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Publication Date

1977-08-01

Submitted to Micron

UC-48
LBL-5636 Rev.
Preprint c.1

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August 1977

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EVENTS SECTION

Prepared for the U. S. Department of Energy
under Contract W-7405-ENG-48

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ANALYSIS OF IRON CONTENT IN INDIVIDUAL HUMAN RED
BLOOD CELLS BY ELECTRON MICROPROBE AND
SCANNING ELECTRON MICROSCOPE

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ABSTRACT

The iron content of single human red blood cells has been assessed using electron microprobe analysis and scanning electron microscope. Cells for microanalysis consist of a fixed-washed and freeze-dried preparation. This preparative procedure improves stability and spatial resolution of the analytic method. The reference standard employs compressed pellets of purified iron-containing human hemoglobin and human albumin, respectively. The cell preparation and reference standard remain stable for long periods of time. Differences in Fe content and its distribution among individual red cells from both normal subjects and sickle cell anemia patients have been measured by the quantitative and semi-quantitative techniques (wavelength and energy dispersive X-ray spectrometry). There are several points which can be made from this study. One concerns the estimate of iron on a cell by cell basis, the average variation among cells being slightly

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over 13%, ranging from 6% to 19.4% by individuals (samples) or from 11% to 14% by ethnic groups. These differences are thus closely comparable across ethnic grouping with a possibility of high level in Asians and lower level in sickle cell anemia patients.

The distribution of iron among individual red blood cells can give a valuable parameter to add to the well-known average values obtained from multicell analyses. In addition, if subpopulations of cells can be distinguished on a morphological basis, e.g., sickle cell anemia preparations, then the distribution of iron between the subpopulations can only be measured by analyzing individual cells.

This study presents a detailed account of a quantitative and semi-quantitative analysis of bound-iron content of individual red cells by means of analytical procedures utilizing electron probe excitation. The physical instruments whose adaptability and sensitivity were explored are (1) electron microprobe analyzer (EMPA), and (2) scanning electron microscope (SEM) fitted with an energy dispersive X-ray spectrometry (EDX) system. A major emphasis is placed on EMPA because of the broad application and extensive theoretical development which it has undergone in the past twenty odd years and personal experience in the study and use of this instrument.

The differences in iron content in individual human red blood cells were determined, and the following issues were chosen for investigation.

- (1) Variation among circulating erythrocytes of the same population (i.e., same subject);
- (2) Variation over a period of time (2 weeks) which would represent an appreciable fraction of an individual's erythrocytic turnover (three samples drawn at approximately weekly intervals span 14 days of the 120-day average red cell life);

- (3) Degree of variation among different individuals who are presumed "euhemic" based on absence of clinical complaints and on microscopic observation of cell form;
- (4) Possible alteration in Fe content in a major blood dyscrasia (i.e., sickle cell).

Sickle cell anemia was chosen, being both genetically and ethnically determined and providing erythrocytes with high morphologic recognition. Thus the groups of normal individuals required to be included were Blacks (for the sickle cell comparison), Whites, and Asian (Chinese).

MATERIALS AND METHODS

Elemental Analysis

Two instruments were used in measuring the iron content in single red blood cells:

- (a) A Materials Analysis Company (Palo Alto, California) electron microprobe analyzer, Model 400S, operated at 15 keV with a 1.2 to 2.0 μm beam diameter with a sample current of 0.05-0.07 μA , and a LiF analyzing crystal for Fe and a PET crystal for Si;
- (b) A Japan Electron Optics Laboratory (Burlingame, California) scanning electron microscope, Model UL operated at 15 keV at a magnification of X50,000 with a raster size of 0.2 μ x 0.7 μ at T.V. rates, interfaced with a Nuclear Equipment Corporation (Los Altos, California) liquid-nitrogen cooled

Si(Li) semi-conductor X-ray detector and Qanta/Metrix (Sunnyvale, California) EDX/80S energy dispersive X-ray analysis system.

Cells

Human circulating erythrocytes were obtained from normal subjects and sickle cell anemia patients. Normal cells were collected by conventional finger-puncture methods; patients' cells were collected into heparinized syringes and prepared for analysis within 24 hours of collection. This blood was prepared in the following manner: a single drop of blood was placed directly onto the surface of a 3-inch parafilm square that was immediately inverted over the mouth of an Ehrlenmeyer flask containing 50 ml of a 2.5% glutaraldehyde solution in 0.15 M cacodylate buffer at pH 7.4. The edges of the square were pressed down securely and held by hand around the neck of the flask so as to render it liquid-tight; and the cells were then mixed by gentle, repeated inversions of the flask enough times until visual observations had confirmed thorough dispersion in the fixative. After 45 minutes to an hour fixation time, the cells were washed; and a ml portion of the fixed cell suspension was spun down in a table model IEC clinical centrifuge at about 5,000 rpm for 3 minutes. The supernate was discarded, and the remaining cells were resuspended in a ml of distilled water and centrifuged. This suspension and centrifugation procedure was repeated three times. After this wash procedure, the cells were resuspended in 1/2 ml to 1 ml of water to which a very small amount ($\sim \mu\text{g/cc}$) of scientific grade gelatin (Difco) had been added. These

