THE HAEM PIGMENTS OF HUMAN BLOOD

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PREFACE

In view of the nature of the project undertaken and the results obtained, it seemed desirable to divide the present work into two distinct sections and to consider each separately.

Part I of this thesis therefore deals with the determination of the specific extinction coefficients of the haemoglobins of the adult and foetus, while Part II represents a study of the reports of a new method for the determination of total haem pigment which it is claimed avoids many of the defects of methods hitherto used.

Inasmuch as several systems of nomenclature of the blood pigments are in existence, a single pigment may be denoted by a variety of names. For example, the pigment formed when pyridine is added to a reduced solution of blood in sodium hydroxide is known as pyridine haemochromogen, pyridine ferrous haemochromogen, dipyridine ferroporphyrin or pyridine haemochrome.

The term "haemochrome" is the one recently proposed by Lemberg and Legge (1949) and replaces the names "haemochromogen" and "ferrous haemochromogen". These pigments, they point out, are not "chromogens" in the chemical sense, i.e., colourless compounds. Lemberg and Legge also suggest the term "haemichrome" (oxidized haemochrome) as a replacement for the earlier names "parahaematin" and "ferric haemochromogen".

The terms "haemochrome" and "haemichrome" have been adopted and will be used throughout this work.

PART I

DETERMINATION OF THE SPECIFIC EXTINCTION COEFFICIENTS OF THE HAEMOGLOBINS OF THE ADULT AND FOETUS

SECTION A - PRELIMINARY CONSIDERATIONS

INTRODUCTION

As long ago as 1888, Von Kruger found that when adult blood was treated with dilute sodium hydroxide the red colour rapidly changed to brown, while, with foetal blood the effect of sodium hydroxide was much slower, many hours elapsing before the red colour was changed to brown.

It is now known that these differences are due to the fact that haemoglobin is rapidly converted to another pigment (denatured globin haemichrome) while with foetal haemoglobin this change takes place much more slowly. By a quantitative study of this reaction Brinkman and Jonxis (1935) found two fractions in adult haemoglobin differing in their rate of reaction with sodium hydroxide, while two different fractions occurred in foetal blood. Similar experiments have been reported by Baar and Lloyd (1943) and Ponder and Levine (1949). More recently White, Delory and Israels (1950) confirmed the existence of two fractions in adult blood, but observed that three fractions could occasionally be identified in foetal blood. As a result of their findings these workers suggested that the names adult haemoglobin and foetal haemoglobin (previously used) be abandoned since they had become meaningless and they suggested that the three fractionsin the

foetal blood be termed f_1, f_2, and f_3, while those of adult blood be called a_1 and a_2.

All the quantitative experiments referred to above, depended on the colorimetric measurement of the rate of change of colour of blood treated with sodium hydroxide. Although the correctness of their results was dependent on the fact that the specific extinction coefficients of the two forms of haemoglobin were the same, definite proof was lacking, and consequently an attempt has now been made to provide that proof by a controlled study and comparison of the specific extinction coefficients (optical density per unit concentration per unit length) of the haemoglobins of the adult and foetus.

Ideally the various haemoglobin fractions should be isolated and the specific extinction coefficients determined for each fraction. However, it is not yet possible to do this due to the speed with which haemoglobin is converted to methaemoglobin. The work was confined therefore to a study of adult and foetal blood since if these gave the same results there could hardly be any significant difference in the specific extinction coefficients of the fractions themselves.

Since the specific extinction coefficient is the optical density per unit concentration per unit path length, at the chosen wave length, the experimental work fell into two parts -- (i) the determination of the optical density of the haemoglobin solutions and (ii) the determination of the haemoglobin concentration; the latter being established by iron analysis which as King et al (1948) have shown is the most satisfactory method for making this determination.

THE DETERMINATION OF BLOOD IRON

In an attempt to find a method of analysis for the iron in blood suitable for the needs of this project, two standard methods were tested and compared. These methods, which have been reviewed by Delory (1943), are the titanous chloride titration method and the alpha alpha' dipyridyl method.

1. The Titanous Chloride Titration Method

The titanous chloride titration method was first adapted to the determination of iron in biological materials by Jahn (1911). It has now become a standard macro method for such estimations. The method is based on the reduction of ferric thiocyanate to the ferrous compound by titanous chloride.

The necessity of excluding oxygen during the titration has generally been recognized. Jahn (1911) protected the titanium reagent from oxidation by an atmosphere of hydrogen. Subsequent workers (Thornton and Chapman, 1921) have used carbon dioxide. The apparatus employed in the present procedure for titrating in an atmosphere of carbon dioxide was described by Klumpp (1934).

Apparatus

The set-up of the apparatus is shown in Figure 1. It consisted essentially of a storage bottle A for the titanous chloride, which was kept under an atmosphere of carbon dioxide supplied by the Kipp generator B. The layer of petrolatum on top of the titanium reagent further protected it. C is a 5 ml. micro burette, the top of which

FIGURE — 1

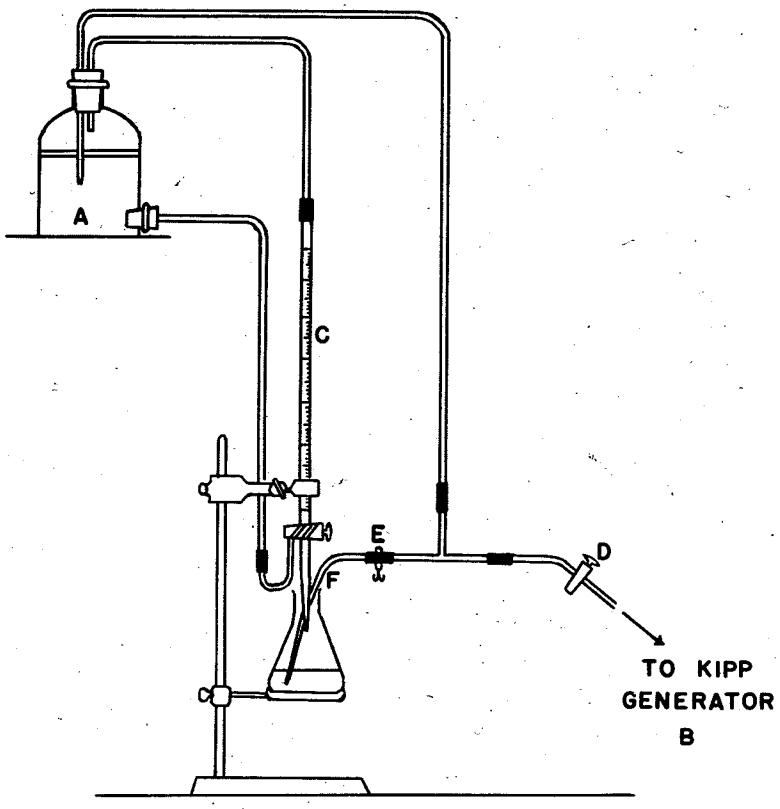

Figure 1

Apparatus for Titrating Titanous Chloride in an Atmosphere of Carbon Dioxide

is connected by tubing with the storage bottle A in order to maintain the solution in the burette under an atmosphere of carbon dioxide. By means of the stop cock at D a stream of carbon dioxide could be bubbled into the solution in A, or when the clamp at E is open, the stream could be directed to the glass nozzle F through which it passes into the solution being titrated.

Reagents

1. Titanous chloride -- the 15 per cent commercial solution was used to make up the stock solution in the following way.

To 2 litres of freshly boiled distilled water was added 20 ml. of concentrated high-grade hydrochloric acid. A suitable volume of 15 per cent commercial solution of titanous chloride was added such that about 3 or 4 ml. was required to titrate 2 ml. of the standard solution. The stock solution was quickly transferred to the storage bottle A and carbon dioxide bubbled into it for fifteen minutes to remove the oxygen dissolved in it and to displace the oxygen above it. The stop cock of the burette was left open during this operation.

2. Standard iron solution, containing 1.0 mg. of iron per ml. of solution. Ferric ammonium sulphate (4.318 grams) was dissolved in about 250 ml. of water in a 500-ml. volumetric flask. Fifty ml. of high-grade concentrated hydrochloric acid were added and the solution made up to the mark with distilled water.

3. Potassium thiocyanate -- a 20 per cent solution.

Analytical procedure

Five ml. of oxalated whole blood were pipetted into each of

two 100-ml. beakers. Three ml. of 2N nitric acid were added slowly and with constant stirring, and the beakers then placed in a constant temperature oven. When charring was complete they were removed and placed in a muffle furnace at 500° C until a red ash remained. Ten ml. of high-grade concentrated hydrochloric acid were added and the beakers heated over a low heat until the ash had dissolved. An equal volume of water was then added. Two ml. of the standard iron solution were placed into each of two other 100-ml. beakers. To each of the four solutions was added 1 ml. of the potassium thiocyanate solution. They were then titrated until colourless with titanous chloride. One standard solution was titrated before, and one after the test solutions, and the mean value used in the calculation.

In order to drive oxygen from the solution being titrated, carbon dioxide was bubbled through nozzle F during the titration. This not only served to prevent the absorption of oxygen at the surface of the liquid, but also helped to stir the solution as the titrant was being added.

The stop cock at E was left open permanently, even when no titrations were being performed.

