INFLUENCE OF AGE AND SEX ON HEMOGLOBIN

A SPECTROPHOTOMETRIC ANALYSIS OF NINE HUNDRED AND NINETEEN CASES *

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To the thoughtful clinician it has long been a matter of keen regret that the very examination of the blood which is perhaps most frequently made in routine practice, namely, the estimation of the percentage of hemoglobin, is the one from which we generally derive the most unsatisfactory results. Aside from the very glaring defects in some of the instruments in common use, this has been due to the fact that we have had few accurate data as to the variations in the amount of hemoglobin at different ages. What knowledge we possess on the subject dates from the work of Leichtenstern.[1] This author recognized very clearly that before any conclusions could be drawn as to whether a person of a given age and sex had a normal amount of hemoglobin, it was first necessary to determine the norm for that age and sex. In addition to studying the effects of a number of other physiologic conditions on the hemoglobin content of the blood, Leichtenstern made determinations of the hemoglobin in a number of normal persons of both sexes, and at various ages, in order to determine the variations due to age and sex. Practically all of the knowledge of these factors which we possess is derived from this work. There are, however, a number of reasons why Leichtenstern's work is inadequate. In the first place, at that time it was not possible to crystallize human oxyhemoglobin, and therefore his results were only relative. Any absolute figures attributed to him have been obtained by using the extinction coefficients given by him with a factor obtained years after by others. Since, as was shown later by Butterfield, this factor may vary materially with slightly different conditions of experiment, and especially with different instruments, the propriety of this is more than questionable.

In addition, his observations were made on too few persons to give anything more than approximate results, a fact which he himself plainly states. For example, he examined no infants on the first day of life, and his values for infants up to four days are based on only four determinations, three in boys and one in a girl, the youngest being

* Submitted for publication June 13, 1916.

1. Leichtenstern: Untersuchungen über den Haemoglobingehalt des Blutes in gesunden und kranken Zuständen, 1878.

only 36 hours old. For three age periods (from 51 to 56, from 56 to 60 and over 60) he based his figures on a total of only five cases, for both men and women, so that in these instances the figures for one sex are based on the examination of only two persons. Such results are, of course, entirely unreliable. He did not carry his age periods beyond the sixtieth year.

In view of the inadequacy of Leichtenstern's work, and in view of the absence of any extended investigations, with methods of precision, bearing on these points, it seemed very necessary that the entire matter should be reinvestigated on an amply large material, and employing a method of acknowledged and demonstrable accuracy.

The fact that Leichtenstern's work deals entirely with persons living under conditions as they then existed in Germany, and who might or might not present hemoglobin concentrations identical with those of our patients in America, seemed a further reason for undertaking this research.

METHOD OF STUDY

Of all the methods for the estimation of hemoglobin which have been proposed, the spectrophotometric is unquestionably the most accurate. In addition to its great accuracy it possesses the important advantage of requiring only a small amount of blood, and the technic of using the spectrophotometer, when once acquired, is relatively simple, although somewhat time-consuming. Even in the comparatively primitive form, as used by Vierordt, it was a very exact instrument, and in the improved form, as used by Huefner, it is even more so. Our instrument was a new Huefner apparatus, as made by Albrecht of Tübingen.

Some months of time were devoted to attaining the necessary familiarity with the instrument so as to insure a sufficiently high degree of accuracy. A critical study of the possible sources of error was made, which resulted in the development of a technic which could be depended on to give results of a marked degree of uniformity and exactness. Through the kindness of Prof. George Dreyer, head of the department of physiology, the facilities of the physiologic laboratory were placed at our disposal. In addition, it is a pleasure to acknowledge our indebtedness to him for much advice and assistance during this preliminary work, which was begun early in 1914.

QUANTITATIVE SPECTROPHOTOMETRIC DETERMINATION OF HEMOGLOBIN: PRINCIPLES INVOLVED

Without attempting to go into the general subject of spectrophotometric examinations, the principles of which must be assumed to be already known, it may be in place to give briefly the salient points. For each colored substance the ratio, concentration to extinction

coefficient, is a constant. This constant is known as the absorption ratio. This law is generally expressed $C/E=A$. The concentration C in the case of blood is the number of grams of oxyhemoglobin in 1 c.c. of the solution examined. The extinction coefficient is the reciprocal of the thickness of solution required to reduce light of the intensity one to the intensity one tenth. In the Huefner apparatus the thickness of the layer of solution remains constant, and E is determined by measuring the intensity of the remaining light. This is effected by rotation of the analyzing with respect to the polarizing Nicol. Now it can be readily shown that the extinction coefficient, E, is equal to -2 log. cos. O, where O is the angle of rotation just referred to. In order to obtain E we measure the angle of rotation, O, necessary to produce equality in the upper and lower fields. This angle is read off on a vernier, and from this the extinction coefficient, E, is computed. Buerker has computed a curve of extinction coefficients for angles from 60 to 82 degrees, which greatly facilitates this computation. For solutions of one and the same substance, in our case oxyhemoglobin, the concentrations are directly proportional to their respective extinction coefficients, so that these are used as a convenient method of expressing relative concentrations. To convert relative into absolute values a solution of oxyhemoglobin of known strength must be prepared, its extinction coefficient, E, determined, and then $A=C/E$, or $C=E\times A$. In other words, the extinction coefficient of any solution multiplied by the absorption ratio thus obtained gives the absolute concentration of the solution. This absorption ratio was determined by Huefner for oxyhemoglobin for the spectral region from 531.5 to 542.5, and found to be 0.001312. Butterfield[2] found it to be 1.18×10^{-3} for the spectral region from 533.5 to 542.

The exact position in the spectrum, the width of slit and several other factors exercise a great influence on the value of A, as Butterfield has shown, so that it is essential, in order to be sure of the results, for each investigator to determine the value of A for his exact conditions of experiment. Since the publication of the preliminary report of this research we have done this, and the absolute values, as given in this paper, represent the relative values previously given multiplied by the factor A, with, of course, the proper correction for dilution. It is unnecessary to describe the Huefner apparatus. We had a special dark room at our disposal and the entire apparatus was further enclosed in a large hinged light-proof case, so constructed that when in use no extraneous light could reach the eye. This is a matter of much impor-

2. Butterfield, E. E.: Ueber die Lichtextinktion, das Gasbindungsvermögen und den Eisengehalt des menschlichen Bludfarbstoffs in normalen und krankhaften Zuständen, Ztschr. f. physiol. Chem., 1909, lxii, 173.

tance, since eye fatigue is the principal source of error in the method. The sector scale was calibrated in the usual manner, so that the divisions on it could be converted into wave lengths. To assure ourselves that the positions actually chosen really represented the positions of maximum and minimum absorption we determined the degree of absorption for the entire area. The positions found were from 542 to 534 and from 566.5 to 558.5. Our collimator slit was set at 0.05 mm., and remained unchanged throughout all the work. The eye piece slit was set at 8.