resuspended cells were then mounted on top of a quartz disk by transferring about 1/8 ml to 1/4 ml of suspension drop by drop with a disposable pipette, the total number of drops having been determined by the size of the counting area desired, but not to exceed the size of the disk in order to avoid spillage. After allowing the cells to settle for 15 minutes, the excess water was drawn off by capillary action, using a corner of a gauze square. The preparation was immediately quenched in liquid nitrogen and quickly transferred to a pre-cooled castle (i.e., a specially constructed, aluminum-lidded container for freeze-drying biological materials) which was placed onto the pre-cooled stage in an Edwards-Pearse Tissue Dryer, Model EPD2 (Edwards High Vacuum, Inc., Grand Island, NY), equipped with a liquid nitrogen tray fitted into the pumping line and dried under vacuum for 24-48 hours at -60°C with a P_2O_5 desiccant present in the drying chamber. This freeze-dried preparation was slowly heated to room temperature for removal from the drying chamber, then put into a Varian Evaporator (Varian Associates, Palo Alto, California), Model Ve-10, equipped with a liquid nitrogen cold trap in a pumping line, and coated with carbon to improve conduction and reduce charging. During coating, the sample revolved simultaneously in opposite directions in two different planes. Though the thickness of the carbon coating was not determined, the amount was established and controlled by evaporating the 3/16-inch-long tip of a presharpened spectroscopically pure carbon rod onto the sample. The preparation, ready for analysis, was stored in a dessicator jar under vacuum.

Reference Standard

The standards used for analysis were pellets pressed from purified powders of human hemoglobin (Hb) and serum albumin (Alb). These were prepared by placing about 5 to 10 mg of granulated, spectroscopic grade carbon into the bore of a mold-plunger assembly. The plunger was put into place, and the carbon was packed tightly by hand pressure only. The plunger was withdrawn, and 40 mg of Hb was poured on top of the carbon through the bore of the mold. The plunger was again replaced, and the mold-plunger assembly was put into a vacuum unit which permitted the application of mechanical pressure while under vacuum. A Black Hawk Hydraulic Press (Black Hawk Collision and Repair Div., Milwaukee, Wisconsin) applied 3,000 to 4,000 psi to the Hb, forming a disk-shaped pellet 1/4 inch in diameter. After about 3 to 5 minutes, the mechanically applied pressure was released and the vacuum was removed. The mold-assembly was then taken from the vacuum unit, and the Hb pellet was carefully removed from the mold. This same procedure was repeated with the Alb. The Hb and Alb pellets thus obtained were mounted next to each other in the center of the quartz disk (Figure 1) with colloidal graphite in alcohol (Dag, Acheson Colloids Co., Port Huron, Michigan). This standard reference preparation was carbon-coated along with the cell preparations described in the previous section.

EMPA

In the electron microprobe, analysis was performed on each erythrocyte while under visual observation. The cell was counted for

a total of 5 minutes (in five 1-minute intervals). Two elements (Fe and Si) were analyzed simultaneously. The X-ray backgrounds (i.e., background counts at the iron peak energy when the beam is moved off of the cell as distinguished from background counts at the energy of the iron peak due to Bremsstrahlung) were measured in noncell areas of the supporting quartz disk with the spectrometers held in their respective peaks. Probe standards, pure Fe and SiO₂, were used to maintain similar probe conditions from experiment to experiment and for probe stability checks. Intensities of the probe standard and reference standard and their respective backgrounds were measured before each series of ten cells.

The absolute concentration values in the red cells were estimated by using the average value of the net intensity of the cells in conjunction with net intensity values and known concentrations of the reference standards as provided by this mathematical relationship: $C_C = I_C/I_S \times C_S$ where C_C is the relative mean iron concentration in the rbc; C_S is the known average iron concentration value of the reference standard in weight per cent (i.e., g/100 g), I_C is the mean net Fe K_α intensity obtained from an rbc, and I_S is the mean net Fe K_α intensity obtained from the standard. The concentration values calculated in this manner were not corrected for ZAF differences between the sample and the standard since these effects should have been small due to the similarity between the cell and the reference standard in composition and nature of matrix.

The detecton sensitivity of the EMPA was computed according to the equation of Liebhafsky et al. (9). In addition to the three

sigma computation above, a 95% confidence interval was also computed.

SEM-EDX

In the scanning electron microscope, selected post-EMPA and thin film (Formvar) preparations were examined in the secondary electron image and X-ray modes. The images were recorded by making a 40 and 70 sec scan. Energy dispersive X-ray spectra were obtained by using an attached liquid nitrogen-cooled Li-drifted-Si conductor X-ray detector, operating at an accelerating voltage of 15 keV, probe current 10^{-9} to 10^{-10} A, and a takeoff angle of 45° . The condenser lens and scan coil settings were maintained at a constant setting. Estimates of Fe K-radiation per cell were obtained under the same SEM operating conditions as those for the plotted spectra except for the X-ray count rate integration periods (e.g., five 1-minute intervals for computed runs). Subtraction of background and integration of remaining peaks of the X-ray data were obtained automatically by a coupled mini-computer interfaced with the scaler of the SEM X-ray detector. The print-out was recorded by an ASR-33 teletype and spectra plots were recorded by an X-Y input-output plotter.

