# STUDIES IN HISTOCHEMISTRY

## LVII. Determination of the Total Dry Mass of Human Erythrocytes by Interference Microscopy and X-ray Microradiography

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### ABSTRACT

The total dry mass of human erythrocytes was determined by both interference microscopy and x-ray microradiography. The determination of mass per unit area, and calculation of total dry mass per cell were simplified by changing the shape of the cells to spheres which were then flattened to discs of constant thickness when smeared on glass slides for measurement of fixed cells by interferometry, and to oblate spheroids when smeared on parlodion-coated slides for measurement of fixed cells by x-ray absorption. From x-ray measurements of 100 smeared and alcohol-fixed cells a mean dry mass per cell of $33.7 \times 10^{-12}$ g was obtained. Interference measurements of 100 fresh cells suspended in isotonic saline gave a mean value of $32.4 \times 10^{-12}$ g while interference measurement of 100 smeared and alcohol-fixed cells gave a mean value of $30.8 \times 10^{-12}$ g. The first two values compare well with a mean corpuscular hemoglobin of $31.2 \times 10^{-12}$ g, obtained from determinations of erythrocyte count and hemoglobin, since 95 per cent of the dry mass of the cell is hemoglobin. The difference in interference values between the fixed and fresh cells is possibly due to a difference between the specific refractive increment of alcohol-denatured hemoglobin and that of the unmodified substance. The value for the latter was used since that of the former is unknown.

### INTRODUCTION

The purpose of this study is to compare the values obtained for the total dry mass of individual human erythrocytes by interference microscopy and by x-ray microradiography, and to compare these values with those for mean corpuscular hemoglobin obtained from determinations of erythrocyte count and hemoglobin. The interference method permits measurement of dry mass in fresh (wet) cells, while the x-ray method requires that the cells be dried since the presence of water contributes to the value obtained.

Previous interferometric studies concerned with the dry mass of red cells have been those of Hale (1), who measured the optical retardation of human erythrocytes, Lagerlöf *et al.* (2) who measured the formation of heme and dry mass per

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unit area during the development of rat erythrocytes, and Mellors (3) who found a great variation in the dry mass of normal human erythrocytes from the same individual. After the present investigation was under way, a report by Ponder (4) appeared in which the concentration of apparent protein in human red cells was measured by interference microscopy.

The red blood cell is especially well suited to measurement of dry mass by both the x-ray and interferometric methods, except for one feature, its shape as a biconcave disc. Since the determination of cellular dry mass by both methods is dependent upon precise definition of cell geometry, variations in thickness in different parts of the cell result in difficulties in measurement and calculation. Fortunately, the shape of the red cell can be easily altered so that x-ray and interferometric measurements and calculation of total dry mass become relatively simple. When, *e.g.*, red cells are suspended in isotonic solution and placed between glass slide and coverslip, they undergo an immediate transformation from biconcave discs to spheres without change in volume (5, 6). This disc-sphere transformation has been shown to be due to an increase in pH of the suspending solution due to the alkalinity of the glass slide and coverslip (6), in addition to the absorption of crystalbumin from the cells by the glass surfaces (7). Identical shape changes occur during the early stages of hemolysis of red cells with slow acting hemolytic agents (5, 8), and Ponder (4) used a non-hemolyzing lecithin to alter red cell shape to be able to calculate the concentration of dry mass in individual red cells. During the present investigation it was observed that the transformation of red cells from biconcave discs to spheres also occurs in suspensions of washed cells in isotonic solutions. Sphering results when diluted suspensions of washed cells are allowed to stand for 48 hours at 4°C. Approximately 90 per cent of the cells are then spheres and no visible hemolysis has occurred.

