THE RELATION BETWEEN RED BLOOD CELL DENSITY AND CORPUSCULAR HEMOGLOBIN CONCENTRATION

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This investigation is concerned with determinations of red blood cell density and mean corpuscular hemoglobin concentration in man, and with their bearing on the problem of red cell structure. At first it seemed that both the density and the quantity of hemoglobin in unit volume of red blood cells would be simple to measure, but a detailed investigation brought several unexpected difficulties to light, including inadequacies in some of the standard methods.

The samples of blood were obtained by vein puncture from persons acting as blood donors, and preoperatively and postoperatively from a series of patients. None of the cases showed blood dyscrasia, but no attempt was made to confine the investigations to those with a hemoglobin concentration in the normal range. On the contrary, blood from cases of anemia from hemorrhage and of the comparatively mild postoperative anemia formed about half of the material, since variations in hemoglobin concentration were sought rather than avoided. The blood samples were withdrawn into small bottles containing heparin.

Methods

Density—The heparinized whole blood is centrifuged at 4000 R.P.M. for 2 hours in a large International centrifuge. The plasma is drawn off by suction, and the upper layers of cells together with plasma which always remains near the surface are removed with capillary pipettes and blotting paper. If the removal is thoroughly done, the mass of cells remaining is at least 98 per cent packed, as can be shown by spinning a sample in a high speed hematocrit.

A 0.5 ml. micro pipette ( "to contain" ), with a ground tip and a constriction in the region of the 0.5 ml. mark, is thoroughly dried and weighed to 0.1 mg. A rubber teat is attached, and the pipette is introduced with the teat compressed into the bottom of the tube containing the packed red cells. It is important to compress the teat before the pipette is dipped into the cell mass, and not after, for if air bubbles are expelled they may not rise through the viscous mass, and, drawn up into the pipette where they may remain undetected because of opacity, may affect the density determinations. The sample of the cell mass should be drawn up no further beyond

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the mark than can be helped; this is easy, as the mass is very viscous. The teat is removed, and the volume adjusted to the mark by tapping the tip of the pipette on filter paper; the pipette is then wiped off on the outside with a moist cloth, dried, and weighed without delay.

The pipette with its contents is introduced into a 100 ml. flask, the teat reapplied, and the contents forced out. The pipette is then rinsed with three successive 1 ml. volumes of water, so as to transfer all of the sample to the flask without loss.

The weight of the sample of packed cells being known, and the weight of the same volume of water having been found by a calibration of the pipette, the density of the cells can be calculated. Repeated determinations on the same sample of cells show that the variation in the weight of successive samples does not exceed ±0.5 mg.; this means that the density can be determined with an accuracy of ±0.001. When the pipette is filled with water, as in calibrating it, the accuracy is not quite so good, for the water, being so much less viscous than the packed cells, is more difficult to adjust to the mark. I have therefore used the average of ten determinations as the weight of water which the pipette holds. The densities are calculated with water at the same temperature taken as unity.

Iron Determinations—Wong's method (1) is modified as follows: The sample of packed cells with the added 3 ml. of water is swirled around in the flask until it is hemolyzed and spread evenly over the bottom and sides. This swirling should take several minutes, for it is most important to obtain homogeneity of the material, which tends to form lumps. 4 ml. of concentrated H$_2$SO$_4$ (Fe-free) are added drop by drop, and the swirling is continued, so that the final mixture is quite homogeneous. After it has stood for about 2 hours, 4.0 ml. of saturated potassium persulfate are added, again with swirling, and the mixture is allowed to stand for another hour. 50 ml. of water are added, and following this 4 ml. of Fe-free sodium tungstate (Klett Manufacturing Company). After another hour of standing, water is added to the 100 ml. mark, and the contents of the flask are filtered through a medium filter paper. The filtrate should be clear and colorless, and when placed in the colorimeter should read substantially the same as water. 2

To 10 ml. of the filtrate are added 0.5 ml. of saturated potassium persulfate

1 I have found that the best results with Wong's iron method are obtained if the sulfuric acid and persulfate are allowed to act for these long times, and that quite irregular results are obtained if Wong's original directions (agitating the mixture for a minute or two, cooling under the tap, etc.) are followed.

2 The filtrate may show a brown tinge if too little acid or tungstate is used, and may become slightly opalescent if allowed to stand too long before color is developed. In either case it must be discarded.
and 2 ml. of 3 N potassium thiocyanate. The red color develops almost instantly, and is read in a Klett-Summerson colorimeter against water set at 0. After some 10 minutes or so, the color slowly fades.

