Mechanism of Vitamin B\textsubscript{12} Uptake by Erythrocytes *

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Pitney, Beard, and Van Loon (2) and Ostrowski, Skarzynski, and Zak (3) reported in 1954 that vitamin B\textsubscript{12} is bound primarily to \(\alpha\)-globulin in normal serum. Pitney and his colleagues (2) also noted that \(\beta\)-globulin can bind B\textsubscript{12} added \textit{in vitro}, but they considered this as "free B\textsubscript{12}" because, unlike B\textsubscript{12} bound to \(\alpha\)-globulin, \textit{Euglena gracilis} is able to utilize it without prior heat treatment. Miller (4) showed that B\textsubscript{12} added to serum \textit{in vitro} binds predominantly to \(\alpha\)- and \(\beta\)-globulin and that this fraction is nondialyzable. Hall and Finkler (5) confirmed the presence of two main B\textsubscript{12}-binding globulins in serum, which Miller and Sullivan (6) and Weinstein, Weissman, and Watkins (7) had shown to be constituents of the seromucoid fraction.

Little is known about the transfer of B\textsubscript{12} from plasma to tissues. Callender and Lajtha (8) reported that partial maturation of megaloblasts \textit{in vitro} can be produced by cyanocobalamin only when gastric juice or serum is present, thus suggesting the importance of a transferring protein. Miller, Raney, and Hunter (9) and Herbert (10) demonstrated that hog intrinsic factor promotes the uptake of B\textsubscript{12} by rat liver slices; human serum has a similar effect (11). Cooper and Paranchych (12) found mouse Ehrlich ascites tumor cells and HeLa cells able to absorb B\textsubscript{12} only in the presence of human serum and ascites fluid; human gastric juice and hog intrinsic factor do not show such an effect. These workers subsequently suggested that the B\textsubscript{12}-binding fraction of ascites fluid may be a mucoprotein (13). Finkler, Hall, and Landau (14) reported that B\textsubscript{12} uptake by HeLa cells in tissue culture is specifically increased by the B\textsubscript{12}-binding \(\beta\)-globulin of human serum and that liver uptake of B\textsubscript{12} from plasma seems to occur more rapidly when the vitamin is bound to \(\beta\)-globulin than when bound to \(\alpha\)-globulin (15).

We have studied cyanocobalamin transfer to tissues by investigating erythrocyte uptake of \textsuperscript{57}Co-labeled B\textsubscript{12} (B\textsubscript{12}\textsuperscript{57}Co), in a test system previously used by Herbert (16) and Herbert and Sullivan (17). It has been reported that mature erythrocytes do not take up significant amounts of B\textsubscript{12} (18, 19), but uptake increases with a rising reticulocyte count (17).

**Methods**

**Materials**

\textit{Reticulocyte-rich blood} was collected in heparinized Vacutainers \(^1\) tubes from patients with hemolytic disease or iron deficiency anemia responding to treatment. In all cases plasma B\textsubscript{12} levels were determined by coated charcoal assay (20). Initially the ABO and Rh blood types of test cells were determined to exclude possible agglutination reactions when serum was added to the test system. However, we found that blood group incompatibility between serum and cells did not cause agglutination under the conditions of the test, due presumably to the relatively high content of red cells. Reticulocyte counts were done by standard methods with brilliant cresyl blue stain.

Test cells were thrice washed with 2 vol physiological saline containing 10 mM calcium chloride (CaCl\textsubscript{2}-NaCl). Washing with saline instead of CaCl\textsubscript{2}-NaCl was later shown not to affect results. Washed cells were finally suspended in equal volumes of CaCl\textsubscript{2}-NaCl, and a microhematocrit was performed on each working suspension.

\textit{Normal blood} with a reticulocyte count less than 1.5\% was used as a control; cells were prepared and suspended as above.

\(^1\) Purchased as Vacutainers (#3208 KA), 20-ml capacity, from Becton Dickinson, Rutherford, N. J.
Experiments were performed in duplicate and accompanied by two controls: 1) test serum replaced by an equal volume of saline, and 2) reticulocyte-rich erythrocyte suspension replaced by a 2-ml suspension of erythrocytes with a normal reticulocyte count. Occasionally reticulocyte-rich and reticulocyte-poor suspensions were obtained from a single sample by differential centrifugation with 30% bovine albumin (22).

