2. A flocculate of silver sulphide, left for several hours in contact with a dilute solution of silver nitrate, acquires considerable and highly selective adsorbent properties.

3. A new quantitative method for separating natural amino-acids is based on these properties. Different types of adsorption are observed which can be regarded as examples of more general phenomena which occur on the surface of mineral precipitates.

4. These investigations indicate new preparatory and analytical possibilities in the sphere of microbiochemistry and suggest methods for eliminating the adsorption of organic compounds at the surface of mineral precipitates, a phenomenon which so often invalidates the determinations of these compounds.

Thanks are due to Prof. Dubuisson for the very valuable help he has given in the course of this work, and to Miss M. Horsman (Wool Industries Research Association, Leeds) for her kindness in translating the text.

REFERENCES

Bailey, K., Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 360.
Böttger, W. (1939). *Physikalische Methoden der analytischen Chemie*, **3**. Leipzig: Akadem. Verlagsges.
Burnstein, R. (1927). *Z. anorg. Chem.* **164**, 219.
Fajans, K. (1941). *J. appl. Phys.* **12**, 306.
Fajans, K. & Erdey-Gruz, T. (1931). *Z. phys. Chem.* **158**, 97.
Fajans, J. & von Beckerath, K. (1921). *Z. phys. Chem.* **97**, 478.
Fajans, K. & Wolff, H. (1924). *Z. anorg. Chem.* **137**, 221.
Freudenberg, K., Walch, H. & Molter, H. (1942). *Naturwissenschaften*, **30**, 87.
Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1943). *Biochem. J.* **37**, 79.
Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1944). *Biochem. J.* **38**, 65.
Hahn, O. (1926). *Ber. dtsch. chem. Ges.* **59**, 2014.
Hahn, O. (1930). *Z. angew. Chem.* p. 871.
Hamoir, G. C. M. (1943). *C.R. Soc. Biol., Paris*, **137**, 734.
Karrer, P., Keller, R. & Szönyi, G. (1943). *Helv. chim. Acta*, **26**, 38.
Kolthoff, I. M. (1934). *Kolloidzschr.* **68**, 190.
Kolthoff, I. M. (1936). *J. phys. Chem.* **40**, 1027.
Kolthoff, I. M. & Lingane, J. J. (1936). *J. Amer. chem. Soc.* **58**, 1524.
Kolthoff, I. M. & MacNevin, W. M. (1936). *J. Amer. chem. Soc.* **58**, 725.
Kolthoff, I. M. & Rosenblum, C. (1933*a*). *J. Amer. chem. Soc.* **55**, 2656.
Kolthoff, I. M. & Rosenblum, C. (1933*b*). *J. Amer. chem. Soc.* **55**, 2664.
Kolthoff, I. M., Von Fischer, W. & Rosenblum, C. (1934). *J. Amer. chem. Soc.* **56**, 832.
Lucas, C. C. & Beveridge, J. M. R. (1940). *Biochem. J.* **34**, 1356.
Martin, A. J. P. & Synge, R. L. M. (1941). *Biochem. J.* **35**, 1358.
Paneth, F. (1914). *Phys. Z.* **15**, 924.
Schramm, G. & Primosigh, J. (1943). *Ber. dtsch. chem. Ges.* **76**, 373.
Sheppard, S. E., Lambert, R. H. & Walker, R. D. (1939). *J. Chem. Phys.* **7**, 265.
Tarte, P. (1945). *Bull. Soc. Sci. Liége*, **4**, 231.
Trillat, J. J. & Aubry, A. (1941). *Cahiers de Physique*, **1**, 35.
Trillat, J. J. & Merigoux, R. (1941). *C.R. Acad. Sci., Paris*, **212**, 160.
Turba, F. (1941). *Ber. dtsch. chem. Ges.* **74**, 1829.
Turba, F. & Richter, M. (1942). *Ber. dtsch. chem. Ges.* **75**, 340.
Turba, F., Richter, M. & Kuchar, F. (1943). *Naturwissenschaften*, **31**, 508.
Van Slyke, D. D. (1929). *J. biol. Chem.* **83**, 425.
Verwey, E. J. W. (1933). *Proc. Acad. Sci. Amst.* **36**, 225.
Verwey, E. J. W. & Kruyt, H. R. (1934). *Z. phys. Chem.* **167**, 137.
Vickery, H. B. & Leavenworth, C. S. (1930). *J. biol. Chem.* **86**, 129.
Wieland, T. (1942*a*). *Hoppe-Seyl. Z.* **273**, 24.
Wieland, T. (1942*b*). *Naturwissenschaften*, **30**, 374.
Wieland, T. (1942*c*). *Ber. dtsch. chem. Ges.* **75**, 1002.
Wieland, T. & Wirth, L. (1943). *Ber. dtsch. chem. Ges.* **76**, 823.

