison with Haemin (mg.)	Found Fe as % of O_2 eqv. Fe
S (human) ..	11·94	29·8	39·5	132
N ,, ..	19·75	49·4	65·1	132
B ,, ..	18·27	45·7	55·6	122
N ,, ..	19·45	48·6	63·6	131
M ,, ..	19·8	49·5	63·6	128
T ,, ..	20·3	50·7	61·7	122
R ,, ..	19·9	49·7	63·6	128
D ,, ..	23·0	57·5	69·5	122
F ,, ..	22·2	55·5	68·5	123
Horse ..	20·1	50·2	63·5	127
			Average =	127

1 atom Fe (56 g.) ≡ 1 mol O_2 (22·4 l.)
1 mg. Fe ≡ 0·4 c.cm. O_2

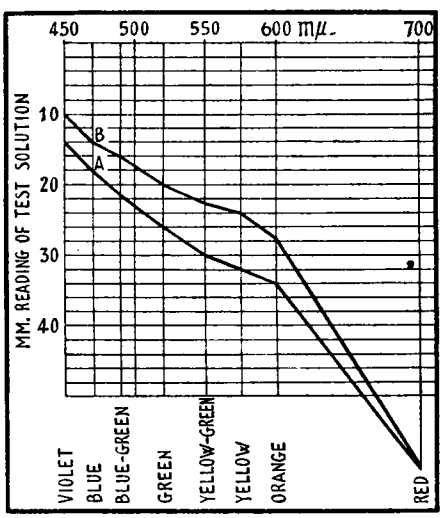

FIG. 2.—Light-absorption curves of haemin in sodium hydroxide (A) and of haemoglobin heated in sodium hydroxide (B); Ilford spectral filters, neutral grey screen, Duboscq colorimeter.

capacity—a far greater difference than could be accounted for by the presence of small amounts of inactive haemoglobin in the normal blood samples investigated.

The colour of alkaline haematin derived from blood had been assumed to be identical with that derived from haemin,

but these results suggested that this was not the case. Although the colours were similar to the unaided eye, and had the same broad band at 606 to 608 mμ when viewed in the Hartridge reversion spectroscope, they showed a slight dissimilarity in absorption in the red. Solutions derived from each source were examined in a colorimeter against a neutral grey screen, using a range of light filters covering the visual spectrum. The absorption curves are plotted from these data in Fig. 2, which indicates that the colours differ slightly in the red portion of the visual spectrum.

Several samples of human haemoglobin were then prepared by precipitation with alcohol as described by Delory (1942). By this method large yields of dried human haemoglobin were readily obtained in a semi-pure state. An iron analysis of quantities of about 1 g. was performed in duplicate on each sample. As in the case of haemin, solutions of alkaline haematin were prepared by dissolving the haemoglobin in N/10 soda. The extinction coefficient was measured and the absorption curve studied. This was found to agree with that derived from blood and to differ in the same way from that of haemin. The solutions were compared with solutions of haemin in N/10 soda and the ratio of colour intensity per mg. of iron in each solution calculated. In each case this ratio was found to be close to the mean of 1.32 for the five samples (Table VI).

TABLE VI.—*Colour of Various Haemoglobin Preparations*

Haemoglobin Preparation No.	% of Iron	Es*	Hb/Haemin Colour Ratio†
1 (human)	0·262	1·03	1·29
2 ,, ...	0·284	1·00	1·32
3 ,, ...	0·278	0·97	1·33
4 ,, ...	0·284	0·98	1·32
5 (ox)	0·249	1·00	1·33

* Es = specific extinction coefficients for 1 mg. haemoglobin Fe/100 c.cm. Photo-electric colorimeter, Chance green filter.
† Colour of haemoglobin solutions against a single haemin standard.

It appears, therefore, that although the brown colours produced in sodium hydroxide from blood and from crystalline haemin are seemingly the same on visual inspection and are similar over most of the visual spectrum, they are not in fact identical. There is a quantitative discrepancy of approximately 30% and a slight qualitative difference. The chemical reasons for the discrepancy are beyond the scope of this paper; suffice it to say that it can be adequately explained by the fact that the alkaline haematin solutions derived from haemoglobin and blood contain both haematin and globin, whereas that prepared from haemin contains only haematin and no globin. Haemin, then, cannot be used as a theoretical standard in the sense that four molecules of haemin should give the same colour as one molecule of haemoglobin. But it can be employed perfectly satisfactorily as an artificial standard. The colours produced by haemin and haemoglobin in soda are similar to the eye in any light,* and in green light they are easily and accurately matched and there is a constant ratio of colour intensity by whatever means the reading is made.

Methods for the Determination of Haemoglobin by the Alkaline Haematin Procedure

The brown colour of blood in sodium hydroxide solution is compared with that of a solution of crystalline haemin of known iron content.

Method 1.—0.05 c.cm. of blood is diluted to 5 c.cm. with N/10 NaOH, heated in a boiling-water bath for 4 to 5 minutes, cooled, and read against the haemin standard in a suitable colorimeter (Duboscq, photo-electric, etc.), using a green light filter.

