THE HAEM PIGMENTS OF HUMAN BLOOD

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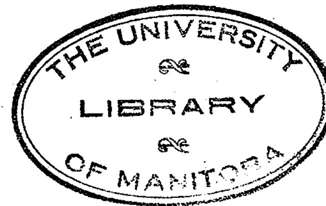

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PREFACE

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

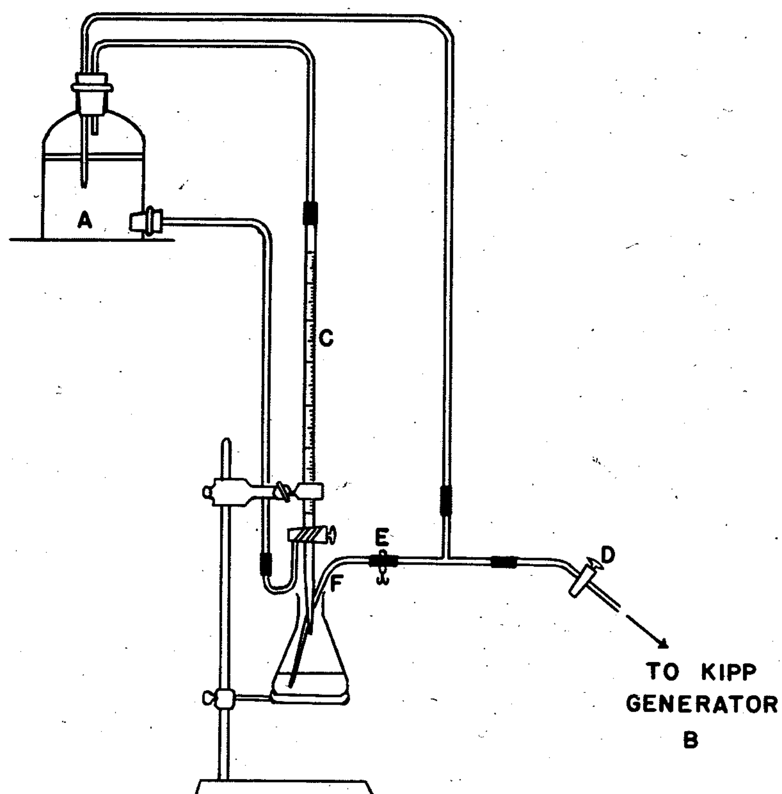

Figure 1

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%