ELECTRON MICROSCOPE AUTORADIOGRAPHY OF ERYTHROID CELLS USING RADIOACTIVE IRON

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ABSTRACT
The "circle analysis" method of Williams (1969) and a new improved method employing hypothetical grains described in the previous paper have been used to analyze the distribution of autoradiographic grains over erythroid bone marrow cells labeled with radioactive iron, 55Fe. The resolution obtainable with this isotope was determined by measuring the distribution of grains about a thin line source. This distribution was also used in calculation of the circle size for the Williams's analysis and the distances of hypothetical grains for the new method. The new method provides estimates for the amount of activity in the regions of condensed and extended nuclear chromatin and for the concentration of isotope at the junction between these two areas. The possible significance of activity in this junctional region is discussed.

INTRODUCTION
Radioactive iron, 55Fe, is a relatively new isotope in autoradiographic work, although Forberg et al. (1964) suggested from theoretical considerations that the resolution should be high, and Orlic (1968) showed experimentally, using red cell ghosts, that this was the case. This isotope decays by electron capture by the nucleus, creating a vacancy in the inner shell which is then filled by an outer electron. This releases energy either in the form of X rays or by ejection of another shell electron—the so-called Auger emission. The spectra differ from those of tritium in that they are line spectra (see Fig. 1). The Auger electrons have energies of 5-6 keV, and some of 0.5-0.6 keV (Forberg et al., 1964).

The interpretation of electron microscope autoradiographs depends on a knowledge of the expected grain distributions around radioactive sources, and in a recent paper a calibration specimen has been described using tritium and the resolution values for electron microscope autoradiography calculated under different geometric and photographic conditions (Salpeter et al., 1969). Resolution in this case was defined as the distance from a radioactive line source in which half the developed grains would fall. More recently, this work has been extended to 14C (E_max 155 keV) and since the emission energy of 35S (E_max 167 keV) is similar, the experimental resolution values determined are also applicable to this isotope (Salpeter et al., 1971).

Since radioactive iron, 55Fe, is now becoming more widely used in autoradiography and its usefulness at the electron microscope level has been demonstrated, a calibration source similar to that of Salpeter's was prepared for 55Fe and the experimental resolution value obtained was used to analyze the distribution of activity within erythroid cells from mouse bone marrow. The autoradiographs were analyzed both by the "circle analysis" method of Williams (1969) and by the new "hypothetical grain" method described in the preceding paper.

Radioactive iron is a convenient label for the porphyrin moiety of the hemoglobin molecule and so can be used in the study of hemoglobin synthesis.
in developing red blood cells (Parry and Blackett, 1970).

METHODS

Resolution Source

A radioactive hot line source of $^{55}$Fe was prepared as follows.

The isotope was incubated with serum in vitro for 30 min in order to bind it to the transferrin present. A thin smear was made of differing dilutions of the serum onto a prepolymerized sheet of Araldite and allowed to dry. This was fixed in 1% osmium tetroxide, rinsed in methanol, and then a second layer of Araldite was poured over this and allowed to harden at 60°C, giving a sandwiched layer of radioactive iron between two layers of Araldite.

The blocks were cut into pieces and uniform pale gold sections were cut at right angles to the line, so producing a radioactive thin line source. Only blocks which produced thin lines were used and care was taken to select sections showing the same interference color, so being as near the same thickness as could be judged by eye. Sections were picked up by using a platinum loop and were transferred to carbon-coated slides.

Autoradiography

Sections were covered with a closely packed monolayer of silver halide crystals (purple interference color) by a modification of the dipping method (Bachmann and Salpeter, 1965). The required dilution of emulsion which would give the purple interference color was determined experimentally, and dipping was then standardized by the use of a dipping apparatus designed to give a steady and regular motion. Standardization is necessary since the speed of removal of the slide from the emulsion and the dilution of the emulsion determine the thickness of the film.

The slides were exposed for 4 wk in light-tight boxes containing silica gel in a desiccator at room temperature and then developed in Microdol X for 3 min at 20°C, fixed in Amfix, and washed in distilled water.

The carbon film was then floated off the glass onto distilled water and sections were picked up on grids. If the carbon film proved difficult to remove from the glass, a score was made around the section in the carbon film and a small drop of dilute (~3%) hydrofluoric acid was pipetted into it. The gentle etching of the glass causes the carbon to lift up onto the surface of the drop and it is then easily picked up with a platinum loop. At no time does the hydrofluoric acid come into contact with the section. The sections were lifted onto distilled water and picked up on grids and viewed unstained in the electron microscope.

