THE PREPARATION, PROPERTIES, AND METABOLISM OF ^{14}C-BICARBONATE-LABELLED TRANSFERRIN*

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Summary:

^{14}C-bicarbonate-labelled transferrin was prepared in order to study the role of bicarbonate in the cell-mediated release of iron from transferrin. ^{14}C-bicarbonate bound to transferrin only in the presence of iron and with a ratio of bound bicarbonate to bound iron of one. The transferrin-^{14}C-bicarbonate complex was very stable in Tris-HCl buffered at pH 7.5-9.0 even in the presence of excess non-radioactive bicarbonate. However, oxalate, citrate, and phosphate promoted a rapid exchange of transferrin-bound ^{14}C-bicarbonate with bicarbonate present in the medium.

Rabbit reticulocytes effected a temperature-dependent release of ^{14}C-bicarbonate from transferrin at the same rate at which they incorporated ^{59}Fe from transferrin -- suggesting the existence of a coordinated mechanism in the cells for the release of both iron and bicarbonate from transferrin.

Introduction:

Transferrin is the serum protein which transports iron to developing erythroid cells for utilization in hemoglobin synthesis. Although the protein has been well characterized chemically, the mechanism whereby iron is released from transferrin by cells is unknown.

Under physiological conditions apotransferrin can bind two atoms of ferric iron and for each Fe^{+++} bound one molecule of bicarbonate is also bound (1), forming a complex with a characteristic salmon-pink color having a broad absorption peak with a maxiumum around 465 mμ. The iron in this complex has an apparent stability constant greater than $10^{24}M^{-1}$, which would

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appear to exclude simple diffusion as a mechanism for the release of iron from transferrin (2).

Since the availability of intracellular iron may play a significant role in controlling the rate of hemoglobin synthesis (3), an understanding of the mechanism of release of iron from transferrin may be important in studying red cell formation in both normal and pathological states.

Because there is an absolute requirement for bicarbonate (4) (or certain other anions; for example, citrate or oxalate (1)) in the binding of iron to transferrin, $H^{14}CO_3^-$- labelled transferrin would be an ideal tool for studying the role of the anion in the cell-mediated release of iron from transferrin. In this paper we present data on the stability of the transferrin-Fe^{+++}-$H^{14}CO_3^-$ complex as studied under a variety of conditions, including the release of iron from transferrin by reticulocytes incubated in vitro.

Materials and Methods:

$NaH^{14}CO_3$ (42.4 mCi/m mole) and ^{59}Fe (3-30 mCi/mg) as ferric chloride in 0.1N HCl were purchased from Amersham/Searle Corp.

Transferrin was purified from rabbit serum and charged with iron as previously described (5).

Reticulocytes from anemic rabbits were washed and purified on dextran gradients (6) and incubated as previously described (5).

^{59}Fe was measured in a Nuclear Chicago automatic gamma counter. Samples containing ^{14}C-bicarbonate were either applied to aluminum planchettes which were pretreated with Triton X-100 (Sigma Chem. Co.) and counted in a Nuclear Chicago low background counter or dissolved in NCS Solubilizer (Amersham/Searle) and counted in a Nuclear Chicago Unilux II-A liquid scintillation counter.

It had been determined that when samples containing ^{14}C-bicarbonate are dried on planchettes at room temperature, free ^{14}C-bicarbonate is rapidly lost into the atmosphere, while transferrin-bound ^{14}C-bicarbonate remains on the planchettes. On the other hand, NCS dissolves and fixes both free and transferrin-bound ^{14}C-bicarbonate.

TABLE I

	Transferrin* (mμ Moles)	$H^{14}CO_3^-$ Bound* (mμ Moles)	% Saturation* with iron	Molar Ratio $H^{14}CO_3^-$/Transferrin
-iron	157	1.4	< 0.5	0
+iron	473	867	92	1.8

*After dialysis for 24 hours against 0.05 M tris-HCl buffer, pH 7.5.