**Results**

\( \text{B}_{12-57}\text{Co} \) uptake by erythrocytes varied from experiment to experiment even with the same reticulocyte count and test serum. For a given experiment with a single source of serum and reticulocytes, the uptake of \( \text{B}_{12-57}\text{Co} \) was quite constant; in three separate experiments the coefficient of variation was 5.4% (nine observations), 3.9% (four observations), and 3.3% (four observations). As indicated in Figure 1, serum-mediated \( \text{B}_{12-57}\text{Co} \) uptake by reticulocyte-rich erythrocytes was consistently greater than by reticulocyte-poor erythrocytes, with a fairly constant uptake slope.

Saline-mediated transfer of \( \text{B}_{12-57}\text{Co} \) showed no significant reticulocyte dependence and was quantitatively less than serum-mediated transfer (Figure 2). Occasionally, relatively high saline-mediated transfer occurred, which may have been due to small amounts of serum trapped in an inadequately washed test cell suspension.

Transfer of \( \text{B}_{12-57}\text{Co} \) to erythrocytes appears to be governed both by extracellular factors in the
transferring medium and cellular factors in the erythrocytes.

Extracellular factors

The rate of $B_{12}^{57}$Co uptake by erythrocytes. A diluting volume of 3 ml cold saline (4° C) was added to incubating mixtures after incubation periods ranging from 2 to 60 minutes. Specimens were then immediately centrifuged and washed, and radioactivity of the hemolysate was counted.

In Figure 3 the uptake curve from normal serum is compared with that of saline. It is evident that at least three quarters of the total serum-mediated $B_{12}$ transfer takes place during the first 5 minutes. Transfer is maximal at approximately 20 minutes. Uptake from saline is quantitatively much less and shows a slight progressive increase over 1 hour after an initial rapid uptake phase.

The role of ionic calcium, magnesium, and strontium in $B_{12}$ transfer. The effect on the test system of 0.5 ml 0.1 M Ca EDTA, Mg EDTA, Sr EDTA, and Na$_2$ EDTA was determined (Table I). The finding that Na$_2$ EDTA greatly diminished $B_{12}$ transfer whereas Ca EDTA and Mg EDTA did not affect it significantly suggests that ionic calcium or magnesium is essential for the reaction. Strontium appears to partially replace these cations in this system.

When test cells were preincubated with 10$^{-1}$ M Na$_2$ EDTA for 30 minutes, thrice washed with 10 mM CaCl$_2$-NaCl, resuspended in this medium, and then used in the standard $B_{12}^{57}$Co transfer experiments, $B_{12}^{57}$Co uptake was unimpaired. This showed that Na$_2$ EDTA did not per se cause irreversible damage to red cells. The uptake of 1 ng $B_{12}^{57}$Co from 0.5 ml saline was not decreased by the addition of 0.5 ml 10$^{-1}$ M Na$_2$ EDTA (Table I).

The effect of pH and temperature changes. On adjusting the pH of the test system with 1 N sodium hydroxide and 1/3 N hydrochloric acid and checking both initial pH and final pH at the end of the 1-hour incubation, we found maximal $B_{12}^{57}$Co transfer to occur in the pH range 7.2 to 8.2. Outside this range hemolysis rendered experimental conditions progressively less reliable.

Incubation at 4° C, 23° C, 37° C, and 45° C, respectively, after the test system had been allowed to equilibrate at these temperatures for 15 minutes before addition of $B_{12}^{57}$Co, demonstrated that maximal $B_{12}$ transfer occurred at 37° C, with progressive but moderate decrease in uptake at lower and higher temperatures (Figure 4).

The transfer of $B_{12}^{57}$Co. In Figure 5, the transfer of $B_{12}^{57}$Co added to pernicious anemia serum (native $B_{12}$, 37 picograms (pg) per ml; UB$_{12}$BC, 1,728 pg per ml) containing varying concentrations of the radioactive vitamin (200 pg

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**TABLE I**

<table>
<thead>
<tr>
<th>EDTA (10$^{-1}$ M, 0.5 ml) added to 0.5 ml uptake medium</th>
<th>$B_{12}^{57}$Co uptake by 1 ml erythrocytes as % of uptake from control*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.† None (Control)</td>
<td>100</td>
</tr>
<tr>
<td>Ca EDTA</td>
<td>97.6 ± 1.8</td>
</tr>
<tr>
<td>Mg EDTA</td>
<td>94.7 ± 3.7</td>
</tr>
<tr>
<td>Sr EDTA</td>
<td>68.6 ± 2.1</td>
</tr>
<tr>
<td>Na$_2$ EDTA</td>
<td>18.0 ± 0.8</td>
</tr>
<tr>
<td>B.‡ None (Control)</td>
<td>100</td>
</tr>
<tr>
<td>Na$_2$ EDTA</td>
<td>105.6 ± 7.3</td>
</tr>
</tbody>
</table>

* Mean ± standard error of five determinations.
† Serum used as uptake medium.
‡ Saline used as uptake medium.