RESULTS

Elemental Analysis

The selection of the proper computer program for quantitation of the EMPA intensity data was a difficult matter because of the nature of biological specimens. One should also take into account their

thickness. The computer routine used was DATATEXT, a general one, designed for the behavioral sciences to perform basic statistics, and ANOVA. The advantage of the use of such a scheme is low cost and availability of a computer center for batch loading of IBM card file data. Large quantities of data can be run in seconds for under \$10 at current rates.

Whole Blood Preparation

A preliminary experiment was carried out with air-dried whole blood smears employed as the sample preparation for EMPA. This seemingly ideal specimen preparation from the point of view of minimizing iron loss proved to be most undesirable for the following reasons: significantly higher mean background X-ray signals; corresponding low signal intensities from the cells; and, more importantly, a relative increase in the electrical instability (charging) of the sample itself. Lechene (8) reported similar problems when EMPA was performed on a specimen preparation of the same type as above. Figure 2 shows examples of the mentioned preparation. Salt crystals (mostly sodium) can be clearly seen, and flattened shrunken cells show evidence of cracks encircling cells' periphery. This surface discontinuity contributes to sample instability, i.e., a charging artifact that is denoted by the peripheral halo seen at all magnifications.

Sample Stability and Durability

Standard deviation of the mean was obtained through manual calculations as well in order to detect variation in multiple readings on the same cell and, in particular, to look for systematic change in successive readings which would reflect deterioration of the sample by the beam.

Table I, showing sample stability and durability under analysis, yields the following conclusions:

- (1) variation among successive readings on the same cell or pellet (reference standard) sample is random and small within limits of acceptability for statistical comparisons of samples;
- (2) no significant degradation of the cell under probe took place during successive readings;
- (3) in terms of stability of cell preparation, there was no measurable instability when analysis time was extended three-fold.

Characteristics of Background (N_B)

Two considerations entered into examining N_B traits: to determine N_B as affected by the support-- SiO_2 , i.e., the inseparable Bremsstrahlung and environmental sources; and the other was the organic matrix Alb, e.g., signal specificity.

The data in Table II -- Characteristics of Background (N_B) --

can be summarized as follows:

- (1) the support and the preparative procedures and milieu yield a background so low as not to obscure the N_L derived from the analysis;
- (2) the pellet provides the better background characteristic.

Energy Operating Potential

To determine the optimal instrumental applied potential, a sufficiently broad range was tested.

Table III, which indicates choice of energy operating potential (i.e., 15 keV), shows the maximal and minimal levels and the intermediate level selected as optimal for use. These results were based on actual measurement of low Fe concentration in single cells.

Cell Population

Findings on circulating erythrocytes of the same blood sample were determined from normal subjects. In each normal subject, the sample was divided among three disks. Ten cells were measured on each disk. Values were determined from five points on each cell (in cpm). Table IV, neatly condensing the experimental design, shows the distribution of ethnic and related groups.

It was concluded that the coefficient of variation among cells on the same disk was slightly over 13%, ranging from 6% to 19.4% (Table IV-a). Whether considered by individuals (Table IV-b) or by groups

(Table IV-c), the coefficient of variation was 11% to 14%, thus giving added confidence to the objective of studying ethnic and related groups; the initial analysis was done without ethnic group breakdown.

Effects of Time Lapse in Sampling Sequence

The possible variation over a 2-week period was examined by breaking up the foregoing determinations on normal subjects into data from the three successive samplings in that time period (see Table V). The effects of time of sampling were minor and followed no systematic change for differences among mean readings yielded in the time period specified.

Cells of Normal Subjects and Sickle Cell Anemia Patients

Table VI, the statistical treatment of data, defines further that a constancy among cells of normal subjects and sickle cell anemia patients exists; these data, when viewed by disk, revealed no significant difference between the groups. Inspection of the ranges and standard error of the data shown yields the following conclusions and suggestions:

- (1) there was no significant difference in the Fe K_a total intensities from the sickle cell samples and full complement of normal samples;
- (2) normal cell samples from Asians (top three sets in Table VI) tended to run appreciably higher than similar samples from Blacks and Whites; the latter two groups (middle and

lower euhemic sets) were essentially similar;

- (3) the suggestion of somewhat high iron concentration in Asian erythrocytes (at least among subjects used in this study) became stronger when analyses of the three disks per person were combined into figures representing individual subjects; under these circumstances there was no overlap between the values in Blacks and the values in Asians, and only slight overlap between Whites and Asians;
- (4) continuing to treat the data from individual subjects, the Black normal and Black sickle cell groups were virtually identical.

Thus, with all necessary reservations regarding the adequacy of sampling numbers in the various groups, it appears that the average iron content of red cells and its distribution among individual red cells is closely comparable across ethnic groups; furthermore, in the blood dyscrasia chosen (sickle cell anemia) the iron content of individual cells remains within the normal range.