DETERMINATION OF THE EXTINCTION-RATIO (E'_0/E_0)

It is well known that Vierordt and his school have, through a long series of investigations, enunciated the law on which the qualitative spectrophotometric determination of hemoglobin, as well as its derivatives, is based. If E' be the extinction coefficient in one part of the spectrum, and E be the extinction coefficient in the other part of the same spectrum, then the extinction ratio E'/E is constant for one and the same hemoglobin derivatives, even at different concentrations; while on the other hand, the ratio E'/E is different for each derivative and characteristic of it. For oxyhemoglobin the ratio $E'o/Eo$ has been found by Huefner to be 1.578, or as it is generally expressed in round figures, 1.58.

Bohr[3] has called into question the identity of hemoglobin from different sources and considers that there are several hemoglobins. His researches do not bear out the contention of Huefner that the ratio $E'o/Eo$ is one and the same under identical conditions of experiment.

Aron and Müller[4] in 1906 also found an inconstancy of this ratio. On the other hand, Butterfield, using the Huefner apparatus, conducted an extensive series of experiments, and came to the conclusion that the light extinction, iron content and oxygen-binding capacity of human hemoglobin are, within the limits of error of the method employed, constants. He further found that these constant values applied, not only to the blood of normal human beings, but also to those bloods derived from patients with anemia, chlorosis, scorbutus and pseudoleukemia. While it was not the principal purpose of this research to determine the constancy of this coefficient, yet inasmuch as the value of the spectrophotometric qualitative analysis depends on the extinction ratio, it was felt to be highly desirable to determine this constant anew under the conditions of our experiments, in spite of the

3. Bohr: Nagel's Handbuch der Physiologie des Menschen, i, 51 (Bibliography).

4. Aron and Müller: Ueber die Lichtabsorption des Blutfarbstoffs, Arch. f. Physiol., 1906, Suppl., p. 109; Ztschr. f. physiol. Chem., 1906, lvi, 443.

apparently conclusive work of Butterfield. To this end, in each and every observation, readings were taken not only in the position of maximum, but in the position of minimum absorption, and from these values the extinction ratio, that is, *E'o/Eo,* was calculated for each determination. Because of the large number of determinations made by us, it was felt that the average of all of these would give a very close approximation to the real value. Every observation is included except those in which the reading in the region between the bands was below 60 degrees, since we, in common with all observers, found the accuracy in reading diminishes rapidly below this point. These observations numbered in all 838, and the ratio was found to be perfectly constant within the limits of error of the method, and to average 1.5813. Our results, therefore, corroborate in fullest measure those of Huefner and Butterfield, not only in regard to the constancy of the extinction ratio, but also in regard to its actual value. Huefner's[5] determinations average 1.578, as against our value of 1.5813.

CONSTRUCTION AND CALIBRATION OF PIPET

It is obvious, in view of the high degree of accuracy attained in the actual reading of the spectrophotometer, that the error involved in measuring and diluting the blood is likely to be several times as great as the error of reading. Leichtenstern recognized this, and came to the conclusion that it might be ten times as great. Our preliminary work led to the same conclusions, and to reduce the error in obtaining and diluting the blood to a minimum, considerable experimentation and thought were given to the question of a suitable pipet. In this matter we were rendered invaluable aid by Professor Welker, to whose kindness we are indebted for the construction and calibration of the pipet and to whose advice and skill the accuracy of this part of the work is entirely due.

In its final form the pipet consisted of a piece of heavy thermometer tubing with thick walls, but of fine caliber, and yet sufficiently large that when it was filled with blood and held vertically the column would flow down with that degree of rapidity best suited for accurate measurement. It was approximately 7 inches in length, with a circular mark about 4 inches from the tip, the latter being drawn out so as to constrict the opening to the least possible extent. Two separate calibrations of the pipet were made, with defibrinated blood, in the most careful manner, and the average of the two determinations gave the weight of blood delivered by it as 0.03865.

5. Huefner, G.: Neue Versuche zur Bestimmung der Sauerstoffkapazität des Blutfarbstoffs, Arch. f. Physiologie, 1894, p. 130.

The specific gravity of the blood was determined with two separate pycnometers, each of a different type, and found to be 1.0601 and 1.0597 respectively, the average being 1.0599. The volume of blood delivered by the pipet was, therefore, $0.03865/1.0599 = 0.03646$ c.c. It was found that this amount could be readily obtained from either the finger in adults or ear in children without resorting to undue manipulation. The advantages of having the pipet of a larger capacity than those in common use are obvious.

TABLE 1.—Comparison of Two Dilutions to Determine Accuracy of Pipet

Solution A		Solution B	
77.9	77.8	77.4	78.0
77.3	77.5	78.0	78.0
77.9	77.6	77.5	77.4
77.4	77.7	77.4	77.6
77.8	77.9	78.0	77.5
77.6	77.6	77.6	77.4
77.9	78.0	77.4	77.4
77.4	77.4	77.4	77.7
78.0	77.6	77.4	77.9
77.4	77.5	77.4	77.6
776.6	776.6	775.5	776.5

A further check of the accuracy of the calibration was made in the following way. A pipetful of defibrinated blood was added to 4 c.c. of 0.1 per cent. sodium carbonate solution, giving a dilution of 1 in 110.709. Similarly 1 c.c. of the same blood was added to 109.709 c.c. of sodium carbonate solution, and these two dilutions were then compared spectrophotometrically. The result of the comparison is shown in Table 1.

The averages were 77.66 and 77.60, respectively with the two solutions, and the extinction coefficients were 1.340 and 1.336 respectively.

The concentrations of the two specimens, as measured by these two methods, are proportional to their extinction coefficients, that is, the dilutions are to each other as 1.340 to 1.336, which gives further evidence of the accuracy of the pipet calibration. One and the same pipet was used throughout all the work. All dilutions were carried on in precisely the same manner, using a Mohr normal 5 c.c. pipet for that purpose. In the comparison given a Mohr 1 c.c. normal pipet and a normal buret were used, all bearing the standardization mark of the German Reichsanstalt.

METHOD OF OBTAINING AND DILUTING THE BLOOD

Considerable preliminary experimentation convinced us that the most satisfactory source of blood, when uniformity is the great desideratum, is the finger pulp. We used in all cases the middle finger of the left hand, except in young children, in whom the lobe of the ear was chosen. The manner of making an incision is of importance. We used cataract knives of the best quality, which were sent to the maker at short intervals for sharpening. A deep stick gave a very large flowing drop of blood, from which the pipet was filled to a point just a trifle above the mark, and then with the pipet held vertically the column was allowed to sink down exactly to the mark, the excess of blood on the tip being removed by the thumb and forefinger. This blood was then blown into 4 c.c. of 0.1 per cent. sodium carbonate solution, which had been previously measured with a Mohr 5 c.c. normal pipet into a 50 c.c. long-necked, glass-stoppered flask. Our pipet was graduated as a delivery pipet, and by blowing with force each time for ten seconds and withdrawing the tip of the pipet from the fluid, while still blowing, a high degree of uniformity was secured, as will be seen from the accuracy determinations below. The solution must be absolutely clear.