An Artificial Standard for Use in the Estimation of Haemoglobin

By Q. H. GIBSON and D. C. HARRISON, *Department of Biochemistry, Queen's University, Belfast*

(*Received* 17 *August* 1945)

Colorimetric procedures for determining haemoglobin have the disadvantage that they must normally be standardized by oxygen capacity or iron determinations on blood, and this process must be repeated at intervals to guard against deterioration of the standard. To avoid the necessity for these measurements, artificial standard solutions have been proposed for some of the commoner methods of estimating haemoglobin. They have not been widely adopted because they are suitable only for use in visual colorimeters under definite conditions of illumination. The absorption spectra of the

standards have usually been so widely different from those of the blood derivatives that any marked variation in the quality of the lighting of the colorimeter has been inadmissible, and they are not suitable for use in modern absorptiometers working with approximately monochromatic light.

The introduction of haemin as a standard substance (Rimington, 1941; Clegg & King, 1942) offered a prospect of considerable improvement in some of these respects. The latter authors stated that haemin could be weighed out to give a stable solution in dilute alkali, that the intensity of colour of the solution was proportional to the iron content of the haemin sample used to prepare it, and that the absorption spectrum of the standard solution was similar to that of blood converted to alkaline haematin by heating with dilute alkali.

During the course of work involving the accurate estimation of haemoglobin in many blood samples, we were impressed by the convenience and reproducibility of results obtained by the alkaline haematin method described by Clegg & King (1942). We have found, however, that some samples of haemin give a colour much in excess of that to be expected from their iron content, that the solutions change appreciably with time, and that there are significant differences in absorption spectrum between individual samples of haemin.

The principal qualities of a satisfactory standard for general use in haemoglobinometry are that it should be reproducible, stable, easily prepared, and have an absorption spectrum closely similar to that of the blood derivative with which it is to be compared. We have prepared a standard solution with certain inorganic salts which can readily be obtained in a pure form. It is stable, and, over a considerable range of wave-lengths, reproduces closely the absorption spectrum of blood treated with dilute alkali. We have assigned a haemoglobin value to this solution by comparison with a number of blood samples, the haemoglobin content of which we determined by very careful estimations of oxygen capacity and iron content.

METHODS

Apparatus. All volumetric apparatus was calibrated. Pipettes used to deliver blood were calibrated by delivery of blood of known specific gravity. Weights were checked against a National Physical Laboratory standard set.

Blood samples. 50 ml. of venous samples were taken from normal male medical students, usually about 2–3 hr. after breakfast. A stainless needle was used, and coagulation prevented by an oxalate mixture (1 mg./ml. of blood), consisting of three parts ammonium oxalate and two parts potassium oxalate. The blood was mixed well and the portions required for the various analyses taken at once.

Oxygen capacity. Triplicate determinations were made by the method of Van Slyke & Neill (1924). Oxygen was not determined by absorption, but the total volume of extracted gases (other than CO_2) was measured, and corrected for O_2 and N_2 in physical solution according to Sendroy, Dillon & Van Slyke (1934) and Van Slyke, Dillon & Margaria (1934). The advantage of this method is that any carboxyhaemoglobin present will be included in the estimation. The average deviation of a determination from the mean was 0·05 g. of haemoglobin/100 ml. of blood. The apparatus was calibrated physically according to Peters & Van Slyke (1932), and chemically by the liberation of nitrogen from hydrazine by iodate. In two cases, CO capacity was measured by the method of Van Slyke & Hiller (1928) as an additional check.

Methaemoglobin. This was determined in all samples by the method of Evelyn & Malloy (1938) as modified by Barcroft, Gibson, Harrison & McMurray (1945). The blood sample was taken from the oxygenator during the O_2 capacity determinations, and the figures given for oxygen have all been corrected by the addition of an amount corresponding to the quantity of methaemoglobin present. In our samples this was never greater than 0·1 g./100 ml. This figure, however, does not necessarily represent the true level of methaemoglobin in normal blood, as reduction of the pigment would be likely to occur during oxygenation of the blood.

Determination of iron in blood. This was carried out on 3 ml. samples of blood by a slight modification of the method of Klumpp (1934). The blood samples were ashed in 25 ml. conical Pyrex flasks, instead of porcelain dishes. This has the advantage that the risk of contamination is reduced, that losses by sputtering are less likely, and that transference of the ash from one vessel to another is avoided. 0·3 ml. of iron-free conc. H_2SO_4 was added to each of the samples, which were then placed in a glass-lined oven at 105° for 4–6 hr. The flasks were then taken singly, supported on an inclined silica triangle under a glass screen in a fume cupboard, and heated with a Bunsen flame. The neck and upper part of the flask was heated first and the heating gradually extended downwards, thus causing the froth to break up. The heating was continued for a few minutes until most of the H_2SO_4 had been driven off and the contents of the flask have become solid. If this precaution is omitted and the flasks put straight into the muffle, many determinations are spoiled owing to the contents pouring over the neck of the flask. Ashing was completed overnight at about 520° in an electric muffle fitted with a removable silica lining, the flasks being placed nearly horizontally to facilitate escape of CO_2. When the residue was quite free from carbon, the sides of the flasks were washed down with 5 ml. 10% (v/v) H_2SO_4, and the ash dissolved by heating at 105° until the last traces of iron oxide had gone into solution. This is a slow process, requiring some hours. The contents of the flasks were then diluted to about 5 ml. with distilled water and heated for a further 20–30 min. to dissolve any $Fe_2(SO_4)_3$ which might have separated out. The titration was completed, after thorough cooling of the flasks, exactly as described by Klumpp (1934). A control flask containing the reagents (including the anticoagulant used for the blood) was always carried through all the stages of the method. The determinations were done in duplicate or triplicate. The average deviation from the means was 0·5% of the amount measured. The blood haemoglobin was calculated on the assumption that haemoglobin contains 0·334% of Fe. In a few cases the values obtained by

$TiCl_3$ were checked by a dichromate method based on that of Bernhart & Skeggs (1943).