Relative Frequency Distribution of Fe K_{α} Net Intensity in Human rbc's

The distribution of the iron values in the two populations of cells measured was computed as the relative frequency of each of the values encountered and plotted as a histogram (Figure 3). It should be emphasized that these counting rates are net values, i.e., above background. The resultant curve for the cell population (i.e., 300 red cells) showed a very close approximation to the normal probability.

The values from the sickle cell anemia samples are added (shown stippled in the histogram). While the sample is small, leaving many open classes, the frequency distribution confirms foregoing conclusions, namely, that the greatest frequencies are found somewhat lower in the scale but are still grouped according to a normal probability distribution.

Scanning Electron Microscopy

Secondary Electron Examination

Some examples selected from many samples studied are presented below to illustrate the potential capabilities that this analytical system, i.e., secondary and EDX modes, affords the biologist.

The SEM micrograph (Figure 4) shows a typical morphology of euhemic human red blood cell. Although this cell consisted largely of water (65%), it tends to maintain more of its overall morphology by this preparative method.

Figure 5 micrographically illustrates the morphology of red cells as they appear after being prepared in a manner similar to the above red cells mounted on SiO₂ except that these are mounted on a thin film, allowed to air dry, and examined without the addition of any conductive coating materials. There are signs of cellular collapse (flattening) and these cells are somewhat distorted.

EDX Analysis

A scanning electron microscope -- energy dispersive system for the semi-qualitative analysis of purified human hemoglobin and human serum albumin (i.e., the reference standard) -- was used in forming the spectrum shown in Figure 6. The spectrum, representing the X-ray energies for the first quarter of the Periodic Table, has prominent peaks for Si and S, and also well-defined peaks for Cl, K, and Fe.

Figure 7 is the spectrum representing the X-ray analysis of a single human rbc mounted on SiO_2 and the corresponding off cell analysis. This spectrum has a strong peak for Si with well-defined Fe and Cu peak as well; Si measurement is against a very high continuous radiation background reflecting the high excitation rate of Si. The Cu peak is present in both on cell and off cell spectra and probably represents the copper parts of the instrument as a remote source.

Figure 8 shows the spectrum representing the analysis of a single human rbc mounted on thin film. The K_α X-ray lines of Fe, Cu, S, Cl, Si, and Al are shown. Iron and sulfur are shown as prominent cell components. Background continuous radiation count has been reduced to 20% of the value when the cell was analyzed on the quartz disk mount. The large Cu peak is due to the supporting copper grid.

Figure 9 illustrates the typical Fe K_α X-ray lines of the X-ray energies for a single human rbc mounted on a quartz disk compared to one mounted on thin film (isolated peaks); the adverse effect of a very high continuous radiation background is explicit.

Data Reduction

X-ray counts for Fe were also made for a series of ten euhemic red cells. Table VII gives the statistical treatment of the mean total X-ray intensity value for each cell and for the white radiation. It was concluded from the statistical treatment of these data that the average variation among cells was a little more than 21% and ranged from a low of 1% to a high of 85%. When an estimated variation for the corresponding off cell analyses on the same sample is being considered, this average value is slightly over 1% less than the variation obtained from the on cell analyses; however, the grand mean for the N_T value in this case is 5% higher than the latter.

The results of the X-ray counts from a blood sample from a sickle cell anemia patient, arranged by cell morphology (sickle and normal-appearing cells), are seen in Table VIII. This analysis yielded similar conclusions when compared with the data above.

Table IX shows the analyses of eight euhemic cell types mounted on thin film. The conclusions drawn from these trials are: (1) the thin film support effects a substantial decrease in the N_B intensity; (2) N_T showed a corresponding reduction; and (3) the N_T/N_B intensities were not significantly improved by this approach (i.e., the signal counts, total counts minus background, is represented by the signal/background).

DISCUSSION

The subject of X-ray spectrometry, as employed by the EMPA system according to Bragg's Law and as utilized by SEM-EDX system according to Moseley's Law, is well established; and information obtained from either system is qualitatively the same -- however, quantitatively, certain differences become apparent.

In practice, then, there are important drawbacks that have become evident through the use of EDS in EMPA and SEM systems in biological studies: increased background signal; serious energy interferences at low X-ray energy; X-ray sources remote from the specimen (backscattered electrons from the specimen generate X-rays indiscriminately upon striking any object inside the specimen chamber as well as the chamber wall); low peak-to-background ratios (P/B). It is important to note that while a rather convincing argument can be made against the use of P/B because they may not tell the whole story in a direct comparison of WDS and EDS systems, the fact remains, nevertheless, that intensity or P/B data are essential to the quantitative aspects of X-ray microanalysis (6).

Noteworthy are the previous studies (WDS and EDS in EMPA) in which accuracy was the utmost concern in WDS systems, and speed and greater spectrometer sensitivity were the prime motivation in EDS systems. Lechene (8) routinely observed a P/B of 50 from his EMPA of 160 mM Na/kg wet weight, while Dorge and associates (3) reported a low 0.3 value (i.e., peak integral divided by the continuum count) taken in a wider band in another part of the spectrum in studying

Na in frog skin as a calibration curve on comparable samples when using EDS on the same samples as Lechene above. The EMPA permitted the better resolution. The work reported here extends this kind of a study by directly examining the Fe content within individual human rbc's when the average P/B was on the order of 2.2:1 for the Fe analysis, while on the SEM-EDX system the analyses of the post-EMPA samples were 1.2:1 at best. The two above X-ray spectrometry systems have been described in (1).