Values used in the calculations:

Transferrin mol. wt. = 76,000 (7)

Apotransferrin: $E^{1\%}_{280\ m\mu} = 11.1$ (8)

Transferrin: $E^{1\%}_{465 m\mu} = 0.6$ (7)

To form ^{14}C-bicarbonate-labelled transferrin, apotransferrin in 1.0 ml of 50 mM Tris-HCl buffer, pH 8.3, was incubated for one hour at room temperature with a six-fold molar excess of $NaH^{14}CO_3$ and a two-fold saturating amount of iron -- the iron solution contained 120 mg of sodium citrate and 5.6 mg of $FeCl_3$ per ml. The solution was then dialyzed for 20 hours at 4°C against two 4 liter changes of 5 mM Tris-HCl buffered at pH 7.5.

Results:

Table I shows that we obtained the expected stoichiometery of binding of $H^{14}CO_3^-$ and Fe^{+++} to apotransferrin (1) with our incubation conditions. It also shows that under these conditions bicarbonate is not bound to apotransferrin in the absence of iron. The transferrin- $H^{14}CO_3^-$ complex was very stable in 0.05M Tris-HCl buffered at pH 7.5. It could be extensively dialyzed against this buffer without appreciable loss of radioactivity, and when the complex was stored at -20°C the radioactivity was lost at a rate of about 4% per month (probably by exchange with atmospheric CO_2). Less than 10% of the radioactivity

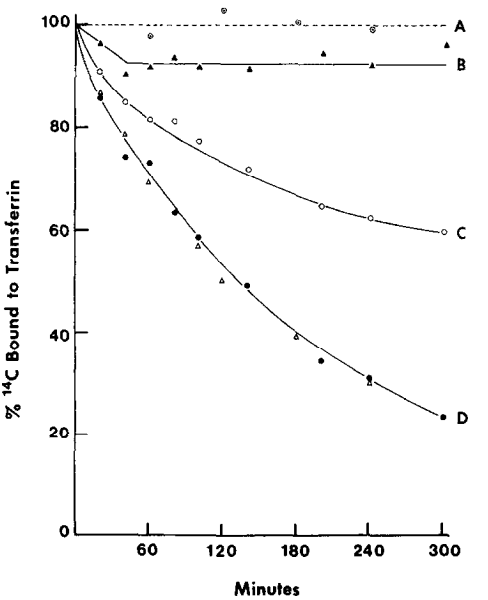

Figure 1:

The effect of various anions on the stability of the transferrin - $H^{14}CO_3^-$ complex. Transferrin - $H^{14}CO_3^-$ solutions, at a concentration of 3.3 mg/ml in 50 mM Tris-HCl, pH 7.5, were incubated at room temperature. At the indicated times samples were removed, dried on planchettes, and assayed for radioactivity as described in Methods. A: control, B: $NaHCO_3$ (16.8 mg/ml), C: 10 mM sodium citrate, D: 10 mM potassium oxalate or 100 mM sodium phosphate.

was lost in two hours when the complex was incubated in Tris-HCl buffer containing a 100-fold excess of $NaHCO_3$.

On the other hand, in 10 mM citrate, 10 mM oxalate, or 100 mM phosphate the radioactivity of the transferrin decreased appreciably (Figure 1); the order of activity of these anions being oxalate > citrate > phosphate (there was no measurable loss of radioactivity in 10 mM phosphate). With all three competing anions there was less than a 6% decrease in the $A_{465m\mu}$ during 5 hours of incubation. Since the complexes of apotransferrin with Fe^{+++} and either citrate, oxalate, or phosphate have a lower extinction coefficient than the complex with bicarbonate (1), we conclude that appreciable concentrations of the complexes with the competing anions were not formed during the experiment, and that iron was not removed from transferrin by these agents. It seems likely that the competing anions promoted a rapid exchange between