## MATERIALS AND METHODS

### *Preparation of Specimens*

Venous blood (human) was immediately placed in vacutubes (Scientific Products, Evanston, Illinois) containing sodium sequestrene (disodium ethylenediamenetetraacetic acid). 0.15 ml. of the blood was then diluted to 15 ml. with buffered 0.15 M sodium chloride (0.01 M phosphate buffer, pH 7.4) and washed in 5 changes of the buffered saline solution by centrifugation at 295 *G* for 10 minutes. The washed cell suspensions were then allowed to stand for 48 hours at 4°C. Following this, sphering of the cells was checked by examination in a hanging drop. A portion of the cells was diluted 1:50 with the buffered saline solution, a drop of the cell suspension was placed on a microscope slide and covered with a coverslip which was sealed with vaseline, and measurements of the cells were made by interference microscopy. The remainder of the cells, diluted 1:10, was used to make smears which were air-dried, fixed in 95 per cent ethanol for 5 minutes at room temperature, and rinsed in distilled water. Measurements on these cells were made by both interference microscopy and x-ray microradiography. The smears for interferometry were made on scrupulously clean slides. A drop of distilled water was placed over a part of each smear and a coverslip rimmed with vaseline was sealed over it. Smears for microradiography were made on slides previously coated with 2 per cent parlodion. A scalpel blade was used to cut approximately 10-mm. squares of the smeared parlodion and they were floated free in distilled water, mounted on doughnut-shaped discs (6 mm. inner diameter, 15 mm. outer diameter) of aluminum foil 30 $\mu$ thick, and air-dried at room temperature.

Values for mean corpuscular hemoglobin were calculated from quadruplicate determinations of erythrocyte count and hemoglobin.

### *Calculation of Cell Mass*

1. *Fresh Cells—Interferometry:* For the determination of total dry mass of fresh red cells by interferometry, the sphered cells were suspended in buffered saline, Fig. 1. The spherical shape was made apparent by gently pressing on the coverslip over a preparation and noting the shape of the cells as they revolved in the suspending solution.

The total dry mass per cell, $M_{\text{rbc}}$, is $M_{\text{rbc}} = (m/v)V$ where $m/v$ is mass per unit volume and $V$ is total volume. This formula can be written as

$$M_{\text{rbc}} = \left(\frac{m/a}{t}\right) V \qquad (1)$$

where $m/a$ is mass per unit area and $t$ is cell thickness. If measurements of optical retardation in the interference microscope are made at the center of the spherical cells, then cell thickness at that point equals cell diameter, $d$, and the formula becomes

$$M_{\text{rbc}} = \left(\frac{m/a}{d}\right) \frac{\pi d^3}{6} = \left(\frac{m}{a}\right) \frac{\pi d^2}{6} \qquad (2)$$

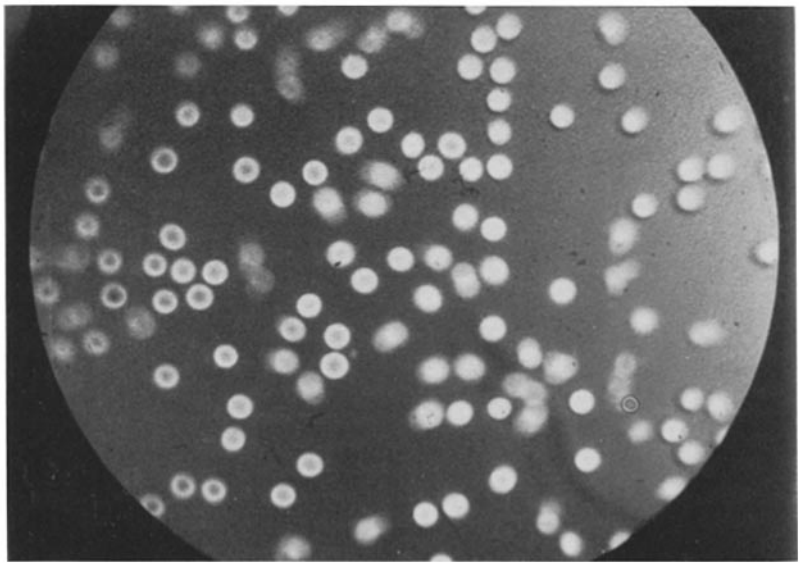

FIGURE 1

Interference photomicrograph of fresh sphered human erythrocytes suspended in isotonic saline. The spherical shape of the cells is indicated by the darker central areas where greater retardation of light through the full diameter of the cell occurs. × 550.