FIG. 3. EFFECT OF INCUBATION TIME ON THE UPTAKE OF $B_{12}^{57}$Co BY SUSPENSIONS WITH VARYING RETICULOCYTE COUNT. Uptake from normal serum (four experiments) and saline (two experiments) is compared. Of the erythrocytes, 10% were reticuloctyes in the highest serum and saline curve, 8% in the next highest serum and saline curve, 5% in the middle two serum curves, and 3% in the lowest serum curve.
per ml, 600 pg per ml, and 1,000 pg per ml) is presented so that transfer from the same total amounts of protein-bound B₁₂⁻⁵⁷Co can be directly compared. It is evident that radioactive B₁₂ is transferred most efficiently from serum protein with the greatest B₁₂⁻⁵⁷Co saturation, even when the total amount of B₁₂⁻⁵⁷Co available to the erythrocytes in the test system is equal. If the quantity of transcobrin molecules is assumed to exceed the number of reticulocyte receptor sites available, this finding would imply that the reticulocyte may not take up the B₁₂⁻transcorrin (23) complex in marked preference to transcobrin alone from a mixture of both free and complexed carrier. Preferential uptake of B₁₂⁻⁵⁷Co-transcorrin over transcobrin alone would be expected to yield similar uptake of B₁₂⁻⁵⁷Co from 1 ml of serum to which was added 200 pg of B₁₂⁻⁵⁷Co as from ½ ml of the same serum containing 200 pg of B₁₂⁻⁵⁷Co (when in both instances transcobrin is not saturated with B₁₂⁻⁵⁷Co).

**Cellular factors**

**Metabolic inhibitors.** The effect of metabolic inhibitors on B₁₂⁻⁵⁷Co uptake by erythrocytes was investigated by adding 0.5 ml 10⁻² M sodium cyanide (NaCN), 10⁻² M sodium fluoride (NaF), and 10⁻² M sodium arsenate (Na₂HAsO₄) to the standard incubation mixtures. No significant decreases in B₁₂ uptake could be demonstrated (Table II). When test cells were preincubated with 10⁻² M NaCN for 15 minutes at 37° C before addition of serum-bound B₁₂⁻⁵⁷Co, similar results were obtained.

**Digitalis glycosides.** The active transfer of sodium and potassium ions across red cell membranes is inhibited by digitalis glycosides (24); this may be due to inhibition of cellular ATPase and the “sodium pump” (25). Five-tenths ml deslanoside (Cedilanid-D, 2 × 10⁻⁴ M concentration) had no effect on B₁₂⁻⁵⁷Co uptake (Table II).

**TABLE II**

**Effect of metabolic inhibitors on B₁₂⁻⁵⁷Co uptake by erythrocytes from serum**

<table>
<thead>
<tr>
<th>Agent (0.5 ml) added to 0.5 ml serum</th>
<th>B₁₂⁻⁵⁷Co uptake by 1 ml erythrocytes as % of uptake from control*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.85% NaCl (Control)</td>
<td>100</td>
</tr>
<tr>
<td>NaCN (10⁻² M)</td>
<td>95.9 ± 4.1</td>
</tr>
<tr>
<td>NaF (10⁻² M)</td>
<td>113.9 ± 5.7</td>
</tr>
<tr>
<td>NaH₂AsO₄ (10⁻² M)</td>
<td>112.2 ± 6.9</td>
</tr>
<tr>
<td>Deslanoside (2 × 10⁻⁴ M)</td>
<td>97.3 ± 4.8</td>
</tr>
</tbody>
</table>

*Mean of five estimations ± standard error.
B₁₂-⁵⁷Co uptake by stored cells. A portion of reticulocyte-rich blood thrice washed with CaCl₂-NaCl and then suspended in this solution was refrigerated at 4° C for periods up to 4 days. Ability to adsorb B₁₂-⁵⁷Co was then assessed and compared with the original uptake. A gradual loss of B₁₂ uptake was evident, but erythrocytes stored for 4 days could still adsorb 58% of the original uptake. Over the same period the reticulocyte count dropped from 13% to 7.6%.

