CHAPTER 9

Red blood cell metabolism, preservation, and oxygen delivery

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A human red cell, mature and released from the bone marrow, lacks a nucleus and mitochondria. It has a life span of 120 days, after which it is removed from the circulation in the natural course of aging.¹ It picks up oxygen in a third of a second transit of the alveolar capillary and delivers oxygen through capillaries with a smaller diameter than its own. It can neither use oxygen for the extraction of energy, nor synthesize proteins or polynucleotides. Its primary functions, transporting oxygen from the lungs to the tissues and carbon dioxide back to the lungs, do not require the expenditure of energy. However, maintaining hemoglobin in an optimal state for delivering oxygen and keeping normal cell flexibility and morphology do require active metabolism and are prerequisites for function and successful transfusion.

During standard storage at 4 °C, significant and in part reversible changes in red cell morphology and metabolism occur, called storage lesions. One striking example is the red cell shape change (Figure 9.1), which can be reversed to a large extent by metabolic rejuvenation of the stored cells.² In order to understand and improve storage, it is essential to understand basic principles of red cell metabolism.

Understanding of red cell metabolism has increased with the application of “-omic” technologies. Proteomics has identified 2200 separate proteins in the red cell that are the products of 5% of all human genes.³ This number includes 340 proteins specifically associated with the red cell membrane. Structural proteins, membrane receptors, metabolic enzymes, heat shock proteins, chaperonins, and others have been catalogued and sorted into pathways and functional groups, and they provide the most complete overview of the molecule-by-molecule functioning of a human cell type. Metabolomics can measure simultaneously the concentrations of several hundred small molecules and follow their activities in view of the molecule-by-molecule functioning of a human cell type. Integration of biochemical metabolism and biophysical discoveries in the last two decades by several research groups shows that many molecular interactions may be missed when the red cell is considered by itself without taking into account the physiologic vascular milieu in which it performs its main biological function.⁴,⁵,⁶

Thus, interaction of hemoglobin and the band 3 protein is influenced by conformational changes that accompany ligand binding to hemoglobin, which modulates the function of band 3, the glycolytic pathway, and the structural architecture of the red cell membrane–cytoskeletal system (Figure 9.2). During storage, concentrations of red cell ATP, DPG (also known as 2,3-bisphosphoglycerate, or BPG), and glutathione decline, leading to cell wall dysfunction and damage. Dysfunction can manifest as stiffer red cells, and damage as membrane loss. Both of these effects can in turn affect the vascular flow of transfused cells. On the arterial side, stiffer cells have trouble absorbing the energy of pulsatile flow and are projected more frequently into the normally cell-free layer of plasma at the vascular surface, where they absorb endothelium-derived nitric oxide and lead to vasoconstriction.⁷ In capillaries, the close contact between hemoglobin and band 3 and carbonic anhydrase in the cell membrane promotes the production of bicarbonate with release of protons, which, via the Bohr effect (see Chapter 10), releases oxygen from hemoglobin where it is needed in the tissues.⁸ On the venous side, damaged membranes can lead to procoagulant and proinflammatory events.⁹ In this chapter, red cell metabolism will be discussed, taking into account intermolecular interactions of importance for the primary function of hemoglobin, which is oxygen and carbon dioxide transport, and followed by a review of the development of red cell storage systems.

Metabolism

Metabolism of glucose

Main glycolytic pathway (Embden–Meyerhof pathway)

Under physiologic circumstances, the energy that the red cell requires is derived through the breakdown of glucose to lactate or pyruvate. The sequence of reactions is generally known as glycolysis or the Embden–Meyerhof pathway.¹⁰ This pathway is phylogenetically very old, and the sequence of reactions is the same in bacteria,

yeast, and vertebrates. Except for the exaggerated production of DPG in the red cell, the pathway is the same in all tissues.

