OBSERVATIONS ON SAHLI'S HÆMOGLOBINOMETER.

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The past two decades has been a period of intensive and fruitful research on the ætiology, diagnosis and treatment of the anæmias. Although diagnostic methods have been extended and elaborated, research has served also to confirm the fundamental value of accurate determination of the hæmoglobin content (Hb) of the blood. Routine estimation of the Hb has revealed the fallacy of assuming that pale people are necessarily anæmic; it has also discovered many cases of undoubted anæmia among those who pride themselves on having a " good colour." Although the finding of a low Hb concentration calls for a detailed blood examination, it will be generally agreed that a practitioner who makes a careful clinical study of his patient and considers his findings in conjunction with the result of the Hb estimation will often be in a position to form a valuable opinion regarding the exact type of anæmia present.

The principle of Sahli's method is simple: " the hæmoglobin contained in a known quantity of blood is converted into acid hæmatin by means of hydrochloric acid. The colour is then compared with a standard tube containing acid hæmatin of known strength " (Whitby and Britton, 1935). The technique is straightforward and can be learned in a few minutes. Despite the simplicity of the method, however, there are numerous sources of error traceable not only to faulty technique but also to unsatisfactory apparatus. The existence of fallacies became apparent while making upwards of five hundred estimations of the Hb in connection with a clinical investigation. The purpose of this article is to make a plea for standardization of both technique and apparatus, and to try to estimate the importance of the various sources of error that are encountered. For reference and discussion the standard method taught to students in this hospital is set out below.

Clean Tube. Brush if necessary.

Volume of Acid. Exact quantity immaterial; 30 per cent. mark is a convenient level.

Obtaining Blood. Sharp, sterile Hagedorn needle. Wipe sweat off lobe of ear with dry swab. Stab skin boldly. Do not squeeze blood out: vigorous percussion of puncture with finger tip promotes blood flow. Do not allow blood to concentrate by drying.

Pipette. Narrow bore most satisfactory, e.g. 20 c.mm. mark about 6 cms. from tip. Absolutely dry and clean. Blood free from air bubbles. If blood passes beyond 20 c.mm. mark, carefully reject excess by tapping tip gently against pad of finger. Wipe blood off outside of pipette.

Transferring to Acid. With tip of pipette just under surface of acid, gently eject blood in stream to bottom of tube. Fill pipette twice with cold water and add washings to tube. Insert stopper into tube: mix blood and acid by inverting: replace tube in colorimeter for *one minute.* While waiting, rinse pipette several times with alcohol, then with ether, and finally dry by sucking air through.

Dilution. As water* is added drop by drop, insert stopper and mix by inverting tube. Do not shake (frothing).

Comparing Colours. Source of light—clear sky or bright cloud: direct sunlight unsuitable. Artificial light less satisfactory: deduct $\frac{1}{12}$ from final reading. Hold colorimeter at arm's length and at eye-level: let it just touch window pane. Keep it

* Distilled water is recommended. Tap water in Glasgow is equally satisfactory.

vertical; tilting produces high readings (glass standards). When finally matched, remove stopper and wait ¼ minute to allow drainage from top. Read. Time taken for dilution and comparison = 1—1½ minutes, making 2—2½ minutes in all.

Accuracy. Using exactly same technique repeat estimation: results of practised worker should not differ by more than 2 per cent. Enter on case-sheet thus: Readings = ... Average = ...

Standards.

It would be an advantage if all makers of hæmoglobinometers would state—preferably on the instrument—the standard that has been adopted, e.g. Av. normal ♂ & ♀ = 85 per cent. = 14.5g Hb per cent. The use of a factor to be applied to the hæmoglobin reading—as in the case of Buechi's colorimeter—introduces an undesirable complication.

