## APPENDIX

## Methods

***Wash solution*** Wash solution was prepared by addition of 4 mL of 50% dextrose (Baxter, Deerfield, IL), 20 mL of 8.4% sodium bicarbonate (American Reagent Laboratories, Inc., Shirley, NY), and 1 mL of sodium phosphates (American Reagent Laboratories, Inc.) to 1 L of 0.9% sodium chloride (Baxter, Deerfield, IL). Total osmolarity of this solution is 356 mOsm/L.

***Sulfo-NHS-biotin solution*** A solution of sulfo-NHS-biotin (approximately 40 µg/mL, Pierce, Rockford, IL) was prepared in wash solution and filter sterilized immediately before labeling RBCs

***($^{125}$I)Streptavidin*** A low-specific activity tracer was prepared by iodination using 100 µg streptavidin (Sigma Chemical Co., St. Louis, MO) at a concentration of 1 mg/mL in 50 mM sodium phosphate buffer, pH 7.4) and 74 Bq (2 mCi) $Na^{125}I$ (Amersham, Arlington Heights, IL) according to the Iodo-Beads technique (Pierce) as previously described (1). The ($^{125}$I)streptavidin product was diluted in 50 mL of wash solution containing 0.1% bovine serum albumin yielding a concentration of approximately 220-370 mBq/mL (7.1-11.8 Bq/mmole, 192-320 Ci/mmole).

***Dextran-metrizoic acid solution.*** Dextran-metrizoic acid solution was prepared by mixing 5 volumes of 6% dextran (Sigma) in wash solution and 1 volume of 32.8% sodium metrizoate (2).

## Labeling red blood cells with ($^{14}$C)cyanate, $^{51}$Cr, and biotin

Approximately 2% of the sheep's estimated blood volume was drawn from the external jugular by venipuncture on the same day as the study. Half the volume was drawn into sodium heparin for ($^{14}$C)cyanate and biotin labeling; half was drawn into CPDA-1 (3) for $^{51}$Cr labeling. RBCs were sedimented by centrifugation at 1000 rcf for 15 min at room temperature. The

plasma was removed by syringe aspiration using sterile technique and saved for later use as described below.

Red blood cells (RBCs) were labeled with ($^{14}$C)cyanate, as previously described (4, 5), using the original technique of Eschbach (6) except that RBCs were washed prior to labeling. The ($^{14}$C)cyanate forms a covalent bond with the amino group of the terminal valine of intracellular proteins, primarily hemoglobin (7), in a carbamylation reaction as follows:

$$R\text{-}NH_2 + HN{=}C{=}O \rightarrow R\text{-}NH\text{-}CO\text{-}NH_2.$$

A volume of blood equal to 1% of the sheep's estimated blood volume was centrifuged and the buffy coat was removed by aspiration and discarded. The centrifuged RBCs were washed 3 times using 4 volumes of wash solution.

RBCs were labeled with $^{51}$Cr according to the standard protocol adopted by the International Committee for Standardization in Hematology (8) as previously described (5). Briefly, $Na^{51}CrO_4$ (Amersham) was mixed with packed RBCs at a ratio of 370 mBq/mL RBCs (10 µCi/mL). This mixture was incubated for 30 min at room temperature. To reduce free $^{51}$Cr, ascorbic acid (10 mg ascorbate/mL RBCs) was mixed with the RBCs and incubated for 30 min at room temperature before infusion back into the same sheep.

The washed RBCs were labeled with sulfo-NHS-biotin; approximately 40 µg of the biotinylating reagent was used per mL of RBCs. Sulfo-NHS-biotin biotinylates RBC cell surface proteins. Prior to addition of the biotinylating reagent, the RBC pellet was resuspended in a volume of wash solution approximately equal to 50% of the packed RBC volume. The RBC suspension was incubated for 1 h at room temperature and then washed four times with four volumes of wash solution to remove unreacted biotinylating reagent and unbound biotin.

The volumes of the ($^{14}$C)cyanate, $^{51}$Cr, and biotin-labeled RBCs were adjusted by addition of the saved plasma to a hematocrit approximately equal to that of the original blood sample. The mass of each the three labeled suspensions was determined gravimetrically, and the three suspensions were mixed together in a sterile plastic syringe. Mass was used to calculate volumes infused based on the density of ovine blood (1.04 g/mL determined empirically in our laboratory).

