A Comparison of the Behavior of $^{111}$In and $^{59}$Fe-labeled Transferrin on Incubation With Human and Rat Reticulocytes

By Michael R. Beamish and Elmer B. Brown

The uptake by human and rat reticulocytes of $^{111}$In and $^{59}$Fe bound to transferrin has been studied. The results indicate a significant difference between the behavior of the two isotopes in both human and rat incubation mixtures. Reticulocyte uptake of $^{111}$In from human and rat serum was 30% and 12% that of the $^{59}$Fe after a 30-min incubation. The process was temperature dependent, inhibited by sodium arsenite, and related to the reticulocyte percentage of the cells in the reaction mixture. Washed reticulocytes, previously incubated for 30 min with either $^{59}$Fe or $^{111}$In bound to serum were reincubated in unlabeled serum. Up to 85% of the $^{111}$In label and less than 10% of the $^{59}$Fe on the reticulocytes were released on reincubation, indicating that, in contrast to $^{59}$Fe, the majority of the $^{111}$In label remained membrane bound. Specific binding of unlabeled In transferrin was demonstrated by its inhibitory effect on $^{59}$Fe-transferrin uptake by rat reticulocytes. Both In- and Fe-transferrin were found to have similar binding affinities for the receptor sites. The $^{111}$In remaining in lysates obtained from washed reticulocytes after incubation with $^{111}$In-labeled serum and reincubation in unlabeled serum did not appear to be associated with either the hemoglobin or heme molecules.

Radioactive indium has been widely used in its ionic and colloidal forms to measure plasma volume, as a scanning agent to visualize body organs and tumors, and more recently as a marrow-imaging agent. Ionic indium in acid solution rapidly binds to transferrin when added to serum in vitro or when injected in vivo. Preliminary reports of the uptake of transferrin-bound indium by human reticulocytes and its incorporation into the heme molecule in human red cells have led to the suggestion that indium behaves in a manner similar to iron. However, other studies using animal sera and reticulocytes have not confirmed these findings. In view of the potential usefulness of $^{111}$In as a specific erythroid marrow-imaging agent and the conflicting preliminary reports as to its biologic behavior, we have investigated the uptake and release of transferrin-bound indium by rat and human reticulocytes. Our findings indicate that transferrin-bound indium binds onto the cell membrane, but there is minimal transfer into the cell or incorporation into heme.

**MATERIALS AND METHODS**

Indium was obtained from Diagnostic Isotopes, Inc. as $^{111}$In chloride, a carrier-free isotope with an initial activity of 1.0 mCi/ml. $^{59}$Fe was obtained in chloride form with a specific ac
ivity of 15.0 mCi/mg from International Chemical and Nuclear Corporation. Reticulocytes were obtained from male Sprague-Dawley rats following repeated venesection and from patients with sickle cell disease or with megaloblastic anemias responding to therapy. The red blood cells were washed three times in ice-cold 0.9% NaCl, 0.01 M NaHCO₃ (pH 7.4) before use. Reticulocyte counts varied between 14%, and 30%. Henceforward, washed reticulocyte-rich erythrocytes are referred to as reticulocytes for the purposes of this study. Serum clotted from blood in iron-free glass tubes was obtained from rats and ABO compatible normal human donors.

Purified, iron-free human transferrin (Behringwerke) was obtained from Hoechst Pharmaceuticals, Inc. and dissolved in 0.9% NaCl, 0.01 M NaHCO₃ prior to use. ¹¹In or ⁵⁹Fe were bound to transferrin of human or rat serum by incubation for 30 min at 37°C. Chromatography with Sephadex G-150 equilibrated in saline bicarbonate buffer was used to confirm that complete binding to transferrin had been achieved.

Procedure for Serum Incubation

Fresh normal rat or human serum was incubated with either ¹¹In or ⁵⁹Fe, diluted 1:3 with saline bicarbonate, and a 0.75-ml aliquot was added to 0.25 ml of washed reticulocytes. Incubation was performed in air at 37°C in a Dubnoff metabolic shaking incubator. Samples were removed at timed intervals and immediately washed twice in ice-cold saline bicarbonate. The cells were lysed in water, and their radioactivity estimated with a Packard gamma scintillation spectrometer.