Calculation

The calculation of blood iron, when 5 ml. of whole blood are employed, is as follows:

$$\frac{V_t}{V_s} \times 2 \times \frac{100}{5} = \text{no. of mg. of iron per 100 ml. of blood}$$

where V_t and V_s are respectively the average volumes of titanous chloride required to titrate the test and standard solutions.

2. The Alpha Alpha' Dipyridyl Method

The alpha alpha' dipyridyl method of Thorp (1941) is based on the pink colour given by dipyridyl with ferrous ions (Hill, 1930).

Reagents

1. Dilute standard iron solution, containing 0.010 mg. of iron per ml. of solution. This was prepared from the standard iron solution containing 1.0 mg. of iron per ml., (the preparation of which is described earlier) by pipetting 10 ml. into a 1-litre flask together with 200 ml. of N hydrochloric acid, and diluting to the mark with water.

2. Alpha alpha' dipyridyl solution -- 1 gram in 100 ml. of N/10 hydrochloric acid.

3. Sodium sulphite solution -- a 21 per cent solution of anhydrous sodium sulphite. This is unstable and should be freshly prepared when required.

Procedure

Into each of two 15-ml. volumetric flasks was pipetted 0.1 ml. of blood followed by about 5 ml. of water. To a third similar flask was pipetted 5 ml. of the dilute standard iron solution, and to a fourth (to be used as a blank) 5 ml. of water.

To each flask was added 0.2 ml. of concentrated sulphuric acid, and the contents heated on an electric hot plate at low heat (to keep

frothing at a minimum) until most of the water had evaporated, and a brownish residue remained. This was followed by the addition of 0.12 ml. of perchloric acid and continued heating on the electric plate, at high heat, until the solutions were clear and colourless. On cooling, 1 ml. of the alpha alpha' dipyridyl solution was added, followed by 3 ml. of the sodium sulphite solution. Water was added to the mark, and the solutions allowed to stand for 15 minutes, after which time, the pink colour which had developed was read in a Beckman spectrophotometer (Model DU) at a wave length of 540 mμ and an effective slit width of 0.05 mm. using 1 cm. Corex cells. The blank solution, which had undergone the same treatment, was used in place of water to set the instrument.

Calculation

From the observed optical density readings of the test and standard solutions, D_t and D_s respectively, the amount of iron in the test solution (C_t) expressed in mg. per 100 ml. of blood is found by the following equation:

$$C_t = \frac{D_t}{D_s} \times 0.05 \times \frac{100}{0.1}$$

$$= \frac{D_t}{D_s} \times 50$$

Comparison of the Two Methods

While the first method, the titanous chloride titration method, probably yields more accurate results, the alpha alpha' dipyridyl

method is easier to carry out. The lack of a good muffle furnace for ashing the blood samples in the titration method considerably lengthened the time of procedure, which proved a real disadvantage where, as in this instance, there were several determinations to make.

It was thus decided to employ the alpha alpha' dipyridyl method for the estimation of blood iron in this and in later experiments. Requiring relatively less time than the titanous chloride procedure this method had the added advantage that several determinations could be carried out simultaneously.

SECTION B - EXPERIMENTAL

PROCEDURE

As mentioned earlier, the specific extinction coefficients were estimated by determining separately (i) the optical density and (ii) the iron concentration of the haemoglobin solutions.

Determination of the Optical Density

Fresh samples of oxalated adult blood and oxalated foetal blood (taken from the umbilical cord immediately following birth) were used. The cord blood was collected in small test tubes containing approximately 10 mg. of potassium oxalate. The blood was centrifuged and the plasma separated from the red cells in order to remove a possible source of error due to the chromogenic properties of the plasma. After removal of the plasma, the cells were washed twice with 0.85 per cent sodium chloride solution. A quantity of water, equal approximately to the volume of plasma removed, was then added to the cells to effect haemolysis. Two ml. of this laked blood were diluted to 100 ml. with water, and kept as the stock solution.

Two 5 ml. samples of this stock solution were further diluted to 25 ml. with the addition of 0.1 per cent sodium carbonate solution (to make a final dilution of 1 in 250) and rotated in a stoppered flask to ensure complete oxygenation of the blood. The optical density of these solutions was read in the Beckman spectrophotometer at a wave length of 578 mμ (the alpha band of oxyhaemoglobin) with an aperture of 0.05 mm.

Estimation of Iron

Duplicate 5-ml. samples of the stock solution (2 ml. diluted to 100) were pipetted into 15-ml. volumetric flasks and analyzed for iron, using the alpha alpha' dipyridyl method, as outlined in Section A.

Measurement of the Specific Extinction Coefficient

The specific extinction coefficient, as already stated, is the optical density, at the chosen wave length, of a unit concentration (in this case 1 mg. of iron per 100 ml.) in a unit length (1 cm.).

The calculation is illustrated by the following example.

Blood (diluted 1 in 250) gave an optical density of 0.492, and the iron concentration was 0.168 mg. per 100 ml. of a solution of blood diluted 1 in 250. The specific extinction coefficient, at a wave length of 578 mμ is therefore

$$\frac{0.492}{0.168} = 2.928$$

RESULTS

The results of the determination of the specific extinction coefficients of a series of 12 samples of adult blood and 12 samples of foetal blood are shown in Table I.

It will be seen that there is a wide range of individual values in both types of blood (from 2.637 to 2.953 in the adult form, and from 2.663 to 2.962 in the foetal form) but that the average value in both cases is almost identical.

CONCLUSION

The above results suggested that there is no systematic difference between the specific extinction coefficient of adult and foetal blood, and hence it was concluded that they were the same.

TABLE I

A COMPARISON OF THE SPECIFIC EXTINCTION COEFFICIENTS OF ADULT AND FOETAL BLOOD

SPECIFIC EXTINCTION COEFFICIENTS	
Adult Blood	Foetal Blood
2.773	2.719
2.685	2.798
2.736	2.701
2.741	2.695
2.637	2.663
2.879	2.786
2.892	2.928
2.785	2.962
2.732	2.781
2.955	2.733
2.764	2.892
2.852	2.793
Mean 2.786	2.788
Coefficient of Variation 3.30%	3.32%

SUMMARY

1. The specific extinction coefficients of adult and foetal blood have been measured and compared.

2. The results obtained indicated that there is probably no significant difference in these specific extinction coefficients.

PART II

A NEW METHOD FOR THE ESTIMATION OF TOTAL HAEM PIGMENT

SECTION A - HISTORICAL

INTRODUCTION

Besides the two main forms of haemoglobin found in circulating blood -- (reduced) haemoglobin and oxyhaemoglobin -- there are certain other inactive haem pigments which often form part of the total haemoglobin content. Chiefly these are methaemoglobin, carboxyhaemoglobin, and sulph-haemoglobin. Ammundsen (1941) has shown that under modern conditions a normal adult may have from 2 to 12 per cent of his total haemoglobin circulating in this inactive form. In the blood of men suffering from certain poisons and intoxications, even larger amounts of this inactive form of haemoglobin may be found. In order therefore to determine the total haem pigment it is necessary to treat the blood so that all the pigments are converted into the same substance.

Before proceeding to discuss the methods available for the determination of total haem pigment, the chemistry of some of the haem pigments will be reviewed.

THE HAEMOCHROMES

A haemochrome is formed when haem (an iron porphyrin) combines with one of a number of basic nitrogenous compounds. The difference between haemochromes formed from the same haem are due to the different basic compounds with which the haem is combined. Many, but by no means

all nitrogenous bases are able to form a haemochrome from haem. They appear to have only nitrogen in common.

Haem also combines with denatured proteins -- the most common being the globin of haemoglobin. When a solution of haemoglobin is treated with a base such as sodium hydroxide and a reducing agent (e.g. sodium dithionite), denatured globin haemochrome is formed.

The haemochromes have not always been regarded as compounds of haem and a nitrogen-containing substance. They were discovered by Stokes (1864) and were considered by him and subsequent observers to be simply "reduced" haem. He identified this substance by its absorption spectrum. When Stokes reduced an alkaline solution of what is now known as haem he discovered a substance having two intense, sharp absorption bands. Since Stokes used an ammoniacal reducing agent, he had in fact produced a haemochrome -- the ammonia haemochrome. Hoppe-Seyler (1878) shortly found that some reducing agents would not form haemochromes unless accompanied by a substance like ammonia or albumin, which he concluded enhanced the reducing action of the system. Since haem had not yet been discovered, it was thought that no reduction had taken place when a haemochrome was not formed. Haem (or "reduced haem" as it was known then) was discovered by Bertin-Sans and Moitessier (1893) who noted that it turned into one of the haemochromes by the action of ammonia, amines, and proteins. This fact was confirmed by some (Menzies, 1894) but denied by others (Laidlaw, 1904).

The view that haemochrome is essentially the same as haem continued to exist as evidenced by the fact that Fischer and Schneller (1923) tried

to make haemochrome by the addition of iron to a porphyrin in the absence of oxygen. Anson and Mirsky (1925) finally showed that haemochrome is a compound composed of haem plus a nitrogenous substance, and that the haemochrome prepared from haemoglobin is a compound of denatured globin and haem.

