Microspectrophotometric Determination of Nonheme Iron in Maturing Erythroblasts and its Relationship to the Endocellular Hemoglobin Formation

By C. A. SONDHAUS AND Bo THORELL

DURING the growth and differentiation of erythroid cells, iron is incorporated and passes from the inorganic state into heme compounds, finally producing the normal hemoglobin complement of the mature red blood cell. It has been suggested\textsuperscript{1-4} that the process presumably involves the chelation of iron by a protoporphyrinogen or reduced protoporphyrin. A heme-forming enzyme in reticulocytes has been described,\textsuperscript{5} and evidence for the location of the enzymic conversion of protoporphyrin and iron to heme within the mitochondria has been presented by Nishida and Labbe.\textsuperscript{6} Vogel, Richert and Schulman\textsuperscript{7} proposed that iron itself plays a specific catalytic role in the sequence of reactions from glycine to protoporphyrin prior to its chelation to form heme. The time course of iron uptake in normal hemopoiesis has been studied radioautographically with Fe\textsuperscript{59} by Austoni,\textsuperscript{8} and by Suit, Lajtha, Oliver and Ellis,\textsuperscript{9} who found uptake to be continuous throughout differentiation, the proerythroblasts showing somewhat higher uptake than the other stages. In recent electron microscope studies, Bessis and Breton-Gorius\textsuperscript{10,11} have demonstrated the presence of ferruginous granules in the spleen and bone marrow reticulum cells and also in the young erythroblasts which frequently cluster around them; in the abnormal states these granules are large and have long been observed in the light microscope as “iron pigments.”

The present study represents an attempt to obtain microspectrophotometric evidence of the presence of inorganic or “ferritin” iron in erythroblasts during normal hemopoiesis, and further, to study its quantitative relation to the extent of hemoglobinization in the individual cells at different maturation states.\textsuperscript{12} The relatively high absorption-coefficients of both constituents at different regions of the ultraviolet and visible spectrum makes it possible in principle to identify each in the presence of the other within a single cell and to draw some conclusions as to their relative amounts as the red cell maturation proceeds.

Material and Methods

Erythroid cells of Salamandra Maculosa\textsuperscript{*} were used as experimental material. These cells arise mainly in the spleen and are larger than their mammalian homologues, being nu-

\textsuperscript{*}The animals were originally from Spain but maintained in excellent condition at our laboratory for years.
cleated and elliptical with major axis about 5 times as large as the diameter of human cells. For these reasons the young cells are relatively well fit for microspectrophotometric analyses; they are favorable optically due to their broad, densely absorbing cytoplasm. Their hemoglobin is spectrophotometrically identical with that of mammalian cells in the actual spectral range.

Specimens were obtained as follows. The animal was placed in a dissection-gas chamber glove box and subjected to an atmosphere of pure carbon monoxide; after death, the spleen was dissected out, and both smears and living preparations in Tyrode physiological salt solution were made on quartz slides under a stream of the gas. The smears were fixed in methanol for 2 minutes, mounted in water-free glycerol and protected with quartz cover slips. Living preparations were covered similarly and sealed with vaseline, and both types of preparation were not removed from the gas-filled glove box until after these steps were completed.

The carbon monoxide, with its affinity for hemoglobin (Hb), was used for 3 reasons. The first was to define as well as possible the absorption spectrum by making the formation of other hemoglobin compounds less likely (cf. oxy- and methemoglobin). The second was to provide a stable enough state in the cells, i.e., a high proportion of carboxyhemoglobin (HbCO), to allow spectral absorption curves to be obtained on several cells in each preparation before oxidation or denaturation had sensibly altered the curve shapes. The third reason was to make optimal the conditions for measuring iron absorption in the ultraviolet region from 310 to 350 m, since HbCO has a lower absorbancy there relative to its Soret peak at 418 m than do the other Hb compounds. The stability of the preparation was always checked by duplicate runs on the first cell measured after an interval of about 3 hours. In all cases the measurements were begun within one hour of dissection.

