Indium-111-Labeled Cellular Blood Components: Mechanism of Labeling and Intracellular Location in Human Neutrophils

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Human neutrophils were labeled with In-111 oxine by incubation at room temperature, and the fate of the oxine and the intracellular location of the In-111 were determined. Neutral (1:3) In-111 oxine complex diffuses rapidly across the cell membrane and then dissociates. Some of the oxine leaves the cell and the In-111 binds intracellularly. After short periods of incubation the label is distributed mostly to four soluble components, a small proportion of which had a distribution similar to that of specific and azurophil granules. After longer incubation periods there was relatively less radioactivity with the soluble components and probably more attached to the particulate material. A small peak of radioactivity also appeared in the region of DNA distribution, but no confirmation could be obtained for the association of the In-111 radioactivity with DNA.

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The major cellular components of blood can be isolated and labeled in high yields with In-111 chelated to 8-hydroxyquinoline (oxine) (*1–4*). The labeled cells have been found to function normally and have been used to study cell kinetics and for the identification of pathologic processes in experimental animals (*1–4,5*), and man (*6,7*). In this study, the fate of the oxine and the intracellular location of In-111 have been examined in labeled human neutrophils.

MATERIALS AND METHODS

Cell labeling. Indium-111 chloride, 5–10 μCi carrier free, was diluted to approximately 1 ml with pyrogen-free water and adjusted to pH 5.5 with acetate buffer. Oxine, 50 μg (0.35 μmoles), dissolved in 0.05 ml ethanol, was added and the resulting complex extracted into chloroform. The extracts were evaporated to dryness and dissolved in 0.05 ml ethanol, to which 0.15 ml 0.15 *M* NaCl were added.

Human neutrophils (about 200 million) were isolated, as described previously (*8*), and finally suspended in 5 ml 0.15 *M* NaCl. The In-111 oxine complex was added dropwise to the cell suspension and the mixture incubated at room temperature (22°C) for 15 min or for other periods as indicated in specific experiments. The cells were centrifuged at 450 g for 5 min and were resuspended twice in 0.15 *M* NaCl and recentrifuged to remove any unbound radioactivity.

Elution of In-111 and oxine from neutrophils. Approximately 10 million labeled cells were suspended in 1 ml human plasma and were incubated at room temperature for varying times up to 2 hr. The cells were centrifuged at 1800 g for 15 min and the radioactivities in the pellet and supernatant plasma measured. In parallel experiments using 0.15 *M*

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NaCl as the suspending medium, released oxine was extracted with chloroform from the supernatant of the centrifuged cells. The chloroform extracts were evaporated to dryness and the residue was dissolved in 50 μl ethanol and then diluted to 1 ml with 0.15 *M* NaCl. Oxine was assayed spectrophotometrically at 240 nm against 0.15 *M* NaCl.

Subcellular fractionation experiments. The technique used has been described in detail previously (*8*). Approximately 200 million neutrophils suspended in 0.15 *M* NaCl were incubated at room temperature with In-111 oxine complex for 15 or 60 min and were then centrifuged at 450 g for 10 min. The washed cell pellet was suspended in 4 ml 0.2 *M* sucrose and homogenized with a tight-fitting Dounce homogeniser. The homogenate was centrifuged at 800 g for 10 min, and 5 ml of the extract subjected to analytical subcellular fractionation on a continuous sucrose density gradient in an automatic zonal rotor. The rotor was run at 35,000 rpm for 60 min, and 15 fractions were collected, weighed and assayed for density, radioactivity, and marker-enzyme activities (*8*). DNA was assayed by the method of Le Pecq and Paoletti (*9*).

Aliquots (0.1 ml) of the subcellular fractions were diluted to 1.5 ml with distilled water and were either extracted with chloroform or dialyzed against 0.15 *M* NaCl for 18 hr at room temperature using a dialysis membrane with a cutoff molecular weight of 2,000. Further aliquots (0.1 ml) of the fractions were diluted to 0.5 ml with distilled water and 0.2 ml of 0.5 *M* trichloroacetic acid added. Centrifugation at 2,500 g for 15 min was used to collect any precipitate.