Structure of the Haemochromes

Anson and Mirsky (1925, 1928) hold the view that in a haemochrome an equilibrium of the following type exists.

$$\text{Haem} + \text{Nitrogen compound} \rightleftharpoons \text{Haemochrome}$$

There is always some uncombined nitrogen compound present. Working with the Hartridge reversion spectroscope they found (1925) that if either haem or nitrogen compound were added, the equilibrium was shifted to the right, and more haemochrome was formed, while if either haem or the nitrogen compound were removed from the system, the equilibrium was shifted to the left and the haemochrome would disappear.

Analyses of "isolated" haemochromes were made as early as 1898 (Von Zeynek, 1898), but according to the above equilibrium it would seem hardly possible to be able to isolate the haemochrome uncontaminated by free nitrogen compound or by free reduced haem.

Hill (1926) found that the addition of two molecules of pyridine was required to convert one molecule of haem into haemochrome. He believed that in his experiments the free pyridine was negligible. Anson and Mirsky (1929), however, do not agree with this and state that the haemochrome probably only contains one nitrogen group per haem radicle.

Pauling and Coryell (1936) showed from magnetochemical investigations that in haemochromes the iron is bound to the four porphyrin nitrogens and to two additional atoms of the combining nitrogenous substances.

Identification of the Haemochromes

A great number of nitrogen-containing compounds such as ammonia, pyridine, nicotine and hydrazine have been employed in the formation of haemochromes.

Some of these compounds (particularly the pyridine haemochrome) have been isolated in crystalline form (Von Zeynek, 1898, 1910) (Hill, 1926, 1929) and found to be definite compounds and not merely complexes.

However, the investigation of the haemochromes has been largely dependent on their spectroscopic and spectrophotometric properties, since these pigments have unusually sharp absorption bands and because exact measurements are easily made. Largely used in this work has been the reversion spectroscope (Hartridge, 1912) and the spectrophotometer. Each haemochrome can be identified by its own absorption pattern. The spectrum is characterized by the sharpness of the alpha or darker band lying in the region of 560 mμ. The exact position of this alpha band depends on the nitrogeous base used in the formation of the haemochrome.

According to Anson and Mirsky (1925) the absorption bands of the haemochrome shift toward the red end of the spectrum under certain conditions. Keilin (1926) says this is due to the aggregation of the molecules of the pigment. He claims that substances like pyridine and ammonia haemochromes show two types of absorption spectra corresponding

respectively to the pigment as a precipitate (where the bands are near the red end of the spectrum) and as a true solution (where the bands are near the blue end).

THE HAEMICHROMES

The haemochromes react with atmospheric oxygen, which converts their bivalent iron to the trivalent state with the production of haemichromes.

Keilin (1926) maintained that haemichromes were stable only at a pH near the neutral point, and in more acid or alkaline solutions dissociated into haematin and the nitrogen compound.

Insofar as the globin haemichrome was concerned, Haurowitz (1927) shared this view, but Anson and Mirsky (1930) and King and Delory (1945) did not.

Anson and Mirsky state that haemoglobin in sodium hydroxide yields a conjugated protein. They found that the substance so formed was precipitated by protein precipitates.

King and Delory, from a comparison of the colour intensities of haemoglobin and haemin in N/10 sodium hydroxide per mg. of iron, concluded that a conjugated protein was formed when haemoglobin is dissolved in sodium hydroxide.

Drabkin and Austin (1935, 1936) also share this view. They state that the conjugated protein formed can exist in both the oxidized and reduced (the haemochrome) forms.

For haemichromes containing other nitrogenous bases, Keilin's

theory has certainly been found not to hold. A study of the absorption spectrum of pyridine haemichrome by Barron (1937) and Lemberg (1943) has definitely shown that haematin combines with pyridine in alkaline solutions. Barron points out that the spectral properties of the haemichromes, unlike those of the haemochromes, change with the hydrogen ion concentration of the solution. There is, he notes, also a change in colour; they are red in neutral solutions and green in alkaline solutions. Holden and Freeman (1929) have even shown that casein and denatured egg albumin form haemichromes only in a strong alkaline solution and dissociate at the neutral point.

Structure of the Haemichromes

The difference of the absorption spectra of the haemichrome with pH is explained by the presence of a hydroxyl group, which, bound to the iron in alkaline solutions, dissociates with increasing acidity. Because of this reaction with hydroxyl ions and the fact that ferric haematin compounds tend to polymerize, along with the experimental difficulties encountered, it has not yet been possible to establish whether haematin combines with one or two molecules of a base to form a haemichrome.

In their spectrophotometric study of pyridine haemichrome, Clark and Perkins (1940) assumed that two molecules of pyridine were added concurrently, but Davies (1940) on the other hand, obtained results that indicated that in the case of nicotine haemichromes only one molecule of the base is concerned.

Analyses of the compositions of solid haemochromes yield results which are no less confusing. Whereas Fischer, Hummel and Treibs (1929) reported

that the collidine haemichrome was found to contain only one molecule of base, Hamsik (1936) isolated some compounds which contained three molecules of base per atom of iron.

Lemberg and Legge (1949) suggest that in an acid or neutral solution two molecules of the base are present but that in alkaline solution one molecule of the base gives way to a hydroxyl group. Clark and Perkins (1940) however, in order to give a consistent account of their results, postulated the existence of a species in which two molecules of pyridine and one of hydroxyl ion were coordinated with the ferriporphyrin.

Absorption Spectra of the Haemichromes

The absorption spectra of the haemichromes have not been as extensively investigated as those of the haemochromes. Barron (1937) has studied the absorption spectrum of pyridine haemichrome in neutral and in alkaline solutions. In neutral solution the spectrum had two bands similar in position to those of pyridine haemochrome. On the whole, though, the two absorption spectra were very different. Barron found that pyridine haemichrome had a weak band at 558 mμ and a stronger band at 530 mμ.

In alkaline solution, he found that the absorption is quite different from that of alkaline haematin. It resembled in shape that of the haemichrome in neutral solution, but the bands were less intense, and shifted toward the red. Drabkin (1942) also found this to be true, while Lemberg (1943) made similar observations.

REVIEW OF EXISTING METHODS

Since 1878, when Gowers measured the haemoglobin in blood by comparing a solution of oxyhaemoglobin with a picrocarmine standard, many different methods have been developed for the estimation of haemoglobin. These will be reviewed under the general headings:

1. Fundamental methods and
2. Methods requiring independent standardization or the use of haemin.

Fundamental Methods

The first group of methods, the fundamental methods, are based on certain fundamental properties of the haemoglobin molecule, and hence do not have to be standardized independently. Although the methods yield accurate results in experienced hands, they are by no means simple, and require considerable time to carry out. They are therefore, primarily, methods for the standardization of instruments or for research purposes rather than for clinical investigation.

1. Method of Iron Estimations

In this method the total iron concentration of blood is taken as a measure of total haem pigment. Bernhart and Skeggs (1943) reported the iron content of human crystalline haemoglobin to be 0.340 per cent. By taking this factor, and carrying out an iron estimation, the haemoglobin content of any blood sample can be determined.

While it is undoubtedly true that plasma contains a small

amount of non-haemoglobin iron, (0.1 mg. per cent) it is generally considered to be negligible with respect to the relatively large amount of iron in the red blood cells.

The analysis of whole blood for iron may be accomplished by several methods. Delory (1943) outlines two standard procedures.

2. Gasometric Methods

In these methods the ability of whole blood to combine with definite amounts of different gases is used to estimate the haemoglobin content of the blood.

The determination of the oxygen capacity of the blood described by Haldane (1899) and the manometric measurement later described by Van Slyke and Neill (1924) depend on the measurement of the volume of oxygen liberated when blood is treated with potassium hydroxide and potassium ferricyanide. This method, however, does not measure the inactive fraction of the blood, and so may provide a false picture of the total haem pigment.

A method based on the carbon monoxide capacity of the blood was introduced by Haldane (1901) and improved by Van Slyke and Hiller (1928).

Methods Requiring Independent Standardization or the Use of Haemin

In all of these methods, haemoglobin is converted into a haem pigment which can be compared colorimetrically with an artificial standard or with a standard solution prepared from crystalline haemin. This is necessitated by the fact that crystalline haemoglobin, the ideal standard, is difficult to prepare absolutely pure. The

use of haemin as a standard is based on the assumption that when haemin and blood are treated identically with certain reagents, they are converted into the same substance. While this assumption is valid for the cyanhaematin method, (in which case haemin may be satisfactorily used as the standard) it does not hold in all cases (e.g. the alkaline haematin method).

Although these methods are less exact than the fundamental methods, they are generally easily and rapidly carried out, and hence are more suitable for clinical use.

1. Oxyhaemoglobin Method

Although this was one of the first methods of haemoglobin estimation, it is still used widely since it is so easily carried out. Essentially the method consists of preparing a dilute solution of blood, shaking to ensure complete oxygenation and reading its optical density. This is then compared to the colour of a solution of blood of known haemoglobin content. This method, however, unlike the other colorimetric methods does not measure total haem pigment.

2. Acid Haematin Method

Developed by Sahli (1931) this method is based on the conversion of haemoglobin to acid haematin (Newcomer 1919) on treatment with hydrochloric acid. The resultant brown colour is compared with that of a glass or gelatin standard, or to a sample of blood of known haemoglobin concentration. Although widely used in the past this method suffers from the disadvantage that

the colour increases in intensity with time up to three hours. Again, commercial standards are often unreliable and may fade, and hence the degree of accuracy desirable may not be attained.