Procedure for Reflux Experiments

¹¹In- and ⁵⁹Fe-labeled sera were each incubated with reticulocytes for 30 min as described above. The reticulocytes were washed three times with ice-cold saline bicarbonate and divided into 0.25-ml aliquots. Duplicate samples were incubated with 0.75 ml of fresh serum diluted 1:3 with saline bicarbonate and with saline bicarbonate solution alone at 0°C and 37°C. The samples were removed from incubation at 5-sec, and 10-, 20-, and 40-min intervals and immediately washed in 15 ml ice-cold saline bicarbonate. The supernatant solution was separated from the cell fraction, and the radioactivity in both was estimated. The radioactivity of the supernatant fraction was expressed as a percentage of the total radioactivity.

Procedure for Inhibition Experiments

The incubation procedures used purified human transferrin and washed rat reticulocytes. The standard reaction mixtures contained 0.5 ml washed reticulocytes and 0.75 ml saline bicarbonate containing ⁵⁹Fe-labeled human transferrin (50%, saturated with carrier iron) in concentrations varying from 0.05 to 1.0 mg transferrin per ml. To each reaction mixture was added 0.25 ml saline bicarbonate containing either 0.25 mg apotransferrin, 0.25 mg transferrin 80% saturated with carrier iron, or 0.25 mg transferrin 80% saturated with carrier indium, respectively. A tube with 0.25 ml of saline bicarbonate alone served as a control. The reaction mixtures were incubated in air at 37°C for 20 min, twice washed with ice-cold saline bicarbonate, and the radioactivity bound to reticulocytes was determined as previously described. The data were plotted using a double-reciprocal plot. The dissociation constant for ⁵⁹Fe-transferrin (Kₘ) and the apparent dissociation constant for apotransferrin, Fe-transferrin, and In-transferrin (Kᵢ) were obtained by extrapolation of the slopes to the abscissa. The actual dissociation constants for the three unlabeled transferrin (Kᵢ) were calculated according to the equation

\[ Kᵢ = Kₘ \left(1 + \frac{i}{Kᵢ}\right) \]

where \(i\) is the concentration of the unlabeled transferrin in the reaction mixture.

Sodium Arsenite Inhibition

Incubation procedures were carried out with sera separately labeled with ¹¹In and ⁵⁹Fe and human reticulocytes obtained as previously described. Sodium arsenite was dissolved in saline bicarbonate and the pH adjusted to 7.8 with 0.1 N NaOH. Aliquots containing 0.5 ml of washed reticulocytes were initially incubated for 15 min at 37°C with 0.65 ml saline bicarbonate alone,
Fig. 1. The uptakes of $^{111}$In- and $^{59}$Fe-labeled sera at 37°C and 0°C by rat and human reticulocytes at timed intervals over an incubation period of 60 min. (---), $^{59}$Fe-labeled serum; (------), $^{111}$In-labeled serum incubated at 37°C. (---), $^{59}$Fe-labeled serum; (-----), $^{111}$In-labeled serum at 0°C.

or containing $7.5 \times 10^{-3}$, $1.5 \times 10^{-2}$, and $4.5 \times 10^{-2}$ M sodium arsenite, respectively. Aliquots of sera (0.35 ml), labeled with either $^{111}$In or $^{59}$Fe, were added to the reticulocyte mixtures and the incubation continued for a further 30 min. The incubations were terminated and the radioactivity in the cells measured as previously described.