The experimental method employed was recording absorption microspectrophotometry, utilizing a xenon arc (Osram XBO 162) as light source, focused on the entrance slit of a Zeiss double quartz prism monochromator. Light from the monochromator exit slit was focused on the preparation by a reflecting condenser, and a reflecting objective (Beck, cond.ap. 0.6, obj. ap. 1.0) formed the image. The image in turn was focused on the cathode of an IP21 photomultiplier tube, and one area of the image was selected by an adjustable aperture in the photometer head. A vibrating mirror alternately projected a neighboring blank reference area onto the same region of the photocathode, and the resulting pair of signals was fed to a high gain d.c. amplifier which was self-compensated for the variation in light intensity and phototube sensitivity over the spectral range used by a dynode feedback circuit. The output of the amplifier was recorded as a function of wavelength, and the resulting absorption curves, of 1.5 sq. m area in the cells, covered a usable wave length range of 310 to 700 m.13,14 The instrument has been described in references 13 and 14.

Absorption curves were obtained which not only determined the concentration of Hb in the cell area measured by its absorbancy at the Soret peak but could also be analyzed to provide information on the chemical state of the Hb and on the presence and proportion of the other absorbing substance. The known curve of specific absorption for HbCO over this wavelength region provided a "calibration" of the system as regards distortion of spectrum shape due to background, scattering, and refractive index boundary effects. The system's calibration was checked by alternate runs on the first cell measured after an interval of about 3 hours.

The preparations were first examined in light of 418 m, and early erythroid cells were located and identified by their faint cytoplasmic absorption at this wave length and by their characteristic cytologic appearance. Cells containing as little as 20 per cent of the mature cell area-concentration of Hb could be reliably located and positioned, and spectral recordings could then be taken of cytoplasmic areas. Mature red cells provided a control, yielding curve shapes and absorption values which served as a basis for comparison.

Absorption curves of Hb and HbCO in solution were also made on a recording differential spectrophotometer (Akerman, unpublished), using preparations made from hemolyzed cells of peripheral Salamander blood. These served as comparison standards in
evaluating the absorption curves made microscopically on the single cells. In addition, absorption curves of inorganic iron in solution were made under the same conditions using Fe$^{+2}$ and Fe$^{+3}$ as chloride solutions, analytical grade, at various pH from 3.7 to 10, and of horse spleen ferritin at pH 6.9.

RESULTS

Figure 1 illustrates some typical erythroid cells at different stages of maturation, with Soret peak and ultraviolet absorption images included. In the first row are absorption images taken at 257 m$\mu$, while the second row illustrates absorption images at 418 m$\mu$. The same cell is shown down each column. The first column shows an early erythroblast, illustrating the faint cytoplasmic absorption at 418 m$\mu$ and the strong nuclear and cytoplasmic absorption at 257 m$\mu$. The second column shows an early erythroblast and a nearly mature erythrocyte under the same conditions. The third column shows two erythroblasts at an intermediate stage ("polychromatic" to "orthochromatic") and a mature erythrocyte, at 418 and 257 m$\mu$. All cells are from dry smear preparations.

Figure 2 illustrates absorption curves from three stages of maturation, expressed in per cent absorption as the data were recorded on the chart paper (see also table 1). The data for curve 3 are representative of mature cells, with Soret peak absorptions of over 80 per cent, of which type of cells about 20 were measured. Curve 2 represents an intermediate stage: about 30 such cells were analyzed, their peak absorption values ranging between 40 and 80 per cent. Curve 1 is that of a young cell, of which 20 were measured; the Soret peaks on these cells absorbed under 40 per cent. Curve B represents...
Fig. 2.—Cytoplasmic absorption curves from erythroid cells at three different stages of maturation: 1, young; 2, intermediate; and 3, mature cells. B = baseline. Compare with table 1 and text.
flat absorption from 310 to 380 mμ, which is seen in curve 1, in the area of cytoplasm which was measured; these cells exhibited the intermediate types of shape of which curve 2 is representative. In this regard, the appearance of granular clusters, presumably with a high concentration of ferritin, which has been noted elsewhere, may explain why a high iron absorption was not invariably found in such a random sampling of cytoplasmic areas in immature cells.

The curves were replotted as log absorbancy (log log absorption per cent) in order to utilize the property of shape preservation which results mathematically from this transformation, thus permitting different concentrations of absorbing material to be compared with regard to identity.