3. Alkaline Haematin Method

Instead of treating haemoglobin with acid, Wu (1922) employed alkali and found that many of the discrepancies in the acid haematin method could be thus overcome. More recently, Clegg and King (1942) showed this method to have considerable promise when they introduced the use of crystalline haemin, of known iron content, in the preparation of a standard. However, the absorption curves for alkaline globin haematin prepared from haemoglobin, and alkaline haematin prepared from haemin differ in the visual spectral range. Clegg and King found that the determination of haemoglobin in blood, based on the assumption that 1 mg. of haemin iron in the standard was equivalent to 1 mg. of haemoglobin iron in the test, yielded results uniformly about 30 per cent higher than the determination based on the oxygen-carrying power of the same sample. They accordingly added a correction factor of 1.34 to the observed colour intensity of the alkaline haematin from haemin.

Collier (1944) however found that this factor was not applicable in all cases since the ratio of the absorption curves depended on the spectral characteristics of the light source, the filter, and the photocell or other detecting device.

4. Cyanmethaemoglobin Method

Introduced by Stadie (1920), this method has since been recommended by Sundermann (1943) and Collier (1944) as a reliable means of estimating total haem pigment. It is based on the fact that when a solution of blood is treated with potassium ferricyanide and potassium cyanide, haemoglobin, oxyhaemoglobin, carboxyhaemoglobin, and methaemoglobin are all transformed to cyanmethaemoglobin. This is then compared to an artificial standard calibrated against haemoglobin measurements by the oxygen capacity methods. Although this is a method of considerable accuracy, there is the hazard of using cyanide routinely.

5. Cyan-Haematin Method

King and Gilchrist (1947) found that by transforming the haem pigments to cyan-haematin by the addition of hydrochloric acid and sodium cyanide, an even more accurate estimation of the total haem pigment was possible. Using crystalline haemin as their standard they found the absorption curves of the cyan-haematins from haemin and haemoglobin to be almost the same.

6. Pyridine Haemochrome Method

This method of Roets (1940) and Rimington (1942) is based on the comparison of the pyridine haemochromes obtained from haemoglobin and from haemin. Haemoglobin and crystalline haemin are treated with sodium hydroxide, pyridine, and sodium dithionite and are thus converted to the pyridine haemochromes. The two solutions are compared colorimetrically and the concentration of

haemoglobin in the test is calculated, on the assumption that each mg. of iron in the haemin standard is exactly the equivalent of one mg. of haemoglobin iron in the test.

FURTHER COMMENT ON METHODS

In a critical appraisal of the different colorimetric methods for the determination of haemoglobin, King et al (1948) found that the oxyhaemoglobin and cyan-haematin methods yielded the most accurate results.

The use of the highly toxic substance, sodium cyanide, however, definitely limits the widespread usage of the cyan-haematin method for routine estimations. Similarly the objectionable odor of pyridine, required in the pyridine haemochrome method, (a method of considerable reliability) proves to be a barrier to the popularity of this method.

It is apparent therefore, that there is a need for a new method for the estimation of total haem pigment. Such a method should possess the following criteria to be of general use in a routine laboratory:

1. It should employ inexpensive, non-toxic and odourless reagents.
2. It should be rapid and easy to carry out.
3. It should yield results that are as accurate as those given by the best colorimetric procedures.
4. It should employ a standard which is easily prepared.

In an attempt to develop such a method, nicotinamide (the amide of pyridine-3-carboxylic acid) was used. Nicotinamide is non-toxic, odourless, inexpensive, and can be obtained pure; hence as a reagent it

is ideal.

Based on the pyridine haemochrome method, nicotinamide was used in this study in the form of its haemochrome. Having produced encouraging results with this nicotinamide compound in preliminary experiments, it was decided to investigate its possibilities more fully.

A presentation of this study is given in subsequent sections.

SECTION B — PRELIMINARY CONSIDERATIONS

INTRODUCTION

The addition of nicotinamide to a solution of blood or crystalline haemin in N/10 sodium hydroxide yielded the bright green nicotinamide haemichrome, and this on reduction, with sodium dithionite, gave the deep rose-coloured nicotinamide haemochrome.

The absorption spectra of these pigments were studied using the Hartridge reversion spectroscope, and their absorption curves determined with the Beckman spectrophotometer by plotting the optical density versus the wave length. While the nicotinamide haemichromes gave no observable absorption bands in the visible part of the spectrum when studied in the reversion spectroscope, their absorption curves (Figure 2) were very similar. There were no peaks in the curves, a fact which confirmed the observations made earlier with the reversion spectroscope. Like most haemochromes, the nicotinamide haemochromes displayed two dark absorption lines in the visible part of the spectrum, and it was noticed that the bands of the haemochrome prepared from haemin were in almost identical positions with those of the haemochrome from haemoglobin. These findings were confirmed by a study of the absorption curves (Figures 3 and 4) which clearly revealed the two absorption bands — the alpha band at 564 mμ and the weaker or beta band at 530 mμ. As in the case of the nicotinamide haemichromes the two curves showed a great similarity.

This suggested that haemin presumably formed the same compound as

FIGURE — 2

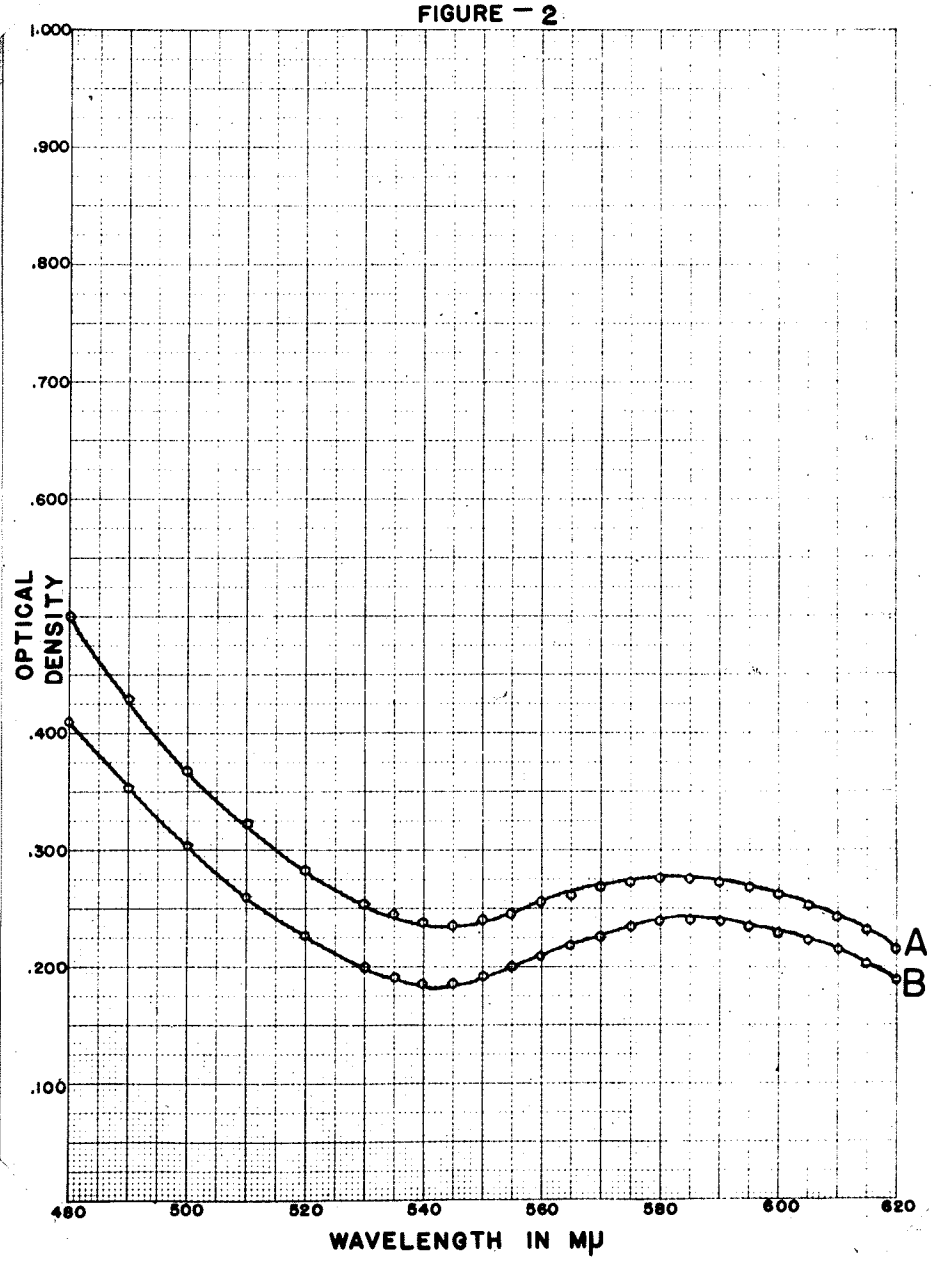

Figure 2

The Absorption Curves of the Nicotinamide Haemichromes Prepared from Haemoglobin (A) and Haemin (B)