A standard, in which color is developed at the same time as in the unknown, is made by taking 5 ml. of a standard iron solution containing 0.1 mg. of ferric iron per ml., adding 4 ml. of H₂SO₄ and 4 ml. of potassium persulfate, making up to the mark, and developing color in a 10 ml. sample in the same way as in the unknown. Under my working conditions, the reading of the standard does not vary by more than ±1 per cent from day to day. A blank is prepared by taking 4 ml. of H₂SO₄, 4 ml. of persulfate, making up to 100 ml. with water, and developing the color in a 10 ml. sample. The reading of the blank is small and constant for the same set of reagents.

The results obtained by this method are reproducible with an accuracy of about ±2 per cent, and the known amounts of iron added to the sample of packed cells may be measured with about the same precision. Beer's law is obeyed over the working range.

To convert the result of the iron determination into values for hemoglobin, I have taken the iron content of hemoglobin as 336 mg. per cent (see "Discussion").

**Difficulties in Measuring Density**—At first I tried to measure the density of the cells from the density of whole blood, then of the plasma, and the percentage volume as obtained by a high speed hematocrit, but the final figure was found to be very sensitive to errors in the hematocrit determination, and so the method was abandoned.

I then tried a method described by Linderstrøm-Lang (2) for the determination of density. I found two sources of difficulty with this method. (a) Droplets of packed cells do not come to equilibrium as quickly as do droplets of the standard, for because of the high concentration of lipid in the packed cells the drop is not entirely insoluble in the kerosene and bromobenzene, nor is the lipid entirely insoluble in it. There is accordingly some doubt about the equilibrium position, but as the final observation can be made at the end of a fixed time, e.g. 5 minutes, this is a minor difficulty. (b) Successive droplets from the same sample of packed cells do not always take up the same equilibrium position, and the variations may be sufficient to affect the third place in the values for density. This may be due to the effect of lipids in attracting bromobenzene into the cells, to inhomogeneities in the samples of packed cells (cf. Ponder (3)), or to both.

**Difficulties in Determining Hemoglobin**—Before finally adopting the modified Wong method described above, I used the Sahli acid hematin method and Wu's alkali hematin method, both of which turned out to be
unsatisfactory and unreliable as compared with the iron determinations. The errors inherent in these methods are the subject of a separate note (4).

Results

Table I shows the data obtained for 60 determinations of red blood cell density (gravimetric method) and of hemoglobin concentration (modified Wong iron method). The relation of these two variables to each other, as expressed by the correlation between them, constitutes the principal conclusion of this investigation.

Table I

<table>
<thead>
<tr>
<th>Data from 60 Determinations</th>
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<tbody>
<tr>
<td>Density, mean value</td>
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<td>S. D.</td>
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<tr>
<td>Mean corpuscular Hb concen.</td>
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<tr>
<td>S. D.</td>
</tr>
<tr>
<td>Correlation coefficient, r,</td>
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<tr>
<td>between density and Hb conc.</td>
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</tbody>
</table>

* Corresponding to 113 ± 5.7 mg. of Fe.

An important statistical problem arises in connection with the interpretation of the correlation coefficient, and I am very grateful to Dr. Joseph Berkson of the Mayo Clinic for supplying me with the following solution. The problem is this: Suppose that there are two attributes, X and Y (e.g., density and hemoglobin concentration), the correlation between which is unity or, more generally, r. If we measure X and Y by any real methods, a series of experimental errors will arise, and these will tend to make it seem that the correlation between X and Y is less than it really is. If the standard deviations are known, what will be the effect of the errors on the value of r?

Let X, Y be the variates.

\[ z = (X - \bar{X}), y = (Y - \bar{Y}) \] (deviations from the mean)

\[ \sigma_X = \sigma_z, \sigma_Y = \sigma_y \] (standard deviations of the variates)

\[ r_{xy} = \frac{\Sigma xy}{\sqrt{\Sigma x^2 \Sigma y^2}} = \frac{(\Sigma xy)^2}{n \sigma_x^2 \sigma_y^2} \]

Introduce an error of measurement \( dx \) into X and \( dy \) into Y.

\[ x' = x + dx, y' = y + dy \] (modified variates)

\[ z' = x + dx, y' = y + dy \] (deviations from the mean)