Cellular membrane changes. a) Enzyme treatment. Powdered trypsin and papain were dissolved in physiological saline in concentrations of 0.1, 0.01, and 0.001%. Thrice washed reticulocyte-rich red cell suspensions were incubated at 37° C for 1 hour with volumes of these enzyme solutions equal to the volume of red cells present. After two additional washings, B₁₂-⁵⁷Co uptake by red cells was determined and compared with uptake by control red cells incubated with saline instead of enzyme.

Results (Table III) indicate that 0.1% enzyme greatly reduced B₁₂-⁵⁷Co uptake; even at 0.001%

<table>
<thead>
<tr>
<th>Agent (0.5 ml) preincubated with erythrocytes</th>
<th>B₁₂-⁵⁷Co uptake by 1 ml erythrocytes as % of uptake from control</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl (0.85%) (Control)</td>
<td>100</td>
</tr>
<tr>
<td>Trypsin (0.1%)</td>
<td>11.6 ± 1.5</td>
</tr>
<tr>
<td>Trypsin (0.001%)</td>
<td>47.8 ± 4.3</td>
</tr>
<tr>
<td>Papain (0.1%)</td>
<td>14.2 ± 2.1</td>
</tr>
<tr>
<td>Papain (0.01%)</td>
<td>45.0 ± 10.9</td>
</tr>
<tr>
<td>Papain (0.001%)</td>
<td>83.4 ± 10.1</td>
</tr>
<tr>
<td>Anti-D coated erythrocytes</td>
<td>97.8 ± 2.4</td>
</tr>
</tbody>
</table>

* Mean of five estimations ± standard error.

concentration, uptake was appreciably decreased by trypsin. Enzyme treatment did not cause visible hemolysis of erythrocytes.

b) Coating of cell surface with antibody. Reticulocyte-rich red cells were collected from a patient with blood group A, Rh positive (CD₆), and incubated at 37° C for 1 hour with high titer anti-D antiserum in volumes equal to the volume of the test erythrocytes. This procedure coats the individual red cell with approximately 24,000 antibody molecules (26) but causes no macroscopic erythrocyte agglutination. Coated cells were twice washed with CaCl₂-NaCl and then tested.

TABLE IV

<table>
<thead>
<tr>
<th>Uptake medium</th>
<th>Elution medium</th>
<th>B₁₂-⁵⁷Co on 1 ml erythrocytes, as % of pre-elution radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. *</td>
<td>Serum None</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Serum Na₂ EDTA (10⁻¹ M, 0.5 ml)</td>
<td>12.5 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>Serum NaCl (0.9%, 0.5 ml)</td>
<td>36.3 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>Serum Normal serum (0.5 ml)</td>
<td>49.6 ± 4.7</td>
</tr>
<tr>
<td></td>
<td>Serum B₁₂-deficient serum (0.5 ml)</td>
<td>48.9 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>Serum Chronic myelogenous leukemia serum (0.5 ml)</td>
<td>49.5 ± 6.1</td>
</tr>
<tr>
<td></td>
<td>Serum Trypsin (0.01%, 1.0 ml)</td>
<td>0.62 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Serum Trypsin (0.001%, 1.0 ml)</td>
<td>9.4 ± 1.4</td>
</tr>
<tr>
<td>B. †</td>
<td>1) Serum None</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Serum Na₂ EDTA</td>
<td>47.5, 21.6</td>
</tr>
<tr>
<td></td>
<td>Saline None</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Saline Na₂ EDTA</td>
<td>101.3, 97.8</td>
</tr>
<tr>
<td>2) Serum None</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Serum Na₂ EDTA</td>
<td>25.4, 21.1</td>
</tr>
<tr>
<td></td>
<td>Saline None</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Saline Na₂ EDTA</td>
<td>120.1, 116.9</td>
</tr>
</tbody>
</table>