The diluted blood was then thoroughly shaken to ensure complete oxygenation, which is considerably facilitated by the relatively large size of the flask. The blood solution was now transferred into the *Trögchen* of the apparatus, which was covered with a glass slip to exclude atmospheric influences, and ten readings were taken, five on each side of the vernier scale, in the selected position (542.0 to 534.0). In all cases readings were also taken in that position of the spectrum lying between the bands (566.5 to 558.5), so that the extinction ratio could be instantly calculated, and any possible deterioration in the specimen thus disclosed. We regard this as an indispensable precaution, since it obviates the possibility of any error due to a reduction of the oxyhemoglobin, or to a possible partial conversion into methemoglobin.

SOURCES OF ERROR

In any research having to do with the establishing of norms, it is highly important to be orientated as to the possible sources of error and the magnitude of these. In our work we must consider the following: (1) error in the calibration of the pipet; (2) error in taking and diluting the blood; (3) error due to a possible reduction of the oxyhemoglobin to reduced hemoglobin or methemoglobin; and (4) error in reading.

1. *Error in Calibration.*—Any error in this respect is of course a constant one. As stated above, the volume of our pipet was obtained by

taking the mean of two calibrations, agreeing closely with each other. In addition this was checked, as above stated, by comparing spectrophotometrically the dilution, as made by our pipet, and a second similar dilution made with normal pipets standardized by the German Reichsanstalt. Two dilutions made in this way gave extinction coefficients, as we have already seen, of 1.340 and 1.336, respectively. The close agreement of these figures, in addition to the trifling variations of the figures of the two standardizations, makes it sufficiently obvious that the error of calibration is an entirely negligible factor. Since any error would be constant, it could have no effect on the relative values.

2. *Error in Taking and Diluting the Blood.*—This is, of course, a variable error. In the calibration of our pipet we proceeded in the following manner: A weighing bottle, with a roll of filter paper, was first weighed, and after the pipet had been filled with blood in the usual manner, this was blown into the bottle, the excess being wiped off on the paper and contents weighed again. Two determinations were thus made, and the weights obtained may be used as an index of the error involved in measuring the blood. They were 0.0385 and 0.0388.

Our pipet was therefore of such construction as to admit of exceedingly accurate measurements.

3. *Error Due to Deterioration of the Oxyhemoglobin.*—As we have already indicated, by taking readings in the positions of both maximum and minimum absorption, we could at once calculate the extinction ratio $E'o/Eo$ for each observation, so that no appreciable deterioration could occur without being detected immediately. All of our readings were made shortly after taking the blood, so that any deterioration was a priori, improbable. Further to test the degree of deterioration dilutions were made in the usual manner, and tested spectrophotometrically, the first immediately, the second at the end of one hour, and the third at the end of two hours. The extinction coefficients thus obtained were (immediately) 1.300, (one hour) 1.290, (two hours) 1.297. This check was made repeatedly, and in no instance did we find a deterioration sufficient to be detected, within two hours' time.

4. *Error in Reading.*—The construction of the Huefner apparatus is such that the readings can be taken with great accuracy after sufficient practice. This will be apparent from a mere inspection of the readings above given. The Gauss method, which Leichtenstern,[1] Reinert[6] and Buerker[7] have employed, enables us to determine the average and probable errors.

6. Reinert: Die Zaehlung der Blutkoerperchen, Leipzig, 1891.
7. Buerker: Tigerstedt's Handbuch, Vol. 2.

Taking a typical example, we find in one accuracy determination (Jan 1, 1915) the readings given in Table 2.

TABLE 2.—READINGS IN AN ACCURACY TEST OF HUEFNER APPARATUS *

Number Readings	Square of Variation	Variation from Average	Reading Left	Reading Right	Variation from Average	Square of Variation
1	0.0289	+0.17	76.6	76.6	+0.17	0.0289
2	0.0529	—0.23	76.2	76.4	—0.03	0.0009
3	0.0049	+0.07	76.5	76.5	+0.07	0.0049
4	0.0169	—0.13	76.3	76.6	+0.17	0.0289
5	0.1849	—0.43	76.0	76.6	+0.17	0.0289
	0.2885	.....	....	....	.....	0.0925

* Average of ten readings 76.43. $Sd^2 = 0.3810$.

Let d equal the variation from the average.

Let Sd^2 equal the sum of the squares of the individual variations.

Let Fm equal the average error of the average value.

Let N equal the number of observations.

The average error of the average value

$$F_m = \sqrt{\frac{Sd^2}{n\ (n-1)}} = \sqrt{\frac{0.3810}{10\ (10-1)}} = \pm 0.0653$$

The angular error is thus plus or minus 0.0653 degrees.

Now, $76.43 + 0.0653 = 76.4953 =$ approximately 76.50
$76.43 - 0.0653 = 76.3647 =$ approximately 76.36

Corresponding to these angles we have the extinction coefficients:

$E'o$ of 76.50 = 1.264
$E'o$ of 76.36 = 1.256

If we calculate by the same method the probable error, we find it to be $\pm\ 0.0653 \times 0.6745 = 0.0440$.

These results justify the conclusion that with ten readings the error of the average of these readings is exceedingly small, and can be safely disregarded.

ACCURACY DETERMINATIONS

Before making any of our record observations, we instituted a series of determinations extending over some time to assure ourselves that the necessary amount of familiarity with the method had been secured to attain a high degree of precision. We employed the following method: A pipetful of defibrinated blood was diluted in the usual manner with 4 c.c. of sodium carbonate solution, and the pipet washed and dried. A second pipetful of the same blood was then taken and diluted in the same way. These were then examined spectrophotometrically, ten readings being taken and the same *Trögchen* being used

in both cases. A typical determination of this sort, made on Oct. 30, 1914, is given in Table 3.

TABLE 3.—A TYPICAL ACCURACY TEST OF THE METHOD

Solution A		Solution B	
78.1	77.9	77.8	77.9
78.4	78.5	78.0	78.0
78.2	77.9	78.0	78.2
78.7	78.3	78.6	78.5
78.0	78.0	78.2	77.9
391.4	390.6	390.6	390.5

The averages were 78.20 and 78.11 for the two solutions, respectively, and the extinction coefficients were 1.379 and 1.372 respectively.

To determine the effect of increasing practice on the degree of accuracy, another series of tests were made several months later, some hundreds of determinations having been made in the interim. A typical series is here given in which on Feb. 7, 1915, three successive dilutions were made in the manner above indicated. In dilution No. 1 the extinction coefficient 1.300 was obtained, and in dilutions Nos. 2 and 3, 1.290 and 1.297 were respectively obtained.

It is obvious from these figures that the degree of accuracy attained is almost precisely the same from the beginning of our recorded observations. In order to eliminate the possibility of any self-suggestion in the reading of similar dilutions, we made further tests in the following manner: One pipetful (A) of blood was diluted with 4 c.c. of sodium carbonate solution, a second (B) with 4.5 c.c. of the same fluid. Calculation shows that the concentrations of these two solutions are to each other as 124.42 to 110.71. These two solutions were then examined spectrophotometrically with the results shown in Table 4.

TABLE 4.—ACCURACY TEST ON DIFFERENT CONCENTRATIONS OF THE SODIUM CARBONATE SOLUTION

Solution A		Solution B	
78.7	78.5	75.6	76.2
78.8	78.7	76.3	75.9
78.4	78.7	76.5	76.2
78.5	78.4	76.5	76.4
78.3	78.6	76.1	76.3
392.7	392.9	381.0	381.0

The averages were 78.56 and 76.20 with the respective solutions, and the extinction coefficients were 1.405 and 1.245.