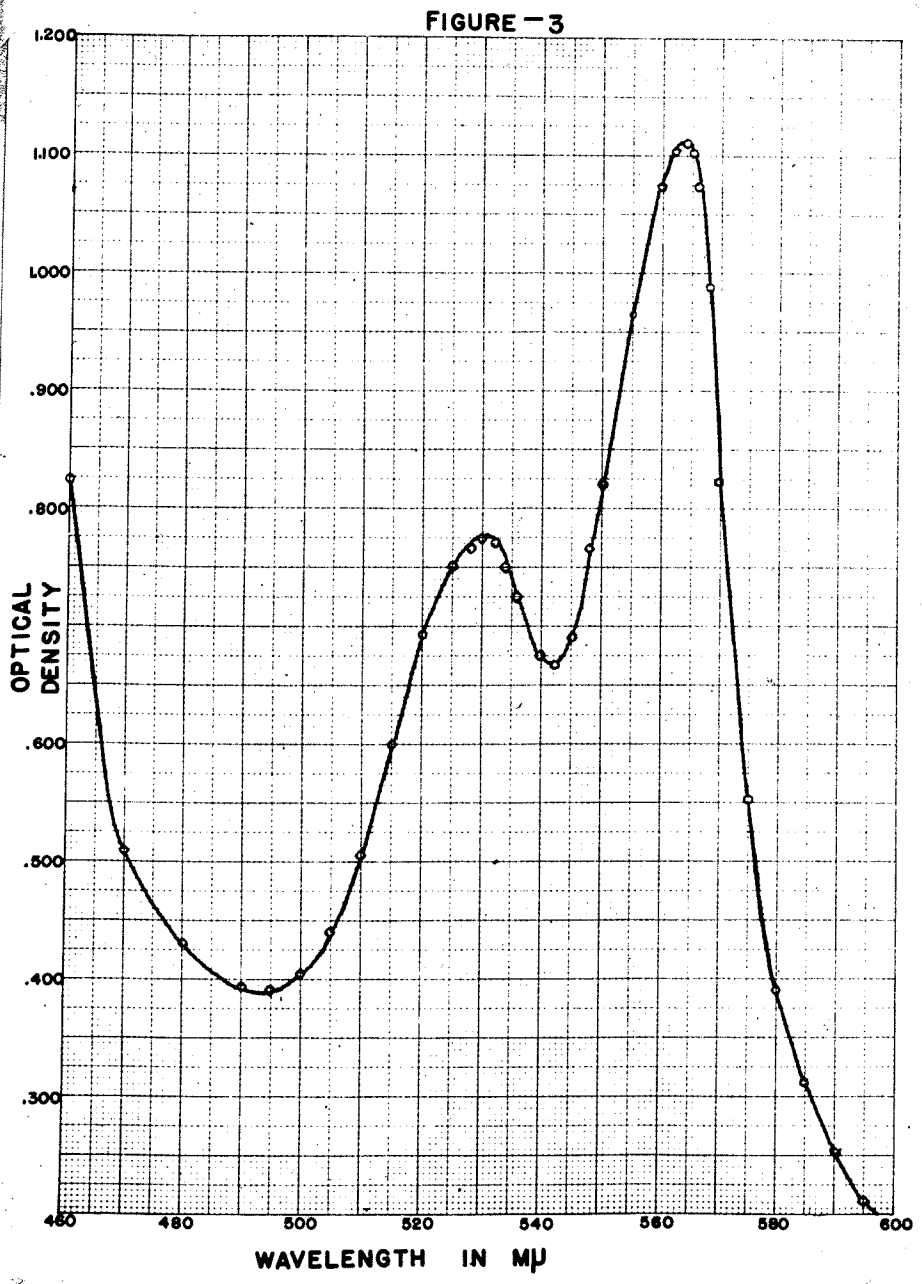

Figure 3

The Absorption Curve of the Nicotinamide Haemochrome Prepared from Haemoglobin

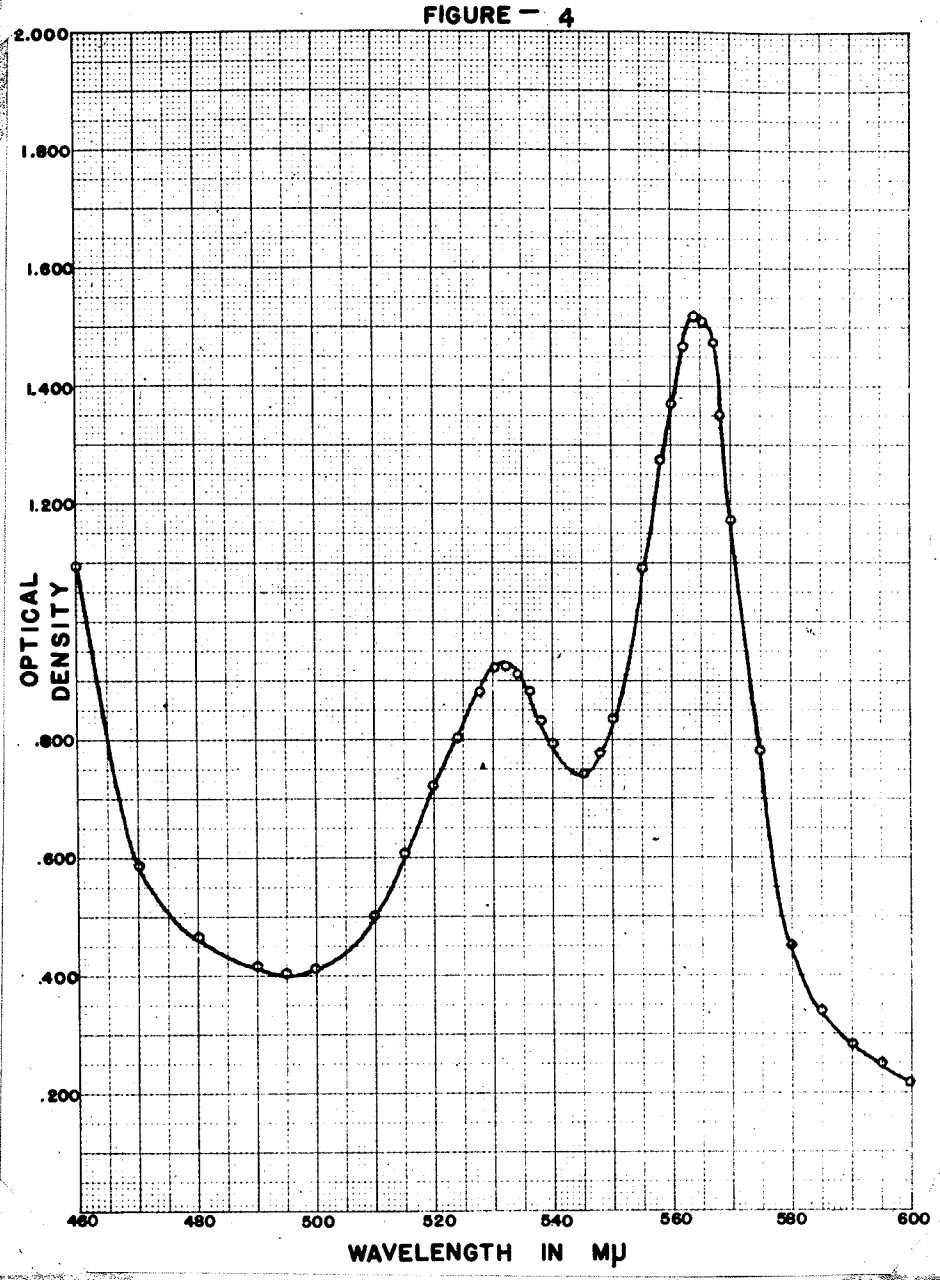

Figure 4

The Absorption Curve of the Nicotinamide Haemochrome Prepared from Haemin

did haemoglobin under these conditions, and that using the easily prepared substance haemin as a standard, the total haem pigment could be estimated by a comparison of the nicotinamide pigment from haemin and haemoglobin.

In order to determine definitely whether the same derivatives were formed from haemin as from haemoglobin it was necessary to show that the absorption curves of the pigments compared on the basis of unit concentration (one mg. of iron being chosen as a unit in this case) were the same. Preliminary experiments had indicated that the absorption curves of the nicotinamide haemichromes compared in this way were not the same, the nicotinamide haemichrome from haemin giving more colour, iron for iron, than the corresponding pigment from haemoglobin. Further experiments, therefore, were devoted solely to the nicotinamide haemochrome.

Before a comparison of the absorption curves of the haemochromes (on the basis of iron concentration) could be made, however, certain factors concerning the preparation of the pigment were considered and will now be mentioned.*

THE RATE OF COLOUR DEVELOPMENT OF THE NICOTINAMIDE HAEMOCHROME

Preliminary experiments had shown that the maximum colour of the

* The blood used in the preparation of this and other pigments was taken (i) from the ear, when small quantities were required and (ii) from a vein in the forearm with a syringe when larger quantities were needed. The crystalline haemin used in these experiments was prepared according to the method outlined by Delory (1943).

nicotinamide haemochrome did not develop immediately after its preparation, a finding which agreed with the observations made by Collier (1944) with the pyridine haemochrome. In order to establish the effect of time on the rate of colour development of nicotinamide haemochrome when prepared in two different ways, the following experiment was performed.