Limits on energy resolution in the EDS impose restraints on the EDS. For example, Lechene reported using WDS to obtain an energy resolution smaller than 10 eV at 5.9 keV whereas the EDS detection in our hands has had a resolution of about 142 at 5.9 keV as measured by Hayes and Pawley (7) for MnK_{α} . In practice this means that the WDS may differentiate between Mg and Na while the EDS system may not. Reports of EDS analyses reflect the peak overlaps of Ca and Sb because of low concentrations of the elements of interest (15, 14), although such overlaps could easily have been recognized with WDS. As another example, Sutfin et al. (16) demonstrated Os interference with P. Figures 7 and 8 show examples of this feature of the EDS. The prominent Cu peaks seen in both spectra have their origin outside the analyzing volume. The copper X-rays are from parts of the instrument, thin film, supporting copper grid, respectively. These problems are of special significance when measuring light elements, e.g., $11 \leq Z \leq 20$, because of X-absorption in the detector window (usually beryllium sheet approximately 10μ thick) and in the lithium-drifted-silicon chip itself; due to such hindrances, these elements are not usually

identified or analyzed by means of energy dispersion (14).

Detectability limits and mass sensitivity are highly controversial subjects in the literature and at scientific meetings. It has been found that calculated limits of detectability for EDS and WDS systems depend as well, among other things, on beam current and matrix. Values of S^2/B (where $S = P-B$) are usually considered because sensitivity of an analysis is calculated in terms of weight-fraction or in terms of the absolute quantity; nevertheless, when signal is much less than background, one can get a very important movement in signal/background with only slight change in N_T/N_B .

In terms of the sensitivity of the above technique, the author's value for minimum detectable limits (MDL) calculated from the SEM-EDX data was 0.19% corresponding to a 95% confidence interval of 0.15% to 0.53% for the Fe value of the rbc (0.34%). However, the experimental intensity data were found to be low: in evidence is a small peak superimposed over a huge background (see Figure 9). The MDL estimate for EMPA was computed in a similar manner: first, for the reference standard and second, for the cell on quartz. It was found to be, respectively, 0.2% -- a 95% confidence interval ranging from 0.32% to 0.36%, and 0.11% -- a range of 0.23% to 0.45% (in cellular analysis, a decrease in sensitivity equivalent to over fivefold when compared to the MDL using the conditions of the reference standard). Under these conditions of EMPA, the intensity data support the calculated minimum detectable concentrations measured experimentally. The results of the analyses shown in Table X clearly demonstrate the contention that the cause of the somewhat lower quantitative accuracy of the

EDS relative to the WDS is directly related to P/B's and the resolution differences of the two instruments. The table also illustrates that the P/B for the SEM application is a factor of two higher for the WDS (i.e., better sensitivity). The low P/B for EDS becomes especially critical when considerations of peak minus background are made.

In this study, the absolute iron concentration values computed in this manner are low. Each mean red cell value represents, according to estimates obtained from multi-cell analyses, an iron content of 0.34% of hemoglobin [which constitutes 95% of the dry cell weight corresponding to about 10^{-13} g, the absolute value calculated for iron per rbc -- based on 3×10^8 molecules as the mean hemoglobin corpuscular concentration by Wintrobe (18)]. In this connection the relative standing of the iron determinations obtained in this present study is somewhat improved. While the determinations have been obtained from analyses of single cells, other measurements by multicellular analyses are available. Those of wet chemistry, absorption spectrophotometry are: Downen (4) reported 0.34 mg 1^{-1} red cell concentration calculated from blood to plasma (blood, 0.27 mg 1^{-1} ; plasma, 0.043 mg 1^{-1} g); Delves et al. (2) 381×10^2 mg per 100 mg and also correlated lowered concentration of iron in whole blood samples with blood pathology (e.g., in anemia); and Schroeder and Nason (13) iron estimates were reported in terms of mg total body blood, plasma, and rbc in reference man: 2,500, 3.6, and 2,400, respectively, with 70.5% in hemoglobin -- indicative of a mean corpuscular hemoglobin concentration of 30% [found over four decades ago by Wintrobe (17)].

The author would like to thank Professor Thomas L. Hayes for his counseling throughout the course of this study; Professor Richard Falk for providing the Qanta/Metrix 80S unit for stages of the work; and Mr. David Eaglesfield for computer user's aid and the use of Harvard Computing Center, Cambridge, MA.

This work was submitted to the Faculty of the University of California at Berkeley in partial satisfaction of the requirement for the Ph.D. on December 10, 1976. The work was supported in part by the U.S. Energy Research and Development Administration; the Associated Western Universities, Inc.; the Graduate Minority Program of the University of California; and a grant from the National Science Foundation.