* After B₁₂-⁵⁷Co transfer (incubation for 60 minutes at 37° C), cells were twice washed and reincubated with elution media (30 minutes at 37° C); B₁₂-⁵⁷Co remaining on erythrocytes was compared with pre-elution radioactivity.
† After B₁₂-⁵⁷Co uptake during 1) 30-minute or 2) 2-hour incubation periods, cells were incubated for 30 minutes at 37° C, with Na₂ EDTA (10⁻¹ M, 0.5 ml), without prior washing. Residual B₁₂-⁵⁷Co on erythrocytes was compared with pre-elution controls.
‡ Only two estimations performed; other values are means ± standard errors of five samples.
for their ability to take up $\text{B}_{12^{-}}^{57}\text{Co}$; results were then compared with uptake by control reticulocytes incubated with saline instead of antiserum. Pre-coating with Rh antibody did not decrease $\text{B}_{12^{-}}^{57}\text{Co}$ uptake by reticulocytes (Table III).

*Elution of $\text{B}_{12^{-}}^{57}\text{Co}$ from test reticulocytes.*

a) **Elution of $\text{B}_{12^{-}}^{57}\text{Co}$ from erythrocytes after serum transfer.** The labeled cells were twice washed with $\text{CaCl}_{2^{-}}\text{NaCl}$ and reincubated for 30 minutes at 37°C with various elution media, in volumes comparable to those used in the standard transfer procedure. Remaining cellular radioactivity was determined after three $\text{CaCl}_{2^{-}}\text{NaCl}$ washes and compared with a pre-elution radioactivity. Elution was maximal with 10⁻¹ M Na₂ EDTA and trypsin, less marked with serum and saline. Normal, chronic myelogenous leukemia, and $\text{B}_{12^{-}}$-deficient serum eluted equal amounts of $\text{B}_{12^{-}}^{57}\text{Co}$ (Table IV).

b) **Comparison of elution of $\text{B}_{12^{-}}^{57}\text{Co}$ from erythrocytes after saline transfer to that after serum transfer.** One-half ml 10⁻¹ M Na₂ EDTA was added to standard test suspensions after red cells had been incubated with $\text{B}_{12^{-}}^{57}\text{Co}$ in saline and serum for 30 minutes and 2 hours. Further incubation of 30 minutes was allowed; the cells were then thrice washed, and remaining radioactivity of the erythrocytes was determined. Results were compared with cellular $\text{B}_{12^{-}}^{57}\text{Co}$ immediately before Na₂ EDTA addition (Table IV). Whereas Na₂ EDTA caused elution of $\text{B}_{12^{-}}^{57}\text{Co}$ from erythrocytes when transferred by serum proteins, $\text{B}_{12^{-}}^{57}\text{Co}$ taken up from saline medium was not eluted.

*Site of $\text{B}_{12^{-}}^{57}\text{Co}$ attachment.* Reticulocyte-rich erythrocytes containing $\text{B}_{12^{-}}^{57}\text{Co}$ taken up from serum and saline media were twice washed with $\text{CaCl}_{2^{-}}\text{NaCl}$ and then hemolyzed in 4 vol distilled water. Toluene, 2 ml, was added; the specimens were shaken intermittently for 5 minutes and then centrifuged at 3,000 rpm for 15 minutes. The red cell stroma was now tightly packed on the under surface of the toluene layer; the hemolysate could be separated from the stroma by gently passing a thin glass pipette down the side of the tube. The radioactivity of the two fractions was determined (Table V).

More than 70% of $\text{B}_{12^{-}}^{57}\text{Co}$ transferred to red cells by serum was present in the stromal layer. Activity in the hemolysate was not significantly greater after 3 hours of incubation than after 30 minutes, suggesting insignificant penetration of $\text{B}_{12^{-}}^{57}\text{Co}$ into the red cell even with prolonged incubation. The percentage saline-transferred $\text{B}_{12^{-}}^{57}\text{Co}$ in hemolysate and stroma was similar in the 30-minute and 3-hour specimens.

