A QUALITATIVE AND QUANTITATIVE STUDY OF THE HEMOGLOBINS IN THE NEWBORN WITH SPECIAL REFERENCE TO NEONATAL JAUNDICE

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SECTION I

GENERAL INTRODUCTION

INTRODUCTION

The etiology of the physiological hyperbilirubinemia of the newborn, though the subject of much experimental work and many theories, is still imperfectly understood. Although bilirubin is a breakdown product of hemoglobin, and although it has been known for many years that the hemoglobin of the foetus and newborn differs in many ways from that of the adult, no attempt has been made to link these two facts in an effort to explain the production of physiological jaundice. It was with this in view, as well as to study these hemoglobins, that the present work was undertaken.

THE GENESIS OF PHYSIOLOGICAL HYPERBILIRUBINEMIA

The rise in the blood bilirubin level following birth may be attributed to (1) an increased production of bile pigment, and (2) an inability of the newborn to excrete bilirubin. Either one of these mechanisms or a combination of both is capable of producing a hyperbilirubinemia.

Increased production. It is well known that bilirubin is a breakdown product of hemoglobin, and that any increase in the rate of hemoglobin destruction could be reflected by a rise in circu-

lating bilirubin. Attention has thus been focused on the fact that the hemoglobin level of the newborn during the first weeks of life is twenty-five to fifty percent higher than that of the adult, and declines slowly towards the adult level.

The hemoglobin level of cord blood measured at the time of delivery has been found to range from fourteen to fifteen grams percent. The high level of hemoglobin (twenty to twenty-three grams) present shortly after birth and through the subsequent weeks may be due to the so called placental transfusion which the baby receives at birth, accentuated by a variable degree of dehydration. The velocity of hemoglobin destruction is most rapid during the first two or three days of life when the hyperbilirubinemia is also usually at its peak. (Weech, 1947). However Waugh *et al* (1940) in a comprehensive study of the problem could find no correlation between the rate of hemoglobin destruction and the degree of hyperbilirubinemia.

Retention of bilirubin. Ylppö (1913) found that the first meconium produced in those babies who developed marked jaundice was of low bilirubin content. The bilirubin content of the meconium of those babies that did not develop jaundice was much higher. This was interpreted to mean that those infants with adequate (mature) liver function could easily cope with the excretion of bile pigment and thus the bilirubin readily found its way from the serum to the meconium. In those infants with immature liver function the bilirubin was retained, did not reach

the meconium, and jaundice supervened following birth.

Attempts have been made to study the ability of the liver to excrete intravenously administered bilirubin. By such a method, Lin and Eastman (1937) found no difference in the excretory function of the liver of jaundiced and non-jaundiced infants. Indeed calculated on a weight basis the rate of excretion was of the same order as that of the adult. Examining this work Weech, Van and Grillo (1941) drew attention to a fallacy in the method of the previous workers in calculating the excretion rates. On taking into account that under all circumstances the rate of excretion of a substance depends on the blood level (load), Weech _et al_ (1941) showed that on this basis infants developing jaundice do have a lesser capacity to excrete bilirubin than do the non-jaundiced infants.

Additional aspects. Although only about half of all normal newborns become jaundiced after birth, hyperbilirubinemia of varying degree is common to all. The fact that the blood bilirubin level required to produce clinical jaundice in the newborn is considerably higher than in the adult has been described by many workers (Weech 1947, With 1947) and may depend on a difference in capillary permeability, or bilirubin binding power of the collagenous and elastic tissues of the skin (With, 1947). Waugh _et al_ (1940) could find no absolute blood bilirubin level at which jaundice developed in all infants.

A direct relationship is known to exist between the bilirubin

level of cord blood (Ylppö, 1913), the iron content of the placenta (Williamson, 1923) and the degree and duration of the neonatal hyperbilirubinemia. A comprehensive review of the subject has been made by Weech (1947).

THE HEMOGLOBINS OF THE NEWBORN

The existence of at least two forms of human hemoglobin has been known for many years. According to Jonxis (1948a) one of these forms known as foetal or refractory hemoglobin occurs only in the blood of the foetus and newborn infant, while the other, termed adult, labile, or later hemoglobin occurs mainly in the adult and in children over four months of age.

The differentiation of these two hemoglobins has been accomplished in many different ways. They differ in their oxygen dissociation curves (Barcroft, 1935), (McCarthy, 1933), (Hall, 1934), the rate of spread of their monomolecular films (Brinkman and Jonxis, 1935), the specificity of their antigenicity (Darrow et al, 1940), and their amino acid composition (Porter and Sanger, 1948). They also differ in their solubility and possess different crystalline structure (Jope and O'Brien, 1948), and differ in their reaction to strong alkali. (Brinkman and Jonxis, 1935), (Trought, 1932). It is now generally accepted on the grounds of evidence derived from these procedures plus the investigations of Haurowitz (1929, 1930), that the differences in these hemoglobins reside in the globin portion of the molecule----the ferroporphyrin

group being identical.

One of the simplest methods of differentiating these hemoglobins is by observing their rates of transformation to alkaline globin hematin when treated with sodium hydroxide. This denaturation occurs rapidly with adult or labile hemoglobin, while foetal or refractory hemoglobin is more resistant to alkali -- its denaturation occurring more slowly. By the use of this fundamental difference it is possible to estimate the amounts of these two substances in minute quantities of blood.

THE PROBLEM

This study arose in an attempt to clarify certain inconsistencies and conflicting findings which have appeared in the literature in the past two years.

Jonxis reported in 1948 that he had found that at birth the blood of the newborn contained only foetal hemoglobin which is replaced at the rate of about 1% a day by the adult form, replacement being complete at approximately 120 days after birth. He claimed that in erythroblastosis the foetal hemoglobin is selectively attacked and broken down -- almost to the exclusion of the adult form and that only in erythroblastotics is adult hemoglobin present at birth.

Later in the same year, Baar (1948) reported that in replacement transfusion studies he had found that the destruction of erythrocytes in hemolytic disease of the newborn was dependent only

upon their sensitization by Rh antibodies and not upon the type of hemoglobin present in the cell.

Though Jonxis postulated that foetal hemoglobin was rapidly destroyed in erythroblastosis, no work had been done towards determining its relationship, if any, to the physiological jaundice of the normal newborn.

SCOPE OF THE THESIS

The problem resolved itself into an attempt to answer the following questions --

1. Is adult hemoglobin present at birth in the blood of the normal full-term newborn?
2. If adult hemoglobin is present at birth in the normal newborn, then is the amount present related to the degree of neonatal bilirubinemia?
3. Is physiological hyperbilirubinemia related to the rate of replacement of foetal hemoglobin by adult hemoglobin?

In the course of this work other problems arose, the investigation of which, while having a direct bearing upon the subject matter considerably widened the scope of the study. It was deemed appropriate to divide this thesis into sections, the results obtained being summarized at the end of each section, rather than attempting to summarize the total results at the end and divorced from the content.

It was thereupon decided to follow quantitatively the

changes in (1) bilirubin, (2) total hemoglobin, and (3) adult and foetal hemoglobin in the blood of the normal newborn, from birth to four weeks of age. The methods of estimation of these substances will be discussed in detail with special reference to the quantitative estimation and properties of foetal hemoglobin.

SECTION II

THE ESTIMATION OF PLASMA BILIRUBIN

INTRODUCTION

As previously mentioned it was necessary as part of this project to be able to measure the bilirubin content of the plasma of newborn infants. Consequently, a study of existing methods was made in order to develop a method which only required 0.5 ml. samples of blood which can be obtained by heel puncture.

HISTORICAL

Ehrlich found that when sulfanilic acid and sodium nitrite were added to a solution of bilirubin a coloured addition product was formed. Van den Bergh and Müller (1916) precipitated the blood protein with alcohol and applied Ehrlich's reaction to this alcoholic extract.

This procedure has undergone many modifications, mostly relating to the use of various coloured substances as artificial standards. Van den Bergh used an ethereal solution of iron thiocyanate, McNee and Keefer (1925) and White (1932) cobalt sulphate, and Haslewood and King (1937) methyl red.

Van den Bergh showed that whereas bile reacted directly with the diazo reagent (direct reacting pigment), plasma bilirubin normally required alcohol for the full production of colour (indirect

reacting pigment). Under certain circumstances the plasma may contain an appreciable amount of both pigments. Many attempts have been made to estimate these fractions but recent work (Gray, 1947) suggests that this differentiation may be an artificial one. In this work we are concerned only with total bilirubin, that is bilirubin reacting in the presence of alcohol.

The Method of Malloy and Evelyn (1937). All previous methods required the precipitation of the serum or plasma proteins. The method of Malloy and Evelyn avoided this precipitation by the dilution of the serum or plasma to ten times its volume with distilled water, since it was claimed that in those methods where the protein is precipitated some of the bilirubin is carried down with the protein. This method utilized the Evelyn photoelectric colorimeter, the solutions being read at a wave length of 540 mμ. A hydrochloric acid blank (in place of diazo solution) is used to obviate any error introduced by the presence of hemolysis or any yellow serum pigments that may have an appreciable absorption at 540 mμ.

The diazo reagent used is that common to many workers and is prepared as follows --

1. Solution A, 1.0 gm. of sulfanilic acid in 15 ml. of concentrated hydrochloric acid and diluted to 1 litre with water.
2. Solution B, 0.5 percent sodium nitrite.

The diazo reagent is prepared by adding 0.3 ml. of Solution B

to 10 ml. of Solution A.

This method requires 1 ml. of serum or plasma.

The Waugh, Merchant, and Maughan Modification of the Method of Malloy and Evelyn (1940). This method differs from the Malloy and Evelyn procedure in two ways.

1. The use of a 1 to 20 dilution of serum or plasma.

2. The use of 60% dilutions of Ehrlich's solutions A and B.

Though this method was devised for use in the estimation of bilirubin in the newborn it requires 1 ml. of serum -- obtained from blood procured by fontanel puncture which limits its value.

METHOD

The method used in this survey is a micro method for the estimation of total bilirubin in plasma, based on the procedure of Waugh et al (1940). It requires only 0.1 ml. of serum (0.5 ml. of blood) which is readily obtained by heel puncture of the newborn infant.

Reagents.

1. Diazo 1 -- 0.6 gm. of sulfanilic acid dissolved in 9 c.c. of concentrated hydrochloric acid and diluted to 1 litre with water.
2. Diazo 2 -- 0.3 percent sodium nitrite.
3. The diazo test reagent is freshly prepared by adding 0.3 ml. of Diazo 2 to 10 ml. of Diazo 1.
4. Diazo blank -- 9 ml. of concentrated hydrochloric acid in

1 litre of water.

5. Absolute methyl alcohol.

Procedure. 0.1 ml. of serum or plasma is diluted to 2 ml. with distilled water. To 1 ml. of this 1 in 20 dilution of serum 0.2 ml. of diazo test solution is added. 0.2 ml. of diazo blank is added to the other 1 ml. of diluted serum -- (this is the blank). 1.2 ml. of absolute methyl alcohol is added to each. The solutions are gently shaken to ensure thorough mixing, corked to prevent evaporation and read at the end of thirty minutes. The readings are taken in the Beckman Spectrophotometer (Model DU) at a wave length of 540 mμ and a slit width of 0.05 mm., the blank being used to set the instrument. The concentration of bilirubin is determined from a calibration curve prepared as described in the following section.

CALIBRATION OF METHOD

The bilirubin used in the preparation of the calibration curve was prepared by the Eastman-Kodak company and though designated as pure bilirubin it was found to be not completely soluble in chloroform. For this reason the following procedure was adopted.

Approximately 10 mg. of bilirubin was placed in a small conical flask together with 10 ml. of chloroform. The flask was then placed on a boiling water bath and the solution heated to boiling. The solution was then filtered hot through a small filter paper into a small weighed evaporating dish. Another 10 ml. of chloro-

form were boiled up in the conical flask and poured through the same filter into the evaporating dish. The evaporating dish was then placed on a water bath and the chloroform carefully evaporated. Following the evaporation of chloroform the evaporating dish was placed in a desiccator and weighed after cooling. The bilirubin (now of known weight and completely soluble in chloroform) was dissolved in chloroform and made up to 100 ml. in a volumetric flask.

Measured samples were withdrawn from this solution and made up in varying dilutions with methyl alcohol. The dilutions ranged from 0.5 mg. to 16 mg. percent and were treated with diazo reagent as outlined above. A blank was prepared and used to set the instrument for each point on the curve. The calibration curve is shown in Figure 1.

COMPARISON OF DETERMINATIONS MADE ON THE SAME PLASMA BY MICRO AND MACRO TECHNIQUES

Determinations of total plasma bilirubin by the micro method were compared with similar determinations done on the same sera using the standard Malloy - Evelyn macro method on the Evelyn colorimeter. Good agreement between the two methods was found, and the results are shown in Table 1.