A fluid medium as the standard is unsatisfactory: it fades in the course of a few years. The rate of deterioration depends partly on the amount of exposure to light; if kept in a box when not in use it fades less rapidly. This difficulty seems to have been overcome by the introduction of solid, coloured, glass rods such as are fitted in the "Nonfade" pattern made by Bergmann and Altmann (Berlin). Matching is easier when the hæmoglobin solution is placed between two standards rather than alongside only one standard. The hæmoglobinometer tube should be long enough to project about 2 cm. beyond the top of the colorimeter; when the end of the tube is flush with the rack it is removed only with difficulty while carrying out the estimation. It should not, however, be longer than the hæmoglobinometer pipette, otherwise there is difficulty in ejecting blood into the acid.

A comparison was made between the standards of three "Nonfade" instruments. The hæmoglobin of one patient was measured three times with each of these colorimeters in the course of one hour, the same pipette and the same diluting-tube being used for all the estimations.

TABLE 1.

Instrument	Hæmoglobin %			
	(a)	(b)	(c)	Average
I	72	71	70	71
II	72	70	72	71.3
III	73	71	71	71.7

It is clear that these colorimeters are identical notwithstanding that No. I and No. II had been in daily use for nearly two years, whereas No. III was new and was being used for the first time.

Adopting the Nonfade instrument (No. I above) as a standard, the Hb content of a sample of blood was estimated using in succession four colorimeters of the old type fitted with fluid standards. The results are given in Table 2 which includes for comparison readings obtained by the Nonfade hæmoglobinometer.

TABLE 2.

Instrument		Hæmoglobin % (a)	(b)	(c)	Average	Date of Instrument
Nonfade		50	49	49	49.3	1937
Fluid Standards	A	51	52	51	51.3	1 : 4 : 37
	B	55	53	55	54.3	14 : 3 : 34
	C	64	63	63	63.3	20 : 1 : 37
	D	60	61	61	60.6	20 : 1 : 31

The difference between the standards was readily demonstrable by slipping the fluid standard tube into the centre compartment of the " Nonfade " colorimeter: A and B appeared to be only a few points lighter, whereas C and D were very much lighter than the colour rods. The differences anticipated in this way were confirmed when the actual estimations were made. In no case was the application of a correcting factor to the reading required by the maker.

It is recommended above that the colorimeter should be allowed to touch the window: in this way the maximum amount of daylight falls on the instrument, thus facilitating matching. If one is standing, hæmoglobinometer in hand, say 12 feet from the window, the final stage of matching the colours is distinctly more difficult. The distance between the " Nonfade " hæmoglobinometer and the eye is also a point of some importance. This is easily appreciated by completing an estimation of the Hb using the standard technique—the instrument being held at arm's length while comparing the colours; if the colorimeter is then brought close to the eyes, it will be found that the solution of acid hæmatin appears to be of a lighter shade and that it no longer matches the standard colour rods. In this connection there are two further points which are of interest but not of practical importance to the clinician; a solution of acid hæmatin which appears to be accurately matched when observed at a distance of about 2½ ft. (colorimeter at arm's length and touching the window) seems to be decidedly *darker* than the standard when seen from a point 12 ft. away (the colorimeter remaining near the window). Again, if the colorimeter is tilted to lie at an angle of 45° to the window pane, the solution appears to be darker than the standards, and if re-matched in the new position by further dilution, the final reading is 5-10 points higher than when the ordinary technique is used. Slight tilting of the colorimeter, say up to 15°, does not produce any appreciable darkening of the standard and it is unlikely that this is a source of error in practice. These peculiar effects are obtained only when glass rod standards are used and are absent from instruments fitted with fluid standards; it is probable therefore that they depend upon the different refractive indices of the media.

Hæmoglobinometer tubes. Errors may arise in a series of observations through using different hæmoglobinometer tubes. That these are often inaccurately graduated will be seen from the following observations. The percentage markings may not be at the same levels on the tubes, but this is not necessarily an indication of faulty graduation, as the calibre of the tubes varies a little. If, however, a stated volume of liquid is run into each of a series of dry tubes, the level of the

fluid should be on the same percentage mark in all of them. Exactly 2.37 mil. of water was delivered into each of 7 dry hæmoglobinometer tubes. The readings were as follows:

TABLE 3.