Determination of iron in haemin. Samples of about 40 mg. were weighed out and treated with 0·3 ml. of conc. H_2SO_4. In this case, preliminary heating was unnecessary, and the samples were placed directly in the muffle. The final volume was 6 ml., and 6 ml. of standard iron solution were used. Otherwise the procedure was the same in the two cases.

Preparation of iron standard solutions. Strong standard solutions were prepared (i) by dissolving electrolytic iron ('H.S.' brand (Johnson, Matthey & Co.), better than 99·9% pure) in 10% (v/v) H_2SO_4, with precautions to avoid loss as spray, and making up to a volume such that the concentration was about 5 mg. of Fe/ml.; and (ii) by dissolving ferric alum in 2N-H_2SO_4. The alum was not regarded as a standard substance. Iron was then determined in these solutions by dichromate titration following reduction of ferric iron by $SnCl_2$, using N-phenylanthranilic acid as indicator. Agreement to within 1 in 1000 was secured between the results of the dichromate titrations and the expected values based on the weight of pure iron taken. Dilute standard solutions were prepared from the iron alum by dilution (1 in 10) with 2N-H_2SO_4. The ferrous iron solutions resulting from the use of the pure iron required preliminary oxidation before dilution. In our hands, the use of $KMnO_4$ oxidation immediately before titanium titration has not led to consistent results. Oxidation was carried out by two methods: (*a*) 25 ml. of strong standard were made alkaline with NH_4OH, and iron-free H_2O_2 added. The mixture was left overnight and boiled for 10 min. to destroy any residual H_2O_2. The precipitate was then dissolved in 2N-H_2SO_4 and made up to volume. (*b*) As a check on the first method of preparation, a dilute standard was prepared by oxidizing 25 ml. of solution with 1 ml. each of conc. HNO_3 and conc. H_2SO_4 in a Pyrex Kjeldahl flask, heating being continued to dryness. The residue was then taken up in conc. H_2SO_4, diluted, cooled, and made up to volume. Dilute standards prepared by the different methods agreed exactly on titration with $TiCl_3$. In the determinations on blood, the volume of standard used was such that the amount of iron contained was roughly similar to that in the blood sample.

Spectrophotometric measurements. In order to obtain approximate absorption curves of the salts used in preparing the artificial standard, the spectra were photographed on Ilford Process Panchromatic plates with a Hilger medium quartz spectrograph and sector photometer. The plates were read with a Hilger microphotometer. In making detailed comparisons between the form of absorption curves of blood solutions and of mixtures of the salts, the spectra were photographed with several different times of exposure and the densities compared with a series of standard densities prepared by photographing the spectrum of the light source on the same plate with varying times of exposure. The light source was a tungsten filament run by storage cells of large capacity.

Measurements in photoelectric absorptiometer. The instrument used was the original type of Hilger Spekker absorptiometer, with the addition of an external mirror galvanometer which permitted the use of relatively dense filters transmitting over narrow ranges of wave-lengths. Measurements were carried out with 1 cm. cells, carefully matched against one another. Variability of the absorptiometer readings was controlled by the use of suitable Ilford neutral density screens which were always read in front of the solvent cell immediately after each solution.

Use of filters. In routine use of the absorptiometer, it is a matter of considerable practical convenience if the graph relating optical density and haemoglobin concentration is linear and passes through the origin, since it may then be replaced by the use of a factor. This point may readily be checked by taking readings of serial dilutions of a blood converted to alkaline haematin. It is desirable to determine if this is the case for any particular filter used, since the results are modified both by the transmission curve of the filter and the spectral response of the photo-cells. In particular, we have found that certain filters did not give a straight line in our instrument because of transparency in the near infra-red (e.g. Wratten 62, Ilford Hg green). We have found the following filters satisfactory from this point of view: Chance OGr. 1, 2 mm., i.e. Hilger H455, no. 5 (maximum at 530 mμ), and (with Hilger H503 2 mm., infra-red absorbing glass) Ilford Spectrum filters nos. 603–606 inclusive (maxima at 490, 520, 550 and 580 mμ respectively). For visual colorimetry, any yellow-green or green filter may safely be used.

Preparation of blood for absorptiometer. 1 ml. blood from an Ostwald pipette was added to 100 ml. of 0·1 N-NaOH in a glass-stoppered volumetric flask. After repeatedly inverting for a few minutes until the solution was perfectly clear, about 10 ml. were poured into a glass-stoppered boiling tube, heated for exactly 4 min. in a vigorously boiling water-bath and cooled immediately by immersing in cold water (Clegg & King, 1942). At the same time, 10 ml. of the standard solution were heated and cooled in exactly the same way.