In order to estimate the level of background to be subtracted from a curve, the ratio of absorbancy near the Soret peak, at 422 mμ, to that at 570 mμ, was obtained from curves of reduced Hb and HbCO made in solution and therefore free from the scatter or refractive index effects which may have been present in the cells. This ratio was then used to successively approximate a position of the background curve below the absorption curve itself as found in a cell. By choosing a background level which caused this ratio to fall within the estimated limits of accuracy, it was usually found that the difference between curve and background level above 650 mμ, was less than 1 per cent transmission unit, as should occur when virtually no absorption exists.

Log absorbancy values were then plotted for each 10 mμ of wavelength. The absorbance values at 340 and 422 mμ, were used for a two wavelength calculation of iron concentration (see following section). Since the 3 wavelengths 340, 422 and 570 mμ are approximate isosbestic points for reduced Hb and HbCO, it can be seen that the ratios of absorbancy due only to either form of Hb at all three points are independent of the relative proportion of either compound in the mixture. This is also approximately true for oxyhemoglobin, but the conditions were such that very little of the latter compound was present.

Figure 3A illustrates the degree of agreement between the absorption curve of HbCO as found in a living Salamander erythrocyte (circles) and that of salamander HbCO in solution (triangles), obtained with the use of the above method of analysis. A published human HbCO absorption curve is included for comparison. It can be seen that the curve as found in the cell can be regarded as that of a pure Hb compound identical to that of human cells (HbCO in this case), and undisturbed by optical artifacts, scattering or other distortions after correction as above. Figure 3B illustrates the conditions found in smears dried in CO and fixed and mounted as described above; in this case, one cell falls almost on the HbCO curve (triangles), while another and more typical cell curve (circles) falls roughly halfway between the HbCO and reduced Hb curves, which are included for comparison. The curves of most cells in the dry smears were found to correspond to mixtures of about half-reduced Hb and half HbCO by the time they were recorded. The agreement with known values from macrodeterminations indicates that reliable curves can be obtained from these cells under proper conditions with this method of analysis. This is most important for this study insofar as it bears on the accuracy with which relative absorbancies of these cells can be determined in the 310 to 450 mμ region, in which comparisons of the curve shape can yield...
Fig. 3A.—O—O: Log absorbancy curve from cytoplasmic area in living erythrocyte. Δ: HbCO in solution. --- ---: HbCO.

information about the probable presence and amount of nonporphyrin iron by using the Hb absorption spectrum as a standard.

Figures 4A and 4B are groupings of log absorbancy curves of types 1 and 3 displaced somewhat vertically to illustrate the degree of agreement among a number of cells of similar type taken from different animals and preparations at different times. The ranges of absorbancies are, however, approximately like those occurring in the cells, the peak absorbancy of the young cells at 418 mμ falling at about 0.3 absorbancy units as seen in fig. 4A while those of the mature cells fall at almost 1.0 units. Intermediate forms were found to fall between these groups both in peak absorbancy values and in curve shapes in the 310 to 380 mμ region. It is seen that in the young cells, there is some 3 times as much absorbancy at 320 mμ relative to 422 mμ as there is in the older cells, which amount is enough to alter the shape of the composite ab-
Fig. 3B.—○○ and △: Log absorbance curves from cytoplasmic areas in dried erythrocytes. ---: HbCO. ---: Reduced Hb.¹

Absorption curve quite strongly up to 410 mμ. It may be remarked here that this difference occurs between curves which are already corrected for background as above; and secondly, that the difference curve is of a quite different shape and greater magnitude than uncorrected λ² (n < 4) scatter alone would produce.

Figure 5A illustrates the decomposition of an average curve for young cells in dry smears (circles) into the sum of an average curve for mature cells in dry smears (triangles) plus a second component; this has been done by subtracting the log values of absorbancy at each wavelength of the mature cell curve from those of the young cell curve. As the figure demonstrates, the re-
suiting difference curve as defined by the points (squares) agrees rather well with the absorption spectrum of inorganic Fe^{+++} in solution (fig. 5B), which has been inserted as the dashed curve in figure 5A. (The positions of the curves are arbitrary, as the log log property allows.) Although not shown, the same difference curve is obtained when the absorption curve of a living mature cell in Tyrode solution is subtracted from that of a young cell under the same condition.