Gel filtration. The 800 g $\times$ 10 min supernatant of the homogenate obtained from 15 min incubation was further centrifuged at 250,000 g for 180 min. A second aliquot of this supernatant was subjected to molecular exclusion chromatography on a 3- by 100-cm column of G200 Sephadex at 4°C. The column was calibrated with a mixture of ferritin (mol. wt. 540,000), bovine serum albumin (mol. wt. 67,000), egg albumin (mol. wt. 45,000), cytochrome C (mol. wt. 12,500), and potassium dichromate (mol. wt. 294). Sodium citrate buffer (0.1 *M*, pH 8.6) was used at a flow rate of 15 ml/hr, and 3.5-ml fractions were collected. Radioactivity was then measured with a gamma counter.

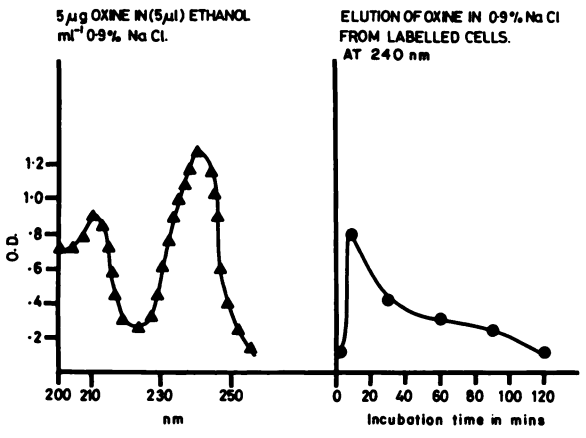

FIG. 1. Left-hand figure shows absorption spectrum for oxine, with a peak at 240 nm. Right-hand figure shows elution of oxine from labeled cells incubated for various times in 0.15 *M* NaCl. Maximal release of oxine occurs at 5 min incubation.