Taking the ratios, we find $1.405/1.245 = 1.128$,

and $124.42/110.71 = 1.124$.

In other words, the two solutions were so prepared that their concentrations were to each other as 1 to 1.124, while the observed concentrations were as 1 to 1.128.

A considerable number of such tests were made with similar results. Our study of the spectrophotometric method leads us to the conclusion that it is capable of quite an extraordinary degree of accuracy, provided that the investigator have the necessary familiarity with the apparatus. With adequate practice, the error of observations made with the precautions we have used, probably does not exceed 1 per cent. We also made determinations, using thirty readings instead of ten, but were unable to increase the accuracy by so doing. Indeed, there is a real danger of lessening it by too many readings, since there is a distinct risk of overtiring the eye, which produces a rapid diminution of accuracy.

In all of these tests for the purpose of determining the accuracy of the spectrophotometric method as employed by us, precisely the same conditions obtained as in the routine determinations. The results, therefore, show the accuracy really attained under our actual working conditions, and not that theoretically possible, or attainable only under especially favorable circumstances.

SELECTION OF MATERIAL

Inasmuch as the concentration of hemoglobin may vary to a considerable degree in individuals who may regard themselves as being perfectly well, the greatest care was exercised to ascertain that the individual examined was in reality normal. The numbers available at all ages were sufficiently great to make it a matter of ease to reject all unsuitable observations. In spite of the painstaking efforts to secure perfectly normal subjects, no attempt was made at any especial selection. In other words, individuals remarkable for their strength or physical development were only chosen as they happened to fall naturally within the scope of our observations. To avoid any possible error due to a preponderance of some particular type of subject, our observations were fairly evenly distributed among all walks of life, so that it is believed that the results represent a fair average. All observations were excluded if the individual had recently had any sickness, was evidently in poor nutrition, or felt below par in any way. In the case of babies and young children only such were taken as were evidently thriving, gaining normally in weight, and who had not had any sickness of conesquence within a year. By far the larger part of our babies were breast fed.

In the age groups included between the 10th and 30th years a considerable number of observations were made on schoolchildren and college students, and these observations were for the most part made shortly after the summer vacation, since the general state of health and nutrition was at this time presumably at its best. In addition to the above precautions, which are of considerable importance if the resulting figures are to establish really normal values, care was taken, as will be evident at a glance from the table, to have substantially the same number of observations in each of the various age groups, and still further to have approximately the same number for each sex. The minimum number of observations on which any figure for an age period is based is thirty, except in the comparison of the sexes, in which, of course, the number for each sex is one half the total for the group. Our results are based on a study of 919 persons, 464 male, and 455 female. With the exception of a few children, all of the determinations were made in the midafternoon hours.

METHOD OF PREPARATION OF HEMOGLOBIN

Several hundred cubic centimeters of blood were obtained from each of two healthy adults by venepuncture, and defibrinated as usual. This was then centrifugalized and the corpuscles washed several times, thus removing the major portion of the serum proteins. It was then laked by the addition of ether, added in successive small portions until a clear solution was obtained. If the solution was too viscid, sufficient water was added to bring it to the proper degree of fluidity. The blood was then treated with an approximately equal volume of aluminum cream, as suggested by Marshall and Welker.[8] The aluminum cream was prepared in the manner described by Tracy and Welker.[9] After thorough mixing and filtering, the filtrate being perfectly clear, this was placed in a refrigerator, cooled down to —3 or —4 C. and then treated with absolute alcohol until the percentage of the latter equaled 25 or 30. This was allowed to remain at the above temperature until crystallization was completed. The mass of hemoglobin crystals on the filter was then transferred to a weighing bottle of known weight, and the moist weight of the mass of crystals determined. From this a suitable sample was removed for spectrophotometric examination, placed in a very small beaker, and then dissolved in a very little 0.1 per cent. sodium carbonate solution, and transferred quantitatively to a 10 c.c. standardized flask, which was then filled up

8. Marshall and Welker: The Precipitation of Colloids by Means of Aluminum Hydroxid, Jour. Am. Chem. Soc., 1913, xxxv, 820

9. Tracy and Welker: The Use of Aluminum Hydroxid Cream for the Removal of Albumin in Nitrogen Partition in Urinary Analysis, Jour. Biol. Chem., 1915, xxii, 55.

to the mark with the same solution. A preliminary reading was made to determine the approximate concentration, and with this as a guide the solution was further diluted so as to bring the readings in the region between 60 and 80 on the scale, since this is the position of optimum accuracy. Readings were then taken in both positions in the spectrum in the way already described.

The weighing bottle with its contents was then placed in a desiccator over sulphuric acid, where it remained until it attained a constant weight. From this the dry weight of the sample was calculated, and this weight, divided by the dilution, gives the concentration, that is, the amount of hemoglobin in 1 c.c. of the solution tested. To make sure that no reduction or conversion of the oxyhemoglobin had taken place, readings were invariably taken with great care in both regions of the spectrum. The ratio of these extinction coefficients gave a constant result of 1.581, within the allowable limits of error. Blood was obtained from two separate individuals, and crystals prepared as above described from each,

DETERMINATIONS OF THE ABSORPTION RATIO (A'_0)

In the preliminary report[10] of this work all the values as there given were relative only, that is, they represented the extinction coefficients obtained by taking —log. cos.^{2} of the angles of rotation. In order to convert these relative into absolute values, that is, to determine the actual hemoglobin concentration, it is necessary to multiply the extinction coefficients by a constant factor, namely, the absorption ratio $A'o$. While the value of $A'o$ has been determined by several authors, notably Huefner, later researches made by Butterfield[2] in Huefner's laboratory give a quite different value. Moreover, Butterfield has shown that the absorption ratio may vary considerably with very slightly different conditions of experiment.

Since the accuracy of the absolute values depends on the correctness of the absorption ratio, it was felt to be important to establish this for our conditions of experiment.

In order to do this it was necessary first to prepare pure crystalline human hemoglobin. After the purity of this had been established spectrophotometrically by determining the extinction ratio, a solution of known concentration was made up. This solution was then placed in the spectrophotometer, the angle of rotation measured and from this the extinction coefficient ($E'o$) of the solution calculated. Since the concentration is known, the absorption ratio ($A'o$) can be determined from the formula $A'o=C/E'o$.

For the preparation of the hemoglobin I am indebted to the courtesy of Professor Welker, and the results are extracted from a joint

10. Williamson: Jour. Am. Med. Assn., 1915, lxv, 302.

paper in process of preparation, in which, in addition to human hemoglobin, a number of hemoglobins from different animals are being studied, Professor Welker undertaking the preparation of them, the present author determining the optical constants.

HUMAN HEMOGLOBIN

Crystallization No. 1: The dry weight of the specimen was 0.0997 gm. This was dissolved in 0.1 per cent. sodium carbonate solution, and made up to 10 c.c. in a standardized flask. From this solution three subdilutions were made, using 1 c.c. of the above for each, and diluting by adding 5.5 c.c. of sodium carbonate solution, making the total dilution 1 in 65.