***Assay for biotin label concentration using ($^{125}$I)streptavidin*** A standard curve was constructed using biotin-labeled RBCs diluted in unlabeled RBCs at ratios of approximately 1/21, 1/42, 1/84, and 1/168 (**Figure 1A**). Exact dilutions of the biotinylated RBCs were calculated from volumes determined gravimetrically and from the hematocrit value determined by a microcapillary technique as previously described (9). Quadruplicate 200 µL aliquots of the dilutions, normal blood, and mixed venous samples were weighed in 12 x 75 mm borosilicate glass tubes. ($^{125}$I)streptavidin (100 µL) was added to each tube, and the contents were mixed by vortexing briefly and then incubating with ($^{125}$I)streptavidin for 1 h at 5°C. Biotinylated RBCs (with bound ($^{125}$I)streptavidin) were then separated from excess free ($^{125}$I)streptavidin by dextran-metrizoic acid density centrifugation as follows. 1.2 mL of wash solution was added to each sample (experimental sample or standard curve sample), the contents were mixed by brief vortexing. Then 1 mL of the RBC suspension was layered gently onto 3 mL of dextran-metrizoic acid solution as described by Takamori (2). The tubes were centrifuged at 2500 rcf for 10 min in a swinging bucket rotor. The aqueous and dextran-metrizoic acid layers containing free ($^{125}$I)streptavidin were removed by vacuum aspiration; any residual liquid was swabbed from the tube walls. The remaining RBC pellet was counted on a 1274 RIA Gamma Counter (Wallac Inc., Gaithersburg, MA) using an energy window of 15-80 keV. For each time, at least triplicate

measurements of label concentration were made. The background radiation from $^{51}Cr$ was $\leq$ 3% of the total cpm of ($^{125}I$)streptavidin and was subtracted. Saturation of $^{125}I$-streptavidin binding to the biotin moieties on the RBCs was demonstrated empirically for each sheep experiment.

***$^{14}C$ assay*** Quadruplicate 50 and 100 µL volumes of 100% $^{14}C$-labeled cells and quadruplicate 0.5 mL volumes of samples and normal blood (as control) were aliquoted into 50 mL tubes and weighed. Total volume of infusate samples was increased to 0.5 mL with normal blood. 2 mL of 0.5 N HCl was added to each tube to lyse red cells and dissociate hemoglobin. Total volume was increased to 45 mL with acetone and mixed. $^{14}C$-labeled proteins were sedimented at 750 rcf for 5 min, supernatant was decanted, resuspend the pellet in 2 mL of water, and washed a second time with acetone. After sedimentation and washing a second time, the $^{14}C$-labeled proteins were dissolved in 7 mL of toluene-based liquid scintillation fluid and decanted into a 20 mL glass scintillation vial. Any residual $^{14}C$ was washed from the tube with an additional 7 mL of liquid scintillation fluid. $^{14}C$ was quantitate using a liquid scintillation counter. Energy windows were set at 0-156 keV, and a quench program was used to compensate for any remaining heme color and to discriminate $^{14}C$ energy from $^{51}Cr$ energy (4).

***$^{51}Cr$ assay*** Sediment the $^{51}Cr$-labeled RBC by centrifugation at 750 rcf for 5 min. Aspirate the supernatant and count the pellet in a gamma counter. Quantitation of the $^{51}Cr$ radioactivity per mL of blood was by gamma counting using energy windows of 0-1000 keV.

***Assay of biotin label using flow cytometry*** Quadruplicate 10 µL aliquots of the standard curve (**Figure 2A**) and of the post-infusion samples were incubated with 50 µL of fluorescein-tagged avidin solution (20 µg/mL in wash solution) for 30 min at 22°C in the dark. To minimize nonspecific binding of fluorescein-avidin to unlabeled RBCs, the mixture was then washed two times with 1 mL of wash solution. The RBCs were resuspended in 1 mL of wash solution and

analyzed with an Epics Profile II flow cytometer (Coulter, Miami, FL) accumulating at least 300,000 events. For each sample, at least triplicate measurements were made.

***RCV by ($^{14}$C)cyanate, $^{51}$Cr, and ($^{125}$I)streptavidin binding*** Calculate and subtract the mean radioactivity determined as dpm/mL of control blood from the mean dpm/mL of the radiolabeled RBCs to be infused and the mean dpm/mL of each sample. Calculate the total amount of radioactivity infused and the concentration of radioactivity in the timed samples.