**Red Cell Lysate Fractionation**

Incubations were performed using sera doubly labeled with $^{111}$In and $^{59}$Fe and reticulocytes obtained from rats and human subjects, using the procedures previously described. The incubations were allowed to proceed for 30 min; the cells were washed four times in ice-cold saline bicarbonate and reincubated in fresh serum diluted 1:3 with buffer for a further 40 min at 37°C. The cells were washed in saline bicarbonate four times and lysed in 10 volumes of distilled water. An aliquot of the whole lysate was retained and the remainder centrifuged at 5000 g for 20 min. The supernatant, membrane-free lysate was collected and the remaining cell membranes washed three times in cold saline bicarbonate. The membrane-free lysate was subjected to Sephadex G-100 chromatography on a 60 x 2.5-cm column equilibrated in 1.0 M NaCl, 0.01 M NaHCO$_3$ (pH 7.8). Heme and globin were extracted from the membrane-free lysate by methyl ethyl ketone and acetone extraction procedures.$^{10,11}$

**RESULTS**

Typical results of the incubation of $^{111}$In- and $^{59}$Fe-labeled sera with human and rat reticulocytes are shown in Fig. 1. There was progressive uptake of $^{59}$Fe into reticulocytes with time at 37°C in both species. The uptake of $^{111}$In after 30 min incubation was 30% and 12% of $^{59}$Fe by human and rat reticulocytes, respectively, and reached a maximum value after 30 min incubation. The uptake of both isotopes appeared to be negligible at 0°C.

The relationship between $^{111}$In uptake and reticulocyte count was investigated by adding $^{111}$In-labeled human serum to mixtures of washed human red cells and reticulocytes, so that the proportion of reticulocytes varied from 2% to 30%. (Fig. 2). The results indicate a linear relationship between $^{111}$In uptake and reticulocyte percentage.

**Reflux Experiments**

The reflux of $^{59}$Fe-transferrin from reticulocytes initially incubated with $^{59}$Fe-labeled plasma and reincubated in unlabeled plasma has been studied by Morgan$^{13}$ and Morgan, Huehns, and Finch.$^{15}$ These authors showed that after an initial incubation time of 1 min, 20% - 40% of the reticulocyte-bound radioiron was released on reincubation with fresh plasma at 37°C. With longer initial
incubation times, the percentage reflux fell to less than 5%, which they attributed to a rapid transfer of iron from the membrane surface into the cell. The reflux of $^{59}$Fe and $^{111}$In from reticulocytes initially incubated in labeled serum for 30 min is shown in Fig. 3. Although there was no appreciable reflux of $^{59}$Fe into either serum or saline-bicarbonate at either 0°C or 37°C, there was up to 85% reflux of $^{111}$In into serum and 50% reflux into saline-bicarbonate at 37°C. Similar results were obtained in reflux incubation experiments with rat reticulocytes. These data indicate that, in contrast with $^{59}$Fe, the majority of the $^{111}$In label remains membrane bound. The process of $^{111}$In reflux was markedly reduced at 0°C and appeared greater in the presence of serum than with saline-bicarbonate.

**Inhibition of $^{59}$Fe-Transferrin Uptake by In-Transferrin**

The ability of In-transferrin to bind to specific reticulocyte receptor sites was studied by competition experiments. Kornfeld has shown that the affinity of various metal-transferrin complexes for physiologic receptor sites on reticulo-
cytes can be determined by their inhibitory effect on the rate of $^{59}$Fe-transferrin uptake determined as a function of concentration.\(^6\) The inhibitory effect of apotransferrin, Fe-transferrin, and In-transferrin on $^{59}$Fe-transferrin uptake is shown in Fig. 4. The degree of inhibition was most marked with In-transferrin and Fe-transferrin and less marked with apotransferrin. The dissociation constants calculated from these data (see Materials and Methods) are 0.748 mg/ml for apotransferrin, 0.025 mg/ml for Fe-transferrin, and 0.021 mg/ml for In-transferrin. These results indicate that In-transferrin is capable of specific binding to physiologic iron receptor sites, and that it has a binding affinity equal to or slightly greater than that for Fe-transferrin. The data also suggest that the nature of the inhibition is essentially similar to that for Fe-transferrin, indicating the presence of competitive, rather than metabolic, inhibition.