Procedure

Into each of two flasks labelled "A" and "B" containing 20 ml. of N/10 sodium hydroxide was pipetted approximately 0.1 ml. of blood. A knife-point of sodium dithionite was added to "A", the flask rotated gently and the resultant pink colour read at timed intervals in the Beckman spectrophotometer. When the readings had become constant, three drops of a 40 per cent solution of nicotinamide were added to the cuvette and the colour of the resultant solution of nicotinamide haemochrome read at intervals from the time of its preparation.

To "B" was added a knife-point of sodium dithionite, immediately followed by 1 ml. of the nicotinamide solution. Here, as before, the time required for the solution to reach maximum coloration was established.

All of the above optical density readings were made at 540 mμ using an effective slit width of 0.05 mm.

Results

The results are shown in Table II. It is seen that solution "A" required thirty-five minutes for full colour development before the addition of the nicotinamide and another twenty-five minutes after its addition, a total of one hour. Solution "B" required only thirty-five

TABLE II

EFFECT OF TIME ON THE RATE OF COLOUR DEVELOPMENT OF THE NICOTINAMIDE HAEMOCHROME

Time in minutes following preparation	OPTICAL DENSITY		
	A		B
	Blood in NaOH + $Na_2S_2O_4$ without nicotinamide	Blood in NaOH + $Na_2S_2O_4$ nicotinamide added 45 mins. later	Blood in NaOH + $Na_2S_2O_4$ nicotinamide added immediately
3	0.564	1.010	0.779
5	0.576	1.020	0.791
8	0.586	1.025	0.800
10	0.590	1.030	0.806
15	0.594	1.034	0.810
20	0.596	1.038	0.815
25	0.598	1.040	0.818
30	0.599	1.040	0.819
35	0.600	1.040	0.820
40	0.600	--	0.820
45	0.600	--	0.820

minutes in all to attain maximum coloration.

Conclusion

The results showed that there was a definite development of the colour of the nicotinamide haemochrome, which was proportional to the time following the preparation of the pigment, and that the production of maximum coloration by procedure "B" was more rapid (and hence better for the present purposes) than by procedure "A".

ORDER OF ADDITION OF REAGENTS

The previous experiment showed that the nicotinamide haemochrome could be most rapidly prepared by adding the nicotinamide and the reducing agent at the same time. This suggested the following experiment to determine whether the nicotinamide should be added before or after the addition of the reducing agent.

Procedure

The nicotinamide haemochrome from haemoglobin was prepared from a 1 in 200 dilution of blood in N/10 sodium hydroxide. The corresponding haemochrome from haemin was prepared from an alkaline haematin solution containing 30 mg. of haemin made up to 1 litre with N/10 sodium hydroxide. To 20 ml. of each of these solutions, 1 ml. of a 40 per cent solution of nicotinamide was added followed by a knife-point of sodium dithionite.

The haemochromes were then prepared a second time, the order of addition of the two reagents being reversed in this case.

The optical densities of these four solutions were read in the Beckman spectrophotometer (slit width of 0.05 mm) at wave lengths from 500 to 600 mμ. The ratio of the optical densities of the haemochromes (from haemoglobin and haemin) was calculated at each wave length investigated. The ratios were established for both cases -- (i) when the sodium dithionite was added before the nicotinamide and (ii) when it was added after the nicotinamide.

Results

The results of this experiment are shown in Table III. The ratios of the two haemochromes where the reducing agent was added before the nicotinamide show a greater constancy. It is interesting to note that the wave lengths where the greatest deviations occur (560 to 580 mμ) are in the region of the alpha band.

Conclusion

It was concluded that better results would be obtained by adding the reducing agent prior to the nicotinamide; in other words by forming the haemochrome directly rather than by reducing the haemichrome.

COMPARISON OF THE ABSORPTION CURVES OF THE NICOTINAMIDE HAEMOCHROME FROM HAEMIN AND HAEMOGLOBIN

As mentioned earlier, the absorption curves of the nicotinamide haemochrome from haemin and haemoglobin could be more directly compared by plotting the optical density per unit concentration (the specific extinction coefficient) versus the wave length. As before, 1 mg. of

TABLE III

THE EFFECT OF THE ORDER OF ADDITION OF REAGENTS IN THE PREPARATION OF THE NICOTINAMIDE HAEMOCHROME ON THE RATIOS OF THE OPTICAL DENSITY OF THE PIGMENTS FROM HAEMIN AND HAEMOGLOBIN

Wave Length in mμ	$Na_2S_2O_4$ added first			Nicotinamide added first		
	Optical Density		Ratio A/B	Optical Density		Ratio A'/B'
	From Haemin A	From Haemoglobin B		From Haemin A'	From Haemoglobin B'	
500	0.381	0.266	1.43	0.407	0.272	1.50
510	0.488	0.339	1.44	0.503	0.341	1.47
520	0.678	0.470	1.44	0.694	0.470	1.47
530	0.764	0.515	1.48	0.799	0.522	1.53
540	0.650	0.448	1.45	0.708	0.458	1.55
550	0.803	0.559	1.44	0.853	0.551	1.55
560	1.120	0.695	1.61	1.175	0.697	1.68
570	0.720	0.548	1.32	0.861	0.582	1.48
580	0.345	0.257	1.34	0.421	0.277	1.52
590	0.245	0.168	1.46	0.307	0.183	1.68
600	0.198	0.121	1.64	0.250	0.131	1.91

iron was adopted as the unit of concentration.

Experimental

The nicotinamide haemochrome was prepared from haemin and haemoglobin, and the optical density of the two solutions was determined with the Beckman spectrophotmeter at wave lengths from 500 to 600 mμ using a slit width of 0.05 mm.

The iron concentration of the solutions was obtained using the alpha alpha' dipyridyl method (fully described in Part I).

The specific extinction coefficient was determined at each wave length by dividing the optical density of the haemochrome solution at that wave length by the iron concentration. The average values of the specific extinction coefficient of the nicotinamide haemochrome from seven different blood samples were plotted against the wave length to give the absorption curve of the pigment from haemoglobin, while the average value of three separate determinations of the specific extinction coefficients of the nicotinamide haemochrome from haemin were utilized in obtaining the absorption curve of the pigment from haemin.

The two curves, drawn side by side, were then compared.

Results

The two absorption curves are shown in Figure 5. It will be noticed that they are not identical, but are very similar at most wave lengths. The greatest discrepancies are in the region of the alpha band (from 560 to 565 mμ) and to a less extent the beta band (530 mμ).

The peaks in the haemin curve are higher than those in the

FIGURE — 5

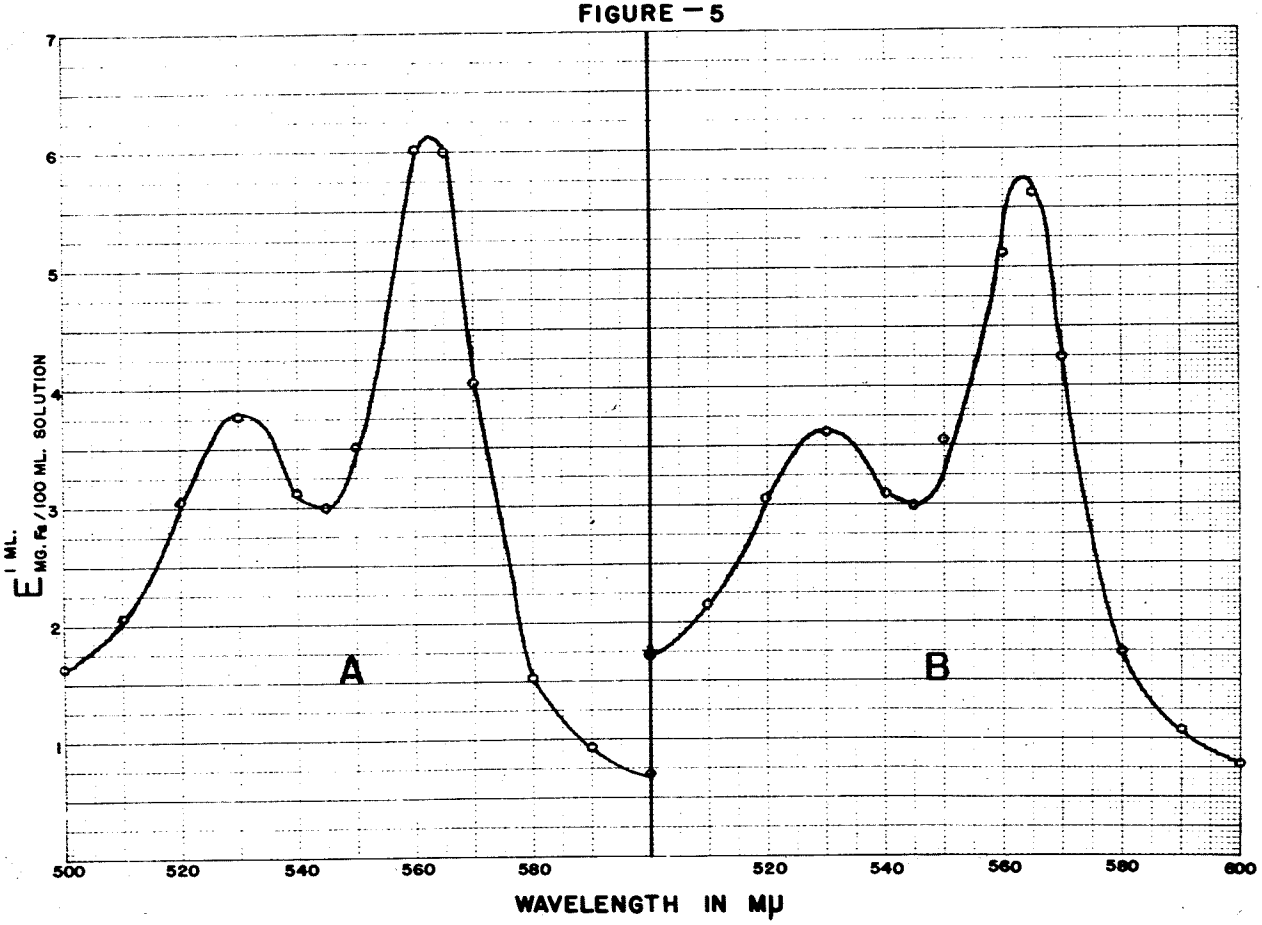

Figure 5

A Comparison of the Absorption Curves of the Nicotinamide Haemochromes from Haemin (A) and Haemoglobin (B) obtained by plotting the Specific Extinction Coefficient versus the Wave Length