Method 2 (Method of Wu; cf. Peters and van Slyke, 1932).—0.05 c.cm. of blood is treated with 4 c.cm. of N/10 HCl, left at room temperature for 40 minutes, and then diluted to 5 c.cm. with N/1 NaOH.

Standard.—The haemin-iron equivalents of the "100% normal" standards are shown in Table VII. An amount of haemin of known iron content, which is the equivalent of the desired standard, is accurately weighed and dissolved in 1 litre of N/10 NaOH. This solution should be preserved in a glass-stoppered bottle in a cool dark place. Kept under these conditions our standards have remained unaltered for 9 months.

Authorities differ as to the percentage of haemoglobin in blood which they regard as a normal figure. The three most commonly used are given in Table VII. There is an increasing body of evidence to show that Haldane's (1900) figure of 13.8 g. of haemoglobin is too low and that the Haden (1922) value of 15.6 g. per 100 c.cm. (20.9 c.cm. O_2) is a more normal figure (cf. Peters and van Slyke, 1932; Wardlaw, 1941). This is the figure we have used and which has seemed adequately to represent our normal healthy cases; it has given colour indices averaging unity for healthy persons and treated anaemias.

TABLE VII.—*Concentrations of "Normal" Blood Standards*

	Haldane	Haden	Sahli
g. haemoglobin/100 c.cm.	13·8	15·6	17·2
O_2 capacity (c.cm./100 c.cm.)	18·5	20·9	23·0
mg. Fe/100 c.cm.	46·2	52·2	57·6
Alkaline haematin equivalents:			
mg. Fe/100 c.cm.*	0·60	0·68	0·75
(i.e., haemoglobin Fe × $\frac{1·3}{100}$)			
Pure haemin, 8·57% Fe (mg. per litre)* ..	70·0	79·4	87·5

* Which give the same colour as blood diluted 1 in 100 in N/10 NaOH.

The alkaline haematin standard we use for routine is weaker than the "100%" standards. It contains an amount of haemin representing 0.45 mg. of iron. This solution matches any haemoglobin solution or blood containing 10.3 g. of haemoglobin per 100 c.cm. when treated with sodium hydroxide to give a dilution of 1 in 100. This standard was chosen because it is close to the average of the concentrations most often encountered in hospital practice. Its relation to the "normal blood" standards is shown in Table VIII. Haemin standards of "100% normal" may of course be used. The figures for these are given in Table VII. The method has now been in use for several months and has proved most satisfactory. Where many determinations are undertaken it is more rapid and more accurate than other methods we have used.

TABLE VIII.—*Alkaline Haematin Standard for Routine Use with Duboscq and Photo-electric Colorimeters*

52.5 mg. of pure haemin (or an equivalent amount of other haemin of known Fe content) is dissolved in 1 litre of N/10 NaOH. This solution contains 0.45 mg. Fe/100 c.cm. and is equal in colour to:

75% of Haldane normal
66% of Haden ,,
60% of Sahli ,,

when blood is diluted 1 in 100 in NaOH.

Approximate Methods

With the Lovibond Comparator.—Through the co-operation of Mr. G. S. Fawcett a Lovibond disk has been prepared to match the alkaline haematin colours. The values are spaced at 10% of the Haden normal—i.e., at differences of 1.56 g. haemoglobin per 100 c.cm. The blood is treated by either of the above procedures, the test-tube is placed in the Lovibond comparator, and the disk is rotated until a colour match is obtained. By careful comparison it is possible to gauge colours intermediate between those in the disk and hence to read the haemoglobin to within about 5%.

With the Hawksley Dilution Colorimeter.—The small dilution haemoglobinometer supplied by Messrs. Hawksley for the Sahli method is easily adapted by the substitution of the coloured glass standard by a tube of alkaline haematin solution (equal to 100% of normal—e.g., Haden). 0.02 c.cm. of blood is added to a little N/10 soda in a calibrated dilution-tube; the mixture is warmed until it is of a clear light-brown colour, and is then diluted by the dropwise addition of N/10 NaOH, with mixing, until a colour match is obtained. The percentage

* The slight qualitative difference in the colours may be apparent if they are viewed through too great a depth of solution. Thus a "100%" blood and the standard will appear identical in 1/4-inch dilution-tubes, but the colours will appear slightly different in wide test-tubes. On the other hand more dilute solutions of haemin and haemoglobin appear identical even when viewed through wide tubes.

is read from the scale on the test-tube. If this method is employed care should be taken to ensure that the tubes used are properly calibrated and of the same diameter.

Summary and Conclusions

The haem-pigments circulating in the blood may be divided into an active fraction, reduced and oxyhaemoglobin, and an inactive fraction chiefly carboxyhaemoglobin and methaemoglobin. A satisfactory method for estimating haemoglobin should measure both fractions.

The Haldane and acid haematin methods may not measure total pigment with the degree of accuracy desirable. Commercial standards are often unreliable and may fade. The pyridine haemochromagen method is rejected because of the objectionable nature of the reagents employed.

Criteria of a satisfactory method are described. These include the measurement of total pigment, reasonable accuracy, simplicity, the use of non-toxic reagents, and some permanent easily reproducible standard.