Addendum

In this present communication we have considered the limits of EDS and WDS and their relative performance in SEM-EDX and in EMPA under conditions as stated. Our SEM-EDX system used a mobile probe 1.5 cm in diameter, at about 2 cm distance from specimen. Recent extensive reports (cf, Shuman et al., Ultramicroscopy 1: 317-339, 1976 and IITRI SEM/1977, 663-672, Vol. 1.) have shown that EDS systems in combination with electron microprobes, under certain conditions, can perform impressively better than either of the systems previously reported.

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FIGURE CAPTIONS

- Fig. 1. Diagram of reference standard preparation: pelleted human hemoglobin and human serum albumin on support.
- Fig. 2. Scanning electron micrograph of an air-dried whole blood smear. X500. (JEOL). Acceleration voltage 10 keV, beam diameter 200 nm, probe current 100 μ A, scan exposure rate 40 sec.
- Fig. 3. Histogram of results for quantitative analysis (EMPA) using data from Table VII. Relative net iron K-radiation distributions are given.
- Fig. 4. Scanning electron image of a euhemic human red blood cell. The bioconcave, rounded shape in appearance is observable. X5,000. Acceleration voltage 10 keV, beam diameter 20 nm, probe current 100 μ A, scan exposure rate 70 sec.
- Fig. 5. Scanning electron microscope shows images of euhemic human red blood cells mounted on thin film (Formvar). These cells are fixed-washed, then mounted, air dried, and examined without any coating of conductive material. X4,000. Acceleration voltage 10 keV, beam diameter 200 nm, probe current 100 μ A, scan exposure rate 40 sec.
- Fig. 6. EDX energy spectra of the reference standard. Acceleration voltage 15 keV; beam diameter 200 nm; probe current 100 μ A; area scanned: $0.2\mu \times 0.7\mu$; T. V. scan rates; integration time 1,000 sec.
- Fig. 7. EDX energy spectra of a single human rbc mouted on SiO_2 . Compare Fig. 8. Acceleration voltage 15 keV; beam diameter 200 nm; probe current 100 μ A; area scanned: $0.2\mu \times 0.7\mu$; T. V. scan rates; integration time 1,000 sec.
- Fig. 8. EDX energy spectra of a single human rbc mounted on thin film (Formvar). Acceleration voltage 15 keV; beam diameter 200 nm; probe current 100 μ A; area scanned: $0.2\mu \times 0.7\mu$; T. V. scan rates; integration time 1,000 sec.
- Fig. 9. A representation of an Fe K_α energy spectrum removed from a typical EDX spectra plot. Integration time: five 1-minute scans at T. V. rates; analyses: on- and off-cell; cell mounts: quartz disks and thin film (Formvar).

Table I

Interaction of Beam with Sample
("Durability" Tests)

Series	N_T^+	N_L^{++}
I. Human hemoglobin (pressed powder pellets)	372	343
	355	307
	347	311
10 tests	369	330
	359	325
	365	325
	372	346
	367	331
	353	314
	354	313
II. Same cell	95	20
	103	30
15 tests as follows:	102	32
	105	34
10 tests	100	25
	98	-
	105	-
	108	-
	109	-
	112	-
30-minute interval		
5 tests	95	-
	103	-
	107	-
	136	-
	82	-

^+N_T = total intensity (i.e., peak of the Fe K_α lines in cpm).

$^{++}N_L$ = net intensity (= corrected for total background contributions by subtraction of N_B).

Table II

Magnitude of Background Contributions

Nature of N _B tested*	Standardization runs	
	Grand mean [†] (normal)	Grand mean [†] (sickle cell)
Support (SiO ₂)	65.584 ± 4.0	62.007 ± 11
Protein pellet (human serum albumin)**	43.351 ± 2.6	46.800 ± 8.5

(See Protocol Appendix B)

*Backgrounds tested at time of running normal and sickle cell preparations.

**See "Materials and Methods" section.

[†]Means are unweighted averages of cell means.

Table III

Sensitivity at Different Accelerating Potentials

Accelerating potential tested	Intensity readings (N_T from cell*)	N_B	N_T/N_B^{**}
10 KeV	80	<u>52</u>	1.269
	68		
	81		
	53		
	48		
15 KeV	183	<u>65</u>	2.994
	163		
	168		
	227		
	212		
18 KeV	172	<u>132</u>	1.606
	199		
	222		
	255		
	209		

*Nature specified in "Materials and Methods" section.

**Peak-to-background ratio.

Table IV

Experimental Design

Group	Number per group	Category	Number of cells per subject	Total number of cells
A	3	Asian	30	90
B	3	Black	30	90
W	3	White	30	90
B(S)	3	Sickled	10	30
Total number of cells analyzed = 300				

Table IV-a

EMPA

Protocol of Computer Basic Statistics, N_T ,
Grouped by Disk (for Euhemic Cells Only), Person, and Group