DISCUSSION

This micro method was found to be quite satisfactory in the

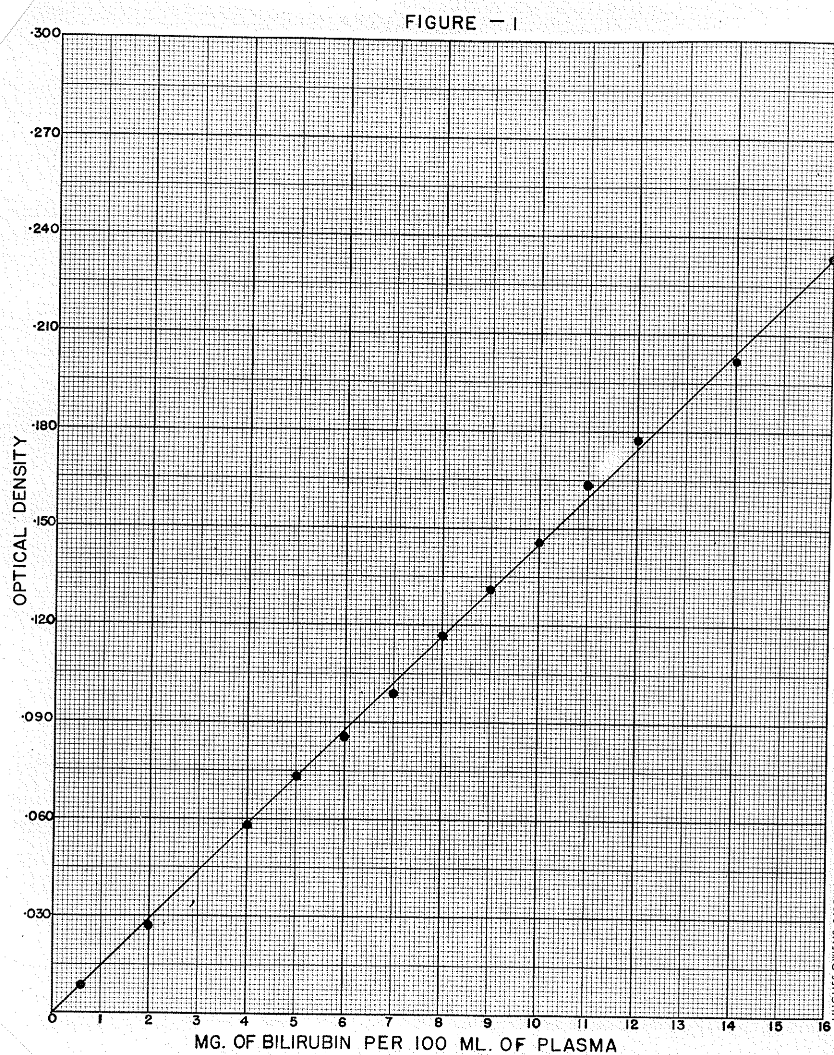

FIGURE 1

Calibration curve for total bilirubin content of plasma.

TABLE I

COMPARISON OF TOTAL BILRUBIN DETERMINATIONS ON THE SAME PLASMA

BY MICRO METHOD AND BY METHOD OF MALLOY AND EVELYN

PLASMA NO.	TOTAL BILIRUBIN (mg. per 100 ml. of plasma)	
	Malloy and Evelyn	Micro Method
1	1.6	1.7
2	1.7	1.8
3	2.6	2.4
4	3.7	3.6
5	4.6	4.9
6	9.5	10.0

series studied. There was no difficulty in obtaining 0.1 ml. of sera from 0.5 ml. of blood after centrifuging.

The blank in this method compensated for the presence of any yellow serum pigments or hemolysis. In those sera showing gross hemolysis a dense precipitate was formed on the addition of the alcohol. These sera were discarded and the results not used in this survey. With the exception of those sera showing gross hemolysis the test and blank solutions were of great clarity and the amount of colour developed was adequate despite the considerable dilution. This adequacy of colour was further enhanced by the use of a sensitive spectrophotometer.

The calibration curve was prepared from a solution of purified bilirubin in chloroform. Some workers (Hunter, 1930) advocate the use of various added buffers in an attempt to simulate more closely the conditions present in the serum. However, in view of Malloy and Evelyn's good recovery experiments on serum using a calibration curve prepared without buffering it was decided to follow their general method in the preparation of the calibration curve.

In this survey no attempt was made to differentiate the so-called direct reacting from the indirect reacting bilirubin. All results are expressed as milligrams of total bilirubin per 100 ml. of plasma.

SUMMARY

1. The literature is briefly reviewed and a method for the estimation of total plasma bilirubin adapted to the needs of the project.

2. The calibration of the method adopted is described.

3. Good agreement was obtained between the developed micro method and a standard macro method.

SECTION III

THE ESTIMATION OF HEMOGLOBIN

INTRODUCTION AND REVIEW OF METHODS

The total hemoglobin content of blood may be determined by various methods which may be classified as follows --

1. Gasometric methods.
2. The determination of blood iron.
3. The conversion of hemoglobin to one of the heme pigments which may be estimated colorimetrically.

The preceding methods were reviewed in an attempt to adopt a method which would be suitable to the needs of the project.

Gasometric Methods. The determination of the oxygen or carbon monoxide capacity of blood offers a precise and physiologically significant method of estimating hemoglobin. The method of gasometric analysis is too time consuming for a routine procedure in a large series of observations. However, many colorimetric methods have been standardized against the oxygen capacity as determined by the vacuum extraction method (Van Slyke and Neill, 1924).

Determination of Blood Iron. The blood iron is confined almost exclusively (except for 0.1 mgm. percent of plasma iron) to hemoglobin and its derivatives. This plasma iron may be eliminated by the use of washed cells. Thus the estimation of blood iron is an accurate, though time consuming method of determining the hemoglobin concentration. It too is very useful for the standardiz-

ation of the more rapid colorimetric methods.

The Colorimetric Estimation of Hemoglobin as a Heme Pigment.

1. Acid Hematin Methods. Blood is treated with dilute hydrochloric acid and the resulting brown pigment (acid hematin) is read against a prepared acid hematin solution or against a coloured glass or gelatin standard. The full development of colour requires at least one hour at room temperature. Acid hematin is insoluble and exists as a colloidal suspension, turbidity and precipitation occurring frequently.

2. Alkaline Hematin Methods. This method was first introduced by Wu (1922) who found that many of the undesirable qualities of the acid hematin method could be overcome by the use of alkali. Clegg and King (1942) placed the method on a more satisfactory basis by the introduction of standards prepared from crystalline hemin of known iron content.

This method possesses the advantage of using a simple standard which may be readily prepared from crystalline hemin. On the other hand the absorption curves for alkaline globin hematin prepared from hemoglobin and alkaline hematin prepared from hemin differ in the visual spectral range. Because of this quantitative discrepancy a correction factor of 1.34 (Clegg and King, 1942) must be applied in the quantitative estimation of hemoglobin.

However later work (Collier, 1945) has shown that this factor is not of general application.

A further disadvantage is met with when hemoglobin determinations are being carried out on blood containing refractory hemoglobin. Refractory hemoglobin is slowly converted to alkaline globin hematin at room temperature though this conversion may be accelerated by heating. As described in Section V, the alkaline globin hematin prepared by heating has a lower optical density, a different absorption curve and a different globin hemochromogen than that prepared at room temperature.

3. Cyanmethemoglobin Methods. This method was advanced by Drabkin and Austin (1935), Collier (1944), and King and Gilchrist (1947) to overcome the disadvantages of the previous method. It depends upon the conversion of total heme pigment to cyanmethemoglobin when blood is treated with hydrochloric acid and sodium cyanide. The use of cyanide is a disadvantage in a method for general routine use.

4. Pyridine Hemochromogen Method. Originally described by Roets (1940) and further developed by Rimington (1942) it depends upon the comparison of the pyridine hemochromogen of the unknown blood against that of a hemin solution of known iron content in a photoelectric colorimeter. The odour of pyridine limits its extensive use in a routine

laboratory.

5. <u>Oxyhemoglobin Methods</u>. Though one of the first methods used in hemoglobinometry it still represents a rapid, simple, and accurate method for the estimation of hemoglobin. Gowers (1878) compared the oxyhemoglobin solution with a picrocarmine standard. More recently the estimation has been adapted to photoelectric methods.

As this procedure estimates oxyhemoglobin, it is only applicable to normal subjects whose hemoglobin is readily converted to oxyhemoglobin by thorough oxygenation. In those persons in whom carboxyhemoglobin or methemoglobin is present the values recorded by this method will be less accurate than in those methods which estimate total heme pigment.

King <u>et al</u> (1948) in a critical evaluation of colorimetric methods for hemoglobin estimations, carried out on the various hemoglobin derivatives, found that the most accurate results were given by oxyhemoglobin and cyanmethemoglobin methods.

METHOD

The method employed in this study depends upon the colorimetric estimation of oxyhemoglobin utilizing a spectrophotometer calibrated by iron determinations on blood. The instrument was calibrated by iron determination rather than gasometrically because of

the fewer technical difficulties of the iron method. The iron determinations were done according to the method of Delory (1942).

This estimation of blood iron and the standardization of the spectrophotometer were carried out as follows:

To approximately 20 ml. of citrated adult blood, a few drops of diethyl ether were added to bring about hemolysis and so reduce the error attendant on the pipetting of blood cells. 5 ml. of laked blood were pipetted into each of two 150 ml. beakers. 1 ml. of twenty percent nitric acid was added and the beakers placed on a boiling water bath for three hours. Following this initial evaporation, the process was completed by heating on a hot plate for three hours. The samples were then placed in a muffle furnace at 400°C for twenty-four hours.

The resultant red ash was dissolved in 10 ml. of concentrated hydrochloric acid and diluted with an equal volume of water. This was titrated with titanous chloride against a standard iron solution (one milligram per millilitre) prepared by dissolving 4.318 grams of ferric ammonium sulphate in 50 ml. of iron free hydrochloric acid and making up to 500 ml. with distilled water.

The titration with titanous chloride was as follows:- 2 ml. of titanous chloride were diluted to 100 ml. with N/10 sodium hydroxide; a 50 ml. burette filled immediately before the titration with this solution, and 1 ml. of 20% potassium thiocyanate added to the iron samples and to two 5 ml. portions of standard iron solution. The titanous chloride was then titrated into the four

solutions until the red colour disappeared, the order of titration being standard, test, test, standard. This order of titration was followed to ensure that no change occured in the titanous chloride during the titration.

The results of a typical standardization are as follows --

The standard solution of iron (5 mg.) required:

(1)	5.58 ml. of $TiCl_3$
(2)	5.60 ml. of $TiCl_3$
Mean	5.59 ml. of $TiCl_3$

5 ml. of blood required:

(1)	2.17 ml. of $TiCl_3$
(2)	2.19 ml. of $TiCl_3$
Mean	2.18 ml. of $TiCl_3$

That is, 2.18 ml. of $TiCl_3$ were equivalent to (2.18/5.59) x 5 = 1.96 mg. of iron.

Thus 5 ml. of blood contained -- 1.96 mg. of iron

and 100 ml. of blood contained -- 39.2 mg. of iron

The iron content of hemoglobin is 0.340 percent (Bernhart and Skeggs, 1943).

That is, 100 grams of hemoglobin contains 340 mg. of iron.

Therefore, the hemoglobin content of the blood was (39.2/340) x 100 = 11.5 grams per 100 ml.

(The low hemoglobin content of this blood was accounted for by the considerable dilution with anticoagulant used in the

collection of blood for transfusion purposes.)

A 0.4% solution of the same blood was prepared in 0.1% sodium carbonate, oxygenated in a Waring Blendor and read in the Beckman Spectrophotometer at a wave length of 578 mμ (the alpha band of oxyhemoglobin) and an aperture of 0.05 mm. The optical density was found to be 0.460.

HEMOGLOBIN ESTIMATION (EXAMPLE)

0.1 ml. of blood is pipetted into 25 ml. of 0.1% sodium carbonate and oxygenated in the Waring Blendor. It is read at a wave length of 578 mμ and a slit width of 0.05 mm. The optical density is found to be 0.600.

Hemoglobin = (0.600/0.460) x 11.5 = 15.0
(G. per 100 ml. of blood)

HEMOGLOBIN SURVEY

This method, and this calibration factor were used in a survey of 394 young adults conducted by the Provincial Laboratory of The Department of Health of the Province of Manitoba. The findings in 273 female and 121 male students of the Faculty of Education of the University of Manitoba and the Winnipeg Normal School are listed in Table II.

SUMMARY

1. Methods of hemoglobin estimation are reviewed and the

TABLE II

HEMOGLOBIN CONTENT OF THE BLOOD OF 394 STUDENTS OF THE FACULTY OF EDUCATION AND NORMAL SCHOOL, WINNIPEG, MANITOBA

HEMOGLOBIN	NUMBER OF STUDENTS	
G. per 100 ml.	Male	Female
9.5 - 11.5	0	7
12.0 - 12.5	0	5
12.5 - 13.0	1	17
13.0 - 13.5	2	32
13.5 - 14.0	3	43
14.0 - 14.5	9	54
14.5 - 15.0	12	56
15.0 - 15.5	40	32
15.5 - 16.0	17	14
16.0 - 16.5	22	11
16.5 - 17.0	5	2
17.0 - 17.5	6	0
17.5 - 18.0	3	0
18.0 - 18.5	1	0

method adopted in this survey is outlined.

2. The results are given of a hemoglobin survey conducted on 394 young adults.

SECTION IV

THE ESTIMATION OF REFRACTORY AND LABILE HEMOGLOBIN

INTRODUCTION

The rate of denaturation as a method of studying hemoglobin was first employed by Korber in 1866, who found that in animals the rate of denaturation varied from species to species, but was constant for a given species. Von Kruger (1888) utilizing the spectroscope, observed the disappearance of the oxyhemoglobin bands on the addition of sodium hydroxide, and found that denaturation took place more rapidly in adult blood than in blood from the umbilical cord. These findings were confirmed by Haurowitz (1929, 1930), Brinkman and Jonxis (1935, 1936), and Bischoff and Schulte (1926).