**Discussion**

This study suggests that serum-mediated $\text{B}_{12^{-}}^{57}\text{Co}$ uptake by the reticulocyte-rich erythrocyte suspension is essentially a calcium (Ca²⁺)- or magnesium (Mg²⁺)-dependent surface adsorption phenomenon or both. The EDTA studies suggest that strontium (Sr²⁺) may partially replace these cations. Similar findings were reported for $\text{B}_{12^{-}}$ uptake by the liver and intestinal systems (10, 27). When the $\text{B}_{12^{-}}^{57}\text{Co}$-labeled test cells were incubated with various elution media, most of the $\text{B}_{12^{-}}^{57}\text{Co}$ could be eluted by trypsin and Na₂ EDTA (Table IV). Na₂ EDTA elution may be due to chelation of essential Ca²⁺ bonds. Reincubation with serum also caused $\text{B}_{12^{-}}^{57}\text{Co}$ elution; no difference was found between normal, chronic myelogenous leukemia, and $\text{B}_{12^{-}}$-deficient serum. Herbert (10) similarly found Na₂ EDTA to cause marked elution of $\text{B}_{12^{-}}^{57}\text{Co}$ from rat liver slices, whereas Jandl, Inman, Simmons, and Allen (28) could demonstrate significant elution of transferrin-facilitated ⁵⁷Fe uptake only when reticulocyte-poor suspensions were used. Trypsin, even in 0.001% concentrations, caused elution of 90.6% of the initial $\text{B}_{12^{-}}^{57}\text{Co}$ taken up by reticulocytes. With toluene separation of red cell stroma and hemolysate, more than 70% of serum-transferred $\text{B}_{12^{-}}^{57}\text{Co}$ was located in the stroma (Table V); radioactivity in the hemolysate was no greater after 3 hours of incubation than after 30 minutes.
The evidence thus suggests that B₁₂⁵⁷Co transferred by serum penetrates the red cell membrane only poorly.

Metabolic poisons such as NaCN and Na₂H₂AsO₄ and an inhibitor of glycolysis, NaF (29), did not decrease B₁₂⁵⁷Co uptake (Table II), indicating that active cellular metabolism is of little importance in the phenomenon under study. Jandl and co-workers (28) reported a pronounced decrease of ⁵⁷Fe uptake by reticulocytes in the presence of these materials. However, in their system, ⁵⁷Fe was actually transported into the cell. Laurell and Morgan (30) found these substances to inhibit in vitro ⁵⁷Fe uptake by rat placenta, and other workers similarly described decreased B₁₂⁵⁷Co uptake by Ehrlich ascites tumor cells (13) and decreased glycine uptake by reticulocytes (31). Herbert (10), on the other hand, found 2,4-dinitrophenol ineffective in reducing B₁₂⁶⁰Co uptake by rat liver slices and concluded that this is a surface adsorption phenomenon. Trypsin and papain, enzymes known to damage the surface membrane, greatly decreased B₁₂⁵⁷Co uptake in the present study (Table III). Jandl and associates (28) and Jandl and Katz (32) found similar results with ⁵⁷Fe uptake by reticulocytes. They also reported that ⁵⁷Fe uptake is impaired when cells are precoated with antibody (28); we were unable to show decreased B₁₂⁵⁷Co uptake by erythrocytes coated with anti-D antibody (Table III). B₁₂⁵⁷Co uptake from saline in the absence of serum showed very different characteristics. It was not reticulocyte dependent, was not affected by Ca²⁺ chelating agents, was quantitatively less than serum transfer, and Na₂ EDTA did not elute "saline-transferred" B₁₂⁵⁷Co from the cell surface. Although uptake from serum increased with a rising reticulocyte count, it is probable that mature erythrocytes also take up significant amounts of B₁₂. [Extrapolation of the "uptake slopes" in Figure 1 shows that the ordinate (0% reticulocyte count) is invariably reached much above the zero uptake level.]