The reactions of the glycolytic pathway are shown in Figure 9.3. In this sequence of reactions, the six-carbon sugar glucose is phosphorylated, isomerized to fructose phosphate, phosphorylated again, and cleaved into three-carbon phospho-sugars. The three-carbon sugars are again phosphorylated. Finally, these carbohydrate-bound high-energy phosphates that have been gained are transferred to adenosine diphosphate (ADP), producing the high-energy compound, ATP. The ATP synthesized is used by ATPases for the pumping of ions against concentration gradients, for the phosphorylation of membrane proteins and lipids, and, very importantly, for the phosphorylation of glucose to make more ATP in the glycolytic pathway.\(^{13}\)

![Figure 9.1](image1) Scanning electron micrographs showing representative red blood cells in various stages of shape change typical of prolonged storage. The cells progress from discocytes (A), through several stages of echinocytes (B–D), to spherocytinocytes (E) and finally to spherocytes (F). Scores, based on the visual appearance of those shown in the lower right of the individual images, can be assigned to several hundred individual stored cells and averaged to produce a morphology score for the unit. Such scores decrease in a linear manner during storage but can be substantially reversed by rejuvenation.

![Figure 9.2](image2) Schemata of red cell components. In the past, it was believed that oxygen transport by hemoglobin and metabolism were cytosolic functions, and that the membrane and cytoskeleton merely enclosed them. We now recognize that the attachments of the membrane to the cytoskeleton from band 3, through ankyrin (An) and proteins 4.2 and 4.1 to spectrin, and from Glycophorin C through protein 4.1 and actin (Ac) to spectrin, are destabilized by 2,3-diphosphoglycerate (2,3-DPG). 2,3-DPG released from deoxy-hemoglobin (d-Hb) binds to band 3 and partially detaches the membrane from the cytoskeleton, allowing lateral movement of membrane structures. This could have implications for red cell flexibility when slipping into peripheral capillaries. O-Hb, oxy-hemoglobin.
The 2,3-DPG shunt (Rapoport–Luebering shunt)

Production of 2,3-DPG is the function of a shunt that branches from the main glycolytic pathway after the formation of 1,3-diphosphoglycerate (1,3-DPG) and returns to it with the formation of 3-phosphoglycerate (3-PGA) (Figure 9.3). The pathway consists of the formation of 2,3-DPG from 1,3-DPG, followed by the dephosphorylation of 2,3-DPG to 3-PGA (Figure 9.4). Both reactions are catalyzed by the same enzyme and are balanced at physiologic pH.14 At higher pH, the enzyme acts only as a mutase whereas a lower than physiologic pH leads to a burst of ATP metabolism.

The production of large quantities of 2,3-DPG is a unique feature of glycolysis in the red cell. Red cells contain approximately equimolar amounts of hemoglobin and 2,3-DPG. Binding of 2,3-DPG to the β subunits of deoxyhemoglobin serves to stabilize the T (tense, low oxygen affinity) state of hemoglobin and thus shifts the oxygen equilibrium curve to the right (favoring dissociation of oxygen). In the R (relaxed, high oxygen affinity) state, approximately 80% of 2,3-DPG is “free,” whereas in the T state over 80% of 2,3-DPG is bound to hemoglobin.5,15 In the “free” state, 2,3-DPG at physiological concentrations modulates properties of the red cell membrane.8 It binds directly to band 3 and thereby interferes negatively in the interactions between protein 4.1, protein 4.2, ankyrin, and band 3.16 2,3-DPG also releases spectrin from the membrane skeleton, and interferes negatively in the interactions between spectrin–actin–protein 4.1 and the glycoporin C complex.8,17,18 This decreases the number of connecting links between the cell membrane and the cytoskeleton, and increases lateral mobility of integral membrane proteins.16,19 The rise and fall of 2,3-DPG concentrations with each pass through the circulatory system therefore result in repetitive destabilization and restabilization of the membrane–cytoskeleton architecture (Figure 9.2). “Free” 2,3-DPG increases cell flexibility by weakening the links between the membrane and the cytoskeleton and facilitates gas exchange by allowing the red cell to slip into narrow capillaries and splenic sinusoids. However, further experiments are needed to clarify the full physiologic implications of the interactions between 2,3-DPG, cell membrane proteins and the cytoskeleton.