Trought (1932) carried out a qualitative spectroscopic estimation of the denaturation rates of the hemoglobin of adult and cord blood. N/4 sodium hydroxide was added to the solution of oxyhemoglobin to be examined, and the time required for its conversion into alkaline globin hematin determined by observing the disappearance of the oxyhemoglobin bands. This denaturation was found to take 45 to 60 seconds in adult blood and 45 to 60 minutes in cord blood.

The quantitative estimation of the two fractions in a blood where both are present or their presence is suspected has been

accomplished in different ways, but most of the methods depend upon the difference in resistance to alkali exhibited by the two forms. The pioneers in this work were Haurowitz, Brinkman and Jonxis, and Baar and Lloyd. Recently Ponder and Levine have described a method based on the same principle.

REVIEW OF PREVIOUS METHODS

The Method of Brinkman and Jonxis (1935). This method is a modification of that of Brinkman, Wildschut and Wittermans (1934). The decrease in optical density attendant on the transformation of hemoglobin to alkaline globin hematin was observed and recorded. Due to the marked difference in the rate of this reaction in labile and refractory hemoglobin, the two forms may be estimated quantitatively.

To 1 ml. of a 5 percent oxyhemoglobin solution they added 0.33 ml. of an alkaline buffer (composition not stated) and the fall in optical density was recorded at one minute intervals over a period of twenty minutes. The reaction took place at pH 11.8 and a constant temperature of approximately 18°C. A calibration curve giving the amount of unchanged hemoglobin for any given optical density was prepared by using a mixture of buffered alkaline globin hematin and oxyhemoglobin in varying proportions.

By plotting the logarithm of the percentage of hemoglobin remaining in solution against the time in minutes two straight

lines were obtained. The first line represented the rapid denaturation of labile hemoglobin, the second line of lesser slope represented the slower denaturation of refractory hemoglobin. By extrapolating the second line to zero time the amount of each form of hemoglobin was obtained.

The Method of Baar and Lloyd (1943). Baar and Lloyd used the Evelyn colorimeter micro-apparatus and a green 540M filter to determine the denaturation rates of the two hemoglobins.

To 1 ml. of an oxygenated hemoglobin solution of known concentration they added 0.2 ml. of N/4 sodium hydroxide, and the denaturation was followed by observing the optical density at one minute intervals for thirty minutes. The amount of hemoglobin at zero time was determined by means of a blank, in which 0.2 ml. of water replaced the sodium hydroxide. No attempt was made to control the temperature at which the reaction took place. The optical density equivalent to complete denaturation was assumed to be proportional to the optical density of a 0.1 percent alkaline globin hematin solution.

The method employed for the calculation of the amount of refractory hemoglobin present was the same as that for the method of Brinkman and Jonxis.

The Method of Ponder and Levine (1949). This most recent method depends upon the precipitation of the alkaline globin hematin at intervals during the denaturation process and obviates the necessity of a sensitive colorimeter.

These authors prepared a ten percent solution of hemoglobin in water after laking the cells by freezing and thawing. 10 ml. of sodium hydroxide-glycine buffer (pH 12.15) were added to 0.2 ml. portions of the hemoglobin solution. The reaction was interrupted at 10, 20, 40, 60, and 80, minutes by the addition of a mixture of saturated ammonium sulphate and hydrochloric acid. This stopped the denaturation and precipitated out the alkaline globin hematin already formed. The precipitate was removed by filtration and the amount of hemoglobin remaining in solution was estimated colorimetrically. By plotting the logarithm of the hemoglobin concentration against time and extrapolating to zero time, the percentage of refractory hemoglobin was determined.

THE DEVELOPMENT OF A METHOD FOR THE ESTIMATION OF REFRACTORY AND LABILE HEMOGLOBIN

A study was made of the factors influencing the form of the denaturation curve and the accuracy of the estimation in methods based upon the serial determinations of the optical density of a hemoglobin solution undergoing denaturation. The following factors were investigated in the course of the development of an accurate method for the quantitative determination of the hemoglobin fractions.

1. The accuracy and dependability of the 100 percent and 0 percent oxyhemoglobin levels.
2. The temperature at which the reaction takes place.

3. The concentration of alkali.

These factors will now be discussed in some detail.

The Establishment of the 100 percent and 0 percent Oxyhemoglobin Level. The accuracy of the method clearly depends upon the exact determination of the optical density of the oxyhemoglobin solution before denaturation (100 percent oxyhemoglobin), and the resulting alkaline globin hematin solution (zero percent oxyhemoglobin). The first of these is an easy matter and is readily obtained by complete oxygenation of the hemoglobin solution and the preparation of a blank using water instead of sodium hydroxide. The optical density of the blank represents by definition an oxyhemoglobin level of 100 percent at that concentration.

The zero percent oxyhemoglobin base line depends on the complete conversion of oxyhemoglobin to alkaline globin hematin. This conversion takes place according to Trought (1932), within one minute in adult blood and within one hour in cord blood. The reaction may be accelerated by heating.

The question arises as to the most accurate method to determine this zero percent base line. It is essential to know whether or not this base line is the same for heated and non heated samples, how long it takes for complete conversion of oxyhemoglobin to alkaline globin hematin, and if the latter is a stable compound.

The following experiment was performed --

Washed cells from adult and cord blood were diluted 1 in 20 in 0.1 percent sodium carbonate and in water. These solutions were

completely oxygenated in a Waring Blendor and filtered to remove cell debris. All solutions were of approximately the same initial hemoglobin concentration. The following solutions were prepared --

1. 20 ml. of hemoglobin solution of adult blood in water.
2. 20 ml. of hemoglobin solution of adult blood in 0.1% Na_2CO_3.
3. 20 ml. of hemoglobin solution of cord blood in water.
4. 20 ml. of hemoglobin solution of cord blood in 0.1% Na_2CO_3.
5. 20 ml. of hemoglobin solution of adult blood in water plus 4 ml. N NaOH.
6. 20 ml. of hemoglobin solution of adult blood in water plus 4 ml. N/4 NaOH.
7. 20 ml. of hemoglobin solution of adult blood in 0.1% Na_2CO_3 plus 4 ml. of N NaOH.
8. 20 ml. of hemoglobin solution of adult blood in 0.1% Na_2CO_3 plus 4 ml. of N/4 NaOH.
9. 20 ml. of hemoglobin solution of cord blood in water plus 4 ml. of N NaOH.
10. 20 ml. of hemoglobin solution of cord blood in water plus 4 ml. of N/4 NaOH.
11. 20 ml. of hemoglobin solution of cord blood in 0.1% Na_2CO_3 plus 4 ml. of N NaOH.
12. 20 ml. of hemoglobin solution of cord blood in 0.1% Na_2CO_3 plus 4 ml. of N/4 NaOH.

The optical densities of these solutions were read at hourly intervals for five hours and then every twenty-four hours for ninety-six hours following the addition of alkali. Identical alkaline globin hematin solutions were prepared (Nos. 5 to 12 above) and heated on a boiling water bath for five minutes, their optical density read immediately and then every twenty-four hours for ninety-six hours. During the course of the experiment the solutions were kept at room temperature (23°C. to 26°C.). All readings were made on the Beckman Spectrophotometer at a wave length of 578 mμ and a slit width of 0.05 mm.

The results obtained are shown in Figures 2, 3, and 4. On inspection of these results the following is noted --

1. There is a rapid fall in the optical densities of the hemoglobin solutions of both adult and cord blood at room temperature. This fall is much more marked in the water solution than in the sodium carbonate solution. (Figure 2).

2. In those solutions to which alkali had been added (Figures 3 and 4) --

   (i) The optical densities of the heated solutions are consistantly lower than the optical densities of the non-heated solutions.

   (ii) The heated solutions are only slightly more stable than the non-heated solutions.

   (iii) The solutions of adult blood are slightly more

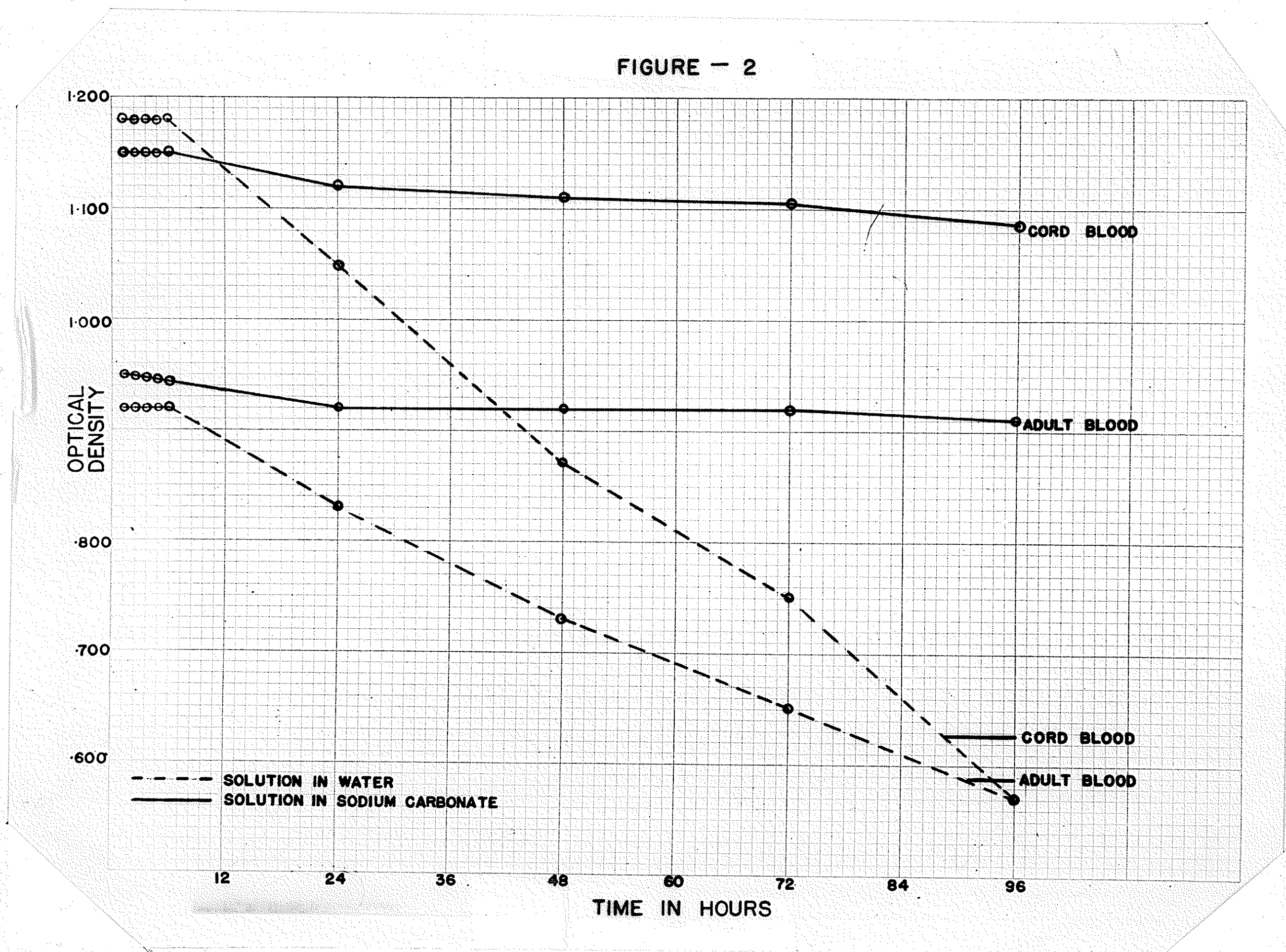

FIGURE 2

The changes in optical density at wave length 578 mμ of oxy-hemoglobin solutions from adult and cord blood in water and in sodium carbonate kept at room temperature for 96 hours.