Disk

Disk number	Mean	SD	V*	N	Variance (SD) ²
1	135.900	14.209	10.46	10	201.897
2	152.020	12.658	8.33	10	160.226
3	138.940	10.082	7.27	10	101.651
4	177.000	34.343	19.40	10	1179.459
5	147.620	12.583	8.52	10	158.321
6	134.320	12.301	9.16	10	151.321
7	138.640	11.475	8.28	10	131.607
8	156.380	13.043	8.34	10	170.117
9	149.700	14.055	9.39	10	197.545
10	124.000	8.429	6.80	10	71.056
11	137.200	24.049	17.53	10	578.374
12	128.460	10.632	8.28	10	113.032
13	140.900	10.881	7.70	10	118.393
14	134.600	13.253	9.85	10	175.641
15	131.080	15.581	11.69	10	242.764
16	131.600	13.363	10.14	10	176.567
17	126.080	9.040	7.17	10	81.727
18	131.040	11.004	8.40	10	121.084
19	117.060	11.075	9.46	10	122.651
20	135.720	8.610	6.34	10	74.140
21	132.140	11.483	8.69	10	131.855
22	130.340	7.854	6.03	10	61.687
23	132.940	14.938	11.24	10	223.134
24	143.300	14.748	10.24	10	217.507
25	150.360	14.554	9.68	10	211.833
26	143.180	12.866	8.95	10	165.527
27	129.000	12.772	9.90	10	163.113
	-----	-----	-----	---	-----
	138.138	18.503	13.39	270	342.344

*Pearson's Coefficient of Variation.

Table IV-b

Person

Cell type	Person number	Mean	SD	V	N	Variance (SD) ²
Euhemic	1	142.287	14.265	10.03	30	203.491
	2	152.980	28.536	18.65	30	814.329
	3	148.240	14.831	10.00	30	219.955
	4	129.887	16.858	12.99	30	284.207
	5	135.527	13.980	10.32	30	195.429
	6	129.640	11.557	8.91	30	133.556
	7	128.307	13.226	10.31	30	174.923
	8	135.527	14.099	10.40	30	198.782
	9	140.847	16.091	11.42	30	258.917
Sickle	10	137.240	21.211	15.46	10	449.911
	11	122.020	17.934	14.70	10	321.620
	12	131.900	10.962	8.31	10	120.166
		-----	-----	-----	-----	-----
		137.363	18.634	13.57	300	347.212

Table IV-c

Group

Group	Mean	SD	V	N	Variance (SD) ²
A	147.835	20.778	14.05	90	431.729
B	131.684	14.553	11.05	90	211.785
W	134.893	15.404	11.42	90	237.283
B(S)	130.387	18.357	14.09	30	336.978
	-----	-----	-----	-----	-----
	137.363	18.634	13.57	300	347.210

Table V
Effects of Time Lapse in Sampling Sequences
Among Asian Subjects

Week	Subject sample	Mean, N_T	SD	V	(SD) ²	N
I	A	135.900	14.209 $\pm$ 4.5	10.45	201.897	10
	B	177.000	34.343 $\pm$ 11	9.40	101.651	10
	C	138.640	11.475 $\pm$ 4.6	8.28	131.667	10
II	A	152.020	12.658 $\pm$ 4	8.33	160.226	10
	B	147.620	12.583 $\pm$ 4	8.52	158.321	10
	C	156.380	13.043 $\pm$ 4	8.34	170.117	10
III	A	138.940	10.082 $\pm$ 3	7.26	101.651	10
	B	134.320	12.301 $\pm$ 4	9.16	151.321	10
	C	149.700	14.055 $\pm$ 4	9.39	197.545	10

Table VI

Net Intensity (N_L) Values—
Corrected for SiO_2 Background

Group		Means	
	Disk	Person	Group
Euhemic	72.500	73.4	80.524
	71.020		
	76.740		
	116.800	89.1	
	79.420		
	71.120		
	79.640	80.0	
	83.180		
	77.300		
	62.800	65.0	
	67.800		
	64.380		
	73.900	69.9	67.140
	72.140		
	63.720		
	68.200	66.5	
	64.880		
	66.440		
	58.689	65.3	
	67.320		
	69.760		
	64.260	68.0	70.060
	68.140		
	71.700		
89.760	76.9	72.577	
76.780			
69.200			
Sickle	78.140	78.140	68.380
	58.900	58.900	
	68.100	68.100	
			72.113

Table VII

Total Intensity (N_T) Values for Fe K_α in Euhemic Cells on Quartz

	Mean	SD	V	(SD) ²
Cell	3395	132	3.89	17,424
	3249	145	4.46	21,025
	4012	121	1.06	14,641
	3935	47	1.19	2,209
	3836	150	3.91	22,500
	3844	136	1.54	18,496
	3885	2213	56.96	4,897,369
	2178	1777	81.59	3,157,729
	1493	73	4.89	5,329
	2530	2150	84.98	4,622,500
	----	----	----	----
	3236	694	21.46	482,191
Support	3708	230	6.20	52,900
	3627	107	2.95	11,449
	4220	139	3.29	19,321
	4147	53	1.28	2,809
	4044	142	3.51	20,164
	4200	84	2.00	7,056
	3840	2136	55.62	4,562,496
	2304	1957	84.94	3,829,849
	1495	50	3.34	2,500
	2450	1942	79.27	3,771,364
	----	----	----	----
	3403	684	20.09	467,856

Table VIII

Total Intensity (N_T) Values for Fe K_α in Red Cells
from Sickle Cell Anemia Patients on Quartz