For each timed sample, the radioactivity per mL of blood was calculated and was plotted versus time. The t = 0 intercept of the linear regression was determined. The linear regression typically contained four samples drawn between 2 and 30 min; at each time, at least triplicate measurements of label concentration were made as described below.

Blood volume (BV) was calculated according to the dilution principle (8) as follows:

$$BV = \frac{A_S}{C_S} = \frac{(L - B) \times W}{(S - B) \times G}$$

where $A_S$ = total radioactivity infused; $C_S$ = radioactivity/mL sample at t = 0; L = radioactivity per g of labeled blood; B = background radioactivity per g of unlabeled RBCs; W = weight of labeled RBC suspension infused (g); S = radioactivity/g mixed venous blood; G = specific gravity of blood (g/mL).

**Sequential determinations of red cell volume (RCV)**

Multiple determinations of RCV in the same individual over a limited interval of time implies substantial or even accumulating concentrations of residual label. Sequential RCV can be determined by correcting for residual radioactivity using the following equation as previously published (5):

$$RCV = \frac{V_i \times C_a}{(C_{k+1} - C_k)}$$

where $V_i$ equals the volume of labeled cells infused on the $k+1^{th}$ infusion, $C_a$ equals the concentration of label on the $k+1^{th}$ infusion, $C_k$ equals the residual concentration of the label from all previous measurements, and $C_{k+1}$ equals the final concentration of label after mixing of the $k+1^{th}$ infusion.

**Derivation of Equation for Repetitive Measurements of RCV**

Using symbols as above, let

RCV = red cell volume

$C_k$ = the residual concentration of label from the k previous measurements

(cpm of $^{14}C$ bound per mL of packed blood cells)

$C_{k+1}$ = the final concentration of label after the $k+1^{th}$ infusion

(cpm of ($^{125}I$)streptavidin bound per mL of packed blood cells)

$T_k$ = Total amount of label in circulation <u>before</u> the $k + 1^{th}$ injection

$T_{k+1}$ = Total amount of label in circulation <u>after</u> the $k + 1^{th}$ injection

$T_a$ = total amount of label in the $k + 1^{th}$ injection

$V_i$ = volume injected on the $k + 1^{th}$ injection

$C_a$ = concentration of label in the $k + 1^{th}$ injection

Conservation assumption: The total amount of label is constant. Restated, the total amount of label in circulation after the $k + 1^{th}$ injection is equal to the sum of the residual label plus the added label.

1) $T_{k+1} = T_k + T_a$

By definition $C_k = T_k/RCV$

$$C_{k+1} = T_{k+1}/RCV$$

$$C_a = \frac{T_a}{V_i}$$

Rearranging, we have $T_{k+1} = RCV * C_{k+1}$

$$T_k = RCV * C_k$$

$$T_a = V_i * C_a$$

Substituting these definitional equations into the conservation equation yields

2) $$RCV * C_{k+1} = RCV * C_k + V_i * C_a$$

Solving for RCV yields

$$RCV = \frac{V_i * C_a}{C_{k+1} - C_k}$$

Each parameter on the right side of the equation is determined experimentally. Of course, the equation reduces to the standard dilution equation for the first measurement:

$C_o = 0$

$$RCV = \frac{V_i * C_a}{C_1}$$

References

1. Mock DM 1990 A sequential, solid phase assay for biotin based on $^{125}$I-avidin. In Wilchek M, Bayer EA (eds) Methods in Enzymology. Academic Press, Inc., San Diego, CA, pp 224-233
2. Takamori K, Yamashita T 1980 Biochemical properties of polymorphonuclear neutrophils from venous blood and peritoneal exudates of rabbits. Infect Immun 29:395-400
3. Beutler E, West C 1984 Measurement of the viability of stored red cells by the single-isotope technique using $^{51}$Cr. Transfusion 24:100-104
4. Mock DM, Strauss RG, Lankford GL 1997 $^{14}$C-cyanate labeling of sheep red cells: Covalent binding to hemoglobin continues in vivo for a day. Pediatr Res 41:424-429
5. Mock DM, Lankford GL, Burmeister LF, Strauss RG 1997 Circulating red cell volume and red cell survival can be accurately determined in sheep using the [$^{14}$C]cyanate label. Pediatr Res 41:916-921
6. Eschbach JW, Korn D, Finch CA 1977 $^{14}$C-cyanate as a tag for red cell survival in normal and uremic man. J Lab Clin Med 89:823-828
7. Cerami A, Manning JM 1971 Potassium cyanate as an inhibitor of the sickling of erythrocytes *in vitro*. Proc Nat Acad Sci USA 68:1180-1183
8. International Committee for Standardization in Hematology (ICSH): Panel on Diagnostic Applications of Radioisotopes in Haematology 1973 Standard techniques for the measurement of red-cell and plasma volume. Br J Haematol 25:801-814
9. Brace RA 1983 Blood volume and its measurement in the chronically catheterized sheep fetus. Am J Physiol 244:487-494