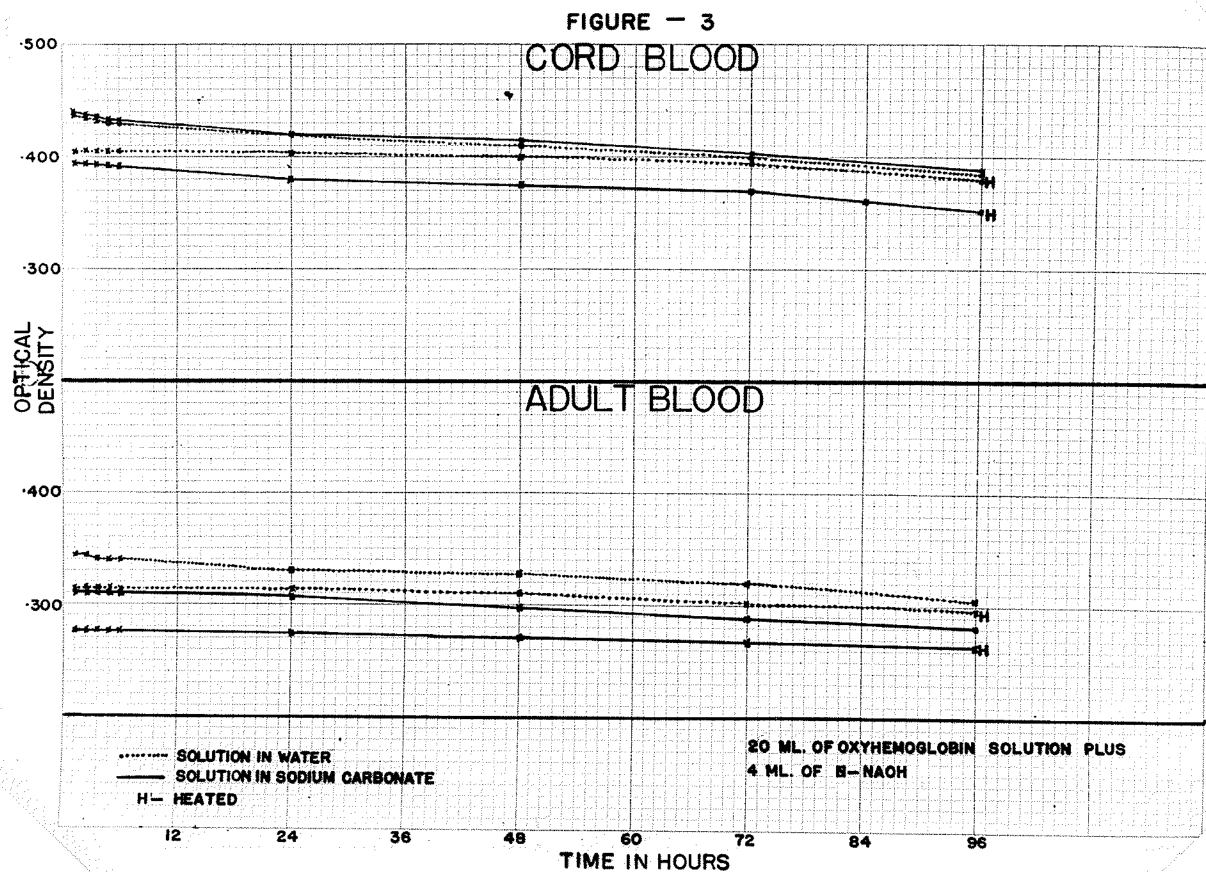

FIGURE 3

Changes in optical density at wave length 578 mμ of solutions of alkaline globin hematin from adult and cord blood in N NaOH at room temperature. H - denotes the heated solutions.

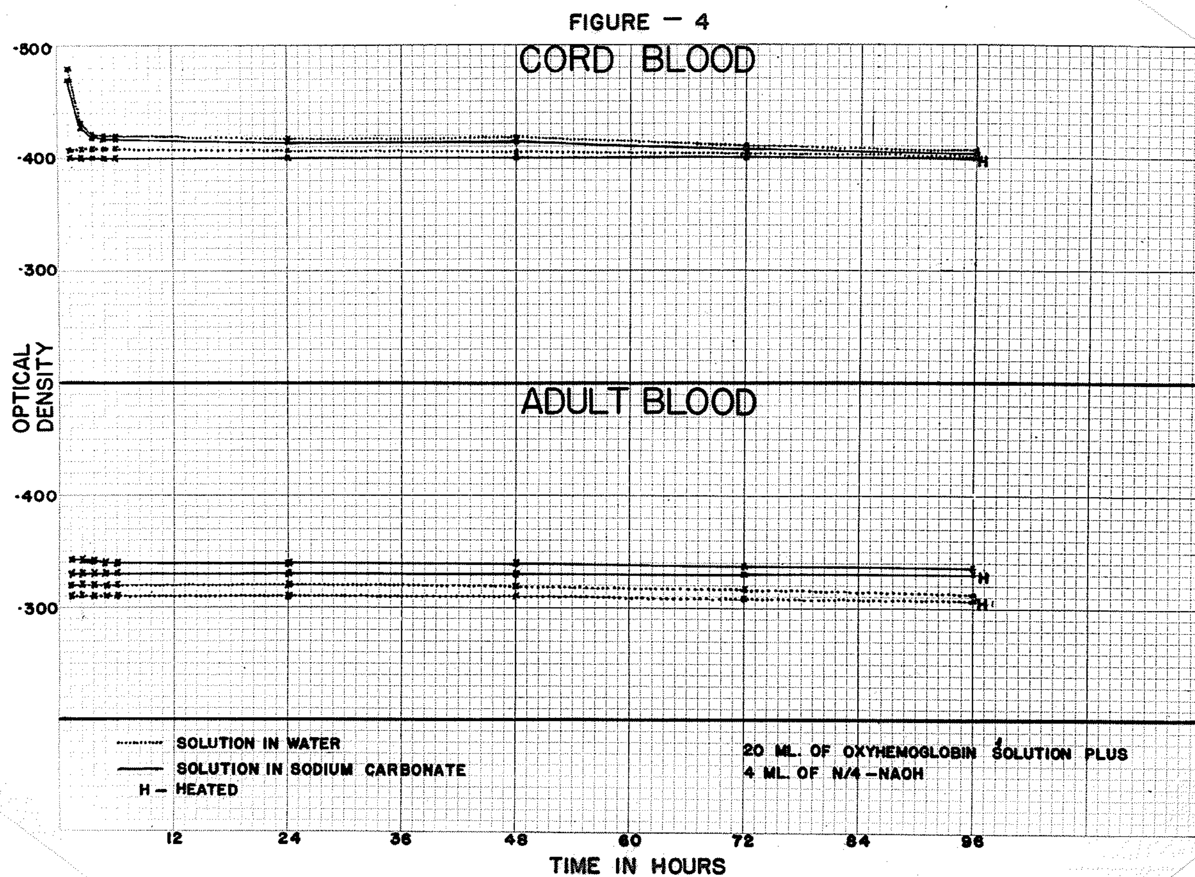

FIGURE 4

Changes in optical density at wave length 578 mμ of alkaline globin hematin solutions from adult and cord blood in N/4 NaOH at room temperature. H - denotes the heated solutions.

stable than the solutions of cord blood.

(iv) There is little difference in the stability of the solutions in water and in carbonate.

(v) The most striking difference is the decrease in optical density in N/6[1] sodium hydroxide compared with that observed in N/24[2] sodium hydroxide from twenty-four to ninety-six hours. In those solutions of N/24 sodium hydroxide the optical density remains stationary from five hours to forty-eight hours, and then begins to fall slowly. The N/6 sodium hydroxide solutions fall steadily and rather rapidly from the one hour level as far as they are followed (ninety-six hours).

It may well be that the N/6 sodium hydroxide besides converting oxyhemoglobin to alkaline globin hematin goes on to destroy this product, as evidenced by the progressive fall in optical density. Investigation of this interesting phenomenon is continuing.

It is apparent that the zero percent (twenty-four hour) base line should bear a constant relationship to the 100 percent starting level. The ratios of the optical densities representing 100 percent and 0 percent oxyhemoglobin should be constant. The

---

[1] N NaOH is equal to N/6 NaOH in final dilution

[2] N/4 NaOH is equal to N/24 NaOH in final dilution

results obtained in twenty denaturation experiments carried out in N/6 and N/24 sodium hydroxide are shown in Table III. The ratios obtained by the use of N/24 sodium hydoroxide are fairly constant, and range from 2.4 to 2.9 with a mean value of 2.69.

In the N/6 sodium hydroxide solution the ratios range from 2.2 to 4.5 with a mean value of 2.9. That is the variation in strong alkali was greater with a lower mean zero percent value as evidenced by the greater D_1/D_2 ratio. Again it seems that the stronger alkali may cause some destruction of the alkaline globin hematin.

From the above evidence it was decided to use N/4 sodium hydroxide, which gives a concentration of N/24 in final dilution. The zero base line is taken as the optical density of the alkaline globin hematin solution after denaturation was allowed to proceed for twenty-four hours at room temperature. The optical density obtained by heating the solution was found to have little more stability than the optical density obtained by allowing the solution to remain at room temperature. Moreover by heating one introduces the factor of denaturation through heating as well as by alkali. A 0.1% solution of sodium carbonate was adopted for preparing the hemoglobin solutions because of the greater stability of oxyhemoglobin in this solution than in water. The carbonate solution is also a much clearer solution because of its solvent action on the stroma of the erythrocytes.

<u>The Effect of Temperature</u>. Brinkman <u>et al</u> (1934) and Brink-

TABLE III

RELATIONSHIP OF THE OPTICAL DENSITIES OF THE ZERO PERCENT BASE LINE AND THE ONE HUNDRED PERCENT LEVEL USING DIFFERENT CONCENTRATIONS OF ALKALI

Oxyhemoglobin Solution in N/6 NaOH			Oxyhemoglobin Solution in N/24 NaOH		
D_1 100% Oxyhemoglobin	D_2 0% Oxyhemoglobin	Ratio D_1/D_2	D_1 100% Oxyhemoglobin	D_2 0% Oxyhemoglobin	Ratio D_1/D_2
803	356	2.2	875	320	2.7
910	309	2.9	895	313	2.9
803	301	2.7	990	355	2.8
990	360	2.7	358	299	2.6
935	329	2.8	840	298	2.8
927	333	2.8	713	296	2.4
582	130	4.5	713	296	2.4
937	271	3.4	760	296	2.6
840	216	3.9	880	330	2.7
945	336	2.8	870	315	2.8
792	306	2.6	810	311	2.6
1000	270	3.7	860	336	2.6
818	294	2.8	880	328	2.7
923	363	2.5	920	332	2.8
820	297	2.8	840	326	2.6
915	352	2.6	885	321	2.7
1045	350	3.0	813	296	2.7
800	314	2.6	970	350	2.8
905	335	2.7	820	307	2.7
810	260	3.9	848	296	2.9

man and Jonxis (1935) took elaborate precautions to keep the temperature of the reaction constant to within one half degree Centigrade. Baar and Lloyd (1943) on the other hand make no mention of temperature in the description of their method.

The variations in environmental temperatures during this series of estimations of refractory hemoglobin ranged from 20°C. to 28°C. The temperatures of the test solutions fell within the range of 23°C. to 29°C., with an average temperature of 27°C.

A series of four denaturation rates were carried out on the blood of a three day old infant. The temperature of the reactions were 15°C., 20°C., 25°C., and 30°C., and were controlled by immersing the cuvette in a large insulated container filled with water at these temperatures. The temperature in each case was constant for each individual reaction. The denaturation curves are shown in Figure 5.

It is seen that the slope of the line increased appreciably as the temperature was increases. However upon calculating the values of refractory hemoglobin obtained by extrapolation the following results were obtained --

Temperature	% Refractory Hemoglobin
15°C.	75.5%
20°C.	75.0%
25°C.	74.0%
30°C.	73.5%

It is seen that a rise in temperature of 15°C. introduced a

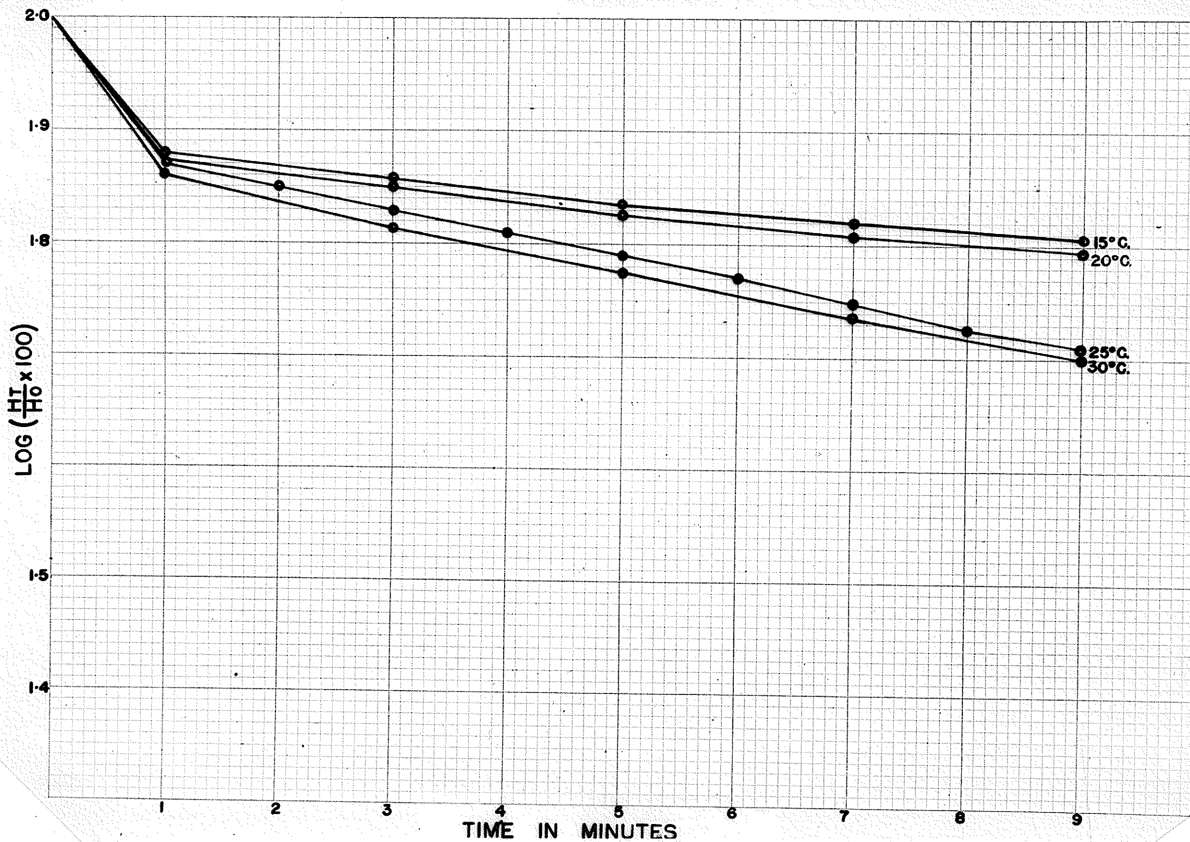

FIGURE 5

The effect of temperature on the denaturation curve of the hemoglobins of cord blood.

difference of 2% in the value obtained for the amount of refractory hemoglobin present. In this work the temperature range was only 6°C. between the various experiments, but did not vary more than 0.5°C. during the course of any one experiment. Thus no attempt was made to control the temperature of the reactions more rigorously.