The Pentose shunt (hexose monophosphate shunt)

Under normal, steady-state conditions, most glucose is metabolized in red cells by way of the glycolytic pathway, but there is another important metabolic pathway called the pentose shunt or hexose monophosphate shunt (Figure 9.3). Some of the glucose-6-phosphate (G-6-P) formed when glucose is phosphorylated in the hexokinase reaction enters this pathway. Glucose-6-phosphate dehydrogenase (G-6-PD) catalyzes the oxidation of G-6-P to 6-phosphogluconate, reducing nicotinamide adenine dinucleotide phosphate (NADP) to NADPH. After hydrolysis of the lactone to 6-phosphogluconolactone, another oxidative step reduces additional NADP to NADPH, and releases carbon dioxide from the six-carbon compound, forming the pentose sugar ribose-1-phosphate. After a series of rearrangements, two normal intermediates of the main glycolytic pathway, fructose-6-phosphate and glyceraldehyde-3-phosphate, are formed and rejoin the main metabolic stream.
The pentose shunt is important to the red cell as a source of NADPH. It is this reduced nucleotide that maintains glutathione in its reduced form. Reduced glutathione is important for the elimination of peroxide, protection of protein SH groups, limitation of lipid peroxidation, and detoxification processes. The pentose shunt also plays an important role for the red cell by providing ribose-5-phosphate needed for the production of phosphoribosyl pyrophosphate (PRPP), an essential substrate for the synthesis of adenine nucleotides required for continuing ATP synthesis (discussed further in this chapter).

**Alternative substrates for red cell metabolism**

Glucose is the natural substrate for human red cell energy metabolism, but red cells are also capable of metabolizing other sugars (i.e., fructose, mannose, galactose, and the three-carbon sugar dihydroxyacetone). However, none of these other sugars have proven to be useful in the design of blood preservatives.

The presence in red cells of the enzyme nucleoside phosphorylase makes it possible for red cells to use nucleosides such as inosine to support ATP synthesis:

\[
\text{Inosine} + P_i \rightarrow \text{Ribose} - 1 - P + \text{Hypoxanthine}
\]

In this reaction, ribose-1-phosphate is formed without the expenditure of ATP. Ribose-1-phosphate is then readily converted to fructose-6-phosphate by the pentose shunt, which feeds into the glycolytic pathway leading to the generation of ATP. In the rejuvenation of red cells (see later in this chapter), inosine allows ATP-depleted red cells to prime their metabolic pump (the glycolytic pathway). It has not been possible to include inosine in blood preservatives because the product of its metabolism, hypoxanthine, is rapidly converted to uric acid in the body. Because many patients who receive blood transfusion have impaired liver function or may already have hyperuricemia because of hereditary or acquired factors, a blood product that increases plasma uric acid level cannot be considered safe.

**Regulation of energy metabolism**

**Glucose metabolism**

In nucleate cells, metabolic regulation is dependent on protein synthesis, which in turn is regulated by increasing or decreasing the rate of DNA transcription or the translation of messenger RNA. Red cells do not have these options. Instead, the rate of glucose metabolism is regulated by feedback mechanisms acting on the glycolytic enzymes. However, in spite of extensive studies, our understanding of the control of glucose metabolism by red cells is still incomplete.

The N-terminal cytoplasmic domain of the band 3 protein binds hemoglobin, cytoskeletal proteins, and glycolytic enzymes. Based on current evidence, de Rosa et al. point out that interaction between hemoglobin in the T state and band 3 causes a release by displacement of glycolytic enzymes, which results in increased activity of the main glycolytic pathway. On the contrary, hemoglobin in the R (highly oxygenated) state is associated with increased activity of the pentose shunt because the enzymes of the main glycolytic pathway show reduced activity when bound to band 3.

The rate of glucose metabolism by red cells is influenced by many factors other than basal enzyme activity. Negative feedback mechanisms are involved in the control of the glycolytic pathway. Both hexokinase and phosphofructokinase are inhibited by hydrogen ion (low pH). The principal reason that the rate of glycolysis slows markedly during red cell storage is the accumulation of lactic acid.

**2,3-DPG concentration**

The concentration of 2,3-DPG depends on the rate of its formation and degradation. Many effectors determine whether the mutase or the phosphatase activity of the diphosphoglycerate mustase–phosphatase predominates (Figure 9.4). The hydrogen ion concentration is the most important physiologic modulator. At low pH, phosphatase activity is stimulated and mutase activity is inhibited. Thus, high pH favors 2,3-DPG maintenance and production during storage at the expense of ATP formation, whereas low pH leads to the rapid loss of 2,3-DPG with an increase in ATP production as long as DPG lasts. Modulation of red cell metabolism by elevating pH has been the principal means used to retard the decline of 2,3-DPG levels that occurs during liquid storage of red cells (see later in this chapter). After depletion of ATP during storage, 2,3-DPG levels in transfused red cells return to 50% of normal in 7 hours and almost to 95% at 72 hours.