Preparation of artificial standard

Precise method of preparation

Potassium dichromate. Dry about 5 g. of $K_2Cr_2O_7$ (Analar), in an oven at 100° or above. Weigh out 0·690 g. and dissolve in about 80 ml. of distilled water.

Chromium potassium sulphate. Weigh out 3·42 g. of $K_2Cr_2O_7$, dissolve in about 100 ml. of distilled water in a 500 ml. conical flask, pipette in 25 ml. N-H_2SO_4 (accurately prepared) and then add slowly, from a measuring cylinder, a freshly prepared saturated solution of SO_2, until reduction of the dichromate is complete, i.e. when the solution becomes pure green, free from any trace of yellow. Then add a further equal volume of SO_2 solution to ensure excess. A total of 50–60 ml. of SO_2 solution is usually sufficient. It is most conveniently prepared from a siphon of liquid SO_2.) Add a few glass beads to prevent bumping, and boil briskly for 45 min. or more until all traces of SO_2 have been removed and the volume is considerably reduced. A wide-stemmed funnel should be placed in the neck of the flask to prevent loss by splashing. Dilute to about 200 ml. and allow to cool.

Cobaltous sulphate. Heat about 30 g. of $CoSO_4.7H_2O$ (Analar) in a small porcelain dish for about 2 hr. in an oven just below its m.p. (96°). Stir frequently with a glass pestle or the bottom of a wide glass test-tube and break up the coarser particles. Then heat overnight at about 400° in an electric muffle. Transfer the product, which should be a uniform lilac powder, while still hot, to a stoppered bottle, and when it is cool, weigh out 13·10 g. The substance is hygroscopic, and this weighing should be done as quickly

as possible. Dissolve in about 80 ml. of distilled water with the aid of heat.

If a muffle with pyrometer is not available, the salt may be prepared by drying at less than 96° as above, then covering the dish with another similar one and placing on a loose pad of asbestos about 1 cm. thick between two larger porcelain dishes which are then directly heated by a fairly strong Bunsen flame for several hours. The salt should be stirred and coarse particles broken up at intervals. A thermometer placed in the salt should read at least 300°, but the heat must not be strong enough to cause local blackening. Alternatively the water content may be determined on a sample of the hydrated salt, and an equivalent amount of this weighed out.

To complete the preparation of the standard, transfer the solutions of $CoSO_4$ and $K_2Cr_2O_7$ quantitatively to the flask containing the chromium, and transfer the mixture to a litre volumetric flask, washing out the conical flask and making up to the mark with distilled water.

Simplified method of preparation

Weigh out 11·61 g. of $CrK(SO_4)_2.12H_2O$ (Analar), using crystals free from any signs of whitening due to efflorescence, 13·10 g. of anhydrous $CoSO_4$, prepared as above, and 0·690 g. of $K_2Cr_2O_7$; dissolve in about 500 ml. of distilled water, add 1·8 ml. of N-H_2SO_4, and heat to boiling. Boil for 1 min., cool well and make up to 1 l. with distilled water. Several preparations have been made by this method, and all have given readings in the absorptiometer in close agreement with those given by the standards made by the precise method.

Notes. (i) In the freshly prepared standard (precise method), the ratio of the two forms of chromium sulphate is different from that in the standard as used. The standard is relatively greener, and on standing for several weeks, becomes reddish. Heating for 4 min. in a boiling water-bath before use is always necessary to obtain the correct equilibrium between the two forms of chromium sulphate.

(ii) The standard should be stored in a glass-stoppered bottle free from grease and organic material. Ethanol or acetone should not be used for drying tubes and bottles used to contain the standard.

(iii) The amount of acid has been defined precisely since the green chromium sulphate is considerably hydrolyzed and large changes in the amount of acid would alter the colour. The pH of a freshly heated and cooled solution is 2·15 at 18°, most of the acid coming from hydrolysis.

(iv) The indirect method of preparation of chromium potassium sulphate has been used because it is not easy to prepare the anhydrous salt in soluble form or to determine the water content of samples of the alum without risk of loss of SO_3.

RESULTS

Haemin as a standard substance in haemoglobinometry

Iron content and light absorption. Clegg & King (1942) found that the extinction of solutions of crystalline haemin in 0·1 N-NaOH was proportional to their iron content. In the thirteen samples examined by them, the coefficient of variation of the specific extinction was 1·18 %. Their measurements were made with a photoelectric absorptiometer with a Chance green filter, and with a visual colorimeter with Hg green illumination. We have repeated these observations on six samples of haemin, and have found that in two of them there was a substantial discrepancy between iron content and light absorption with the Chance green filter. The results are shown in Table 1. While certain of the samples behave as predicted by Clegg & King, errors of up to 8 % may be involved in the acceptance of iron content as a basis for the use of haemin as a standard for haemoglobinometry.