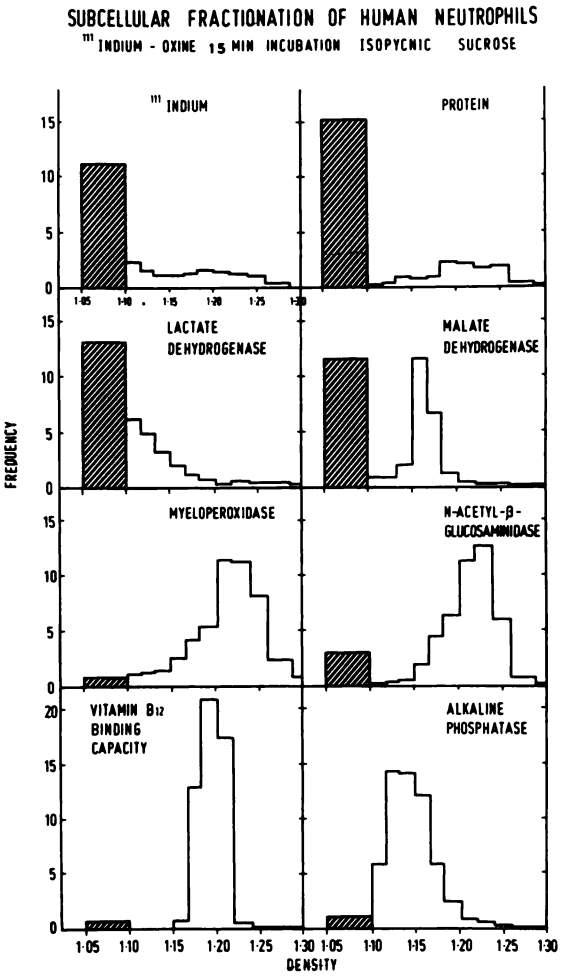

FIG. 2. Isopycnic centrifugation of postnuclear supernatant from neutrophils prelabeled with indium-111 and incubated in 0.15 *M* NaCl for 15 min at room temperature. Graphs show frequency-density histograms for marker enzymes, protein, and indium-111. Frequency is defined as the fraction of total recovered activity present in subcellular fraction, divided by density span covered. Cross-hatched areas represent, over arbitrary abscissa interval, enzyme remaining in sample layer. Percentages of recovered activity are: indium-111, 75; protein, 103; lactate dehydrogenase (cytosol), 108; malate dehydrogenase (mitochondria), 85; myeloperoxidase (azurophil granules), 70; N-acetyl-β-glucosaminidase (lysosomes), 84; vitamin B_{12} binding capacity (specific granules), 80; alkaline phosphatase (endoplasmic reticulum), 73.

RESULTS

Elution of In-111 and oxine from neutrophils. More than 95% of the In-111 chelated to oxine was taken up by the cells during the 15-min incubation at room temperature. The labeled cells incubated in either plasma or 0.15 *M* NaCl released less than 1% of the radioactivity into the medium over a 2-hr period, but detectable amounts of oxine were eluted as shown in Fig. 1. It appears that maximum release of oxine occurs during the first few minutes of incubation. At each subsequent incubation period the measurable quantity of oxine decreases.

Subcellular fractionation experiments. The analytical subcellular fractionation experiments indicate that after 15 min of incubation with In-111 oxine approximately 80% of the radioactivity was found in the cytosol fraction, following the distribution of lactate dehydrogenase, the marker enzyme for this compartment. The remaining activity was distributed in a broad peak and corresponded to the distribution of protein (Fig. 2, top right). Also distributed in this region (density 1:18–1:22) were azurophil and specific granules (Fig. 2, lines 3 and 4, left) as determined by the distribution of myeloperoxidase and vitamin B_{12} binding capacity respectively. Approximately 3% of the radioactivity in the gradient fractions was extracted with chloroform, suggesting that only very small amounts of the label remained bound to oxine. Less than 10% was dialysable, indicating that most of the radioactivity was associated with compounds of molecular weight greater than 2,000. Less than 5% of the radioactivity was precipitated by trichloroacetic acid.

The analytical subcellular fractionation experiments indicate that, after a 60-min incubation with In-111 oxine, approximately 40% of the activity is in the cytosol with the remainder sedimenting into the denser gradient with a broad peak (Fig. 3). A part of this radioactivity coincides with the distribution of DNA in the gradient (density span 1:25–1:27) and the remainder is associated with particulate materials such as cell nuclei and cell debris.

Gel filtration. The supernatant from 180 min at 250,000 g contained 55–60% of the radioactivity. Of the fraction loaded on the column, about 80–85% of the radioactivity was recovered. The pattern of elution of radioactivity (Fig. 4) indicates that the radioactivity in the cytosol fractions is associated with two major and two minor components. The apparent molecular weights, and the proportion of the radioactivity associated with each component, are listed in Table 1.