Further Effects of the Concentration of Alkali. Discussion of this will be deferred until after the method adopted in this survey is described.

EXPERIMENTAL

The method used is an adaptation of that employed by Baar and Lloyd (1943). In this work the Beckman Spectrophotometer is used in place of the Evelyn colorimeter. The use of this more sensitive and accurate instrument allows more precise selection of a narrow spectral band. The denaturation was observed at a wave length of 578 mμ - the alpha band of oxyhemoglobin. This wave length was selected in place of 540 mμ because of the greater difference in optical density between oxyhemoglobin and alkaline globin hematin at this point (Figure 6).

Baar and Lloyd depended upon the use of an accurately prepared solution of hemoglobin of known concentration for which the optical density before and after complete denaturation was accepted empirically from data obtained by previous investigations, but concerning which no details are given. It is well known that the micro-

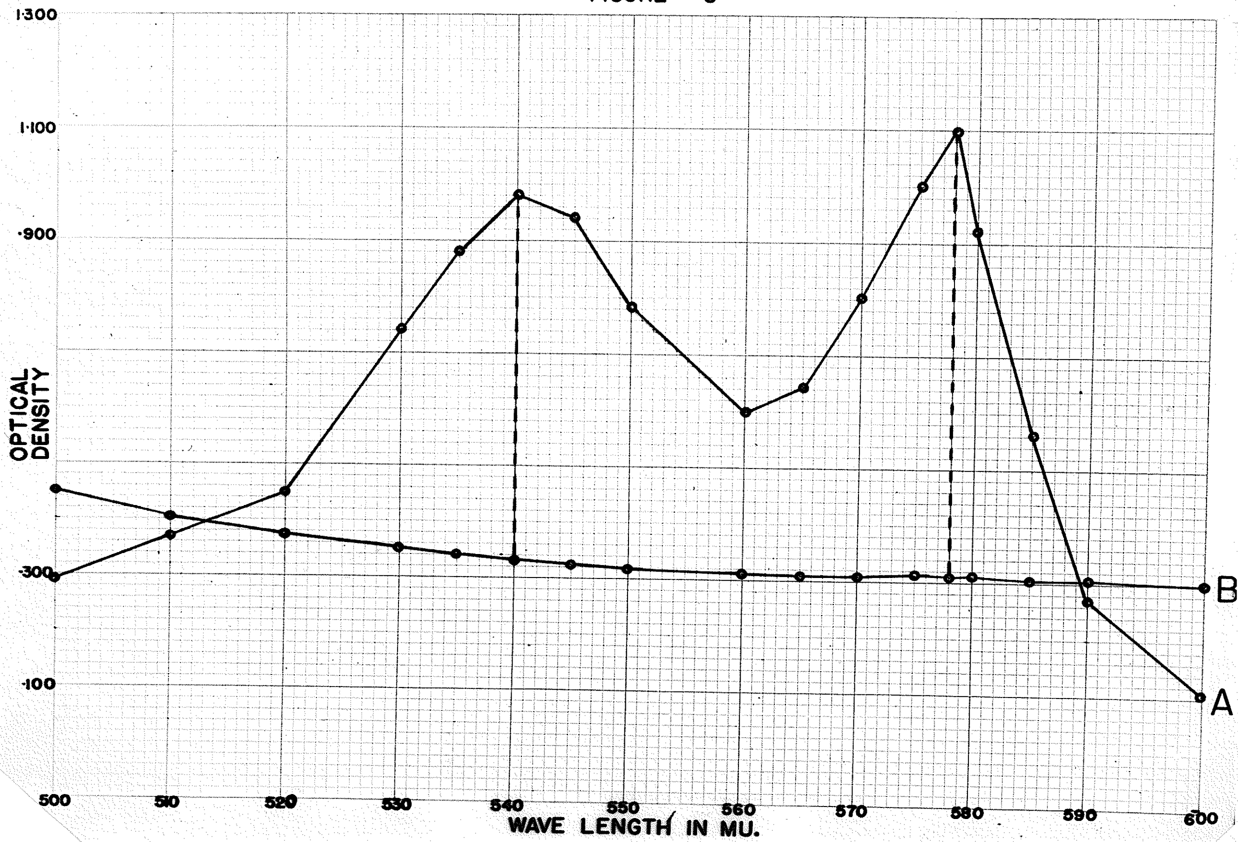

FIGURE 6

The absorption curves of: A -- oxyhemoglobin, and B -- alkaline globin hematin from cord blood.

pipetting of red cells is subject to many sources of error, so that the preparation of a one percent hemoglobin solution is a difficult task even if the total hemoglobin content of the blood being examined is known. The method to be described determines the optical densities experimentally before and after denaturation and does not depend upon the precise preparation of a solution of known hemoglobin content.

In place of whole blood, packed cells centrifuged free of plasma are used. This eliminates any error which may be introduced by icteric plasma producing an increase in optical density.

Method. The oxyhemoglobin content of the blood is determined as indicated in Section III.

A solution of oxyhemoglobin is prepared as follows --

0.5 ml. to 1 ml. of packed red cells are placed in approximately 100 ml. of 0.1 percent sodium carbonate, thoroughly mixed and completely oxygenated in a Waring Blendor. The solution is then filtered to remove any extraneous matter. The optical density is determined at a wave length of 578 mμ and an aperture of 0.05 mm. The dilution is adjusted so as to yield an optical density between 0.900 and 1.200.

2 ml. of this solution is accurately pipetted into each of two cuvettes. To the first cuvette 0.4 ml. of water is added -- this is the blank. To the second cuvette 0.4 ml. of N/4 sodium hydroxide is added using a Becton-Dickson One Ml. Tuberculin Syringe for accurate measurement and rapid manipulation, and the cuvette

thoroughly shaken to insure adequate mixing. A stop watch is started at the moment the sodium hydroxide is added. The cuvette is rapidly placed in the spectrophotometer and the optical density read, and recorded at one minute intervals for ten minutes. The blank is read and a thermometer inserted into the cuvette to record the temperature at which the reaction took place. The test solution is then allowed to stand for twenty-four hours at room temperature and its optical density determined at that time. The reaction takes place at a pH of 11.8.

All readings are made at a wave length of 578 mμ and a slit width of 0.05 mm.

Calculation. The addition of sodium hydroxide brings about the complete conversion of oxyhemoglobin to alkaline globin hematin. The optical density of the blank (2 ml. of oxyhemoglobin solution plus 0.4 ml. of water) represents 100 percent oxyhemoglobin and 0 percent alkaline globin hematin at that dilution. The optical density at twenty-four hours, represents complete denaturation - 0 percent oxyhemoglobin and 100 percent alkaline globin hematin. In the twenty-four hour period 100 percent of the oxyhemoglobin has been destroyed. If the optical density of the blank is taken as 100 percent oxyhemoglobin and the optical density at twenty-four hours taken as 0 percent oxyhemoglobin, then the optical densities read at the minute intervals are proportional to the amount of hemoglobin present at the time of the reading. If the percentage of oxyhemoglobin is plotted against

the time in minutes on an arithmetic grid the curves obtained are as shown in Figure 7.

Derivation of the Logarithmic Formula. If we assume that the rate of change of the oxyhemoglobin to alkaline globin hematin is proportional to the amount of unchanged hemoglobin present at any given time, then --

$$\frac{dH}{dt} = -kHt$$

Where Ht equals the amount of hemoglobin at any given time and k is a constant.

or

$$\frac{dH}{Ht} = -kdt$$

By Integration

(1) $\log_e Ht = -kt \text{ plus } C$ Where C is the constant of integration.

but when $t = 0$, Ht is equal to its original value (H_o).

The equation now becomes --

(2) $\log_e H_o = C$

From (1) and (2) $\log_e Ht - \log_e H_o = -kt$

or $\log_e \frac{Ht}{H_o} = -kt$

then $\log_{10} \frac{Ht}{H_o} = -Kt$ Where K is a different constant.

If $H_o = 100$ Then $\frac{Ht}{H_o}$ = percentage of hemoglobin present at time t.

By graphing $\frac{Ht}{H_o}$ against time "t" in minutes the curves now become as in Figure 8.

It can be seen that if the denaturation follows the law of

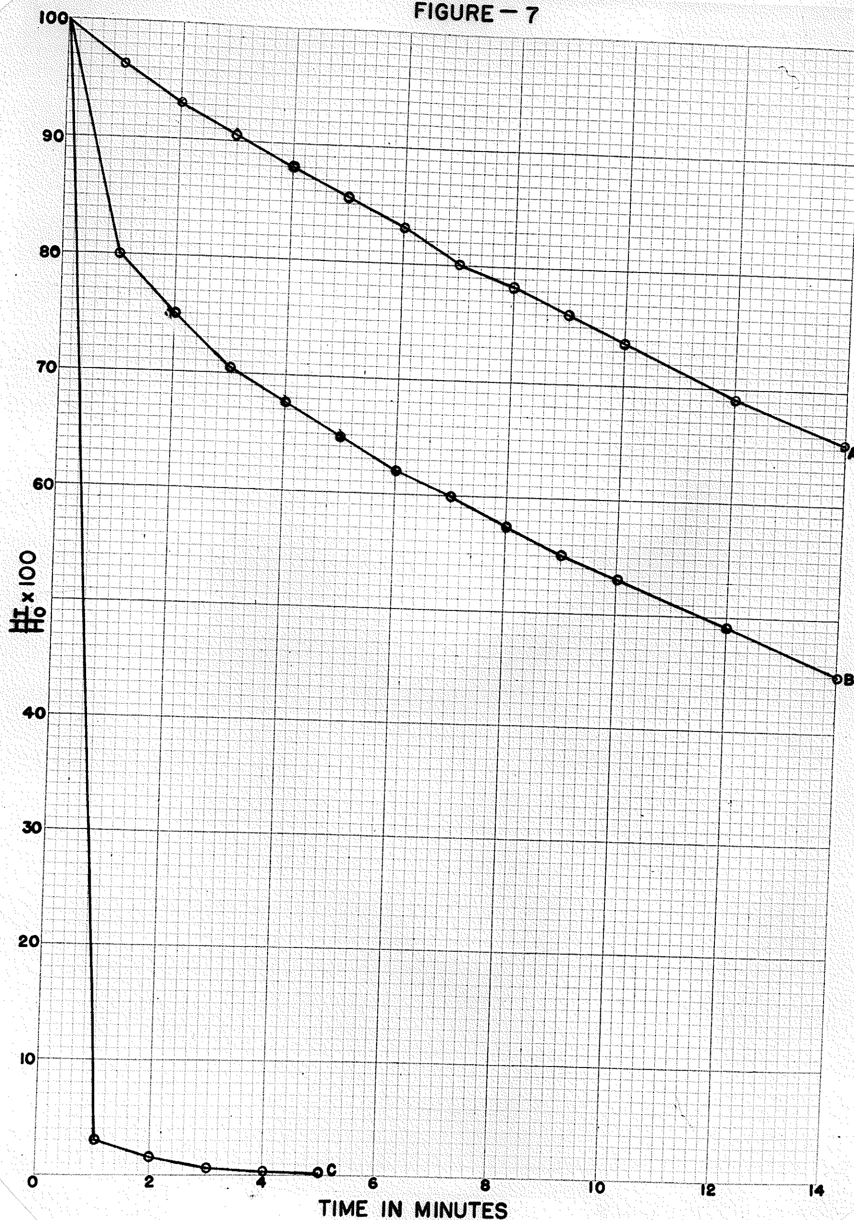

FIGURE 7

Denaturation curves of: A -- a prepared refractory hemoglobin, B -- the hemoglobin of cord blood, and C -- the hemoglobin of adult blood. $\frac{Ht}{H_o}$ x 100 is the percentage of hemoglobin present at any given time "t".