Table 1. *Iron content and optical density of haemin samples*

(The specific extinction, E_s, is the optical density of a solution containing 1 mg. of haemin Fe in 100 ml. of 0·1 N-NaOH solution, measured in a 1 cm. cell with a 2 mm. OGr. 1 filter (Chance) in the absorptiometer.)

Haemin sample	Description	Fe content (%)	E_s	Deviation from average (%)
A	Crystalline	7·97	0·776	−3·4
B	Amorphous	8·18	0·815	+1·5
C	Crystalline	8·29	0·786	−2·1
D	Crystalline	8·23	0·792	−1·4
E	Amorphous	8·00	0·865	+7·8
F	Crystalline	8·17	0·784	−2·2
			Average	0·803±3·1

E was a sample of haemin issued by a commercial firm for the purpose of haemoglobin standardization. The other samples were made by workers in several different laboratories.

Light absorption of blood as alkaline haematin and of haemin solutions in different spectral regions. If a standard for haemoglobinometry is to be suitable for use with a variety of filters, its absorption spectrum must be similar to that of the blood derivative with which it is to be compared. We have investigated this point by measuring the extinction of haemin solutions and of blood, both in 0·1 N-NaOH, with the absorptiometer over the range covered by the Ilford spectrum filters nos. 603–606 inclusive. Using ten samples of normal blood, we measured the extinction with each filter and calculated the average ratios of extinctions with the different filters as compared with the Chance green filter OGr. 1. We carried out a similar series of measurements with solutions of each of the haemin specimens and similarly calculated the ratios of the extinctions with the different filters. In general, the ratios with the haemin solutions differed considerably among themselves and differed also from those obtained with blood. Arbitrarily taking the haemoglobin equivalent of each haemin solution with the OGr. 1 filter as 15·6 g. Hb/100 ml. and comparing the filter ratios from blood with those

from each haematin specimen, we calculated the haemoglobin equivalent of each haematin solution for all the other filters. The results are shown in Table 2. It is clear that the absorption spectrum of

Table 2. *Haemoglobin equivalents of haemin solutions with various colour filters*

Haemin sample	Ilford filter no. Y606 (g. of haemoglobin/100 ml.)	YG605	G604	BG603
A	14·5	14·8	16·8	15·8
B	15·1	15·4	16·1	15·9
C	14·7	15·1	16·8	15·8
D	15·6	15·4	16·7	16·2
E	15·6	15·1	16·1	16·8
F	15·1	15·3	16·5	16·1
Artificial standard	15·7	15·4	15·6	15·7
Blood	15·6	15·6	15·6	15·6

The figures give the haemoglobin equivalents of each haemin solution with the various filters, taking the haemoglobin equivalent with the OGr. 1 (2 mm.) Chance filter as 15·6 in each case.

haemin differs appreciably from that of blood, and that individual haemins differ from one another. It follows that errors will be introduced by changes in the colour of light used in the measurements, and these are additional to those which may arise through lack of proportionality between colour and iron content. It should be noted that these comparisons were made on haemin solutions prepared only 24 hr. previously, for we have found that haemin solutions change markedly in colour and absorption spectrum on prolonged storage, even in the dark. Visual inspection of most haemin solutions and of blood treated with alkali showed an obvious difference in colour, the blood being greenish, the haemin reddish brown with a slightly violet tinge. Solutions of the two amorphous samples of haemin (B and E in Table 1), however, had a colour very closely similar to that given by blood. On keeping for some time, the brown colour of the haemin solutions gradually changed to the greenish colour given by blood.

The development of an artificial standard

It is clear that the use of haemin as a standard substance for haemoglobinometry may lead to considerable errors. We therefore investigated the possibility of preparing a standard solution from a mixture of inorganic salts. Such a standard, we hoped, would at least have the advantages of reproducibility and stability. Since the more accurate colorimetric and absorptiometric methods for estimating haemoglobin usually involve measurements with filtered light of a fairly narrow range of wavelengths, we aimed at the preparation of a mixture whose spectral absorption was similar to that of blood treated with alkali over a considerable portion of the visible spectrum, regardless of whether or not there was also a good visual match. We prepared rough absorption curves for various salts to aid us in choosing the components for our standard, and used, finally, chromium sulphate in its green modification (giving a band similar to the α-band of alkaline haematin), together with cobaltous sulphate (supplying a broad band filling the gap between the bands of chromium sulphate in the yellow and blue), and a small quantity of potassium dichromate to increase the absorption in the blue-green. The exact amounts of the components were settled by trial. The closeness of fit finally achieved may be indicated by the fact that if the haemoglobin equivalent with a 2 mm. Chance OGr. 1 filter be taken as 100, the values with Ilford filters were: no. 603, 100·8, no. 604, 99·8, no. 605, 98·7, no. 606, 100·8. These results compare very favourably with those obtained with haemin (cf. Table 2).

Some properties of the artificial standard

Reproducibility. As the method of preparation of the standard involves the weighing out of relatively large amounts of two substances, one of which, $K_2Cr_2O_7$, is a primary analytical standard, and the other, $CoSO_4$, is used for weighing in the gravimetric analysis of cobalt, it might be expected that the reproducibility of the standard would be good. This point has been confirmed by the preparation of eight samples of standard from different specimens of the reagents.