Each of these subdilutions was placed in the *Trögchen* and ten readings taken in each of the two positions in the spectrum already alluded to. Table 5 gives the results.

TABLE 5.—DETERMINATION OF $A'o$

Subdilution	Average of Ten Readings (542.0 to 534.0)	Extinction Coefficient E'_o	Average of Ten Readings (566.5 to 558.5)	Extinction Coefficient E_o
No. 1	77.19	1.309	67.40	0.831
No. 2	77.36	1.320	67.04	0.818
No. 3	77.21	1.310	68.13	0.858

The average $E'o = 1.313$; and the average $Eo = 0.836$. The concentration $= 0.0997/65 = 0.001534$. $A'o = C/E'o = 0.001534/1.313 = 0.001168$. $Ao = C/Eo = 0.001534/0.836 = 0.001835$.

That the solution was actually a pure oxyhemoglobin solution is shown by the extinction ratio. $E'o/Eo = 1.313/0.836 = 1.571$.

Crystallization No. 2: The dry weight of the specimen was 0.02479 gm. This was dissolved in 0.1 per cent. sodium carbonate solution, and made up to 10 c.c. in a standardized flask. From this solution three subdilutions were made until the final dilution was 1 in 17.

TABLE 6.—DETERMINATION OF $A'o$

Subdilution	Average of Ten Readings (542.0 to 534.0)	Extinction Coefficient E'_o	Average of Ten Readings (566.5 to 558.5)	Extinction Coefficient E_o
No. 1	75.73	1.216	65.91	0.778
No. 2	76.25	1.248	66.01	0.782
No. 3	75.62	1.210	66.07	0.784

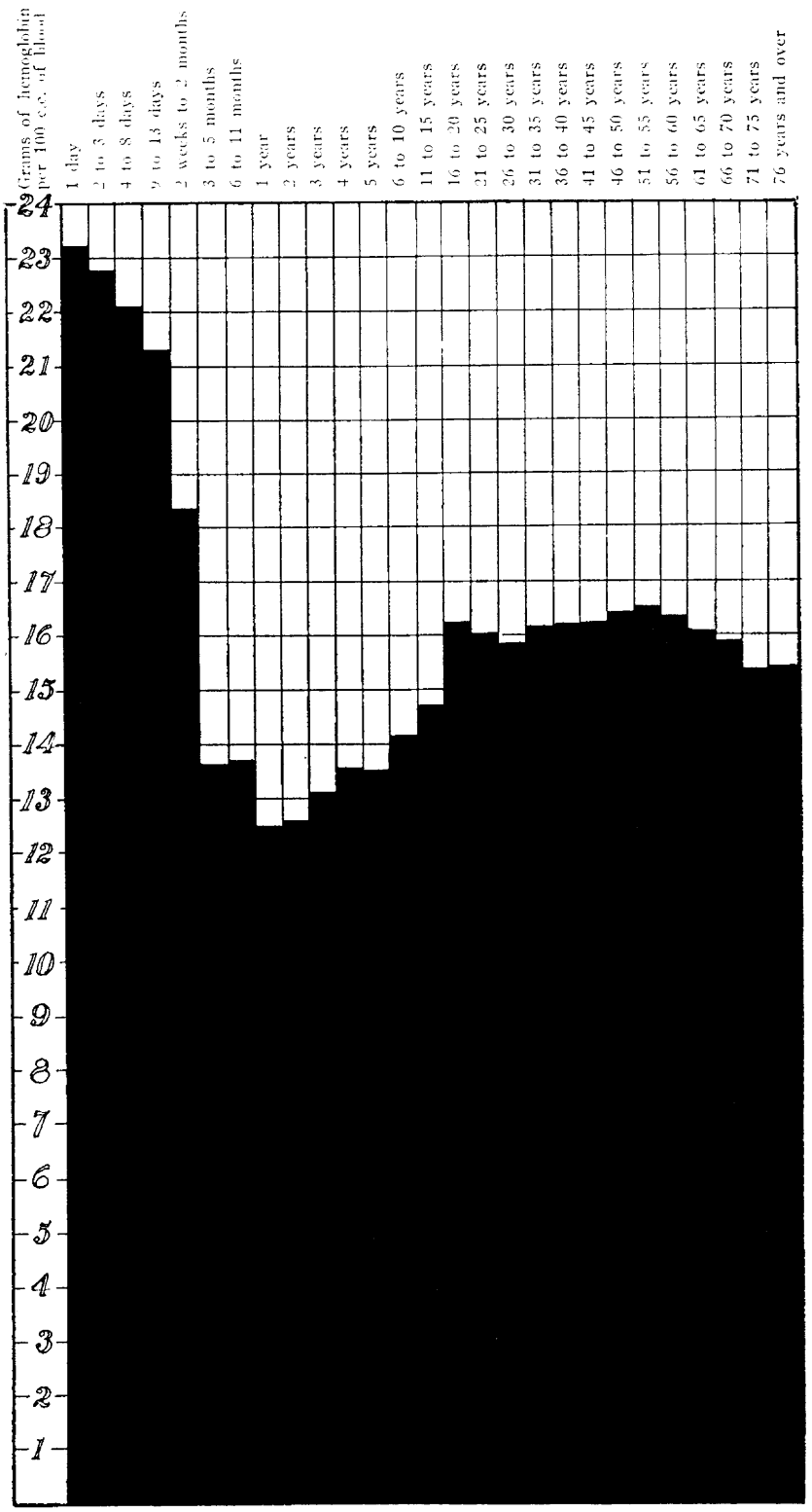

Fig. 1.—Grams hemoglobin per 100 c.c. of blood in persons ranging in age from 1 day to over 76 years.