**Discussion**

The early erythroblasts of the Salamander exhibit a complex spectral absorption curve which, compared to the spectral absorption of the mature red blood cell, contain an additional absorption between 300 to 400 μm. The shape and magnitude of this absorption correspond very closely to that of inorganic ferric ion or ferritin at physiologic pH. Nonporphyrin pyrrols, a possible alternative with somewhat similar spectral properties, absorb at significantly shorter wavelength. It can be concluded, therefore, that the cytoplasms of the early erythroblasts are relatively rich in nonheme iron.

The correction of the data into single cell absorption curves relatively free from distortion due to scattering and other nonspecific losses of light permits the estimation of relative values of inorganic iron concentration at different maturation stages in terms of total hemoglobin concentration. The absorbancy
per unit area at 422 mμ. \((E_{422})\) may be taken as a measure of the Hb concentration per unit area, since nonheme iron contributes only 2 per cent to the total absorbancy at that wavelength in mature or young cells respectively.\(^{20}\) The ratio of \(E_{340}/E_{422}\) in turn is a measure of the relative amount of nonheme iron and Hb per unit area, the former contributing up to 50 per cent of the absorbancy in young cells. If \(E_{340}/E_{422}\) is plotted against \(E_{422}\), figure 6 results, in which the vertical brackets indicate the range of values of the ratio found at each value of \(E_{422}\). Each point is at the average \(E_{422}\) for about 12 cells. It can be seen that the ratio \(E_{340}/E_{422}\) has already fallen almost to its mature cell value by the time the Hb synthesis is about one-third complete.

It follows from the Beer-Bouger law that the ratio of mass per unit area of iron \((m_{AF})\), to that of Hb \((m_{AH})\), is given by

\[
m_{AF}/m_{AH} = \frac{K_{340} - K_{422}(E_{340}/E_{422})}{K_{422}(E_{340}/E_{422}) - K_{340}}
\]

where the ratio of absorbancies is as above and the \(K\)'s are the absorption coefficients of Hb and iron at 340 and 422 mμ. One may also calculate the values of \(m_{AF}\) and \(m_{AH}\) in similar fashion. A brief summary of representative values of these quantities is given in table 2, together with estimated values of cytoplasmic area for mature cells and for the earliest cell types observed. It should be emphasized that many of the youngest cells had higher values of nonheme iron concentration than the average used, and that the cytoplasmic distribution, as mentioned above, was probably quite nonuniform. Thus it would be necessary to perform an area scanning measurement of \(E_{310}/E_{422}\) over each cell image to compute a more exact total amount. The total amount per cell given in the table must therefore be regarded only as an estimate; much further work would be necessary to increase its accuracy.

On the basis of these values, some tentative conclusions can be made. First, it appears that in normal erythroblasts the youngest cells measured may already have taken up enough inorganic or ferritin iron to provide for the subsequent Hb synthesis and Hb content of a mature cell, although the cytoplasmic areas of the young cells are small in comparison. Second, however, the fact that one or probably more maturation divisions intervene must at least double this requirement, indicating, in agreement with the Fe⁵⁰ uptake studies of Austoni\(^{6}\) and of Suit et al.,\(^{7}\) that a continuous further uptake of Fe must take place during differentiation. Third, an excess of nonheme iron relative to Hb content on a molecular basis seems to be present in the cell throughout its growth period; this result would tend to support the finding of Vogel et al.\(^{5}\) that the synthesis of Hb requires an excess of free Fe as catalyst. Fourth, the data thus suggest that the erythroid cell series itself may contain a pool or reserve storage site of nonheme iron.

**Summary**

The strong near-UV absorption of inorganic iron makes possible the microspectrophotometric determination of nonheme intracellular iron as distinct
from hemoglobin-bound iron. Erythroid cells of *Salamandra Maculosa* were therefore studied microspectrophotometrically in the near ultraviolet and visible region. Absorption curves from cytoplasmic areas in living and dried cells were obtained from 310 to 700 m\(\mu\), and two component analyses were made to determine the presence and amount of hemoglobin and nonhemoglobin absorption. Under the experimental conditions, intracellular hemoglobin was present largely as the carbon monoxide derivative, a stable form which provided a well defined standard to correct for nonspecific absorption and other spectral distortion due to the optical properties of single cells.