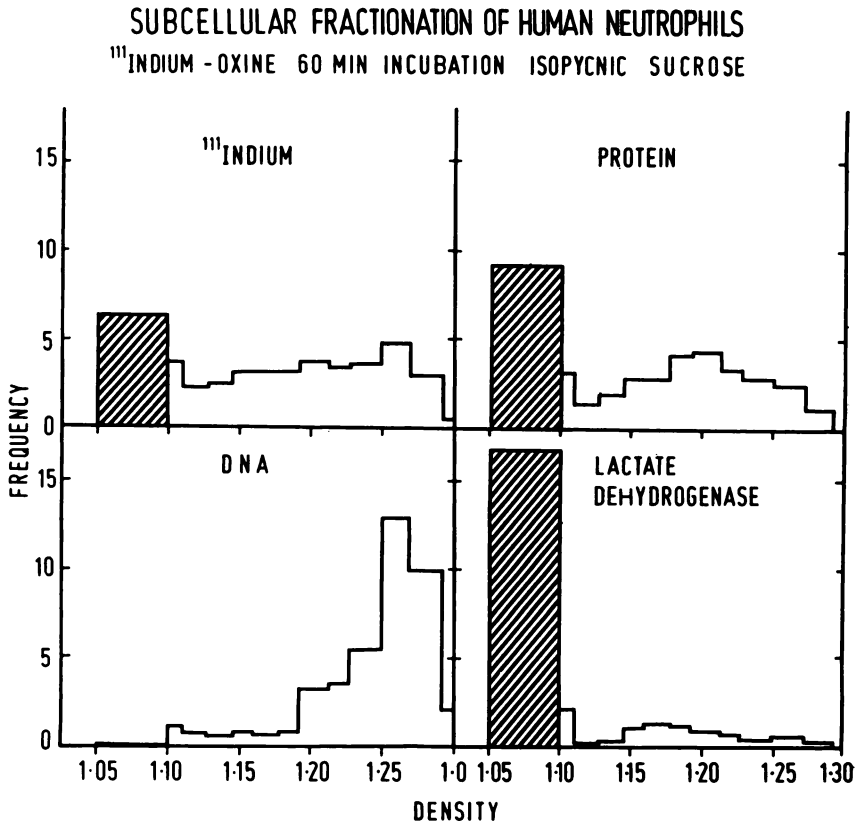

FIG. 3. Isopycnic centrifugation of postnuclear supernatant from neutrophils prelabeled with indium-111 and incubated in 0.15 *M* NaCl for 60 min. Details as in Fig. 2. The percentages of recovered activity are: indium-111, 79; protein, 88; DNA, 85; lactate dehydrogenase, 108.

TABLE 1. PERCENT OF RADIOACTIVITY IN INDIUM-111 COMPLEXES SEPARATED BY GEL FILTRATION AND THEIR APPARENT MOLECULAR WEIGHTS

Peak No.	Radioactivity (%)	Mol. wt.
1	16	560,000
2	32	80,000
3	48	3,600
4	4	200

DISCUSSION

Indium-111 forms a saturated (3:1) complex with oxine similar to that of (3:1) oxine:iron complex (*10*). The complex is neutral and lipid-soluble, which enables it to penetrate the cell membrane (*5*). The estimated stability constant (*5*) of the complex is approximately 10^{10}. This low stability may be the cause of the rapid displacement of the indium from the complex and its subsequent binding to cytoplasmic components as occurs in the formation of In-111 transferrin complex (stability constant 10^{30}). Breakdown of the 3:1 (oxine:iron) complex has been described by Albert (*10*). The apparent maximum release of oxine occurs within a few minutes of neutrophil incubation. Since 200 million cells were labeled with 50 μg oxine, and each incubated fraction comprised about 10 million cells, each fraction had a maximum of 2.5 μg oxine. In the second fraction (Fig. 1), around 2.5 μg of oxine was detectable. Ideally, after prolonged incubation of each subsequent fraction the amount oxine released should have remained the same. Nevertheless, the measurable amount of oxine in each following fraction decreased. This apparent loss of oxine from the suspending medium during prolonged incubation of the neutrophils may be due either to slow reaccumulation of free oxine by the cells or to formation of further complexes with metal ions present in the suspending medium. Therefore, the actual amount of oxine released from the cells in these fractions may be substantially higher than that measured. It would have been extremely useful to know the actual quantity of oxine released from the cells in each fraction including the oxine initially associated with In-111. This could not be firmly established due to the reutilisation of the released oxine. The fact remains, however, that at least some of the oxine was observed in saline in which labeled cells were incubated, and that most of the In-111 activity was found in association with various intracellular components and not with oxine.