2. *Fixed Cells—Interferometry:* When spherical cells are smeared on glass slides they become flattened, presumably owing to adherence of the undersurface of the cells to the slide. Values for mean diameters of fixed and fresh cells in the preparations for interferometry in Table I illustrate this. The diameter of the smeared cells, 7.6 ± 0.03 $\mu$, even after shrinking due to fixation, is still greater than that, 5.8 ± 0.02 $\mu$, for the cells in suspension, Fig. 1, and 2. The smeared cells have the same mean diameter as that of smeared and fixed unaltered red cells which are biconcave discs, 7.5 ± 0.03 $\mu$, Fig. 3.

To determine the shape of the flattened cells, the cell images in a negative of an interference photomicrograph were spot scanned with the microdensitometer used by Ottoson *et al.* (10), which is similar to that of Lessler and Charipper (9). Fig. 4 represents a densitometric tracing along the diameter of a single cell. Relative thickness at points along the cell diameter were calculated from density values, and the central vertical cross-section of the cell was constructed, Fig. 4. The flattened cells are discs with the edges rounded off. However they differ little from true discs, (sections of cylinders) and there-

TABLE I

*Dry Mass of Human Erythrocytes**

| Method of measurement | Mean diameter | Standard error of mean | Uncorrected (for $\Delta v$) mean mass/area | Standard error of mean | Corrected mean mass/area | Mean mass/cell | Standard error of mean |
|---|---|---|---|---|---|---|---|
| | $\mu$ | | $10^{-12}$ g/$\mu^2$ | | $10^{-12}$ g/$\mu^2$ | $10^{-12}$ g | |
| Alcohol-fixed cells (x-ray) | 6.7‡ | 0.06 | 1.47 | 0.02 | 1.47 | 33.7 | 0.4 |
| Fresh cells (interference) | 5.8 | 0.02 | 1.80 | 0.01 | 1.85 | 32.4 | 0.2 |
| Alcohol-fixed cells (interferference) | 7.6 | 0.03 | 0.68 | 0.01 | 0.68 | 30.8 | 0.1 |

* 100 cells measured by each method.

‡ Diameter based on measurement of 200 cells from microradiogram.

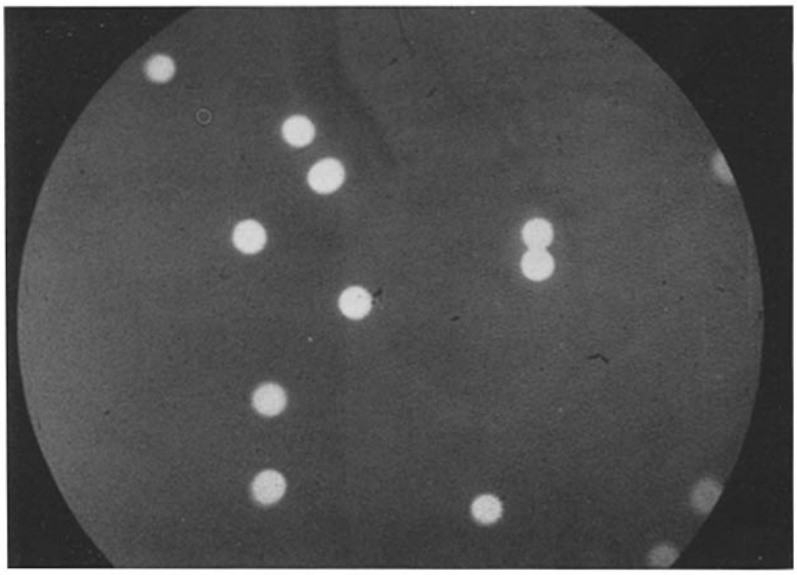

FIGURE 2

Interference photomicrograph of smeared alcohol-fixed sphered human erythrocytes. The even retardation of light over the entire surface of the cells indicates that they approximate discs of almost constant thickness. Comparison with Fig. 1 shows the increase in cell diameter from flattening. × 550.

fore it was considered an adequate approximation to assume a true disc shape for the calculations. From equation (1)

$$M_{\text{rbc}} = \left(\frac{m/a}{t}\right)\frac{\pi d^2 t}{4} = \left(\frac{m}{a}\right)\frac{\pi d^2}{4} \qquad (3)$$

where $\frac{1}{4}\pi d^2 t$ is the volume of a cylinder of diameter, $d$, and thickness, $t$. Interferometric measurements were made at the center of the cells and equation (3) was used to calculate total dry mass.