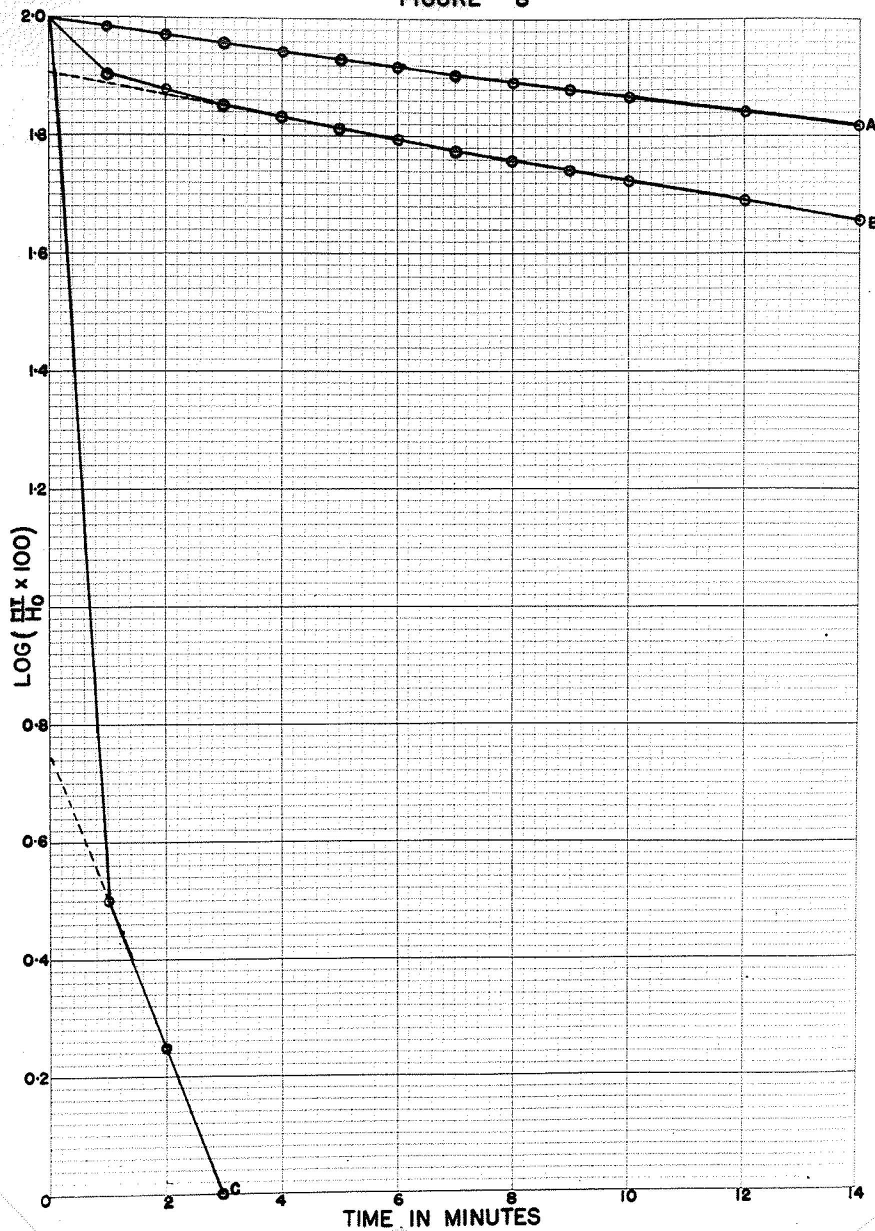

FIGURE 8

The logarithmic representation of the denaturation curves of: A -- a prepared refractory hemoglobin, B -- the hemoglobin of cord blood, and C -- the hemoglobin of adult blood. Log $\frac{Ht}{H_o}$ x 100 is the log of the percentage of hemoglobin present at any given time "t". On extrapolating to zero time the amount of refractory hemoglobin in the sample is obtained.

monomolecular reactions the curves will resolve themselves into straight lines.

On extrapolating the last straight line to zero time the value of $\log \frac{H_t}{H_o} \times 100$ is obtained at the point where t equals zero. The antilog of this value is the percentage of refractory hemoglobin in the blood sample.

Recovery Experiment. The following recovery experiment was performed --

Hemoglobin solutions of cord and adult blood were prepared and their denaturation curves determined. The amounts of refractory and labile hemoglobin were determined for each solution. These solutions were then mixed in varying proportions and the amounts of labile and refractory hemoglobin estimated according to the method described above. The values obtained were compared with the calculated values. The results are shown in Table IV. The agreement between the experimental and calculated values was thought to be satisfactory.

SUMMARY

1. The previous methods for estimating refractory and labile hemoglobin are reviewed and a modification adapted to the present study.

2. The effect of the concentration of alkali on the base line and the effect of variations in temperature on the accuracy of the method have been studied.

TABLE IV

RECOVERY EXPERIMENT

HEMOGLOBIN MIXTURE		PERCENT REFRACTORY HEMOGLOBIN	
Solution of:		Experimental	Calculated
Cord Blood	Adult Blood	Value	Value
10 ml.	0 ml.	73	--
10 ml.	2 ml.	61.5	61
10 ml.	5 ml.	53	49
10 ml.	10 ml.	36.5	37
5 ml.	10 ml.	22	24
2 ml.	10 ml.	13	12
1 ml.	10 ml.	9	7
0 ml.	10 ml.	0	--

3. A recovery experiment using the adopted method gave good results.

SECTION V

ADDITIONAL STUDIES OF THE HEMOGLOBINS OF CORD BLOOD

INTRODUCTION

In the foregoing section certain rather interesting phenomena were related in the discussion of the development of the method for the estimation of refractory hemoglobin. Though not directly related to the development or the application of the method it was thought that a study of these factors would make some contribution to our knowledge of the hemoglobins of cord blood.

The following subjects will be discussed --

1. The difference in the alkaline globin hematin formed from oxyhemoglobin and sodium hydroxide on heating, and that formed at room temperature.
2. The relationship of the concentration of alkali to the form of the denaturation curve.
3. The possible existence of a third form of hemoglobin.
4. The preparation of a completely refractory hemoglobin.

THE ALKALINE GLOBIN HEMATIN OF CORD BLOOD

It was noted in the previous section that in those experiments carried out for the establishment of a zero percent base line the optical density of the alkaline globin hematin which was heated was consistently lower than the optical density of

that which was allowed to denature at room temperature for twenty-four hours. This suggested that there may be some difference in the structure and behaviour of the product which had been heated. These differences were studied by - (1) the absorption curves, and (2) the hemochromogens.

The Absorption Curves. The absorption curves of the following substances at the same concentration were -- prepared.

1. Alkaline globin hematin formed by the addition of 4 ml. of N NaOH to 20 ml. of a 1% solution of cord blood in 0.1% Na_2CO_3 and allowed to remain at room temperature (approximately 23°C) for twenty-four hours.
2. Alkaline globin hematin formed by the addition of 4 ml. of N NaOH to 20 ml. of a 1% solution of cord blood in 0.1 Na_2CO_3, heated on a boiling water bath for five minutes and allowed to remain at room temperature for twenty-four hours.

The absorption curves were determined on the Beckman Spectrophotometer from a wave length of 430 mμ to 630 mμ, using an aperture of .05 mm. The curves are shown in Figure 9.

Clegg and King (1942) noted that the absorption curves formed by the action of sodium hydroxide on blood (alkaline globin hematin) and on hemin (alkaline hematin), though similar were not identical. King and Delory (1944) investigated this further and suggested that this discrepancy was due to the presence of globin in one compound and not in the other.

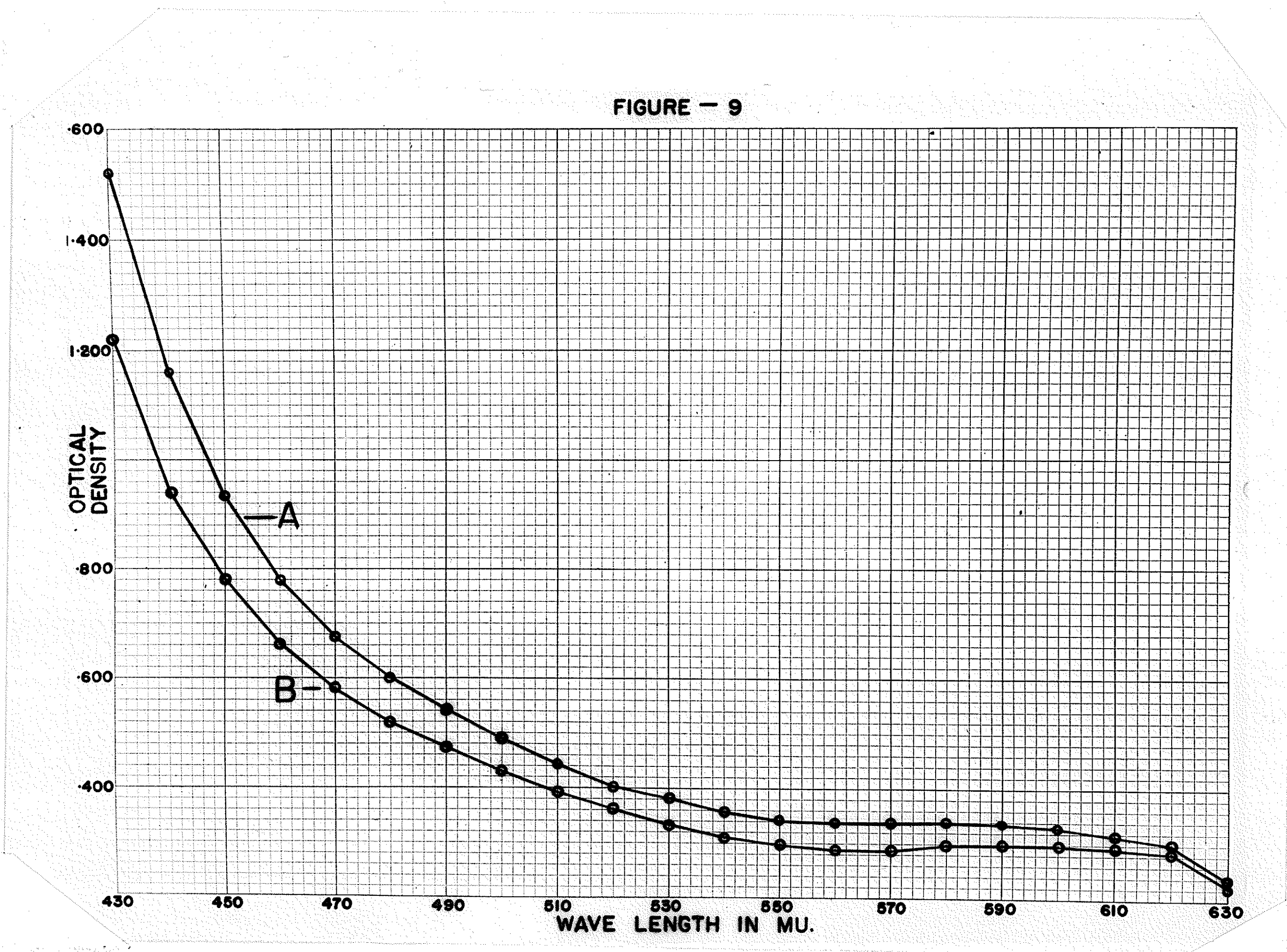

FIGURE 9

The absorption curves of alkaline globin hematin of cord blood: A -- not heated; B -- heated.

In Figure 9, we note that the absorption curves of alkaline globin hematin derived from the same sample of cord blood differ when one is formed on heating and the other allowed to form at room temperature. The curves though similar are not identical and converge in the region of 620 mμ. The curves do not touch or cross. The optical density of the heated form is at all times lower than is that of the non-heated.

The Hemochromogens. Following King and Delory the alkaline globin "hematins" of cord blood were studied by preparing their globin hemochromogens.

40 ml. of an approximately 1% solution of blood in 0.1% Na_2CO_3 was prepared and divided into two 20 ml. portions. 4 ml. of NaOH was added to each. One solution was allowed to remain for twenty-four hours at room temperature; the other was heated on a boiling water bath for five minutes and then allowed to remain for twenty-four hours at room temperature. A series of five cord and five adult bloods were prepared in the above manner. To each solution a pinch of Sodium Hydrosulphite was added to form the globin hemochromogen. The absorption bands were measured in the Beck-Hartridge reversion spectroscope. The results are shown in Table V.

In the alkaline hemochromogen formed from adult blood no change is found in the position of either the alpha or beta band on heating. The alpha band is located at 5570 A and 5571 A, the beta band at 5246 A.

TABLE V

THE ABSORPTION BANDS OF THE GLOBIN HEMOCHROMOGENS OF ADULT AND CORD BLOOD

ADULT BLOOD

Blood No.	Not Heated		Heated	
	alpha	beta	alpha	beta
1	5572 A	5244 A	5573 A	5243 A
2	5572 A	5246 A	5570 A	5248 A
3	5567 A	5247 A	5570 A	5250 A
4	5571 A	5243 A	5574 A	5241 A
5	5570 A	5247 A	5568 A	5520 A
Mean	5570 A	5247 A	5571 A	5246 A

CORD BLOOD

Blood No.	Not Heated		Heated	
	alpha	beta	alpha	beta
1	5574 A	5252 A	5569 A	5269 A
2	5571 A	5249 A	5571 A	5265 A
3	5563 A	5251 A	5570 A	5268 A
4	5570 A	5251 A	5571 A	5274 A
5	5569 A	5250 A	5573 A	5273 A
Mean	5569 A	5250 A	5571 A	5270 A

Cord blood, however, yields a very interesting result. In the non-heated samples the alpha band is located at 5569 A, and the beta band at 5250 A, which are in good agreement with those values found for adult blood. (The 4 A difference in the beta band is within the limits of experimental error). In the heated globin hemochromogen, although the alpha band is found at 5571 A, in good agreement with both adult blood and the non-heated cord blood form, the beta band is located at 5270 A -- a position significantly different from the other forms.

Discussion. No reference could be found in the literature describing differences in alkaline globin hematin of cord blood, depending upon its method of preparation.

It may well be that in the process of heating the hemoglobin of cord blood in the presence of strong alkali, an alkaline globin hematin is produced which differs somewhat from that produced when no heat is employed. This is evidenced by a change in the absorption curve and in a shift of the beta band of the globin hemochromogen.