haemoglobin curve ~~=~~ while to the right and left of the peaks the haemoglobin curve is slightly higher. The minimal points at 545 mμ are coincident.

Conclusion

Since the readings of the alpha band were so high, the difference between the two curves appears greater than a similar difference would be at a lower reading. This is due in part to the use of the Beckman spectrophotometer, an instrument which allows a precise selection of a narrow spectral band. Hence it was concluded that the use of an instrument with a filter, which transmitted a broad band would yield overall absorptions of the two nicotinamide haemochromes which would agree very closely. In such a case the two nicotinamide haemochromes (from haemin and haemoglobin) could be directly compared at all wave lengths and haemin could be employed as the standard in the estimation of the total haem pigment content of blood.

The following section is devoted to the proposed new method for this estimation.

SUMMARY

1. The possibility of using the nicotinamide haemochrome as a means of estimating total haem pigment (employing haemin as the standard) has been considered since the two substances, haemin and haemoglobin, appeared to form the same derivative with nicotinamide.

2. Certain factors affecting the preparation of the nicotinamide haemochromes have been considered.

3. The absorption curves of the nicotinamide haemochromes from haemin and haemoglobin have been compared directly using their respective iron concentrations as the basis of comparison. The results showed that the two curves are approximately the same.

4. The use of an instrument which transmits a broad spectral band, for reading the colour of the nicotinamide haemochrome solutions, has been recommended, to minimize the error in reading the peaks of the curve.

SECTION C – PROPOSED METHOD FOR THE ESTIMATION OF TOTAL HAEM PIGMENT

PRELIMINARY CONSIDERATIONS

This method, which arose from a study of the absorption curves of the nicotinamide haemochromes, essentially compares the colours of the pigments prepared from haemin and blood (whose total haem pigment is to be measured) in a photoelectric colorimeter which has a broad spectral band. Before an outline of the method is given, certain factors governing the preparation of the haemin standard are considered.

Preparation of the Haemin Standard

The nicotinamide haemochrome from haemin, utilized as the standard in this procedure, was prepared from a solution of crystalline haemin in sodium hydroxide (alkaline haematin). Although Clegg and King (1942) found that a solution of alkaline haematin was stable for nine months, Horecker (1946) observed that a solution of alkaline haematin prepared from N/10 borate buffer pH 9.4 was even more stable, and had the further advantage that it would not react appreciably with glass. King (1947) investigated Horecker's claims and found them to be fully substantiated. It was decided therefore to test this borate buffer in the preparation of alkaline haematin solution.

Use of Haemin, Stabilized by Borate, in the Preparation of the Nicotinamide Haemochrome

Following Horecker, a standard haemin solution was prepared using a borate buffer. Thirty mg. of haemin were made up to 1 litre with

TABLE IV

THE POSITION OF THE ABSORPTION BANDS IN THE HARTRIDGE REVERSION SPECTROSCOPE OF THE NICOTINAMIDE HAEMOCHROMES FROM HAEMOGLOBIN, HAEMIN IN NaOH, AND HAEMIN IN BORATE BUFFER

Nicotinamide Haemochrome Source	Wavelength in Å	
	Alpha Band	Beta Band
Haemoglobin	5637	5298
Haemin in NaOH	5635	5301
Haemin in Borate Buffer	5508	5234

Sorenson's borate buffer pH 9.4 (900 ml. of 0.05 M borax plus 100 ml. of N/10 sodium hydroxide). However, from observations made with the Hartridge reversion spectroscope, it was apparent that when the nicotinamide haemochrome was prepared from the haemin in borate buffer solution, the absorption bands were shifted toward the blue end of the spectrum. The absorption bands of the nicotinamide haemochromes prepared from haemin in N/10 sodium hydroxide, on the other hand, were in almost identical positions with the bands of the nicotinamide haemochromes from haemoglobin (Table IV).

As a result of these observations, it was clear that borate could not be used and therefore it was decided to employ freshly prepared solutions of the haemin whenever a standard was required.

THE PROPOSED METHOD

As a result of the work previously carried out, the following method was developed to estimate the total haem pigment of a sample of blood.

Preparation of the Test Solution

Samples of oxalated whole blood were diluted 1 in 200 with N/10 sodium hydroxide to form denatured globin haemichrome. This was well shaken to ensure adequate mixing. To 20 ml. of this solution a knife-point of sodium dithionite was added, and the flask gently rotated until it had all dissolved. One ml. of a 40 per cent solution of nicotinamide was then added and thirty-five minutes allowed to elapse before the deep rose colour of the resultant nicotinamide haemochrome was read.

Preparation and Analysis of the Standard Solution

Thirty mg. of haemin were accurately weighed and dissolved in N/10 sodium hydroxide to form alkaline haematin. This was shaken well and allowed to stand until all of the haemin had dissolved. The solution was made up to 1 litre with more N/10 sodium hydroxide. To 20 ml. of this solution, a knife-point of sodium dithionite was added, followed by the addition of 1 ml. of a 40 per cent nicotinamide solution. After waiting thirty-five minutes the colour of this solution was determined.

The haemin was analyzed for iron as follows. Thirty mg. of haemin were dissolved in N/10 sodium hydroxide and the solution made up to 250 ml. with more alkali. Five ml. of this alkaline haematin solution were pipetted into a 15-ml. volumetric flask, and its iron content determined by the alpha alpha' dipyridyl method (See Part I).

Reading of the Solutions

The optical densities of the test and standard solutions were read in the Hilger absorptiometer using a green filter.

Calculation

Since the iron content of the haemin was 8.15 per cent, the haemoglobin equivalent of the standard solution was 0.245 mg. per cent (the alkaline haematin solution contained 30 mg. per litre).

Since the blood in the test solution was diluted 1 in 200, and since the iron content of total haem pigment is 0.340 mg. per cent (Bernhart and Skeggs, 1943) the total haem pigment content of the blood (in grams per 100 ml.) is expressed as:

$$\frac{D_t}{D_s} \times \frac{0.245 \times 200}{1000} \times \frac{100}{0.340}$$

or $$\frac{D_t}{D_s} \times 14.4$$

where D_t and D_s are respectively the optical densities of the test and standard solutions.

An example follows:

Optical density of the test solution (prepared in duplicate)

(i) $D_t = 75.5$

(ii) $D_t = 75.9$

Mean $D_t = 75.7$

Optical density of the standard

$D_s = 69.0$

Total haem pigment $= \frac{75.7}{69.0} \times 14.4 = 15.8$ mg. per 100 ml. blood

SUMMARY

1. The use of alkaline haematin, prepared with a borate buffer, has been found to be unsatisfactory for the preparation of the standard nicotinamide haemochrome solution since the absorption bands of the pigment are shifted toward the blue end of the spectrum.

2. A new method for the estimation of total haem pigment, which compares the colours of the nicotinamide haemochromes from haemin and blood, has been outlined.

SECTION D - AN EVALUATION OF THE PROPOSED METHOD

INTRODUCTION

In order to assess the value of the nicotinamide haemochrome method for the estimation of total haem pigment, the method was used to estimate the haem pigment content in a sample of blood of known total haem pigment concentration. The known haem pigment content of blood was determined by employing two different methods -- one, a fundamental method and the other, a colorimetric procedure. They were respectively --

1. The method of iron analysis.
2. The cyan haematin method.

1. USE OF IRON ANALYSIS TO ESTIMATE THE KNOWN TOTAL HAEM PIGMENT OF BLOOD

By analysis, the iron concentration, and hence the total haem pigment content, of blood samples was determined. Employing the newly-proposed nicotinamide haemochrome method, the total haem pigment was again estimated on the same blood samples. The results were compared and correlated.

Procedure

Fresh samples of whole blood were used. The iron analyses were made using the alpha alpha' dipyridyl method, (with 0.1 ml. of blood). Knowing that haemoglobin contains 0.340 per cent iron (Bernhart and Skeggs, 1943), the total haem pigment of the blood was calculated.