**Sodium Arsenite Inhibition**

Reticulocytes were initially incubated at 37°C for 15 min with varying concentrations of sodium arsenite or with saline-bicarbonate alone, then $^{111}$In- or
Fig. 6. The distribution of $^{59}$Fe and $^{111}$In in membrane-free lysate, washed cell membranes, and heme, obtained from a lysate of washed human reticulocytes following initial incubation with $^{59}$Fe- and $^{111}$In-labeled serum and reincubation with unlabeled serum. The activities in the respective fractions are expressed as a percentage of the activity in the whole lysate.

$^{59}$Fe-labeled serum was added and the incubation continued (Fig. 5). The uptake of $^{59}$Fe was inhibited 80% and 90% with $7.5 \times 10^{-1}$ and $4.5 \times 10^{-2}$ M sodium arsenite, respectively, while the $^{111}$In uptake was inhibited 30% and 60% at these concentrations. These results indicate that at least 60% of the $^{111}$In uptake is energy dependent, while the remaining fraction probably represents a process of adsorption which is unaffected by arsenite inhibition.

**Red Cell Lysate Fractionation**

The distribution of the $^{59}$Fe and $^{111}$In label in membrane-free lysate solutions, washed reticulocyte membranes, and in heme and globin fractions obtained from a lysate of reticulocytes after 30-min incubation with serum doubly labeled with $^{111}$In and $^{59}$Fe, washing, and 40-min reincubation in unlabeled serum and washing in saline-bicarbonate in order to remove membrane-bound $^{111}$In is shown in Fig. 6. Following centrifugation, 77% and 56%, respectively, of the $^{59}$Fe and $^{111}$In label present in the reticulocyte lysate were recovered from the membrane-free lysate and 10% and 16%, respectively, recovered from the washed reticulocyte membranes. Ninety-four per cent of the $^{59}$Fe label in the membrane-free lysate was recovered as heme extracted with methyl ethyl ketone. However, virtually none of the $^{111}$In label was recovered from either heme or globin obtained by separate extraction procedures. Sephadex G-100 chromatography of the membrane-free lysate yielded an expected peak of $^{59}$Fe radioactivity, which corresponded with the visible hemoglobin peak, but no corresponding peak of $^{111}$In radioactivity.

**DISCUSSION**

The reaction between Fe-transferrin and reticulocytes has been characterized by a minimum of four steps. There is an initial adsorption of transferrin onto specific reticulocyte receptor sites. This reaction has been shown to be temperature independent, instantaneous, and unaffected by metabolic inhibitors. Adsorption is followed by a firmer association reaction which is characterized by temperature dependence and inhibition by metabolic inhibitors. Both these reactions represent phases of binding by the reticulocyte receptor.
sites and are readily reversible, as demonstrated by the reflux of \(^{59}\)Fe-transferrin from reticulocyte surfaces on reincubation with unlabeled serum or plasma at 37°C.\(^{13,15}\) The final phases are represented by the transfer of iron into the cell, and its incorporation into heme and nonheme proteins, with the consequent release of transferrin from the cell surface.\(^{12,14}\)

While transferrin is capable of binding a variety of heavy metals,\(^{7}\) Kornfeld\(^{16}\) has shown that the particular metal bound to transferrin influences the binding of the protein to its receptor site. In the case of Cr\(^{3+}\)-transferrin, the reticulocytes fail to take up the metal, although specific binding to specific receptor sites occurs.