3. *Fixed Cells—X-Ray:* For x-ray measurements the spherical cells were smeared on glass slides coated with 2 per cent parlodion. These cells were also flattened, but less than the smeared cells in the preparations for interferometry. This is illustrated in Table I where the mean cell diameter, 6.7 $\mu$, in the x-ray preparations, Fig. 5, can be seen to be considerably smaller than that, 7.6 $\mu$, for the fixed cells in the interferometric preparations. This difference is probably related to the fact that less of the cell surface adheres to the parlodion film, which is hydrophobic, so that the cells more closely approximate spheres.

Microdensitometry along the diameter of the photographic image of a single cell in the primary microradiogram provided the density tracing shown in Fig. 6. Construction of the central vertical cross-section of the cell from the density values revealed it to be an ellipse which, when rotated about its minor axis, forms an oblate spheroid. If measurements of density of the images of the cells in the primary microradiogram are taken at the center of the cell images, then cell thickness, $t$, at that point is the minor axis of an oblate spheroid. From equation (1)

$$M_{\text{rbc}} = \left(\frac{m/a}{t}\right)\frac{4\pi}{3}\left(\frac{d}{2}\right)^2\frac{t}{2} = \left(\frac{m}{a}\right)\frac{\pi d^2}{6} \qquad (4)$$

where $\frac{4}{3}\pi(d/2)^2\ t/2$ is the volume of an oblate spheroid with a major axis of $d$, cell diameter, and a minor axis of $t$, cell thickness.

## *X-Ray Microradiography*

The preparation and mounting of the reference system, the microradiography, densitometry, and calculation of mass per unit area and total dry mass per cell was that employed previously in this laboratory (10, 11), based on the work of Engström and Lindström (12) and Lindström (13). In this study the x-ray apparatus developed by Engström and coworkers was used (14). The microradiograms were made on Kodak spectroscopic film 649 GH by exposure for 10 minutes at 1.2 kv. and 1.0 ma.

Principal constituents of red blood cells in per

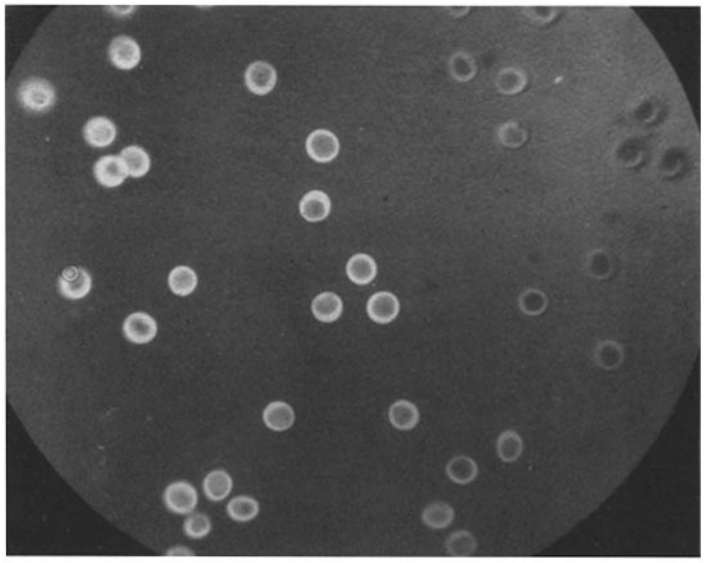

FIGURE 3

Interference photomicrograph of smeared alcohol-fixed unaltered human erythrocytes. The biconcave shape of the cells is indicated. Comparison with Fig. 2 shows the same cell diameter. × 550.

cent of total dry weight, were calculated from data given by Wintrobe (15): hemoglobin and methemoglobin, 96.4 per cent, stromal protein, 1.4 per cent, and lipid, 1.36 per cent. Since no values for the mass absorption coefficients of hemoglobin, methemoglobin, and stromal protein are listed in the data of Lindström (13), and the effect of the slight amount of lipid present is negligible, the average mass absorption coefficient for animal protein was used. This appears to be justified by the great similarity in elementary composition, on which the mass absorption coefficient depends, of various types of protein (13).