A difficulty arises in connexion with the reproducibility of the standard since, in solution, the two modifications of chromium sulphate, green and violet, exist in equilibrium with one another. At low temperatures, the equilibrium mixture contains considerable amounts of the violet salt, but at 100° the green form predominates. It has been found that a definite equilibrium is reached very rapidly at 100°. In practice, 4 min. on a boiling water-bath is sufficient. Heating the standard, besides being essential for this reason, has the advantage that, since the blood must be heated for a similar period in the method of Clegg & King (1942), possible losses from evaporation will be compensated.

Stability. (*a*) *Immediate stability.* After the standard has been heated and cooled, the green and violet forms of chromium sulphate are no longer in equilibrium, and on standing, the amount of the violet form increases slowly. We find that at room temperature the change in absorption in the green region is only about 1 % in the first hour, and after that the rate of change is considerably less. (The total diminution in absorption after prolonged standing is of the order of 10 %.) It is quite safe, therefore, to use the standard within ½–1 hr. of

heating. We recommend that a fresh portion of standard be taken on each occasion to eliminate the risk of cumulative errors due to evaporation during the heating.

(*b*) *Long-term stability.* We have found that the following treatments do not cause changes exceeding 1 %: (*a*) standing under ordinary laboratory conditions for 8 months; (*b*) exposure for 6 hr. in a quartz tube to the light of a quartz Hg vapour lamp at a distance of 10 in.; (*c*) south exposure in a quartz tube to outdoor conditions for 3 months; (*d*) heating in a sealed tube at 95–101° for 48 hr. (heating to temperatures above 105° caused formation of a precipitate); (*e*) boiling down six times to one-fifth of initial bulk and making up to original volume. In view of the resistance of the standard to these adverse conditions, we consider that it may be expected to remain stable indefinitely under ordinary laboratory conditions.

Beer's Law. It is desirable that the standard should, on dilution, behave in the same way as blood, and that the same haemoglobin value be obtained for a given blood when several dilutions of the blood are compared with several dilutions of the standard. To test this point, blood was diluted with various amounts of 0·1 N-NaOH, up to four times the usual amount. The resulting solutions were heated and cooled as usual and compared with similar dilutions of the standard made, after heating and cooling, with distilled water. The solutions were compared, each one with all the others, in the absorptiometer with the OGr. 1, and Ilford Spectrum filters nos. 603–606 inclusive with Hilger H. 503. The results indicated that, within the range examined, Beer's Law is obeyed both by the solution and by blood. The standard may be used for direct comparison with samples of blood having haemoglobin values widely different from that of the standard. If it be preferred, however, a diluted standard may be prepared for use with bloods low in haemoglobin by addition of distilled water to the standard after heating and cooling.

Haemoglobin equivalent of the standard

To determine this figure, we have measured the light absorption of a number of samples of normal blood of known haemoglobin content, diluted 1 in 101 and treated by the method of Clegg & King (1942). The results have been compared with those given by the standard.

These measurements have been carried out in the absorptiometer with Ilford Spectrum filters nos. 603–606 inclusive, and with the Chance green, OGr. 1. We have also made comparisons in the visual colorimeter, in natural and artificial light, both with and without filters.

The haemoglobin values of the blood samples in the series were determined by measurement of both O_2 capacity and iron content. Table 3 shows the figures obtained for the haemoglobin equivalent of the standard. The average value for the coefficient of variation is 1·6 % derived from O_2 capacity and 1·1 % from Fe. These values indicate a satisfactory parallelism between O_2 and colour and between Fe and colour. Although this variation is small, we consider that the differences between individual bloods lie outside the range of experimental error. In both series the Spectrum Blue-Green filter (Ilford 603) gave rather less consistent results than the other filters. When the value of the standard was being determined, we standardized an Ilford Neutral Grey Screen over the same series of bloods, with the OGr. 1 filter only. In this case the coefficient of variation from oxygen was 0·6 % and from iron 0·5 %.

Table 3. *Haemoglobin equivalents of standard*

(g. of haemoglobin/100 ml.)

	From oxygen capacity					From iron determination				
Filters ...	OGr. 1	606	605	604	603	OGr. 1	606	605	604	603
Blood no.										
1	15·7	15·6	15·4	15·5	15·5	16·1	16·0	15·8	15·9	15·9
2	15·5	15·5	15·2	15·5	15·5	15·9	15·9	15·6	15·9	15·9
3	15·4	15·6	15·3	15·5	15·6	15·9	16·1	15·8	16·0	16·1
4	15·5	15·4	15·2	15·3	15·5	16·1	16·0	15·8	15·9	16·1
5	15·8	16·0	15·6	15·8	16·3	16·1	16·3	15·9	16·1	16·6
6	16·1	16·2	15·8	16·0	16·2	16·4	16·5	16·1	16·3	16·5
7	15·5	15·8	15·3	15·4	15·5	16·0	16·3	15·8	15·9	16·0
8	15·6	15·6	15·4	15·5	15·8	16·0	16·0	15·8	15·9	16·2
9	15·8	15·8	15·4	15·7	15·8	16·1	16·1	15·7	16·0	16·1
10	15·4	15·6	15·3	15·5	15·5	15·8	16·0	15·7	15·9	15·9
Averages	15·6	15·7	15·4	15·6	15·7	16·0	16·1	15·8	16·0	16·1
		Average from O_2 = 15·60					Average from Fe = 16·01			

The figures represent the haemoglobin equivalent of the standard calculated on the assumption that 1 g. Hb combines with 1·34 ml. O_2, and contains 0·334 % Fe.