It is interesting to speculate at this time that just as the difference between alkaline hematin and alkaline globin hematin may reside in the globin portion of the molecule -- so may the process of heating effect some change in the globin constituent of the alkaline globin hematin derived from cord blood, or may remove the denatured globin moiety.

THE RELATIONSHIP OF THE CONCENTRATION OF ALKALI TO THE FORM OF THE DENATURATION CURVE

To study this phenomenon solutions of adult and cord blood were prepared in 0.1% sodium carbonate as described previously and the denaturation curves determined as outlined in Section IV. For each hemoglobin solution the denaturation curve was determined using N-, N/2, N/4, and N/10 sodium hydroxide as the agent of denaturation. The temperature remained constant for the serial determinations on each blood. The results obtained from adult and cord blood are shown in Figure 10.

The purposes of clarity the results obtained in cord blood and adult blood shall be discussed separately.

Cord Blood.

N. Sodium Hydroxide, (N/6 Sodium Hydroxide in final dilution). The denaturation curve consists of two components, a rapid drop to the one minute level and a more gradual decline from one minute onwards. The two parts form two distinct straight lines, the second line is of much greater slope than in those reactions in which weaker alkali was used. On extrapolation the percentage of refractory hemoglobin present was found to be 68 percent.

N/2 Sodium Hydroxide, (N/12 Sodium Hydroxide in final dilution). This curve is quite similar to that described for N Sodium Hydroxide but for the more gentle slope of the second part of the curve. On extrapolation the percentage of refractory hemoglobin was found to

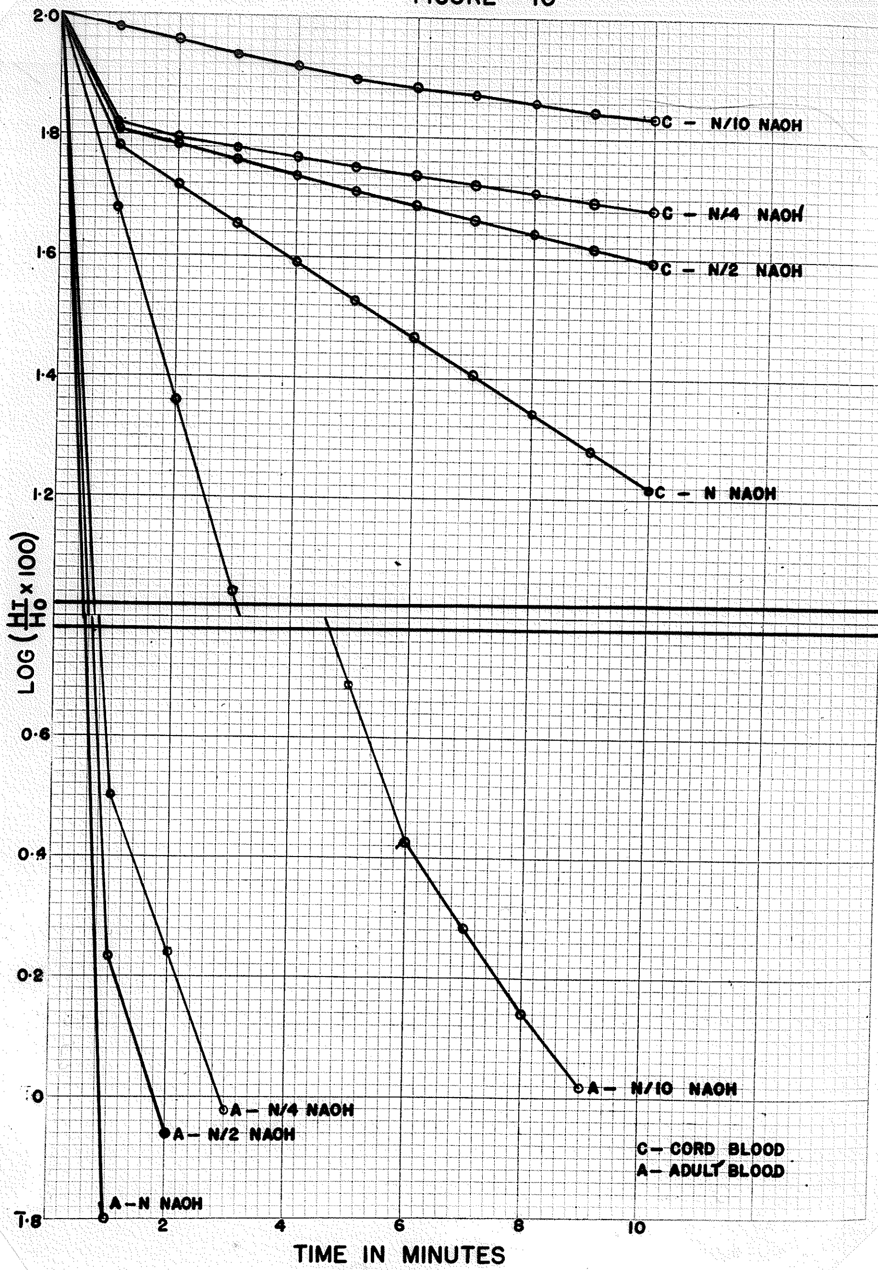

FIGURE 10

The effect of the concentration of alkali on the denaturation curves of adult and cord blood. The double line represents a break in the graph.

be 67.5%.

N/4 Sodium Hydroxide. (N/24 Sodium Hydroxide in final dilution). On careful examination this curve is found to resolve itself into three distinct components -- the steep slope to one minute, the more gradual slope from the one to the two minute level and then a very gradual slope from two minutes onward. The presence of this third intermediate slope will be discussed in detail in the following section. On extrapolation of the third straight line the percentage of refractory hemoglobin was found to be 66%.

N/10 Sodium Hydroxide. (N/60 Sodium Hydroxide in final dilution). This denaturation curve presents no sharp differentiation into two or more straight lines and does not distinguish between the refractory and labile hemoglobins present in the blood sample. The curve is of gentle slope indicative of slow denaturation. No calculation of the relative amounts of labile and refractory hemoglobin was thought justified in view of the form of the curve.

Adult Blood.

In Figure 10, the curves for adult blood are of necessity broken since, because of the range it was thought desirable to include all of the curve and yet not reduce the scale of the graph.

N. Sodium Hydroxide (N/6 Sodium Hydroxide in final dilution). This denaturation process is graphed as a straight line --

that is at this concentration of alkali less than .7% (antilog $\bar{1}.8$) of unconverted hemoglobin remained one minute following the addition of alkali.

N/2 Sodium Hydroxide. (N/12 Sodium Hydroxide in final dilution). This curve resolves itself into two distinct straight lines at the one minute level. The second more gentle slope from 1 to 2 minutes is much steeper than the secondary slopes of cord blood -- but the exaggeration of slope is a function of the logarithmic scale and may not be inherent in the blood itself. On extrapolation of the second slope, the percentage of the more refractory hemoglobin present is found to be 3%.

N/4 Sodium Hydroxide. (N/24 Sodium Hydroxide in final dilution). This curve closely resembles that described for N/2 Sodium Hydroxide but indicates a lesser destruction of hemoglobin per unit time. The amount of more refractory hemoglobin is found to be 6%.

N/10 Sodium Hydroxide. (N/60 Sodium Hydroxide in final dilution). This denaturation curve does not resolve itself into two or more straight lines but into a continuous curve -- showing an ever decreasing rate of hemoglobin destruction. In view of the form of the curve, no estimation of the hemoglobin components was attempted.

Discussion. The primary effect obtained by an increase in the concentration of alkali is an acceleration of the process of denaturation which is reflected in the increase of the slope of

all portions of the curve but is particularly evident in the last part of the curve which represents the refractory hemoglobin component. This is illustrated best in cord blood where there is a relatively greater amount of the refractory form present.

Despite the differences in slope produced by the various concentrations of alkali the calculated value of refractory hemoglobin present in the same sample of cord blood varied only from 66% to 68% with the use of N/4 to N/24 Sodium Hydroxide. The variation in adult blood was somewhat greater, the range being from 3% to 6%.

A finding of considerable interest is the fact that the denaturation curve produced by N/24 Sodium Hydroxide is composed not of two components but three. This was a constant finding throughout this work as the routine estimations done by the method previously described utilize N/24 Sodium Hydroxide.

It was further observed that the hemoglobin of adult blood was not completely of the labile form but contains a not inconsiderable amount of a more refractory hemoglobin which in this work was found to range from 4% to 9%. This refractory hemoglobin component in adult blood was first described by Brinkman, Wildschut, and Wittermans (1934), who considered it identical with the refractory hemoglobin of cord blood. Though the slope of this hemoglobin component seems much steeper than its counterpart in cord blood, as mentioned previously, this is more apparent than real and much of this is due to the logarithmic scale. This is

evident from Figure 7 which shows the denaturation curves of adult cord blood as graphed on a double arithmetic grid.

THE POSSIBLE EXISTENCE OF MORE THAN TWO HEMOGLOBINS IN CORD BLOOD

It was seen in the previous section that N/24 NaOH on cord blood produces a denaturation curve which resolves itself into three distinct portions, (Figures 8 and 10). It may well be that this intermediate slope represents a third form or forms of hemoglobin which possess a rate of denaturation intermediate between what have been designated as labile and refractory hemoglobin. Derrien and Roche (1949) claim to have isolated five hemoglobins from cord blood by salting out methods.

When the denaturation rate is accelerated by the use of stronger alkali this intermediate portion of the curve is masked as it is incorporated into the first portion of the curve representing labile hemoglobin. Weak alkali produces a curve which is difficult to interpret because of its failure to resolve into two or more straight lines when plotted on a logarithmic-arithmetric basis.

THE PREPARATION OF A COMPLETELY REFRACTORY HEMOGLOBIN

It was thought advisable to prepare a hemoglobin which would exhibit no labile constituent(s) when treated with sodium hydroxide. This was prepared from cord blood as follows --

To 50 ml. of a 10% solution of cord blood in 0.1% Na_2CO_3, 10 ml. of N NaOH was added. The ensuing denaturation was allowed to proceed for five minutes at room temperature. The solution was then buffered by the addition of 20 ml. of N $NaHCO_3$. The denatured globin fraction was then precipitated out by the addition of 80 ml. of a saturated solution of Na_2SO_4. This was filtered and the bright red clear filtrate was found to contain only refractory hemoglobin.

The denaturation curves of the filtrate treated with N/4 NaOH are shown in Figures 7 and 8. On calculation the amount of refractory hemoglobin was found to range from 99% to 101.5% of the total hemoglobin present.

The filtrate was found to be free from alkaline globin hematin when ammonium hydroxide and ethyl alcohol were added and the solution examined in the Beck-Hartridge reversion spectroscope. The characteristic band of alkaline globin hematin was detected in aliquots taken after the addition of sodium hydroxide and sodium bicarbonate and in the precipitate following the addition of sodium sulphate. That is the alkaline globin hematin had been largely if not completely removed by the precipitation with sodium sulphate leaving a relatively pure solution of hemoglobin - which was found to be completely of the refractory form.

SUMMARY

1. A study of the absorption curves and globin hemochromo-

gens of the alkaline globin hematin of cord blood suggests that a different compound is produced when the alkaline globin hematin is formed on heating.

2. The relationship of the concentration of alkali to the form of the denaturation curve has been studied.

3. A method for the preparation of a completely refractory hemoglobin has been described.

4. The possibility of the existance of a third form of hemoglobin is advanced.

SECTION VI

COLLECTION OF MATERIAL FOR CLINICAL STUDY

SOURCE OF MATERIAL

The 32 newborns studied in this series were all normal, healthy, babies born at or near full term in the Winnipeg General Hospital from November 1949 to February 1950. They were all public (staff) cases.

FREQUENCY OF SAMPLING

An attempt was made to obtain blood specimens from the umbilical cord at birth, and from the child at three and seven days as well as at three and four weeks after birth. It was not possible to do this in all cases - and it was particularly difficult to get the mothers to bring the child back at three to four weeks of age to obtain a blood sample at that time. This accounts for the smaller number of observations from the seventh day onward after the child had left hospital.

CLINICAL DATA

In all cases the maturity of the newborn is recorded - as computed from the date of the mother's last menstrual period. As this is not too accurate a method of assessing maturity - the birth weight was also recorded as an additional index.

An attempt was made to assess clinically the presence and

degree of jaundice in each newborn whenever a blood sample was taken. This was done by inspection of the infants skin and mucous membranes and graded as follows --

- -no jaundice
- +-questionable jaundice
- +slight jaundice
- ++moderate jaundice
- +++moderately severe jaundice
- ++++severe jaundice

In all cases the mothers records were inspected for any abnormalities likely to influence the pregnancy or foetus. Three mothers showed some signs of toxaemia of pregnancy but their babies were normal on delivery. These cases are included in the series.

THE COLLECTION OF BLOOD FROM THE UMBILICAL CORD

Approximately 1 ml. of 5% potassium oxalate was placed in a 20 ml. test tube and dried in an oven so that a thin film of oxalate was present down the length of the tube. The tube was then wrapped and autoclaved so that it could be handled as a sterile vessel by the obstetrician without causing any break in the aseptic-antiseptic technique of the delivery.

Following delivery of the baby, the cord was clamped and cut after it had ceased pulsating. The placental end of the cord was carefully cleaned and placed in the oxalated tube and the cord

milked gently in the direction of the tube. The blood was gently mixed with the anticoagulant and the tube placed in the refrigerator until the estimations were performed.