The formula for the calculation of total dry mass of the red blood cell by the x-ray method is given by the equation

$$M_{rbc} = E_c \, W \, 1.32 \, \pi d^2/6 \qquad (5)$$

where $E_c$ is the photographic density at the center of the cells expressed in parlodion equivalents and $W$ is the weight of the parlodion in $10^{-12}$ $g/\mu^2$. 1.32 is the ratio between the mass absorption coefficients of parlodion and protein at the voltage used. Cell diameter, $d$, was obtained by direct measurement on the microradiogram at a magnification of 1200 with a micrometer ocular.

## *Interference Microscopy*

A Dyson interference microscope (Cooke, Troughton, and Simms, Ltd., York, England), which has been

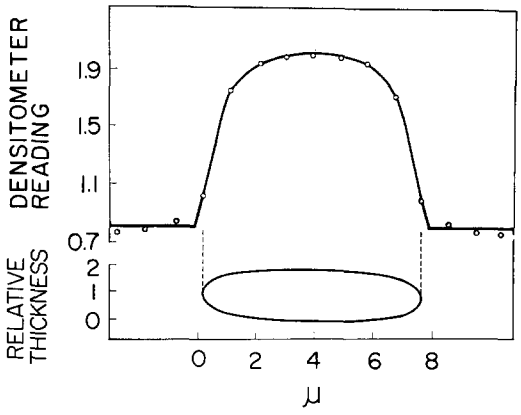


FIGURE 4

Densitometric tracing across the diameter of a cell image on the negative of an interference photomicrograph of a smeared alcohol-fixed sphered human erythrocyte. The figure under the curve represents the central vertical cross-section of the cell calculated from the values along the curve.

fully described by Davies (16) and Hale (17), was used. The light source was a zircon arc lamp equipped with an interference filter transmitting maximally at 5460 A. Measurements of optical path difference were made with a Dyson visual photometer, capable of a setting reproducibility of $\frac{1}{200}$ of a wave length (16), with the microscope wedge plates parallel and the interference bands maximally separated to illuminate the field evenly. Cell diameters were measured with a micrometer ocular.

The basic formula relating optical path difference, $\phi$, expressed in wave lengths of the green light used, to cell mass per unit area is $m/a = \phi/\chi$ where $\chi = 100\alpha$, with $\alpha$ representing the specific refractive increment of the substance being measured. Since the dry substance of the red blood cell is almost entirely hemoglobin, its specific refractive increment, 0.00193, (18) was used.

In the case of fresh spherical red blood cells, suspended in isotonic saline, the total dry mass per cell is given by the mass per unit volume, $m/a/d$, times the total volume, $V$. Because the specific refractive increment for hemoglobin was obtained with reference to water (18), a correction factor, $\Delta v$, for the weight of material in the volume of suspending solution displaced by the cell, was applied to the formula. This correction factor, calculated from the difference in the refractive index of 0.15 M sodium chloride and water at 25°C., is $(0.0016/\chi)$gm./ml. (10, 16) and the correction in total dry mass per cell becomes $(0.0016/\chi)V$ or $(0.0016/\chi)\ \pi d^3/6$. Therefore,

$$M_{\text{rbe}}(\text{corr } \Delta v) = \frac{m\pi d^2}{a6} + \frac{0.0016}{\chi}\frac{\pi d^3}{6} \qquad (6)$$

$$= (m/a + 0.0083d)\pi d^2/6$$

In the case of the fixed cells the medium was distilled water, and no correction factor was necessary.

## RESULTS AND DISCUSSION

The value of $32.4 \times 10^{-12}$ g obtained for the mean total dry mass of fresh erythrocytes by interferometry agrees well with a mean total dry mass of $33.7 \times 10^{-12}$ g obtained by x-ray absorption, Table I. This is in accord with the findings of Davies, Engström, and Lindström (19), who obtained close agreement in results from both the x-ray absorption and interference methods for the mass of a number of biological samples. It is also in accord with data by Ottoson, Kahn, and Glick (10) which show essentially the same values for the mass of rat mast cells as determined by both methods. Differences in values were well within the error, ±5 per cent, of both methods (13, 16). In the case of the x-ray determination, the error contributed by iron in the red cell is negligible since,