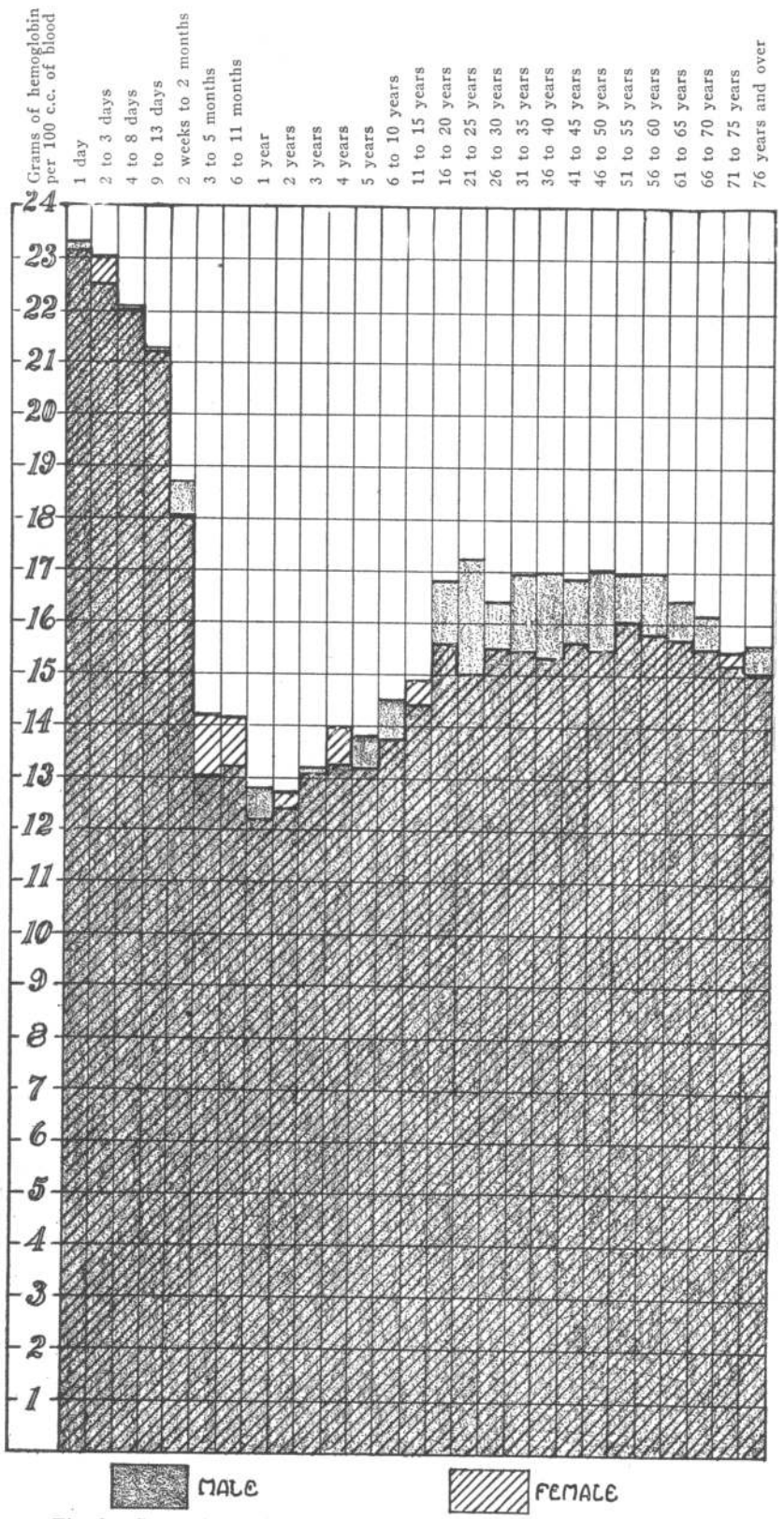

Fig. 2.—Grams hemoglobin per 100 c.c. of blood in the male and in the female, for the ages ranging from 1 day to over 76 years.

The average $E'o = 1.225$; and the average $Eo = 0.781$. The concentration $= 0.02479/17 = 0.001458$. $A'o = C/E'o = 0.001458/1.225 = 0.001190$. $Ao = C/Eo = 0.001458/0.781 = 0.001867$.

That the solution was actually a pure oxyhemoglobin solution, is shown by the extinction ratio. $E'o/Eo = 1.225/0.781 = 1.569$.

TABLE 7.—Results of Examination

Age	Extinction Coefficient, Dilution 1:110.71			Grams of Hemoglobin per 100 C.c. of Blood			Number of Cases		
	Male	Female	Both	Male	Female	Both	Male	Female	Both
1 day	1,7859	1.7763	1.7813	23.31	23.19	23.25	16	15	31
2 to 3 days	1.7239	1.7657	1.7454	22.50	23.05	22.78	15	16	31
4 to 8 days	1.6965	1.6936	1.6949	22.14	22.11	22.12	15	18	33
9 to 13 days	1.6366	1.6342	1.6355	21.36	21.33	21.35	15	15	30
2 weeks to 2 mos.	1.4329	1.3822	1.4109	18.70	18.04	18.42	15	15	30
3 to 5 months	1.0019	1.0915	1.0467	13.08	14.25	13.66	16	16	32
6 to 11 months	1.0129	1.0876	1.0499	13.22	14.19	13.70	18	15	33
1 year	0.9806	0.9367	0.9599	12.80	12.23	12.53	18	16	34
2 years	0.9530	0.9727	0.9631	12.44	12.70	12.57	16	17	33
3 years	1.0121	1.0041	1.0081	13.21	13.11	13.16	15	16	31
4 years	1.0177	1.0710	1.0435	13.28	13.98	13.62	16	15	31
5 years	1.0596	1.0166	1.0375	13.83	13.27	13.54	17	18	35
6 to 10 years	1.1163	1.0498	1.0861	14.57	13.70	14.18	18	15	33
11 to 15 years	1.1090	1.1389	1.1252	14.48	14.87	14.69	17	20	37
16 to 20 years	1.2879	1 1986	1.2447	16.81	15.64	16.25	16	15	31
21 to 25 years	1.3203	1.1515	1.2270	17.23	15.03	16.02	17	21	38
26 to 30 years	1.2572	1.1895	1.2169	16.41	15.53	15.88	19	28	47
31 to 35 years	1.2975	1.1832	1.2403	16.94	15.44	16.19	16	16	32
36 to 40 years	1.3008	1.1771	1.2428	16.98	15.36	16.22	17	15	32
41 to 45 years	1.2913	1.1981	1.2432	16.85	15.64	16.23	15	16	31
46 to 50 years	1.3080	1.1866	1.2588	17.07	15.49	16.43	22	15	37
51 to 55 years	1.2993	1.2323	1.2687	16.96	16.08	16.56	19	16	35
56 to 60 years	1.3000	1.2074	1.2537	16.97	15.76	16.36	15	15	30
61 to 65 years	1.2612	1.2033	1.2313	16.46	15.71	16.07	15	16	31
66 to 70 years	1.2405	1.1880	1.2159	16.19	15.51	15.87	17	15	32
71 to 75 years	1.1663	1.1848	1.1750	15.22	15.46	15.34	19	17	36
76 and over	1.2003	1.1519	1.1793	15.67	15.04	15.39	30	23	53
							464	455	919

Average for the two crystallizations: No. 1 $A'o = 0.001168$; No. 2 $A'o = 0.001190$; average $A'o = 0.001179$. No. 1 $Ao = 0.001835$; No. 2 $Ao = 0.001867$; average $Ao = 0.001851$.

In common with other observers, we find that the readings in the band ($A'o$) are slightly more accurate than those taken between the bands. In accordance with this all our absolute figures have been calculated by multiplying the extinction coefficient of the readings in this position, $E'o$, by the average absorption ratio, $A'o = 0.001179$. This result, multiplied by the dilution 110.71, gives the concentration, that is, the amount of hemoglobin in each cubic centimeter of blood. For convenience sake we have expressed the results in grams of hemoglobin per 100 c.c. of blood, since this permits us to avoid fractions.

CHARACTERISTICS OF THE AGE CURVE

By far the highest values attained are at birth ($E'o = 1.781 = 23.25$ gm.). This substantiates the results of many investigators. Beginning immediately, there is a very rapid decline, so that from the 3rd to the 5th month the average value is nearly down to the minimum ($E'o = 1.047 = 13.66$ gm.) and is far below the average adult figure. From the 5th month onward there is a relatively gradual diminution, but the actual minimum ($E'o = 0.960 = 12.53$ gm.) is not reached until 1 year, that is in the 2d year of life. During the next year also the same minimum value is found, and only after the child has completed its 2d year does the curve rise again. This is then fairly rapid up to the 16th year, and from the 16th to the 55th year the variations in the different age periods are very slight.