Labeling of Erythroid Cells

Mice were injected with 5 µCi/g body weight carrier-free $^{55}$Fe via a lateral tail vein. At the appro-
At the appropriate time, the animal was killed and the bone marrow was taken from the femurs and processed for electron microscopy, using 3% glutaraldehyde as fixative (Sabatini et al., 1963) followed by postfixation in osmium tetroxide, and embedded in Araldite. Autoradiograms were prepared as already described and after exposure were developed in Microdol X and stained with alkaline lead hydroxide (Karnovsky, 1961). Chemical extraction of bone marrow by the method of Teale (1959) was used to determine the proportion of radioactive iron (in this case $^{59}$Fe) incorporated into heme.

RESULTS

Thin Line Source

Grains appeared on both sides of the thin line source at varying distances, and photographs were taken at random along its length (Fig. 2). The distance from the midpoint of each grain (determined as the center of the smallest circle which would enclose the grain) to the center of the hot line was measured for over 1,000 grains to a cut-off distance of 2 µm on each side of the line. A histogram was plotted of the frequency of occurrence of grains at various distances from the line (Fig. 3).

A one-sided histogram was plotted as the grains were approximately symmetrically distributed about the hot line. The distance which includes 50% of the grains (HD value) can be read from the integral curve (Fig. 4) and is found to be 1,200 Å.

Equation 5’ in the Appendix of the preceding paper was then fitted to these results by suitable choice of the constants $a$, $d$, and $c$, and Equation 2’ was then used to obtain the list of random grain distances on a random basis using a simple computer program.

**Figure 2** Radioactive "hot line" of $^{59}$Fe. The smear of labeled transferrin is seen as a thin electron-opaque line just below a somewhat broader dark band. × 8,000.

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FIGURE 3  Histogram showing number of grains at different distances from a thin $^{55}$Fe line source.

Bone Marrow

The uptake by the bone marrow of radioactive iron is largely by the erythroid cells, although small amounts appear in other cell types possibly due to incorporation into heme-containing enzymes (Fig. 5). The label appears in the nucleus as well as the cytoplasm of the erythroid cells, and within the nucleus it gave the appearance of being more confined to the areas of extended chromatin (euchromatin) (Fig. 6).

Biochemical extraction of heme from washed bone marrow cells showed that 95% of the radioactive iron is incorporated into heme.

The circle analysis of Williams (1969) was first applied to a set of random prints of late erythroid cells, using the 50% circle size calculated from the HD value for a line source (circle radius = 1.7 $\times$ 1,200 = 2,040 Å) and using heterochromatin, euchromatin, and junctional heterochromatin/euchromatin (H/E) as separate entities. A chi-squared test was applied to the results (Table I) and with 2$^o$ of freedom the total $\chi^2 = 28.5$ showing that the nonrandom distribution within the nucleus is highly significant at the 0.1% level. For the junctional H/E region, $\chi^2 = 10.2$ also has 0.1% probability of significance. This method of analysis therefore demonstrates that there is a nonuniform distribution of activity but does not provide estimates of the relative activity in different regions.

The new method an analysis using hypothetical grains described in the preceding paper was then applied. A grid of points was superimposed over the autoradiographs and a scale and circle (radius = HR) were used in conjunction with a list of random “distances” and “circles.”

Table II shows the source of the hypothetical grains in the column on the left and in the other columns the region underlying the circle surrounding these grains. The total grains in each column at this stage represent the distribution of grains for a uniform distribution of isotope.

The activity in heterochromatin was then altered (Tables III and IV) and the new distribution of grains was compared directly with the real distribution of grains to see if there is a high probability that the two distributions have been made the same as indicated by the $\chi^2$ test. It will be seen that with a reduction of the grains in the heterochromatin by a factor 2 (Table III) there
are too many hypothetical grains over both heterochromatin and euchromatin. With a factor 4 (Table IV), the hypothetical grains over heterochromatin are about right but those over euchromatin are much too high. The hypothetical grains over the junctional region in each case are too low. The $\chi^2$ analysis shows that the fit is very poor for both these ratios of activity of heterochromatin to euchromatin. No ratio of activities could be found which gave a reasonable fit.