TUBE	I	II	III	IV	V	VI	VII
LEVEL OF WATER (% marking)	112	123	113	122	113	115	122

Nos. I, II, III and IV were from " Nonfade " instruments.

Pipettes. Similarly, it is unwise to assume that all pipettes furnished with Sahli's type of hæmoglobinometer are accurately calibrated to 20 c.mms. Six pipettes were compared by using them in turn to determine the Hb of a sample of blood. The capacities of the pipettes at the 20 c.mms. mark were regarded as being proportional to the results of these hæmoglobin estimations.

TABLE 4.

Pipette	Hæmoglobin %			
	(a)	(b)	(c)	Average
I	62	63	63	62.7
II	65	66	66	65.7
III	65	66	66	65.7
IV	66	67	67	66.7
V	70	70	71	70.3
VI	66	67	67	66.7

This series of readings was obtained between 10 a.m. and 12 noon. In matching the colours I was not aware of increasing difficulty such as may result from retinal fatigue. To check this, I repeated the estimation using pipette I when half way through the series and again at the end of the series: the result—62 per cent. in each case—suggested that there was no retinal fatigue.

Each pipette was then filled with mercury to the 20 c.mms. mark and the metal was carefully weighed. This would seem to be a more accurate method for determining the volume of the pipette. In practice, however, it is difficult for an unskilled person to fill the lumen of the tube with mercury so that it extends exactly from the tip to the 20 c.mms. mark. The indirect method—by hæmoglobin estimation—probably indicates more accurately the relative volumes of the pipettes. In the following table it will be seen that there is a rough relationship between the two sets of figures for pipettes I, III, V and VI but in Nos. II and IV the weight of mercury is unexpectedly high.

TABLE 5.

Pipette	Av. Hb. %	Wt. Hg. mgm. to 20 c.mms. mark Average of 2 readings.
I	62.6	244
II	65.6	270
III	65.6	266
IV	66.6	272
V	70.3	270
VI	66.6	265

Accurate adjustment of the volume of blood to the 20 c.mms. mark is easier when the bore of the pipette is fairly narrow, e.g. the calibration about 6 cms. from the tip. When a wide bore tube is used (short column of blood) the percentage error is increased if the blood is not exactly at the 20 c.mms. mark, and satisfactory transfer of the blood is less easily managed. A wet pipette is an obvious source of error: the blood may be diluted in the lumen by minute drops of moisture condensed on the walls, or, more markedly by water actually filling part of the lumen. The following results were obtained: (i) by the standard technique, using a perfectly dry pipette—68%, 69%, 69%, 69%; (ii) using the same technique but having the inside of the tube wet—63%, 61%, 59%, 66%. The deficit is probably maximal when the blood mixes rapidly with a column of water in the pipette. Furthermore, the upper end of the column of blood becomes ill-defined on account of lysis and this makes accurate measurement impossible. The use of a wet pipette is a gross error unlikely to occur in the hands of an experienced worker. Failure to wipe blood (fresh or dried) off the outside of the pipette is another gross but uncommon mistake; in one case, this raised the hæmoglobin reading from 60% to 70%.

If blood passes beyond the 20 c.mms. mark, the excess is, of course, rejected. Contamination of the inside of the pipette above the mark occurs, however, and on adding the pipette washings to the tube an excess of hæmoglobin is transferred. In practice the additional amount of blood must be small, as no appreciable increase in the final result was noted even when the blood over-ran the mark by 6 cms. (the excess beyond the 20 c.mms. mark being rejected in the usual way).

TABLE 6.