Our data obtained from \(^{111}\)In and \(^{59}\)Fe uptake studies demonstrate a significant difference in the uptake of the two isotopes after incubation with both human and rat reticulocytes. The uptake of \(^{111}\)In-transferrin determined as a function of time showed an initial phase of adsorption, present at 0°C, and similar to that found for \(^{59}\)Fe-transferrin. This phase was followed by a limited increase in uptake, reaching a maximum after 30 min incubation (Figs. 1A and 1B). This second phase of indium uptake was temperature dependent, inhibited by sodium arsenite, and specific for reticulocytes. It was usually 20\(^\%\)–40\(^\%\) and 5\(^\%\)–12\(^\%\) that of \(^{59}\)Fe uptake by human and rat reticulocytes, respectively. Reflux experiments using \(^{59}\)Fe- and \(^{111}\)In-labeled reticulocytes demonstrated 80\(^\%\) reflux of \(^{111}\)In compared to only 10\(^\%\) reflux of \(^{59}\)Fe, suggesting that the majority of the \(^{59}\)Fe was intracellular, while the \(^{111}\)In was still membrane bound after an initial 30-min incubation.

Inhibition of \(^{59}\)Fe-transferrin uptake by In-transferrin (Fig. 4) and analysis of these data by double-reciprocal plot indicate that inhibition is competitive rather than metabolic, and is evidence for binding of In-transferrin to specific reticulocyte receptor sites with an affinity at least equal to that of Fe-transferrin.

The finding that 85\(^\%\) of the reticulocyte-bound \(^{111}\)In was eluted following reincubation with unlabeled sera suggested that the remaining 10\(^\%\) could represent intracellular transfer. This possibility was investigated on reticulocyte lysates obtained from reticulocytes initially incubated with \(^{111}\)In-labeled serum, then reincubated with unlabeled serum and washed in saline to remove membrane-bound indium (Fig. 6). Centrifugation of the resulting lysate resulted in a 23\(^\%\) loss of \(^{59}\)Fe and a 44\(^\%\) loss of \(^{111}\)In. Of the \(^{111}\)In radioactivity remaining in the membrane-free lysate, none was recovered in the hemoglobin fraction on column chromatography or in the heme fraction on methyl ethyl ketone extraction. These results indicate that intracellular transfer of \(^{111}\)In following incubation is minimal and that there is no significant incorporation of indium into the metalloporphyrin ring.

It may be concluded from these results that the binding of indium to transferrin does not materially affect the phases of adsorption and association of the protein onto the specific reticulocyte receptor sites. However, the process of intracellular transfer and incorporation into heme appears to be specific for iron and could not be demonstrated in the case of indium.

The data presented in this paper are at variance with Lilien et al.,\(^{5}\) who report no difference between the uptake of these two isotopes using human reticulocyte incubation mixtures, and of Potchen et al.,\(^{8}\) who demonstrated some
heme incorporation following incubation of $^{113m}$In with mature red cells. Other workers, using rat and rabbit incubation mixtures, have reported limited uptake of $^{111}$In or $^{113m}$In in comparison to $^{59}$Fe uptake and also an absence of indium incorporation into the metalloporphyrin ring. The reason for the discrepancies between the results presented in this paper and those of Lilien et al. and Potchen et al. is not apparent. However, data obtained from clinical studies with $^{111}$In, demonstrating that normal subjects incorporate only about 4% of the injected dose into the circulating red cells after 12 days, support our observations that intracellular transfer of $^{111}$In is limited.

Clinical studies with $^{111}$In have demonstrated that it localizes to sites occupied by erythroid marrow, and, in view of its ideal physical properties and imaging characteristics, $^{111}$In is being used increasingly as a bone marrow-imaging agent. While the cellular distribution of indium uptake by marrow has not been determined, we have been able to demonstrate that In-transferrin has a strong binding affinity for physiologic receptor sites on reticulocytes, but that it is not utilized for metalloporphyrin synthesis. It appears likely that some of the uptake of $^{111}$In into red marrow, as clinically demonstrated by scanning procedures, represents specific binding by the membrane of marrow erythroid cells.

ACKNOWLEDGMENT

The authors wish to acknowledge the helpful suggestions of Dr. Barry A. Siegel and the technical assistance of Ramon Diaz.

REFERENCES


A Comparison of the Behavior of $^{111}$In and $^{59}$Fe-labeled Transferrin on Incubation With Human and Rat Reticulocytes

Michael R. Beamish and Elmer B. Brown