If the junctional region between heterochromatin and euchromatin was well defined, for instance as would be obtained with a membrane, it would be possible to assume a certain activity per unit length for this structure and then determine the distribution of grains about this membrane as described in the preceding paper. However, the junctional region between heterochromatin and euchromatin in erythroid cells is ill defined, so the above procedure is impracticable. An approximate alternative is to assume that the junction is a straight line and then use the experimental curve for such a line shown in Fig. 4, to obtain

the proportion of grains falling outside the limits of the junctional region. The 50% distance (2,040 Å) for a point source (double-dashed line) corresponds to 66% on the curve for a line source. Consequently, 34% of grains will fall outside the junctional region, half (17%) in heterochromatin, and half in euchromatin. Using this extra site of activity, the real grain distribution can in fact be fitted exactly as shown in Table V. Let the activity (disintegrations) in heterochromatin be $x$; and in euchromatin be $y$; and the activity in the junctional region be $z$. From the distribution of hypothetical grains shown in Table II, the grains originating from heterochromatin, falling over heterochromatin will be $\frac{333x}{711}$, over euchromatin $\frac{51x}{711}$ and over the junctional region $\frac{327x}{711}$. Values for euchromatin can be obtained in a similar manner. For the junctional region, 0.17$z$ grains will fall in both the heterochromatin and the euchromatin.

The sum of each column in Table V gives three
**Figure 5** Electron microscope $^{65}$Fe autoradiograph of mouse bone marrow, showing a labeled reticulocyte and granulocyte and several erythroblasts. $\times 13,250$. 
Figure 6  Electron microscope $^{55}$Fe autoradiograph of mouse bone marrow. Label found in both cytoplasm and nucleus and showing nuclear pores. $\times$ 26,400.
TABLE I
Circle Analysis of Mouse Bone Marrow 54Fe Autoradiography Using 2,040 Å Radius Circles

|                      | Heterochromatin | Euchromatin | Junctional H/E | Total  |
|----------------------|-----------------|-------------|----------------|--------|
| Number of grains (O) | 89              | 145         | 296            | 530    |
| Number of random grains (n) | 306          | 309         | 531            | 1,146  |
| Grains expected      | 142             | 143         | 246            | 530    |
| \( n \times \frac{1,146}{E} \) |                 |             |                |        |
| \( \chi^2 = \frac{(O - E)^2}{E} \) | 18.3           | 0.03        | 10.2           | 28.5   |
| Probability          | 0.001           | 0.80        | 0.001          | 0.001  |

TABLE II
Hypothetical Grains for Uniform Activity Throughout the Section

| Site of hypothetical grains | Source of hypothetical grains | Grains per unit area | Heterochromatin | Euchromatin | Junctional H/E | Total grains |
|-----------------------------|-------------------------------|----------------------|-----------------|-------------|----------------|--------------|
| Heterochromatin             | 1                             | 333                  | 51              | 327         | 711            |
| Euchromatin                 | 1                             | 31                   | 363             | 314         | 708            |
| Cytoplasm                   | 1                             | 55                   | 13              | 41          | 109            |
| Total                       | 419                           | 427                  | 682             | 1,528       |

The autoradiographic resolution achieved with the isotope 54Fe is slightly better than the value for tritium. This could be expected since the maximum energy of the Auger electrons is 6 keV, whereas with tritium there is a spectrum of energies with a mean of 5.5 keV (see Fig. 1). The 6 keV X rays might be expected to produce a higher background but the distribution of grains about the line source showed no evidence of this. The HD value of 1,200 Å for 54Fe compares favorably with the value of 1,600 Å for tritium quoted by Salpeter et al. (1969) for a similar section thickness and photographic emulsion.

Visual inspection of 54Fe autoradiographs of erythroid mouse marrow cells did not suggest specific labeling of the junctional regions in the nuclei, and it was only with the application of the circle analysis method of Williams that this became evident. This method does not, however, provide estimates of the relative activity in the various regions considered. The method of analysis described in the previous paper provides this as shown in Fig. 7. At the 95% level of confidence \( P = 0.05 \) there could be negligible activity in the heterochromatin provided that the activity in the junctional region is two to three times that in the euchromatin. The other extreme would be for the heterochromatin to have slightly more than one-half the activity of the euchromatin with the activity in the junctional region about the same as that in the euchromatin.

The maximum range for the activity in the junctional region is from about four times down to one-half the activity present in the euchromatin. It will be seen that in no circumstances is the data consistent with zero activity in the junctional region.