Absorption curves from the youngest cells measured were found to differ
Fig. 5B.—Absorption curves of $4 \times 10^{-5}$ molar Fe$^{+++}$ (1) and the same concentration of iron as horse spleen ferritin (2), both in water solutions at pH 6.9. (The ferritin preparation was kindly supplied by Dr. R. H. Neve, Berkeley).

from those of mature cells, the difference curve agreeing most closely with that of inorganic iron or ferritin in solution. In the youngest cells, nonheme iron appeared to exceed 6 per cent by weight of hemoglobin, or over 20 times molar hemoglobin concentration, dropping and remaining at about twice the hemoglobin molarity from the time hemoglobin synthesis was one-third complete until maturity. Allowing for the increase of total cell mass with growth, the data suggest that although young erythroblasts may already have enough Fe for subsequent Hb synthesis, they continue to incorporate excess Fe throughout maturation and may thus contain a reserve pool of nonheme iron.

**SUMMARIO IN INTERLINGUA**

Le forte absorption, in le ultravioletto proxime, de ferro inorganic rende possibile le determination microspectrophotometric de non-hemic ferro intracellular in distinction ab ferro ligate a hemoglobina. Cellulas erythroide de *Salamandra maculosa* esseva studiate, per consequente, per medio de
Fig. 6.——$E_{340}/E_{422}$ versus $E_{422}$, illustrating the decrease in cytoplasmic nonheme iron with maturation of erythroid cells.

Table 2.—Calculations of the Ratios Non-Heme Iron to Hemoglobin from the Cytoplasmic Absorbancies in Salamander Erythrocytes and Early Erythroblasts

<table>
<thead>
<tr>
<th></th>
<th>Mature erythrocytes</th>
<th>Early erythroblasts</th>
<th>Mature : Early</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{422}$</td>
<td>0.80</td>
<td>0.20</td>
<td>4 : 1</td>
</tr>
<tr>
<td>$E_{340}$</td>
<td>0.18</td>
<td>0.08</td>
<td>2 : 1</td>
</tr>
<tr>
<td>Area of cytoplasm</td>
<td>150 sq.µ</td>
<td>&lt;30 sq.µ</td>
<td>5 : 1</td>
</tr>
<tr>
<td>Hb/area</td>
<td>1.3 µg./sq.µ</td>
<td>0.3 µg./sq.µ</td>
<td>4 : 1</td>
</tr>
<tr>
<td>Hb/cell</td>
<td>180 µg.</td>
<td>9 µg.</td>
<td>20 : 1</td>
</tr>
<tr>
<td>Fe/Hb per unit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>area (molar)</td>
<td>&lt;2</td>
<td>&gt;20</td>
<td>1 : 10</td>
</tr>
<tr>
<td>Fe/Hb per unit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>area (mass)</td>
<td>&lt;0.6 µg.</td>
<td>&gt;0.5 µg.</td>
<td>1 : 10</td>
</tr>
<tr>
<td>Fe/cell</td>
<td>&lt;1.0 µg.</td>
<td>&gt;0.5 µg.</td>
<td>&lt;2 : 1</td>
</tr>
</tbody>
</table>
se concordava le plus proximamente con le curva del ferro inorganico o de ferritina in solution. In le cellulas le plus juvene, il pareva que ferro nonhemic excedeva in peso 6 pro cento del hemoglobina, i.e. plus que 20 vices le concentration molar de hemoglobina. Sequeva un declino con stabilisation a circa duo vices le molaritate de hemoglobina inter le tempore quando le synthese de hemoglobina esseva un tertio complete e le attingimento del maturitate. Si nos prende in consideration le augmento del massa total del cellulas in le curso de br crescentia, le datos suggere que, ben que juvene erythroblastos ha possibilemente satis ferro pro le subsequente synthese de nemoglobina, illos continua incorporar excessos de ferro in le curso de br maturation e contine assi possibilemente un reservoir de ferro nonhemic.

REFERENCES
Microspectrophotometric Determination of Nonheme Iron in Maturing Erythroblasts and its Relationship to the Endocellular Hemoglobin Formation

C. A. SONDHAUS and BO THORELL