The immediate and rapid decline in the amount of hemoglobin after birth and continuing up to the 6th month, and to a less extent up to the 1st year, is a highly characteristic feature of the age curve. Our results are in this respect in accord with those of Leichtenstern. This decline is quite independent of nutrition, since, as has been above stated, the greatest care was exercised to select only infants that were thriving. Moreover, it is not a question of artificial feeding, since the great majority of our cases, especially up to the 6th month, were breast-fed infants.

A noticeable feature of our curve is the fact that in the age period from 16 to 20 years the values have attained practically the maximum ($E'o = 1.245 = 16.25$ gm.) and from this period to middle age the curve is nearly horizontal. The very highest point is attained in the period from 51 to 55, but the variation in this period from the other younger adult values is insignificant. From the 55th year onward the values decline steadily up to the 75th year ($E'o = 1.175 = 15.34$ gm.). After the 76th year there is a rise, but it is so small as to be negligible ($E'o = 1.179 = 15.39$ gm.). The sharp drop found by Leichtenstern in the period from 55 to 60 is seen, on the basis of our larger number of patients, not to exist. The rise in old age found by him, was based on only five cases in all, whereas our figures for the age period over 76 alone is based on a total of fifty-three persons.

INFLUENCE OF SEX

An inspection of Figure 2 shows that the values from birth up to the 15th year are almost identical for both sexes. At birth the difference is so slight that it can just be portrayed graphically on our curve: male $E'o = 1.786 = 23.31$ gm.; female $E'o = 1.776 = 23.19$ gm.

It must be remembered that the figures on which the values for each sex are based, represent, of course, only one-half the total number of cases. It is, therefore, to be expected that slight variations will occur in these curves to a somewhat larger extent than when the figures for both sexes are combined in a common curve. One especially valuable observation was made on twins, a boy and a girl, both perfectly healthy, of approximately the same weight, whose blood was first examined when they were 5 hours old. The values were almost identical. In order to formulate more precisely the relative values for the two sexes up to the age of puberty we have calculated the average extinction coefficient for each sex separately, as shown by Table 8.

TABLE 8.—INFLUENCE OF SEX ON HEMOGLOBIN IN CHILDHOOD

Age	Sum of Extinction Coefficients, Male	Number Cases	Sum of Extinction Coefficients, Female	Number Cases
1 day	28.576	16	26.645	15
2 to 3 days	25.858	15	28.252	16
4 to 8 days	25.447	15	30.486	18
9 to 13 days	24.550	15	24.513	15
2 weeks to 2 months	21.593	15	20.733	15
3 to 5 months	16.031	16	17.464	16
6 to 11 months	18.333	18	16.314	15
1 year	17.650	18	14.988	16
2 years	15.248	16	16.536	17
3 years	15.181	15	16.071	16
4 years	16.284	16	16.065	15
5 years	18.014	17	18.299	18
6 to 10 years	20.093	18	15.747	15
11 to 15 years	18.853	17	22.779	20
	281,711	227	284.892	227

The average extinction coefficient for males is $1.241 = 16.20$ gm.; for females, $1.255 = 16.38$ gm.

From these averages it is clear that the variations in hemoglobin due to sex are, up to the 16th year, hardly greater than the error in the method, and may, for all practical purposes, be neglected.

On the other hand, from the 16th to the 70th year the sex differences make themselves plainly manifest. While the variations are, for either sex, relatively slight within these periods, the values for women are, in every instance, considerably lower than those for men. From the 50th to the 70th year, that is, after the cessation of menstruation, it should be noted that the differences between the sexes still exists, although to a less marked degree than during the child-bearing period.

Table 9 shows the differences due to sex from the 16th to 70th year.

TABLE 9.—Influence of Sex on Hemoglobin in Adult Life

Age	Sum of Extinction Coefficients, Male	Number Cases	Sum of Extinction Coefficients, Female	Number Cases
16 to 20 years	20.606	16	17.979	15
21 to 25 years	22.445	17	24.181	21
26 to 30 years	23.887	19	33.308	28
31 to 35 years	20.759	16	18.932	16
36 to 40 years	22.114	17	17.657	15
41 to 45 years	19.370	15	19.169	16
46 to 50 years	28.778	22	17.800	15
51 to 55 years	24.688	19	19.718	16
56 to 60 years	19.500	15	18.116	15
	202.147	156	186.860	157

The average extinction coefficient for males is $1.296 = 16.92$ gm.; for females, $1.190 = 15.53$ gm.

It will be seen from Table 9 that from the ages of 16 to 60 inclusive the hemoglobin of the two sexes are in the ratio of 1.296 to 1.190; or, otherwise expressed, the female averages 91.8 of the value of the male. Expressed in absolute terms, the men average 16.92 gm., the women 15.53 gm.

TABLE 10.—Influence of Sex on Hemoglobin for the Ages, Sixty-One to Seventy Years

Age	Sum of Extinction Coefficients, Male	Number Cases	Sum of Extinction Coefficients, Female	Number Cases
61 to 65 years	18.919	15	19.254	16
66 to 70 years	21.089	17	17.821	15
	40.008	32	37.075	31