The presence of hemoglobin in the nucleus of erythroid cells is well established for both mammalian (immature) cells and avian and amphibian (mature) cells from ultraviolet absorption and
### Table III

**Hypothetical Grains for Chosen Activities in the Section**

| Source of hypothetical grains | Grains per unit area | Site of hypothetical grains | Total grains |
|-------------------------------|----------------------|-----------------------------|-------------|
| Heterochromatin               | 0.5                  | Heterochromatin             | 166         |
|                               |                      | Euchromatin                 | 26          |
|                               |                      | Junctional H/E              | 164         |
|                               |                      | Total                       | 356         |
| Euchromatin and cytoplasm     | 1.0                  |                             |             |
|                               |                      |                             |             |
| Total (n)                     | 232                  | 402                         | 519         |
| Real grain distribution       | 89                   | 145                         | 296         |
| Expected grains               | 114                  | 182                         | 234         |
| $n \times \frac{(89-114)^2}{114}$ | 5.5                  | $\frac{(145-182)^2}{182}$ | 7.6         |
| $\chi^2$                      | 16.5                 | $\frac{(296-234)^2}{234}$ | 29.6        |
| Probability                   | 0.025                | 0.005                       | 0.0005      |

### Table IV

**Hypothetical Grains for Chosen Activities in the Section**

| Source of hypothetical grains | Grains per unit area | Site of hypothetical grains | Total grains |
|-------------------------------|----------------------|-----------------------------|-------------|
| Heterochromatin               | 0.25                 | Heterochromatin             | 83          |
|                               |                      | Euchromatin                 | 13          |
|                               |                      | Junctional H/E              | 82          |
|                               |                      | Total                       | 178         |
| Euchromatin and cytoplasm     | 1.0                  |                             |             |
|                               |                      |                             |             |
| Total (n)                     | 169                  | 389                         | 437         |
| Real grains                   | 89                   | 145                         | 296         |
| $\chi^2$                      | 0.01                 | 18.6                        | 17.0        |
| Probability                   | 0.9                  | 0.0005                      | 0.0005      |

### Table V

**Hypothetical Grains for Chosen Activities (x, y, z) in the Section**

| Source of hypothetical grains | Grains per unit area | Site of hypothetical grains | Total grains |
|-------------------------------|----------------------|-----------------------------|-------------|
| Heterochromatin               | x 0.468x             | Heterochromatin             | 0.072x      |
|                               |                      | Euchromatin                 | 0.460x      |
|                               |                      | Junctional H/E              | x = 40      |
| Euchromatin                   | y 0.105y             |                             | 0.460y      |
|                               |                      |                             | y = 200     |
| Junctional H/E                | z 0.170z             |                             | 0.660z      |
|                               |                      |                             | z = 290     |
| Real grains                   | 89                   | 145                         | 296         |
|                              | 89 = 0.468x + 0.105y + 0.170z | 145 = 0.072x + 0.460y + 0.170z | 296 = 0.46x + 0.434y + 0.662z |
chemical-staining procedures (Davies, 1961; O'Brien, 1960) and estimates of the proportion of the nuclear hemoglobin in heterochromatin have been reported as 45% and 10% (Tooze and Davies, 1963; Small and Davies, 1970). The presence of labeled hemoglobin in the nucleus is not surprising as there are apparently large pores in the nuclear membrane which could allow easy access of labeled hemoglobin, which has been synthesized in the cytoplasm, and also the appearance of the euchromatic regions is similar to the cytoplasm. It is almost certain that the $^{55}$Fe activity is incorporated into hemoglobin since chemical extraction has shown that more than 95% of the activity in bone marrow cells is in heme, and it is unlikely that any heme remains uncombined with globin in erythroid cells. However, if there is free diffusion of hemoglobin, a concentration of activity would imply a concentration of hemoglobin in this region. The resolution of ultraviolet absorption methods is not sufficient to determine this but it might be possible by electron probe X-ray emission microanalysis using an instrument such as the AEI EMMA-4.

If heme is not concentrated in the junctional region, then an active process would have to be invoked. The synthesis in the nucleus of an essentially cytoplasmic protein such as hemoglobin is not considered likely since many of the enzymes for hemoglobin synthesis, including iron synthetase, the last step in the synthesis of heme, are associated with mitochondria. However, hemoglobin synthesis in isolated nuclei has been reported (Hammel and Bessman, 1964) although questioned by others (Kabat, 1968), so the possibility of a small amount of nuclear synthesis cannot perhaps be ruled out completely. It is clear that the junctional region can be metabolically active since thymidine and uridine have been shown to localize in this region in transforming lymphocytes after stimulation by phytohemagglutinin (Milner and Hayhoe, 1968). These compounds are involved in the synthesis of DNA, and it would therefore be of interest to know where the associated nuclear protein synthesis takes place.

The observation of specific labeling by $^{55}$Fe of

![Diagram](https://via.placeholder.com/150)
the junctional region between euchromatin and heterochromatin in normal mouse bone marrow does not on its own allow any definite conclusion as to its significance. It is hoped that studies under different experimental conditions and in different states of haemopoiesis will provide an explanation of this observation.

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