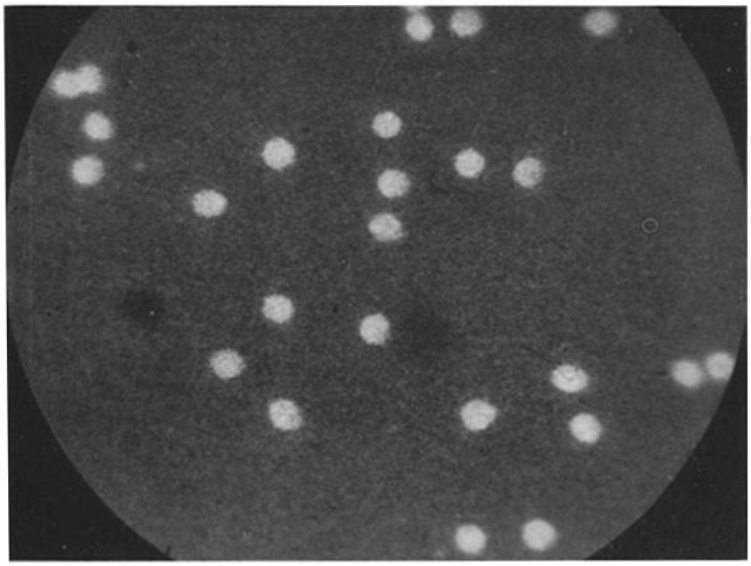

FIGURE 5

Photomicrograph of a microradiogram of smeared alcohol-fixed sphered human erythrocytes. × 600.

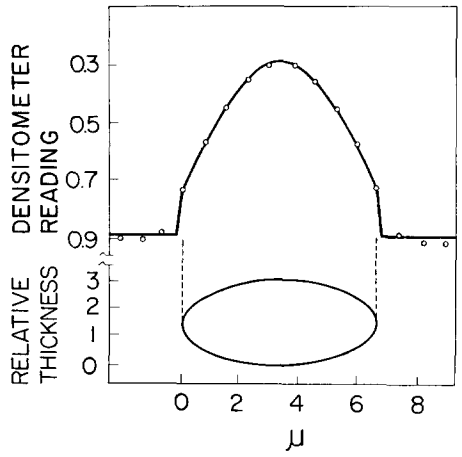


FIGURE 6

Densitometric tracing across the diameter of a cell image in a primary microradiogram of a smeared alcohol-fixed sphered human erythrocyte. The figure under the curve represents the central vertical cross-section of the cell calculated from the values along the curve.

by calculation, it can account for only 0.6 per cent of the total energy absorbed. If the hemoglobin is assumed to be fully oxygenated, this oxygen accounts for an additional 0.5 per cent.

Comparison was made of the total dry mass of the red cells with mean corpuscular hemoglobin, MCH, calculated from quadruplicate determinations of erythrocyte count, $4.81 \times 10^6$/mm.$^3$, and hemoglobin concentration, 15.0 gm. per cent. Since very close to 95 per cent (15) of the dry mass of the human erythrocyte is hemoglobin, $0.95\ M_{rbc} \simeq$ MCH. Values estimated for MCH of $32.0 \times 10^{-12}\ g$ from x-ray absorption and of $30.8 \times 10^{-12}\ g$ from interferometry agree well with value of $31.2 \times 10^{-12}\ g$ for actual MCH.

The mean difference between mean total dry mass per cell and MCH represents the mass of dry material other than hemoglobin in the red cell. Values of $2.5 \times 10^{-12}\ g$ and $1.2 \times 10^{-12}\ g$ for x-ray and interferometry, respectively, are in fair agreement with that of $3.1 \times 10^{-12}\ g$ found by Ponder (4), and support his view that other protein, in addition to hemoglobin, is present in the interior of the red cell, possibly in the form of an internal framework (4, 5).

The mean total dry mass per cell of $30.8 \times 10^{-12}\ g$ for fixed red cells, determined by interference microscopy, is lower than that obtained for fresh cells by the same method, or for fixed cells by x-ray absorption. This difference may result from a difference between the values for the specific refractive increment of native and alcohol-fixed hemoglobin. The value of the latter is unknown and that of the native substance was used. From calculations of $\alpha$ based on experimental data for other dried protein, the mean total dry mass per fixed cell, calculated with $\alpha$ for dilute hemoglobin solutions, would be expected to be lower than that for the fresh unfixed cell (16).

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