If \( dx \) is normally distributed around 0 and uncorrelated with \( dy \) and its S.D. is \( \varepsilon_x \), and similarly if for y the S.D. is \( \varepsilon_y \), then

\[ r_{x'y'}^2 = r_{xy}^2 \left( \frac{1 + \frac{\varepsilon_x^2}{\sigma_x^2}}{\frac{1 + \varepsilon_y^2}{\sigma_y^2}} \right) \]

(\( r_{x'y'}^2 \) is the modified correlation)

\[ \Sigma(x + dx)(y + dy) = \Sigma xy \] (because the \( dx \), or \( dy \) products = 0)

\[ \sigma_{x'}^2 = \frac{\Sigma(x + dx)^2}{n} = \frac{\Sigma x^2 + \Sigma dx^2 + 2\Sigma dx x}{n} = \sigma_x^2 + \varepsilon_x^2 \]

\[ \sigma_{y'}^2 = \sigma_y^2 + \varepsilon_y^2 \]
The average value for density lies between that of Lindeboom (5) or of Macleod (6), the former giving 1.103 for normal humans, and the latter 1.0989. The most interesting result, however, is the low value for \( r \), the coefficient of correlation between corpuscular hemoglobin and cell density. This means that the density is determined only in part by the hemoglobin concentration as measured.

One explanation of the result is that since hemoglobin is produced intracellularly from colorless precursors of about the same density as itself, a deficiency of the pigment may result in its place being occupied by another protein (the precursor) of about the same density. In this series there occur, for example, two specimens with the same density, 1.099, but with mean corpuscular hemoglobin concentrations of 32.7 and 36.4. The difference, 3.7 per cent, gives an idea of the amount of protein other than hemoglobin which might occupy the place of the pigment in the cells with the lower hemoglobin content. The presence of proteins other than hemoglobin has frequently been suggested on many different grounds and the low correlation between red cell density and mean corpuscular hemoglobin may point in the same direction.

An alternative explanation for the result may be that the weight of hemoglobin indicated by 1 gm. of iron varies, for Morrison (10) found the Fe content in fourteen samples of human hemoglobin to be from 305 to 4

\[
\frac{r'}{r} = \sqrt{\frac{(\Sigma xy)^2}{n^2(\sigma_x^2 + \sigma_y^2)(\sigma_x^2 + \sigma_y^2)}} = \sqrt{\left[1 + \left(\frac{\epsilon_x}{\sigma_x}\right)^2\right]\left[1 + \left(\frac{\epsilon_y}{\sigma_y}\right)^2\right]}
\]

So, as in our specific case (see Table I) if without error the correlation is \( r \) and there is introduced into \( x \) an error for which the standard deviation \( \epsilon_x = 0.17\sigma_x \) and into \( y \) an error of measurement for which the s.d. is \( \epsilon_y = 0.25\sigma_y \), \( r' \) the new correlation will be

\[
r' = r \sqrt{\frac{1}{(1 + 0.17^2)(1 + 0.25^2)}} = 0.96r
\]

i.e., the correlation coefficient will not be appreciably affected by the errors of measurement associated with the experimental methods.

Using the mean value of all the density determinations and taking the density of a 1 per cent NaCl solution as 1.0075, we get a value for the density of intracellular hemoglobin of 1.32. Svedberg and Pedersen (7) give a value of 0.749 for the partial specific volume of hemoglobin, based on the work of Svedberg and Fähraeus (8). This corresponds to a density of 1.335. Perutz (9) gives a density of 1.242 for the wet crystals.
338 mg. per cent. I have based my calculations on 336 mg. per cent, which does not allow for variation in the Fe content, and it is possible that this factor, plus experimental errors still remaining in the methods, may account for, or at least contribute to, the low correlation found between hemoglobin content and density. For example, the apparent difference between the hemoglobin contents of the two samples with a density of 1.099, supposed to have 32.7 and 36.4 per cent of hemoglobin on the basis of calculation from the iron values, might be due to the iron content of the hemoglobin of the first sample differing from that of the second by about 10 per cent, which, on the basis of Morrison’s results, is just possible.

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SUMMARY

1. The correlation between red blood cell density and corpuscular hemoglobin concentration, as calculated from the content of iron, is only 0.59, a result which suggests that the place of hemoglobin in the red cell may be taken by a colorless precursor of the same density, that the Fe content of human hemoglobin is inconstant, or both.

2. Difficulty was encountered in applying the Linderström-Lang method of measuring density to packed red blood cells. Successive droplets of packed red cells did not always reach the same equilibrium position, and so seemed to have different densities. The inconstancy was probably largely due to the solubility of cell lipids in the bromobenzene-kerosene medium. A direct gravimetric method proved preferable for cell density.

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