The present findings suggest that the mechanism of serum-mediated vitamin B₁₂ uptake by the reticulocyte-rich red cell suspension is very similar to that for intrinsic factor-mediated vitamin B₁₂ uptake by intestinal mucosa (33). Because serum was preincubated with subsaturating doses of B₁₂⁵⁷Co, no unbound radioactive B₁₂ was present in those experiments testing serum transfer of B₁₂. The red cell surface probably contains receptor sites adapted to receive the transport protein B₁₂ complex but may also accept the transport protein per se, depending on amount and saturation of carrier protein by B₁₂. In spite of equal absolute amounts of B₁₂⁵⁷Co, most radioactive B₁₂ was transferred by the serum with highest B₁₂⁶⁰Co concentration (Figure 5). Ionic calcium probably consolidates the carrier protein bond to the reticulocyte surface (Figure 6). Vitamin B₁₂ uptake in the absence of plasma protein may represent simple diffusion. In the present study B₁₂ transferred by protein entered the test cells in minute amounts at the most. One could speculate that the developing erythropoietic cell probably loses its ability to incorporate B₁₂ as its declining metabolic activity decreases the need for this coenzyme. At the reticulocyte stage, and even with mature erythrocytes, active B₁₂ receptor sites may still be present on the cell surface, but the cell no longer needs B₁₂, and the vitamin is not transferred from the surface to the interior of the cell. The work of Schilling and Meyer (34), who showed that tracer doses of radioactive B₁₂ are incorporated into erythroid cells only at the nucleated precursor stage, conforms with this hypothesis. They found that radioactivity incorporated in this manner is located in the hemolysate rather than stroma and that it progressively disappears from the cell during maturation. B₁₂⁵⁷Co taken up from saline, on the other hand, probably penetrates the cell membrane independent of receptor sites. Our in vitro experimental model thus suggests a dual mechanism for B₁₂ transport to erythrocytes, as exists for transport across the small intestine: a glycoprotein-mediated transport operative primarily in the presence of physiologic quantities of the vitamin, and diffusion operative primarily in the
presence of supraphysiologic quantities of the vitamin. The rapid rate of serum-mediated $^{57}$Co transfer resembles the “primary” phase of the biphasic $B_{12}$ uptake curve found with mouse ascites tumor cells (12). However, we could not demonstrate a “secondary” uptake. With saline transfer the initial rapid uptake may have been facilitated by minute amounts of contaminating serum protein.

A number of workers have studied $^{59}$Fe uptake by reticulocytes (28, 35–37). The most recent evidence suggests the absolute amount of iron present is the critical factor (35–37). In vivo tissue uptake of iron, on the other hand, seems to correlate better with transferrin saturation than with serum iron, per se (38). The present study suggests that the reticulocyte discriminates imperfectly between $B_{12}$-carrying transcinnor and transcinnor alone, since $B_{12}$-$^{57}$Co uptake by reticulocytes was related to the number of $B_{12}$-$^{57}$Co-transcinnor molecules in relation to the number of transcinnor molecules not carrying $B_{12}$-$^{57}$Co (figure 5). However, this problem can only be finally solved by labeling the carrier protein and $B_{12}$ separately in the same experiment. Adding various amounts of $B_{12}$-$^{57}$Co to pernicious anemia plasma may sequentially saturate different binding proteins with subsequent changes in transferring properties. A method for rapid separation of $B_{12}$ binding $\alpha$ from $\beta$-globulin is presented elsewhere (39) as is evidence that the $\beta$ $B_{12}$ binder delivers more $B_{12}$ to reticulocytes than does the $\alpha$ binder (40).

Summary

1. Serum-mediated $B_{12}$-$^{57}$Co uptake by reticulocyte-rich erythrocytes appeared to represent rapid adsorption to the red cell surface; ionic calcium or magnesium was essential for this reaction, but strontium could partially replace these cations. In the test system used, $B_{12}$-$^{57}$Co uptake was maximal after 20 minutes' incubation, with near maximal adsorption during the first 5 minutes. Uptake increased with a rising reticulocyte count, but mature erythrocytes could also adsorb small amounts of $B_{12}$-$^{57}$Co. Trypsin and papain reduced $B_{12}$ uptake, but metabolic poisons had no effect. $Na_3$ EDTA and trypsin could elute virtually all $B_{12}$-$^{57}$Co previously adsorbed to erythrocytes; elution was much less complete with serum and saline.

2. $B_{12}$-$^{57}$Co taken up from a saline medium was less than from serum, did not concentrate in red cell stroma (unlike $B_{12}$-$^{57}$Co from serum), did not show calcium or reticulocyte dependence, and could not be eluted by $Na_3$ EDTA.

3. We suggest that two mechanisms exist for $B_{12}$ uptake by erythrocytes analogous to the dual mechanisms for $B_{12}$ transport across the intestinal mucosa: a) calcium- or magnesium- (or both) dependent, carrier glycoprotein-mediated transfer to receptors on the cell surface, operative primarily in the presence of physiologic quantities of $B_{12}$ and b) simple diffusion independent of receptor sites (primarily operative in the presence of excess unbound $B_{12}$).

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MECHANISM OF VITAMIN $B_{12}$ UPTAKE BY ERYTHROCYTES