## Figures

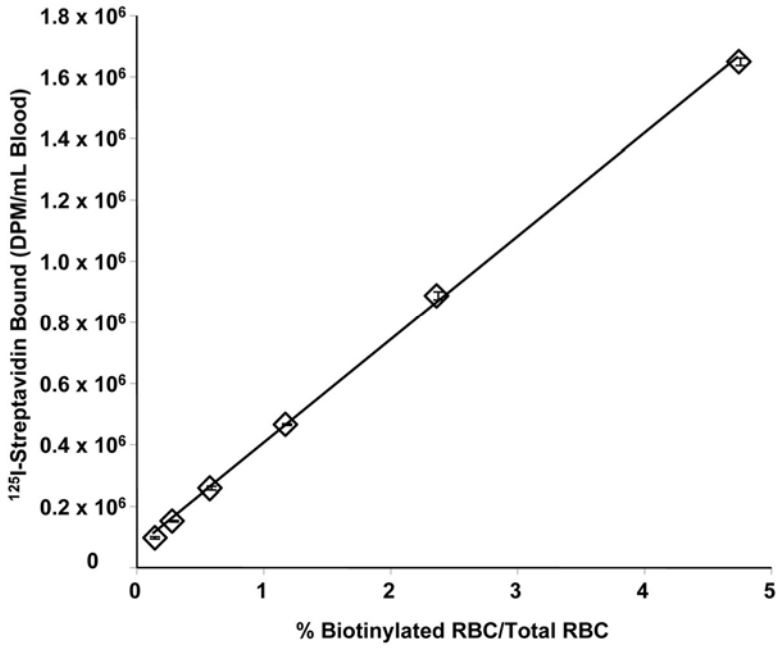


Figure 1A. $^{125}$I-Streptavidin Bound per mL of RBCs as a Function of Percent of Biotinylated RBCs. Error bars depict one standard deviation and are smaller than the graphical symbol.

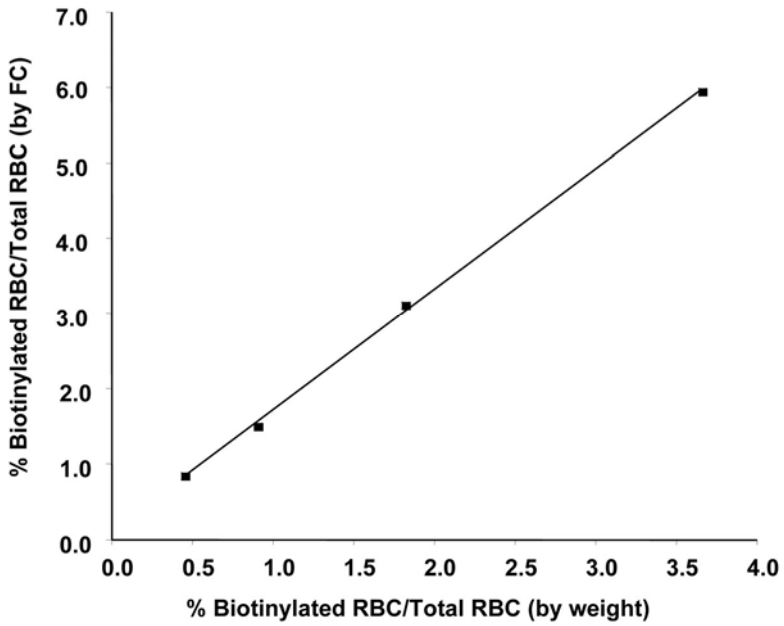


Figure 2A. Percent Biotinylated RBCs Enumerated by Flow Cytometry as a Function of Percent of Biotinylated RBCs.

Error bars depict one standard deviation and are approximately the size of the graphical symbol. The correlation coefficient of the regression line was 1.00. The intercept = 0.00, and the slope was 1.6.