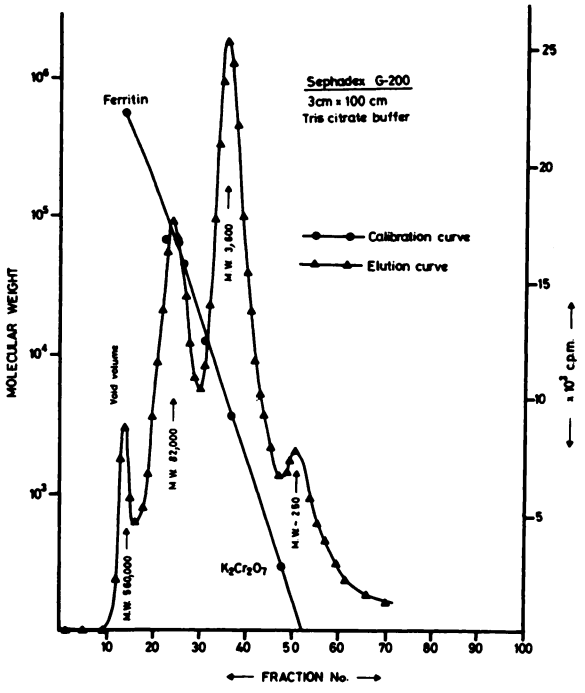

FIG. 4. Elution curve of radioactivity from gel filtration of 250,000 g supernatant from neutrophils prelabeled with indium-111 and incubated at room temperature in 0.15 M NaCl for 15 min. The G200 column was calibrated with ferritin (mol. wt. 540,000), bovine serum albumin (mol. wt. 67,000), egg albumin (mol. wt. 45,000), cytochrome C (mol. wt. 12,500), and potassium dichromate (mol. wt. 294). Sodium citrate buffer (0.1 M, pH 8.6) was used as eluent at 4°C.

The subcellular fractionation experiments indicate that after short periods of incubation, as used in the standard labeling procedure, most of the radioactivity remains in the cytosol. The results of gel filtration indicated that this radioactivity was associated with four components. Although the apparent molecular weights of these components (estimated from the calibration curve) are listed, they may not be accurate enough to identify the components. After longer periods of incubation, such as occurs following reinjection of labeled neutrophils into patients, a relatively smaller proportion of the radioactivity remains in the cytosol and a greater proportion appears in a broad peak, indicating its association with particulate matters in cells (Fig. 3). Some of the radioactivity also appears to have a distribution similar to that of DNA. The possibility of this association of In-111 with DNA may be similar to the binding of cadmium ions to nucleic acids (*11*). This remained unestablished, however, since the first peak in gel filtration (mol. wt. 560,000, Fig. 4) could not be confirmed as DNA by precipitation.

CONCLUSION

From this work it is possible to conclude that (a) the In-111 oxine binds to neutrophils efficiently, (b)

at least some of the oxine leaves the cells subsequently, and (c) the majority of the radioactivity changes in the cells in its initial chemical form, binds to various intracellular components, and provides a stable label. Attempts have been made to obtain as much information as possible on quantitation and intracellular location of the radioactivity. The various figures quoted, however, should be taken only as a guide, since much more work will be needed for their absolute quantitation and for the determination of some of the intracellular components to which the radioactivity is bound.

ACKNOWLEDGMENTS

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ERRATA

The authors of the article "Pharmacokinetics of Technetium-99m Diphosphonate" (*J Nucl Med* 18: 809–814, 1977) wish to point out that Table 2, appearing on p 812, is mislabeled. In the first column under the heading "Population" "NP" should read "PP," and in the last footnote, "NP: Patients with normal bone" should be replaced by "PP: patients with positive bone scans."

In the article "Quantification of Flow in a Dynamic Phantom Using ^{81}Rb-^{81m}Kr and a NaI Detector" (*J Nucl Med* 18:570–578, 1977), the top section of the right-hand column of p 572 should appear as follows:

$$S(H) = S_0 \exp\left[\frac{-(H-\bar{H})^2}{2\sigma^2}\right] + (BH + D),$$

where the Gaussian term of standard deviation σ represents the distribution of pulse heights (H) about the mean ($\bar{H}$), and the linear term (BH + D) represents the contribution from the Compton continuum due to higher-energy photons. Thus, the area under the Kr-81m photopeak at 190 keV may be approximated by a sum over pulse heights of the total counts less the Compton contribution

$$C_2 = \sum_{\bar{H}-\Delta H}^{\bar{H}+\Delta H} [S(H) - (BH + D)]. \quad (7)$$