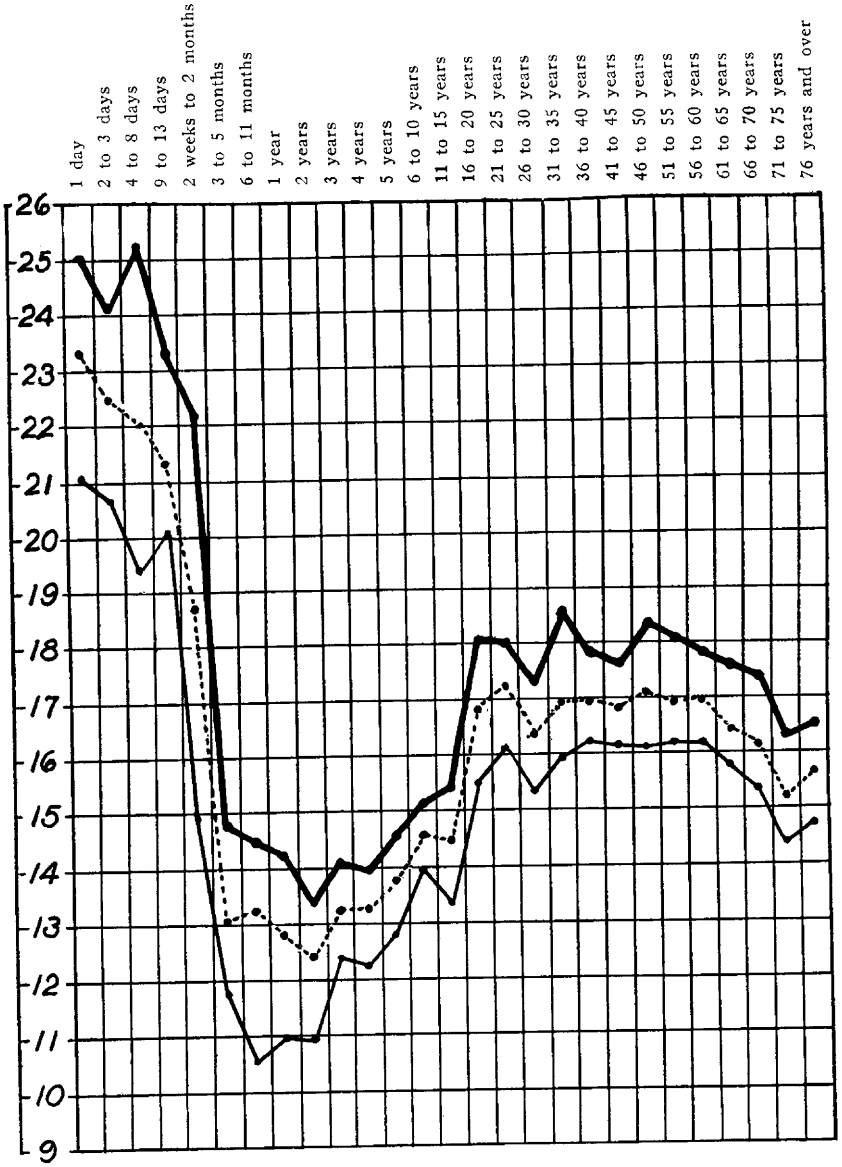

Fig. 3.—The average amount of hemoglobin, and the average amount for those having more than the average and also for those having less than the average, for males ranging in age from 1 day to over 76 years.

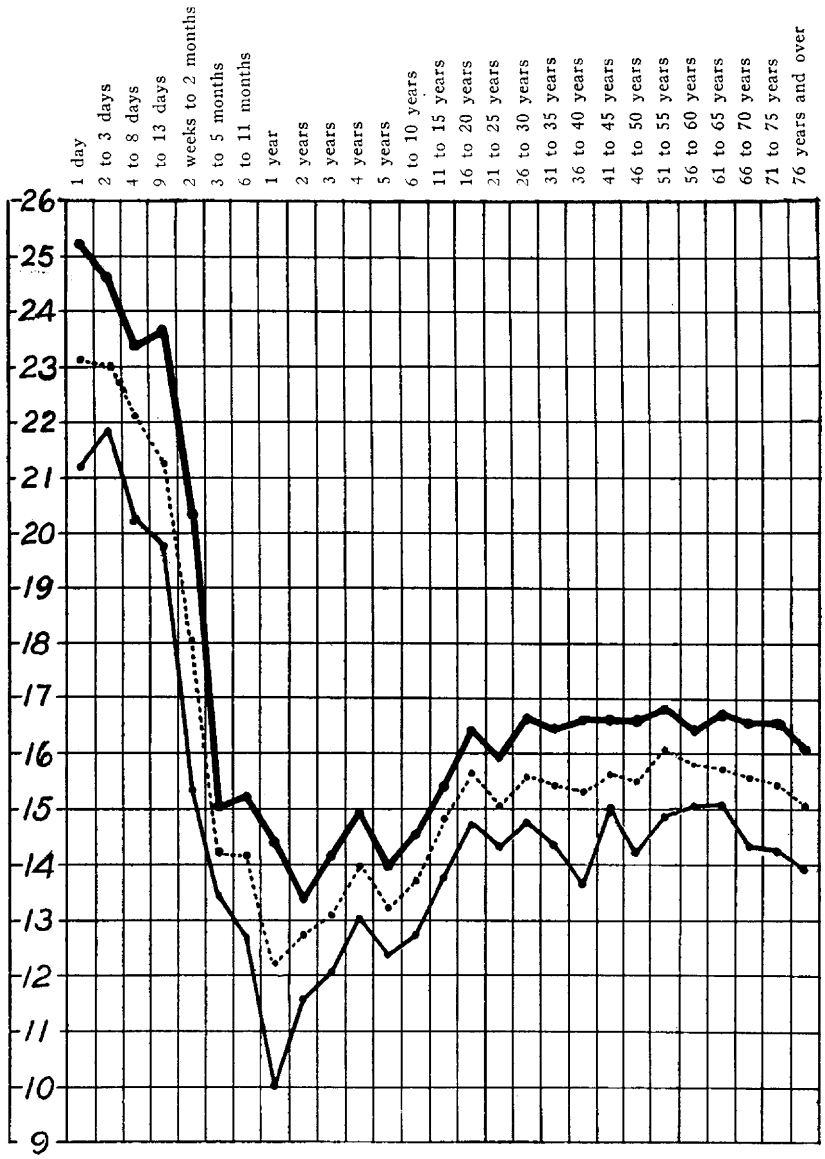

Fig. 4.—The average amount of hemoglobin, the average amount for those having more than the average and also for those having less than the average, for females varying in age from 1 day to over 76 years.

For the ages from 61 to 70 inclusive the values are as 1.250 to 1.196, that is, the value for the women is 95.6 per cent. of the value for the men. Expressed in absolute terms, the men average 16.32 gm., the women 15.61 gm.

TABLE 11.—Influence of Sex on Hemoglobin for the Ages Above Seventy-One Years

Age	Sum of Extinction Coefficients, Male	Number Cases	Sum of Extinction Coefficients, Female	Number Cases
71 to 75 years.....	22.161	19	20.142	17
Over 76.....	36.010	30	26.495	23
	58.171	49	46.637	40

The averages are 1.187 for the men, and 1.166 for the women, or, expressed in absolute terms, 15.49 and 15.22 respectively.

It is, of course, quite desirable to know within what limits the hemoglobin values vary in individuals of the same age and sex. To obtain what might be properly regarded as an average variation we adopted the following method: We first averaged all of the values in a given group, then took all the values above this average, and averaged them separately, repeating the process with all the values below the average. This was done for male and female separately, and the results are shown in Figures 3 and 4.

An inspection of these figures shows that the variations from the average are somewhat greater in very early life. This is, of course, almost self-evident, when we realize how marked a difference in hemoglobin may be produced by a very slight difference in age during the early months of life. From the 3rd or 4th year onward this average variation is fairly constant, and amounts to approximately 1 gm. above or below the average norm.

CONCLUSIONS

From a simple inspection of the Figures the following conclusions are obvious:

1. The amount of hemoglobin in the blood of normal persons varies greatly at different ages, and follows a well-defined curve.

2. These age variations are so great that in determining whether a given blood contains more or less hemoglobin than normal, it is imperative to consider the age. These variations are greatest from birth to the 16th year.

3. Between the ages of 16 and 60 there is a marked difference between the two sexes, this difference growing less after the 60th year.

4. In view of these facts, it is evident that hemoglobinometers should be standardized in absolute terms, most conveniently in grams of hemoglobin per 100 c.c. of blood. (Because of the superior accuracy attained, it is highly desirable that the standardization of hemoglobinometers should be spectrophotometrically controlled.)

5. Whether or not a given blood contains a greater or less amount of hemoglobin than the normal can be determined only by a comparison of the absolute value obtained by a hemoglobinometer thus standardized, with the normal value for that age and sex, as shown by Table 7.