THE COLLECTION OF BLOOD FROM THE INFANT

The blood from the infant was collected in a small (4 ml. to 5 ml.) test tube which had a thin film of dried potassium oxalate down one side.

The baby was placed at the edge of a table so that its leg could be held hanging down and thus produce some congestion of the vessels. The heel was cleansed with alcohol and carefully dried with a cotton sponge. A small stab wound was made on the child's heel with a scalpel blade and the blood allowed to drip into the test tube, turning the tube so as to insure adequate mixture with anticoagulant.

SECTION VII

RESULTS AND CONCLUSIONS

INTRODUCTION

The data assembled in this survey and recorded in Table VI may be assessed from the following aspects --

1. The variations in bilirubin in the blood of the normal newborn.
2. The variations in total hemoglobin in the neonatal period.
3. Refractory hemoglobin levels in the blood of the newborn.
4. The relationship of the total hemoglobin and refractory hemoglobin levels to the degree of bilirubinemia.

BILIRUBIN

The total plasma bilirubin level was found to have a mean value of 1.5, with a range of from 0.7 to 3.5 mg. percent. By the third day of life the mean value had risen to 4.9 mg. with a range of 1.1 to 15.2 mg. percent. On the seventh day the range was 0.9 to 10.0 with a mean value of 4.2 mg. percent. The third week found the mean value (in six cases) to be 1.4 mg. with a range of 0.6 to 2.8 mg. percent. In those newborns returning at four weeks (six) the range was 0.6 to 2.3 with a mean value of 1.2 mg. percent.

This rise after birth reaching its maximum on the third day or

TABLE VI

HEMOGLOBIN -- BILIRUBIN STUDY OF 32 NORMAL

CASE NUMBER	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
MATURITY (weeks)	41	40	44	40	40	39	40	40	40	40	37	39	40	42	41
BIRTH WEIGHT lbs.	6	9	7	9	7	7	8	9	9	6	7	6	6	8	8
oz.	14	4	12	13	7	6	8	5	5	3	3	12	14	6	3
SEX	F	M	M	M	M	F	F	F	M	M	M	F	M	M	F
TOTAL HEMOGLOBIN (G. %)															
cord blood	13.4	13.8	15.0	17.5	15.0	14.5	20.0	13.8	15.2	15.5	14.5	14.6	13.0	17.0	17.
3rd day	18.5	20.0	20.0	18.0	22.8	18.5	21.0	21.0	16.8	19.5	18.2	20.4	19.0	21.0	17.
7th day	14.2	18.9	22.0		19.5	18.1	20.0	20.0	17.7	15.5	16.9	20.5	20.5	19.8	17.
3 weeks					15.6			20.0	17.7	15.5	15.2	18.2			
4 weeks	14.7		19.0												
REFRACTORY HEMOGLOBIN (as % of total)															
cord blood	75.0	77.5	50.0	76.0	82.5	79.5	71.0	69.0	74.0	82.5	80.5	75.0	69.0	76.5	82.
3rd day	67.5	78.5	45.0	77.0	83.0	77.5	61.0	66.0	68.0	79.5	68.5	76.0	72.0	75.0	82.
7th day	65.0	77.0	44.0		80.0	77.5	66.0	55.0	83.0	78.5	75.0	75.0	77.5	74.0	83.
3 weeks					57.0			59.0	65.0	77.5	61.5	69.0			
4 weeks	75.0		40.0												
TOTAL BILIRUBIN (mg. %)															
cord blood	1.3	1.3	.7	.7		.7	1.0	1.6	1.0		2.0	1.5	1.5	1.1	3.
3rd day	4.0	4.1	2.1		2.2	3.6	7.3	3.4	5.9	1.1	15.2	5.5	9.0	2.6	9.
7th day	6.3	1.6	1.1		2.1	3.3	3.9	1.2	4.3	1.2	8.6	10.0	9.0	2.1	6.
3 weeks					1.5			1.8	0.6	0.7	2.8	1.0			
4 weeks	0.8		0.6												
ICTERUS															
birth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3rd day	+-	+	-	++	-	-	+	+-	+	-	+++	+	++	-	+
7th day	+	-	-		-	-	-	-	+	-	++	++	++	-	+
3 weeks					-			-	-	-	-	-			
4 weeks	-		-												

NEWBORNS FROM THE WINNIPEG GENERAL HOSPITAL

	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
	41	40	38	40	42	40	39	38	37	40	36	36	40	40	40	40	40
	7	7	5	7	11	5	6	8	5	7	8	5	7	10	7	6	6
	5	15	0	5	5	14	9	8	0	8	5	15	15	3	14	6	5
	M	M	M	M	M	M	M	M	F	M	M	F	M	F	M	M	M
553	15.2	14.7	15.7		14.9	14.4	11.4	13.0	12.6	15.0	16.0	14.3	16.6	15.7	15.5	14.1	15.1
	22.0	20.1	20.5	19.6	20.7	20.7	19.8	19.4	14.6		18.6	21.5	16.3	21.0	17.3	18.2	21.8
	18.1	21.4	19.5	17.2	17.7	17.4	17.6	15.6	16.6	18.6	15.0	18.5	16.0	16.4	18.0	15.6	22.5
			10.5							16.0			12.8	16.7			
550	77.5	79.5	67.0	69.0	72.5	67.0	81.0	80.0	87.0	82.5	75.5	86.0	85.5	78.0	77.5	86.5	87.0
	74.0	78.5	83.0	76.0	70.0	76.0	81.0	85.0	87.0		77.0	84.0	83.0	78.0	79.5	87.0	81.5
	76.0	78.5	85.0	75.0	66.5	75.0	81.0	84.0	87.0	79.5	75.5	83.0	80.0	78.5	77.0	83.0	81.5
			76.0							79.5			77.0	74.0			
515	2.2	1.8	1.0	1.6	1.0	1.6	2.0	0.6	1.1	0.9	2.0	1.1	2.3	1.2	2.0	2.4	2.1
	6.9	4.2	3.5	3.4	1.7	4.0	5.4	2.5	7.7			3.8	6.6	2.9	4.2		5.4
	5.2	4.3	3.0	3.4	1.0		10.0	1.5	2.0	1.8	4.1	4.3	5.5	0.9	3.0	6.2	7.5
			2.3							2.1			0.9	0.7			
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	++	+	+-	+	-	+	+	-	+		-	+-	++	-	-	+	+
	+	+	-	+	-	+	++	-	-	-	+	+-	+	-	-	++	++
			-							-			-	-			

thereabouts is in agreement with the findings of other workers (Yllpö, 1913, Weech, 1947). The decline from the seventh day onwards is usually rapid. The values for serum bilirubin as given by Davidson et al (1941) are as follows; cord blood - mean 1.6, range 1.4 to 6.0; third day - mean 4.8, range 1.3 to 11; seventh day - mean 3.4, range 0.9 to 10.8 milligrams percent.

In this series of 32 normal newborns, 19 or 59 percent developed a definite clinical jaundice during the first seven days of life. This is within the range of from 50 to 75 percent which is generally quoted (Smith, 1945) and which of course depends on the individual workers clinical concept of jaundice. There was no bilirubin level (threshold) at which all infants became jaundiced but jaundice was present in all infants with a plasma bilirubin greater than 4.5 milligrams percent. It is seen from Table VI that in general the greater the plasma bilirubin in cord blood the more marked and prolonged was the bilirubinemia.

TOTAL HEMOGLOBIN

The cord blood total hemoglobin levels were found to range from 11.4 grams percent to 20.0 grams percent with a mean value of 14.9 grams percent. At the third day the mean value had risen to 19.2 with a range of 14.6 to 22.8 grams percent. By the seventh day the mean value had begun to fall and was now 18.2 grams percent with a range of 14.2 to 22.5 grams percent. Those infants which returned at three weeks of age (six) were found to have a

mean hemoglobin level of 17.3 grams percent and the range was from 15.2 to 20.0 grams percent.

By the 4th week, in those cases returning (six) the mean value had fallen to 14.9 grams percent and the range was 10.5 to 19 grams percent.

Thus, considering the mean values, the total hemoglobin rose sharply following birth and reached its maximum at three days, thereafter declining slowly until it returned to its initial level at four weeks.

The results of this series are quite different from those recorded by Waugh _et al_ (1940) who found the total hemoglobin level of umbilical cord blood to average 15.36 grams percent, to rise to 15.46 grams on the fourth day and thereafter decline to 14.7 grams by the ninth day. However, Faxén (1937) found 23.2 grams on the first day, 21.7 on the seventh day and less than 18 grams at four weeks. Smith (1945) in his survey of several studies states the hemoglobin at birth to be 17.9 grams and to rise to a mean value of 19.0 grams during the first week.

It can be seen that the various series differ markedly and the last word has yet to be said as to the normal values for hemoglobin in the newborn. Indeed perhaps as Clement Smith claims there is no universally acceptable figure.

REFRACTORY HEMOGLOBIN

The cord blood refractory hemoglobin level was found to have

a mean value of 76.2% with a range of from 50% to 87%. The average level at the third day was found to be 76.1% with a range of from 45% to 87%. The seventh day found the range to be from 44% to 87% with a mean value of 76.3%. By the third week the mean value (for six cases) had fallen to 64.7% with a range of from 57% to 69%. The fourth week found the mean value (for six cases) to be 70.2% with a range of from 40% to 79.5%.

Jonxis (1948a) stated that:

> " A normal newborn child has only foetal haemoglobin. During the early months after birth this haemoglobin is gradually replaced by later haemoglobin, at a rate of less than 1 percent a day, so that after 120 days the foetal haemoglobin has disappeared."

Our findings are in complete disagreement with the initial part of this statement as we did not find one infant whose cord blood contained only foetal or refractory hemoglobin. The mean value of 76.2% refractory hemoglobin in cord blood is in good agreement with that reported by Ponder and Levine (1949) of 79.7% -- for a series of fifteen normal newborns.

Later in the same year Jonxis (1948b) had apparently changed his mind and now reported the range of foetal (refractory) hemoglobin in the cord blood of the full-term newborn to be from 75% to 98%. This is quite different to the range recorded in this series of from 50% to 87%. It is interesting to note that this 50% refractory hemoglobin level recorded in Case No. 3 was that of

a baby which was thought to be hypermature (44 weeks) -- thus agreeing with the observation that the more mature the child, the smaller the amount of foetal hemoglobin present in its blood (Jonxis, 1948b).

The steady decline in refractory hemoglobin described by Jonxis was found to be an oversimplification of the facts. It is seen that though the mean values found in the third and fourth weeks are lower than the mean values recorded for the first week of life -- there is no significant difference between the level of refractory hemoglobin in cord blood and that found at seven days.

If three percent is taken as an appreciable variation in consecutive determinations of refractory hemoglobin then only ten of the thirty-two infants showed a consistent decline in refractory hemoglobin after birth. The level in twelve infants remained the same for as long as they were followed. Five newborns exhibited a rapid rise immediately after birth and five others showed an initial decline followed by a subsequent rise.

The statement that the foetal hemoglobin disappears in 120 days is probably based on the hypothesis that no foetal hemoglobin is produced after birth. The life span of the erythrocyte is known to be approximately 120 days and if no production of foetal hemoglobin took place after birth, the last erythrocytes carrying this pigment would be destroyed by that time. However the fact that foetal hemoglobin is produced after birth as evidenced by its rise in many cases after birth would tend to throw doubt on the 120 day

period, which is probably incorrect. Further work in this direction is indicated.

THE RELATIONSHIP OF TOTAL HEMOGLOBIN AND REFRACTORY HEMOGLOBIN TO NEONATAL JAUNDICE

No correlation could be found between the absolute hemoglobin level of cord blood, or its subsequent rise and fall and the degree of bilirubinemia which developed. This is in agreement with the findings of Waugh et al (1940).

Similarly no relationship could be found between the refractory hemoglobin content of cord blood, nor its subsequent rise or fall and the bilirubinemia of the infant. It is readily seen on inspection of Table VI that both high and low cord blood levels of refractory hemoglobin were associated with mild, moderate and severe bilirubinemia.

To by-pass the cause and effect relationship, it might be expected that mature liver function and thus a low plasma bilirubin might be associated with a mature erythropoieses and the formation of a "mature" adult hemoglobin. Although this association was present in some infants, i.e., Baby No. 3, it was not found consistently throughout the group.

Thus no evidence could be found that the "foetal", refractory hemoglobin is more susceptable to destruction than its adult counterpart and it apparently plays no special role in the etiology of neonatal jaundice.

SUMMARY

1. The variations in plasma bilirubin following birth are described, 59% of the infants showed clinical icterus and all infants with a bilirubin level of more than 4.5 mg. percent were icteric.

2. Although the mean hemoglobin content of cord blood was found to be 14.9 grams percent the hemoglobin rose rapidly to the third day and then began to decline slowly. No relationship was discovered between the total hemoglobin values and the bilirubinemia.

3. Labile hemoglobin was found to be present at birth in all the newborns studied. The mean value for the refractory hemoglobin content of cord blood was found to be 76.2%.

4. Contrary to previous reports, refractory hemoglobin does not always undergo a slow, steady replacement after birth and in many instances may rise after the child is born.

5. Foetal or refractory hemoglobin does not seem to be selectively destroyed in the blood of the newborn and would appear to play no special role in the production of neonatal jaundice.

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