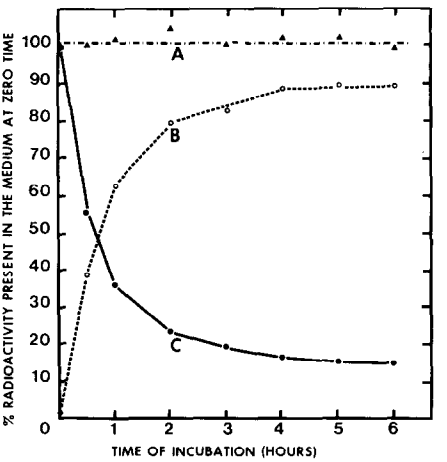

Figure 2:

Incorporation of ^{59}Fe from transferrin and removal of $H^{14}CO_3^-$ from transferrin by rabbit reticulocytes. Purified reticulocytes, as 25% cell suspensions, were incubated in medium (5) containing 0.51 mg/ml of iron-saturated transferrin labelled with either ^{59}Fe or $H^{14}CO_3^-$.

Curve A: The stability of $H^{14}CO_3^-$ transferrin at 0°C. Reticulocytes were incubated at 0°C in medium containing $H^{14}CO_3^-$ transferrin. At the indicated times portions of the cell suspension were removed and the radioactivity determined as described in Methods.

Curve B: Incorporation of ^{59}Fe from transferrin by reticulocytes at 37°C. Reticulocytes were incubated at 37°C in medium containing ^{59}Fe-transferrin. At the indicated times portions of the cell suspension were removed and cooled in an ice bath. The cells were washed five times in normal saline by centrifugation and resuspension at 4°C, after which they were assayed for ^{59}Fe.

Curve C: The removal of $H^{14}CO_3^-$ from transferrin by reticulocytes at 37° C. The procedure was the same as for Curve A except that the suspension was incubated at 37°C.

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transferrin-bound bicarbonate and bicarbonate present in the medium which was formed from dissolved atmospheric CO_2.

To see whether a relationship exists between the cell-mediated removal of iron from transferrin and the transferrin-bound bicarbonate, reticulocytes were independently incubated with transferrin labelled with either $^{59}Fe^{+++}$ or $H^{14}CO_3^-$. In both cases iron-saturated transferrin at a final concentration of 0.51 mg/ml was present in the medium. As can be seen in Figure 2 there was an excellent correlation between the rate of incorporation of ^{59}Fe into the cells and the rate of disappearance of $H^{14}CO_3^-$, which was presumably lost to

the atmosphere. There was no loss of $H^{14}CO_3^-$ from the transferrin in the medium at 37°C in the absence of reticulocytes, or from transferrin in the medium at 0°C in the presence of reticulocytes.

Discussion:

Our finding that for each Fe^{+++} bound to apotransferrin one molecule of $H^{14}CO_3^-$ is also bound confirms the observations of Young and Perkins (1). Like them, we also found that certain anions, in this case oxalate, citrate, and phosphate, can displace bicarbonate from transferrin. However, unlike the previous authors we could not detect the formation of appreciable amounts of transferrin complexed with the competing anions, probably because our experiments were done in the presence of atmospheric CO_2. This suggests that the displacement of radioactive bicarbonate by the competing anions in our system was most likely the result of an exchange reaction between transferrin-bound bicarbonate and bicarbonate dissolved in the reaction medium.

The stability of the transferrin-$H^{14}CO_3^-$ complex, even in the presence of excess $NaHCO_3$, allowed us to examine the behaviour of the complex under conditions in which reticulocytes can actively incorporate iron from transferrin into hemoglobin. The excellent correlation between the kinetics of incorporation of iron from transferrin into reticulocytes and the release of $H^{14}CO_3^-$ from transferrin would seem to indicate that these events are coordinated in developing erythroid cells.

Since bicarbonate is the physiologically significant anion involved in the transferrin-iron complex (2), and since transferrin does not complex with iron in the absence of suitable anions (4), it is conceivable that the enzymatic removal of bicarbonate from transferrin by cells would be sufficient to result in the release of iron from the protein, thereby enabling the cells to further metabolize the iron.

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