The nicotinamide haemochromes were prepared from the same specimens

of blood and from haemin as described above and employed in the determination of the total haem pigment.

Results

The results of a series of nine samples of blood are recorded in Table V. There is good agreement between the results of the total haem pigment obtained from the two independent methods on the same samples. The mean value of the ratio of the two methods is 1.02. The coefficient of variation is 1.96 per cent.

Conclusions

Since the method of iron analysis is an absolute one, it makes a good standard by which to evaluate the nicotinamide method. The wide range of the individual values, and the close agreement observed in each case indicates that the method is applicable to all types of blood.

2. USE OF CYAN-HAEMATIN METHOD TO ESTIMATE THE KNOWN TOTAL HAEM PIGMENT OF BLOOD

The cyan-haematin method of King and Gilchrist (1947) is widely used at the present time in laboratories for the routine determination of total haem pigment, and although it has its drawbacks, it is reported to be one of the most accurate of the rapid and relatively simple colorimetric methods. Hence it was used to estimate the known total haem pigment of blood samples, which were then analyzed by the nicotinamide method.

TABLE V

A COMPARISON OF THE NICOTINAMIDE HAEMOCHROME METHOD AND IRON ANALYSIS FOR THE ESTIMATION OF TOTAL HAEM PIGMENT

Blood Sample	H_b Content (gm./100 ml. blood)		Ratio
	By Iron Analysis (A)	By Nicotinamide Method (B)	B/A
1	11.1	11.3	1.02
2	16.5	16.3	.99
3	13.1	13.7	1.05
4	16.0	15.8	.99
5	12.4	12.5	1.01
6	12.6	12.6	1.00
7	9.7	9.9	1.02
8	12.8	13.0	1.02
9	4.8	5.0	1.04
		Mean . . .	1.02
		Standard Deviation . . .	.02
		Coefficient of Variation . . .	1.96%

Procedure

The cyan-haematins were prepared following the procedure given by King and Gilchrist (1947). Specimens of oxalated whole blood were diluted 1 in 15 with N/10 hydrochloric acid with which it was well mixed. After about ten minutes, the time allowed for the transformation of haemoglobin into acid haematin, 4 volumes of 5 per cent sodium cyanide were added and the solution made up to 20 volumes with water. After mixing thoroughly, the bright red cyan-haematin solution was read in the Hilger absorptiometer, using a green filter, and compared with the standard solution.

The standard solution was prepared by adding an accurately-weighed 30 mg. sample of crystalline haemin (the same haemin sample used in the preparation of the nicotinamide haemochrome standard) to 200 ml. of 5 per cent sodium cyanide in a litre flask. Water was added until the flask was nearly full and the mixture left at room temperature, with occasional shaking until the haemin was all dissolved. Water was then added to the mark.

The total haem pigment of the blood sample was determined by using the calculation outlined for nicotinamide method. From the optical densities, D_t and D_s, of the test and standard solutions respectively, the total haem pigment content is

$$\frac{Dt}{D_s} \times 14.4$$

The newly proposed nicotinamide haemochrome method was now used to estimate the total haem pigment of the same specimens of blood, and the results obtained were compared with those obtained from the

TABLE VI

A COMPARISON OF THE NICOTINAMIDE HAEMOCHROME AND CYAN-HAEMATIN METHODS FOR THE ESTIMATION OF TOTAL HAEM PIGMENT

Blood Sample	H_b Content (gm./100 ml. blood)		Ratio
	By Cyan-Haematin Method A	By Nicotinamide Method B	B/A
1	15.6	15.1	.97
2	14.7	14.8	1.01
3	14.7	14.6	.99
4	15.4	15.6	1.01
5	15.7	15.3	.97
6	16.2	16.0	.99
7	9.7	9.9	1.02
8	15.7	15.4	.98
9	16.3	16.0	.98
10	14.1	14.1	1.00
11	14.7	14.6	.99
12	14.2	14.0	.99
13	13.3	13.2	.99
14	13.2	13.4	1.02
15	8.2	8.9	1.09
16	12.7	13.0	1.02
		Mean	1.00
		Standard Deviation	.0283
		Coefficient Variation	2.83%

cyan-haematin method.

Results

The results of the estimation of the total haem pigment of sixteen samples of blood as determined by the cyan-haematin method and the nicotinamide haemochrome method are shown in Table VI. The individual results of the two methods are in good agreement. The mean of the ratio of the results of the two methods is 1.00, and the coefficient of variation is 2.83 per cent.

Conclusions

Although the cyan-haematin method is not a fundamental one, yet, its relative accuracy makes it a reasonably good yard-stick with which to judge the nicotinamide haemochrome method. Besides yielding reliable results, the nicotinamide method, like the cyan-haematin procedure, is rapid and simple and hence a large number of determinations can be carried out in a relatively short time.

SUMMARY

1. The total haem pigment of blood samples has been determined by the nicotinamide method and compared to the total haem pigment estimated by: (i) iron analysis

(ii) the cyan-haematin method.

2. The results obtained with the proposed nicotinamide haemochrome method are in good agreement with those obtained with the two above methods.

SECTION E - SUMMARY AND CONCLUSIONS

It was apparent from a survey of the existing methods for the estimation of total haem pigment that there was a need for a new method which would be suitable for a routine series of observations. The use of nicotinamide as a reagent in developing such a method arose from a consideration of the nicotinamide haemochromes and haemichromes. Following the usual procedure for a study of this kind, these pigments were investigated in the reversion spectroscope and the spectrophotometer.

The reversion spectroscope showed that the absorption bands of the nicotinamide haemochrome prepared from haemoglobin and from haemin were in the same position. Since the corresponding nicotinamide haemichromes showed no absorption bands in the reversion spectroscope, this instrument was not used for study of these pigments.

Using the spectrophotometer, it was found that the absorption spectra of the nicotinamide haemochromes from haemoglobin and haemin were very similar. This observation was also made in the case of the nicotinamide haemichromes from haemoglobin and haemin. Not only did the maxima and minima occur at the same wave lengths, but the curves in general resembled each other in shape.

By plotting the specific extinction coefficient against the wave length, the absorption curves could be compared directly. It could thus be seen whether one mg. of iron in the haemin solution was exactly the equivalent of one mg. of iron in the haemoglobin solution, at all wave lengths.

Preliminary experiments had shown that the nicotinamide haemichrome from haemin gave more colour, iron for iron than the corresponding haemichrome from haemoglobin. Hence this comparison of the specific extinction coefficients was carried out only with the nicotinamide haemochromes.

Using the Beckman spectrophotometer, an instrument which transmits a narrow spectral band, it was observed that the specific extinction coefficients, and hence the absorption curves, of the nicotinamide haemochromes were not identical at all wave lengths. Around the positions of the peaks the nicotinamide haemochrome from haemin gave more colour than the corresponding compound from haemoglobin. At other wave lengths, however, the agreement was good.

These difficulties were largely overcome, by the use of an instrument which transmitted a broad spectral band. It proved to give better overall agreement between the nicotinamide haemochromes from haemin and haemoglobin, iron for iron.

Following the procedure outlined for the pyridine haemochrome method, a method for the estimation of total haem pigment employing the nicotinamide haemochrome was devised. Using crystalline haemin, of known iron content, as the standard, the nicotinamide haemochrome solutions prepared from it and from haemoglobin were compared colorimetrically in an instrument with a broad spectral band, using an appropriate filter. The use of such an instrument for reading the colour of the nicotinamide haemochromes was thought to be justified since instruments of this type are commonly employed in routine laboratories where this method would be

of greatest value.

Using this method, the total haem pigment of different samples of blood was estimated. The results obtained therefrom were compared to those obtained with reliable and widely-used methods on the same blood samples.

The total haem pigment concentration as estimated by the nicotinamide method on a series of different blood samples agreed very closely with the known value as determined by iron analysis.

Comparing the new method with the cyan-haematin method (one of the less accurate but more rapid colorimetric procedures) the results of the two independent determinations again showed good agreement. The ratios of the two results for each sample of blood were calculated and the average value was found to be unity.

From these results it was concluded that the nicotinamide method is a reliable means of estimating total haem pigment, provided that an instrument with a broad spectral band (the type widely used in routine laboratories) is employed. The method is relatively quick and simple to perform. It requires the use of nicotinamide, an inexpensive, odourless, and non-toxic reagent. It yields results which agree closely with the known values as estimated by a fundamental method, and which also compare favourably with one of the widely-used routine procedures. Moreover, it is equally applicable to anaemic as well as to normal bloods.

This method should be of special value for the routine estimation of total haem pigment in a clinical laboratory, where a large number of determinations are carried out daily.

SUMMARY

1. The absorption curves of the nicotinamide haemochrome when prepared (i) from haemoglobin and (ii) from haemin showed a striking similarity when compared on the basis of unit concentration, showing that a mg. of iron in the haemoglobin solution was almost the equivalent of a mg. of iron in the haemin solution.

2. Using crystalline haemin as a standard it has been shown that the total haem pigment of a blood sample may be determined by the nicotinamide haemochrome method with sufficient accuracy for routine purposes (as evaluated by reliable methods).

3. Other advantages of nicotinamide (its non-toxicity, inexpensiveness, and lack of odour) all seemed to indicate that as a method the nicotinamide haemochrome procedure is better for routine purposes than present-day colorimetric procedures.

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