The use of the artificial standard in visual colorimetry was investigated with a further series of ten normal blood samples. These were diluted and heated with alkali in the ordinary way and read against the standard in two colorimeters, illuminated by daylight and artificial light without filters and with a variety of green filters (Chance OGr. 1, Wratten 62, Leitz green (as supplied with colorimeter), and Ilford 604). At the same time, the haemoglobin values were determined against the standard in the absorptiometer. The results with the visual colorimeters agreed closely in each case with those obtained in the absorptiometer, indicating that the standard is suitable for visual colorimetry.

Although the tint of the standard is not absolutely identical with that of blood treated with NaOH (it appears very slightly greener when freshly heated), yet it is so nearly the same that we found no difficulty in matching with unfiltered light. The haemoglobin equivalent with unfiltered light is slightly higher than that found with filters, owing to deviations in the absorption spectrum of the standard in the red and violet, and should be taken as 16·2 g./100 ml. on an iron basis.

The effect of temperature on the haemoglobin value of the standard

The optical density of the standard depends to a small extent on its temperature. The average values given in Table 3 for the haemoglobin equivalent are valid at 18°, and, for ordinary purposes, the effect of temperature may be disregarded. For accurate work, however, the values given in Table 3 should be corrected by adding the following quantities in g. of haemoglobin/100 ml. for each degree by which the temperature of the standard exceeds 18°. Filters: OGr. 1, 0·03; Ilford 606, 0·015; Ilford 605, 0·03; Ilford 604, 0·04; Ilford 603, 0·02. If the temperature of the standard is less than 18°, corresponding amounts should be subtracted. (Although the relation between density and temperature is not strictly linear, the error introduced is quite negligible.) The temperature coefficient of blood in 0·1 N-NaOH is relatively small and may be neglected.

Application of standard to routine determinations of blood haemoglobin

Pipette 10 ml. of a clear solution of 0·1 N-NaOH into a dry boiling-tube, about 2·5 × 15 cm., fitted with a loosely fitting ground-glass stopper. This may be made in a few minutes by grinding a stopper into an ordinary boiling-tube with moderately coarse carborundum. Ordinary corks must not be used as they give a colour with the alkali. Pure NaOH (Analar) should be used, and the solution should be stored in a Pyrex vessel. Some impure samples of NaOH give an opalescence when heated with blood. Add 0·1 ml. well-mixed blood from a calibrated pipette, stopper and mix gently for a few minutes until the solution is clear. Take about the same volume of standard in a similar tube and immerse the two tubes (with stoppers loosened) in a vigorously boiling water-bath so that the liquid in them is just covered by the water. Heat for exactly 4 min. At once cool thoroughly by immersing in cold water and mix by inversion after wiping the outside of the tubes.

Visual colorimeter. (*a*) *Used as colorimeter.* Set standard at 10 mm. and compare, preferably with a green filter over the eyepiece. Readings should be taken as quickly as possible before the eye becomes fatigued. Haemoglobin value for standard = 16·0 g./100 ml. using filters and 16·2 g./100 ml. using unfiltered light (both on an iron basis).

(*b*) *Used as a photometer.* Place a neutral screen of about 0·5 density on one side, and the standard solution on the other. View the fields through any sharply selective light filter (such as an Ilford spectrum filter or Hg green filter) transmitting maximally near the middle of the spectrum. A series of readings is taken, the average of which (*s* mm.) will correspond to 16·0 g. of haemoglobin/100 ml. on an iron basis. If now a blood solution is substituted for the standard, and gives a reading of *B* mm., the haemoglobin value of the blood will be $\frac{16{\cdot}0 \times S}{B}$ g./100 ml. For accurate work, the screen and solution should be used alternately on both sides of the colorimeter and the readings averaged. Once the haemoglobin value of the screen has been determined, the standard need be used only to check the screen at intervals.

Photoelectric absorptiometer. The standard and the blood solution are read alternately in similar 1 cm. cells against water, using the Chance OGr. 1 (identical with Hilger H 455, no. 5) or other suitable filter (see under Methods). It is convenient to use the standard to calibrate an Ilford neutral grey screen of about 0·5 density, as suggested by King, Gilchrist & Delory (1944). With the colour filter in position the standard is read against water and then the grey screen is read in front of the water cell. Having assigned a haemoglobin value to the screen by several comparisons with the standard, it may then be used with that particular filter at any time for comparison with different bloods, and a check-up with the standard need only be done occasionally. We would mention, in this connexion, that the density value marked on the filters cannot be taken as the reading to be expected on any particular absorptiometer, as its value depends on the optical conditions of measurement. It should be emphasized that the haemoglobin value assigned to the screen is only valid with the particular colour filter used for its standardization.