(i) Standard technique.	Hb = 62% and 60%.
(ii) Blood over-ran 20 c.mms. mark by 1 cm. Excess rejected.	Hb = 61%.
(iii) Blood over-ran 20 c.mms. mark by 6 cms. Excess rejected.	Hb = 60%.

Having expelled the measured volume of blood into the acid, it is a matter of routine to rinse the pipette with water and add the washings to the acid and blood in the tube. Neglect of this point produces readings a little lower than those obtained by the standard technique, but the difference is much smaller than had been expected.

TABLE 7.

(i) Standard method. Hb = 66%, 66%, 66%.

(ii) Pipette washings omitted. Hb = 65%, 65%, 64%.

The effect of squeezing blood from the skin puncture was investigated. The results show that if squeezing causes the blood to flow freely so that only momentary pressure is necessary to obtain an ample supply, the hæmoglobin concentration is unaffected. On the other hand, if the patient does not bleed easily, and vigorous and sustained squeezing is needed to collect just enough blood for the estimation, the hæmoglobin concentration may be considerably reduced; presumably this is brought about by diluting the cells with an excess of tissue fluid.

TABLE 8.

Patient A. Bled freely after one needle stab.

(i) Standard method. Squeezing practically nil.
Hb = 62% and 63%.

(ii) Gentle squeezing *which produced rapid blood flow.*
Hb = 62% and 62%.

Patient B. Bled freely.

(i) Standard method. Squeezing practically nil.
Hb = 60% and 60%.

(ii) Vigorous squeezing 5 minutes later. Blood flow fair.
Hb = 55% and 54%.

Patient C. Bled poorly even after 3 needle stabs.

(i) Standard method. Squeezing slight.
Hb = 72%, 72% and 74%.

(ii) Vigorous and sustained squeezing required to obtain minimum volume of blood.
Hb = 57%, 48% and 50%.

Patient D. Bled moderately after 2 needle stabs.

(i) Standard method. Squeezing slight.
Hb = 65% and 67%.

(ii) Vigorous and sustained squeezing.
Hb = 57% and 58%.

If the blood is allowed to remain on the skin for any considerable time before being taken up into the pipette, it evaporates and becomes more or less concentrated, thus giving rise to spurious high readings. In a routine blood examination, taking blood for the hæmoglobin estimation is often deferred until both hæmocytometer pipettes have been filled; not infrequently the same drop of blood is then

used for measurement of the hæmoglobin. To investigate the effect of evaporation, the blood was allowed to remain on the lobe of the ear for one minute: this raised the hæmoglobin concentration from 60 per cent. (standard method) to 70 per cent. on one occasion and to 67 per cent. on another. The temperature of the ward was 65° F.; the humidity was not noted. In three other patients, however, the difference was much smaller. (Table 9.)

TABLE 9.

	Hæmoglobin %				
Standard method	60 ; 60	61 ; 61	60 ; 60	50 ; 48	51 ; 52
Evaporation 1 min.	67 ; 70	63 ; 64	59 ; 62	48 ; 51	55 ; 54
Patient	A	B	C	D	E
Ward Temp. F . .	65	60	60	60	60

On adding the blood to the N/10 hydrochloric acid lysis begins at once and is complete within half a minute. Thereafter the formation of acid hæmatin proceeds rapidly, and after a further half minute the colour of the solution does not seem to darken appreciably. It is well known, however, that the amount of acid hæmatin actually continues to increase for at least 30-40 minutes (Whitby and Britton, 1935). If the solution is set aside for 40 minutes before proceeding to dilution and matching, appreciably higher readings will be obtained than if the test is completed within 4 minutes of adding the blood to the acid. It will be generally agreed, however, that a method of estimation which cannot be completed in less than three-quarters of an hour will not find favour among medical practitioners. Teaching on this point is not uniform: the interval recommended varies from 1 minute to 5 minutes. From the following observations it would appear to be justifiable to allow only 1 minute to elapse before beginning dilution of the acid hæmatin, and to aim at completing the test in 2-2½ minutes.