The alkaline haematin method of Wu eliminates errors which arise in the acid haematin procedure due to lipids, the colloidal nature of acid haematin, and the presence of inactive haemoglobin. Alkaline haematin has the advantage of being a true solution and is easily prepared both from haemoglobin and from crystalline haemin. Haemin is easy to prepare in a pure state from haemoglobin, and its iron content may be estimated with accuracy. Thus it appeared to satisfy our criteria for a suitable standard, and was investigated for this purpose.

Sixteen samples of crystalline haemin were prepared from ox and human bloods and the iron content of each determined. Solutions of alkaline haematin were prepared from each sample. The specific extinction coefficients were found to agree closely. The intensity of colour was proportional to the concentration of haemin iron, and impurities failed to influence the colour.

Several forms of haemoglobin occur in normal blood, some of which are resistant to alkali denaturation. Whatever the proportion of alkali-resistant haemoglobin in a blood sample, if a 1 in 100 dilution in decinormal soda is prepared and placed in a boiling-water bath for five minutes all the haemoglobin is converted into alkaline haematin.

Similar treatment converts all inactive forms of haemoglobin into alkaline haematin. The colour developed is not significantly influenced by the amount of lipoid or plasma in the sample.

Determinations of haemoglobin in blood samples based on the assumption that 1 mg. haemin iron in the standard was equivalent to 1 mg. haemoglobin iron in the test yielded results uniformly about 30% higher than determinations based on the oxygen-carrying power of the same sample. It was then found that solutions prepared from haemin and blood gave colours whose absorption curves differed slightly in the red portion of the spectrum.

Samples of human and ox haemoglobin were prepared and their iron content determined. Solutions of alkaline haematin were prepared from each sample and their specific extinction coefficients and absorption curves determined. The curves derived from the haemoglobin solutions were identical with that from blood, and the colour produced was 30% higher than would be anticipated from the iron content, when compared with haemin standards.

Thus haemin is not a theoretical standard in the sense that four molecules of haemin should give the same colour as one molecule of haemoglobin; but it can be employed satisfactorily as an artificial standard. The colours of the solutions are almost identical in the concentrations used for the test, and are matched with ease and accuracy. The standard appears to be unchanged during several months.

Details of methods for the estimation of total haemoglobin using haemin as a standard are given, together with instructions for the preparation of standard solutions equivalent to 100% of the Haldane, Sahli, and Haden scales.

We have employed this method for many months and have found it to give excellent results. Where many determinations are made daily it is quicker and more reliable than other methods we have used.

Our thanks are due to Dr. R. G. Macfarlane of Oxford and Dr. J. M. Peterson of Cardiff, who have carried out independent investigations of the alkaline haematin method, for helpful discussions and advice; to Mr. G. E. Delory for his invaluable assistance with the iron analyses; to Dr. J. McMichael for the estimations of O_2 capacity; to Mr. G. L. Widd for his assistance in comparing the alkaline and acid haematin methods; and to Dr. G. A. D. Haslewood for valuable advice.

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LOUSE-BORNE TYPHUS FEVER

BY

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AND

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(From a Prison Camp in Germany)

The articles on this subject that appeared in the *British Medical Journal* of Dec. 6 and 20, 1941, have induced us to record our observations on a series of 21 cases occurring in a prison camp in Germany, of which 3 (14.28%) were fatal; 16 (76.18%) arose in men employed in handling clothing at the camp disinfector. In only 2 cases was it possible to determine the incubation period, and this was in accordance with the usual period of 12 to 14 days.

Onset

The illness was in some cases ushered in by severe frontal headaches, shivering, occasionally epistaxis, soreness of the throat, and thirst, followed by a general feverish feeling, like influenza. In milder cases, on the other hand, the patients felt quite well during the first 3 or 4 days in spite of their temperature being in the region of 102° F.

One patient gave a history of an attack of influenza for one day. He felt better the next day, but was cold and shivery; on the fourth day he suddenly developed acute abdominal pain, vomited twice, and was admitted to hospital as a case of acute appendicitis. His temperature was 102.2°, and his pulse 100; his urine contained nothing abnormal. A laparotomy was performed and the next morning his temperature and pulse fell to normal, but the same evening they rose to 102.9° and 112 per minute respectively, and remained about this level. On the third day after his operation the rash appeared—i.e., 6 days after his initial attack of influenza.

General Appearance immediately before Occurrence of Rash.—The conjunctival vessels were injected and the eyes assumed a heavy appearance reminiscent of measles without photophobia and lacrimation. The face was flushed and a dusky tinge or distinct cyanosis was present in the skin. The tongue was dry and thickly coated with white or brownish-white fur. Sordes of the mouth was very marked until frequent cleansing was adopted. The facial expression was worried, with a continual frown which was aggravated by questioning or outside interference. Speech was sluggish, and the patients appeared to have difficulty in expressing themselves. In some cases there was delirium immediately before the appearance of the rash, and all became increasingly drowsy. Acute dyspnoea usually occurred towards the end of this phase, and was suggestive of bronchopneumonia with only a few crepitations in the lungs.