DISCUSSION

The consistent difference between the haemoglobin values calculated from Fe determinations and oxygen capacity measurements is not due to carboxyhaemoglobin or methaemoglobin, since both of these pigments were included in the figures for oxygen capacity. In view of the special care exercised in standardizing our iron and oxygen methods, and the consistent difference found with each blood, we consider the discrepancy a real one, indicating the presence of some 3 % of the iron in a non-functional form. It is not due to neglect of plasma iron, since the amount of this in normal

blood (one-eighth % of total iron; Fowweather, 1934) is too small to account for the difference. It is interesting that similar results have recently been obtained by Macfarlane & O'Brien (1944) on a series of samples of male blood.

The artificial standard has the disadvantages that it has a relatively large temperature coefficient and that it must be heated shortly before use. On the other hand, it has the following advantages over haemin as a standard: it is stable and reproducible, being made of inorganic substances easily obtained pure; it does not require the use of a very sensitive balance in its preparation; it gives a better colour match with alkaline solutions of blood and more closely follows the blood spectrum over the middle range of the visible spectrum; its use eliminates the need for the lengthy and difficult analysis of iron involved in the use of haemin. Our experience leads us to believe that the systematic error involved in using the standard as described should not exceed $\pm 1{\cdot}5$ %, i.e. $\pm 0{\cdot}2$ g. of haemoglobin/100 ml. in a normal blood. In view of the results of Mole (1945) on the variability of blood samples from the same individual, it would appear that no practical advantage would be gained from a greater degree of precision.

Although we have taken every care in determining the haemoglobin value of the standard, it is possible that further work may lead to a small revision of the figures given. In that case, any necessary correction could easily be applied to haemoglobin determinations already made by the method. We consider that the reproducibility of the standard is such that it should provide a useful basis for haemoglobin surveys carried out at different times or in different places.

It is hoped that the introduction of this standard will make relatively accurate haemoglobinometry possible in any ordinarily equipped laboratory. It may be pointed out that, in addition to its use in the routine estimation of haemoglobin as alkaline haematin, the standard may be employed to standardize bloods for checking other colorimetric methods of haemoglobinimetry.

SUMMARY

1. Errors may arise in the use of haemin as a standard substance in haemoglobinometry, (*a*) because the colour intensity of various samples of haemin dissolved in 0·1 N-NaOH is not strictly proportional to their iron content, (*b*) because the absorption spectrum of haemin solutions differs significantly from that of blood converted to alkaline haematin.

2. To meet this difficulty, an artificial standard has been prepared from inorganic salts which is stable, reproducible, and follows the absorption spectrum of blood in 0·1 N-NaOH between the yellow and blue-green regions.

3. Methods of application of the standard to routine haemoglobinometry are described.

4. The haemoglobin equivalent of the standard is $15{\cdot}6 \pm 0{\cdot}2$ g. of haemoglobin/100 ml. of blood, based on oxygen capacity measurements, and $16{\cdot}0 \pm 0{\cdot}2$ based on Fe determinations.

5. Determinations of Fe in ten consecutive samples of male blood gave consistently higher results (average 3 %) than the values expected from oxygen capacity measurements. This difference was not due to the presence of carboxyhaemoglobin or methaemoglobin, and appears to indicate the presence of 'inactive' iron in normal male blood.

REFERENCES

Barcroft, H., Gibson, Q. H., Harrison, D. C. & McMurray, J. (1945). *Clin. Sci.* **5** (in the Press).
Bernhart, F. W. & Skeggs, L. (1943). *J. biol. Chem.* **147**, 19.
Clegg, J. W. & King, E. J. (1942). *Brit. med. J.* **2**, 329.
Evelyn, K. A. & Malloy, H. T. (1938). *J. biol. Chem.* **126**, 655.
Fowweather, F. S. (1934). *Biochem. J.* **28**, 1160.
King, E. J., Gilchrist, M. & Delory, G. E. (1944). *Lancet*, **1**, 239.
Klumpp, T. G. (1934). *J. biol. Chem.* **107**, 213.
Macfarlane, R. G. & O'Brien, J. R. P. (1944). *Brit. med. J.* **1**, 248.
Mole, R. H. (1945). *J. Physiol.* **104**, 1.
Peters, J. P. & Van Slyke, D. D. (1932). *Quantitative Clinical Chemistry*, **2**, 238. London: Ballière, Tindall and Cox.
Rimington, C. (1941). *Brit. med. J.* **1**, 177.
Sendroy, J., Dillon, R. T. & Van Slyke, D. D. (1934). *J. biol. Chem.* **105**, 597.
Van Slyke, D. D., Dillon, R. T. & Margaria, R. (1934). *J. biol. Chem.* **105**, 571.
Van Slyke, D. D. & Hiller, A. (1928). *J. biol. Chem.* **78**, 807.
Van Slyke, D. D. & Neill, J. M. (1924). *J. biol. Chem.* **61**, 523.