TABLE 10.

(i) Blood added to HCl.
Dilution begun after 1 minute.
Hb = 68%, 68%, 68%, 66%.

(ii) Blood added to HCl.
Dilution begun after 2 minutes.
Hb = 68%, 68%, 67%.

(iii) Blood added to HCl.
Dilution begun after 3 minutes.
Hb = 68%, 68%, 69%.

This experiment was varied slightly as follows: a sample of blood was matched carefully in the colorimeter and then put aside to allow the further formation of

acid hæmatin. It was examined at intervals and re-matched, if necessary, by further dilution, the new readings being noted. The following series were obtained:

TABLE 11.

Patient A.			*Patient B.*	
Minutes.	Hb%.		Minutes.	Hb%.
1	51	(Standard	1	75 (Standard
6	53	Method)	10	81 Method)
11	54		25	84
21	56		38	85
			53	85
			61	85
			91	85

It is clear that this matter of the time of "exposure" of the blood to the action of hydrochloric acid has an important bearing also on our *standards* of normal hæmoglobin concentration. Thus, according to current views (Whitby and Britton) the healthy male having on an average 16 g. of hæmoglobin per cent. is said to have a Hb of 90 per cent. Sahli. If this percentage is obtained by exposing the blood to hydrochloric acid for 40 minutes—or until the maximum amount of acid hæmatin has formed—it must be discounted to some extent when we are concerned with clinical hæmoglobinometry.

Colour matching. With practice it is possible to obtain remarkably uniform results in estimating the Hb; frequently a series of three readings vary by no more than 1 per cent. Nevertheless, several observers, using the same sample of blood and the same technique, may arrive at widely differing results, though consistent in their respective series.

To investigate this I made three estimations of the Hb in one patient and noted the results. Two experienced colleagues accustomed to measuring Hb concentration were then invited to undertake the matching as I repeated the test, using exactly the same technique and the same instruments throughout. They were not previously informed of my own findings, nor were their own results shown to them until they had completed three estimations each. The following figures were obtained:

TABLE 12.

Observer	Hb%			Av.
S.A.	63	62	62	62.3
A.S.R.	71	74	71	72.0
W.F.A.	73	77	77	75.7

While gradually diluting the acid hæmatin solution for these observers I was able to note the stage at which the matching satisfied me, and I was interested to observe that on both occasions my colleagues were in no doubt that further dilution was required. When the matching had been completed to their satisfaction, the acid

hæmatin solution appeared to me distinctly lemon yellow in colour compared with the relatively orange shade of the standard colour bars. As a final control, having noted my colleagues' readings, I repeated the estimation matching the colours myself: the reading was 63 per cent.—the same as my original readings. The experiment was repeated with the co-operation of three other colleagues using different samples of blood on each occasion.

TABLE 13.

Observer	Hb %			Av.
S.A.	71	71	70	70.6
A.S.M.	78	75	75	76
S.A.	72	73	71	72
A.J.S.	79	78	79	78.6
S.A.	67	67	66	66.6
M.A.F.B.	68	67	66	67

It is noteworthy that the differences are less than in the first series and in one instance (M.A.F.B.) the average readings are almost identical.

Summary.

1. A standard technique for estimating the hæmoglobin by Sahli's method is given.

2. The need for checking hæmoglobin colorimeters and all accessories is stressed.

3. When serial readings are being made it is essential that they should be undertaken by a single observer using the same instruments and a standard method. In view of the diurnal variations which are known to occur it is also important that the estimations should be carried out at approximately the same hour of the day. Under these conditions the accuracy of the Sahli method is confirmed.

4. Certain sources of error in the Sahli method are mentioned and an attempt is made to assess their importance. Standardization and careful checking of the instruments would contribute greatly to the reliability of the test.

I desire to thank Professor Noah Morris for his helpful criticism while carrying out this work.