	Mean	SD	V	(SD) ²
<u>Cell</u>	3636	1399	38.48	1,957,201
	1292	37	2.86	1,369
<u>Sickle</u>	1254	65	5.18	4,225
	1239	47	3.79	2,209
	1310	80	6.11	6,400
	1213	43	3.54	1,849
	-----	-----	-----	-----
	1657	278	9.99	328,875
<u>Normal</u>	3633	1677	46.16	2,812,329
<u>appearing</u>	1368	43	3.14	1,849
	1223	36	2.94	1,296
	1229	69	5.61	4,761
	1304	67	5.14	4,489
	1242	52	4.19	2,704
	-----	-----	-----	-----
	1666	324	11.20	471,238
	-----	-----	-----	-----
	1661	201	10.59	400,056
<u>Support</u>	3370	1400	41.54	1,960,000
	1485	59	3.97	3,481
	1415	68	4.81	4,624
	1365	48	3.52	2,304
	2061	1476	71.62	2,178,576
	1375	77	5.60	5,929
	-----	-----	-----	-----
	1845	521	21.84	692,486

Table IX

Intensity Values for Fe K_a of Euhemic Cells on Thin Film

	N _T	N _B	N _L	N _T /N _B
Cell	574	393	181	1.46
	2370	1977	393	1.20
	799	623	176	1.28
	766	588	178	1.30
	869	605	264	1.44
	836	571	265	1.46
	803	536	267	1.50
	766	499	267	1.54
	---	---	---	---
	973	724	249	1.40

Table X

Fe K_a Intensity Values Comparison, EMPA and SEM-EDX

Iron K _a X-radiation					
EMPA			SEM-EDX		
Cell type	*	Quartz	Thin film	Quartz	
Euhemic	N _T	147 21 ± 0.2	973 571 ± 71	3236	694 ± 69
	N _B	68	724	2902	
	N _L	79	249	291	
	MDL	0.1%	0.1%	0.2%	
	P/B	2.2	1.3	1.1	
Sickle		132 18 ± 0.6	Quartz		
			Sickle		Normal appearing
			1657 278 ± 46	1666	324 ± 54
			1486	1548	
			118	100	
			0.3%	0.4%	
	N _T	132 18 ± 0.6	1657 278 ± 46	1666	324 ± 54
	N _B	64	1486	1548	
	N _L	68	118	100	
	MDL	0.1%	0.3%	0.4%	
	P/B	2.1	1.1	1.1	

*Legend: Total counting peak: N_T counts
 Background: N_B counts
 Net count in peak: N_L counts
 MDL (wt%) 60 sec: MDL
 P/B ("Gross peak"-
 to-Bkgd ratio): N_T/N_B

XBL768-9205

Fig. 1. Diagram of reference standard preparation: pelleted human hemoglobin and human serum albumin on support.

XBB 767-6574

Fig. 2. Scanning electron micrograph of an air-dried whole blood smear. X500. (JEOL). Accelerator voltage 10 keV beam diameter 200 nm, probe current 100 μ A, scan exposure rate 40 sec.

Fig. 3. Histogram of results for quantitative analysis (EMPA) using data from Table VII. Relative net iron K-radiation distributions are given.

XBL 769-10656

XBB 768-6876

Fig. 4. Scanning electron image of a euhemic human red blood cell. The bioconcave, rounded shape in appearance is observable. X5,000. Acceleration voltage 10 keV, beam diameter 20 nm, probe current 100 μ A, scan exposure rate 70 sec.

XBB 769-8498

Fig. 5. Scanning electron microscope shows images of euhemic human red blood cells mounted on thin film (Formvar). These cells are fixed-washed, then mounted, air-dried, and examined without any coating of conductive material. X4,000. Acceleration voltage 10 keV, beam diameter 200 nm, probe current 100 μ A, scan exposure rate 40 sec.

XBL7611-10710

Fig. 6. EDX energy spectra of the reference standard. Acceleration voltage 15 keV; beam diameter 200 nm; probe current 100 μ A; area scanned: $0.2\mu \times 0.7\mu$; T. V. scan rates; integration time 1,000 sec.

00004607043
-41-

Fig. 7. EDX energy spectra of a single human rbc mounted on SiO₂. Compare Fig. 8. Acceleration voltage 15 keV; beam diameter 200 nm; probe current 100 μ A; area scanned: 0.2 μ x 0.7 μ ; T. V. scan rates; integration time 1,000 sec.

XBL7610-9360

Fig. 8. EDX energy spectra of a single human rbc mounted on thin film (Formvar). Acceleration voltage 15 keV; beam diameter 200 nm; probe current 100 μ A; area scanned: 0.2 μ x 0.7 μ ; T. V. scan rates; integration time 1,000 sec.

XBL7610-9361

Fig. 9. A representation of an Fe K_{α} energy spectrum removed from a typical EDX spectra plot. Integration time: five 1-minute scans at T. V. rates; analyses: on- and off-cell; cell mounts: quartz disks and thin film (Formvar).

This report was done with support from the Department of Energy. Any conclusions or opinions expressed in this report represent solely those of the author(s) and not necessarily those of The Regents of the University of California, the Lawrence Berkeley Laboratory or the Department of Energy.

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