**The pentose shunt activity**

The rate of the pentose pathway is influenced by the availability of NADP and the concentration of NADPH. Under oxidative stress, NADPH is oxidized to NADP and the activity of the pentose pathway increases, which is consistent with the observation of increased pentose shunt activity when hemoglobin is in the R state.
Adenosine triphosphate
In the red cells, ATP is synthesized by the glycolytic pathway, but its regulation is complex. ATP is used in a number of different metabolic pathways, particularly by the kinases that phosphorylate sugars (i.e., hexokinase and phosphofructokinase) and proteins, and by ATPase-driven ion pumps (i.e., Na⁺-K⁺-ATPase, Mg²⁺-ATPase, and Ca²⁺-ATPase). Moreover, red cell membrane shape and rigidity are controlled by the ATP-dependent cytoskeleton.

\[ \text{ATP} + \text{AMP} \leftrightarrow 2 \text{ADP} \]

ATP is in equilibrium with ADP and adenosine monophosphate (AMP), as shown above. Production of ATP from ADP in glycolysis normally keeps AMP concentrations low. As the level of AMP increases when glycolysis slows, some is converted to AMP. AMP, in turn, is deaminated in the AMP-deaminase reaction and the total red cell pool of adenine decreases during storage, which leads to the depletion of ATP if adenine is not added to the anticoagulant and/or additive solution.

Guanine nucleotides
Red cells contain and turn over guanine nucleotides, which perform at least two functions in red cells. First, G proteins play a role in the signal transduction of membrane shear into secretion of the local vasodilators, cyclic AMP and ATP. Second, high concentrations of GTP inhibit red cell transglutaminase, a primitive coagulation system that also interacts with the cytoplasmic domain of band 3 and with protein 4.1. The GTP concentration is reduced when red cells age so that transglutaminase, which has a factor XIII-like activity, can facilitate the removal of senescent red cells by binding them to fibrin clots.

Synthetic processes
Red cells are only able to assemble a limited repertoire of important molecules from simpler precursors. They retain the capacity to synthesize nucleotides through a “salvage pathway” and to synthesize glutathione from its precursor amino acids. Adenine is able to enter the erythrocyte, and purine nucleotides are synthesized through the adenine phosphoribosyl transferase reaction:

\[ \text{Adenine} + \text{PRPP} \rightarrow \text{AMP} + \text{PP} \]

This reaction is critical in blood storage. The beneficial effect of addition of adenine to stored blood depends on it. Phosphoribosyl pyrophosphate (PRPP), one of the substrates for the formation of AMP in this reaction, is synthesized from pentose-phosphate formed in the pentose shunt. Guanine nucleotides form in an analogous reaction and are catalyzed by a different enzyme, hypoxanthine-guanine phosphoribosyl transferase. Red cells also actively synthesize a number of other small molecules, including reduced glutathione, nicotinamide adenine dinucleotide (NAD), and S-adenosyl-L-methionine. The phospholipid asymmetry is maintained by the ATP-dependent flipase (aminophospholipid translocase) activity. This activity counteracts phospholipid scrambling, which moves PS from the inner to the outer cell surface. Flipase activity decreases during storage but can be corrected by rejuvenation of the red cells. Phospholipid scrambling is normally low during storage but can be enhanced by photodynamic treatment for pathogen inactivation.

Maintaining the iron of hemoglobin in a reduced state is a prerequisite for effective oxygen transport. The enzyme involved is methemoglobin reductase, which is NADH driven and reduces Fe³⁺ to Fe²⁺. Protection of the SH groups of hemoglobin and membrane proteins from oxidation is also a crucial function. This is accomplished by maintaining adequate amounts of reduced glutathione (GSH) by reduction of NADPH to NADP. The oxidation of GSH to GSSG catalyzed by peroxidase reduces H₂O₂ to H₂O and Fe³⁺ to Fe²⁺. Adequate levels of ATP, NADH, NADPH, and 2,3-DPG for these metabolic functions are secured by the glycolytic pathway with its pentose and 2,3-DPG shunts. In addition, the synthesis of NAD and NADP from nicotinic acid must also occur.

Membrane metabolism
The red cell membrane is composed of a phospholipid bilayer containing cholesterol molecules and membrane proteins. The phospholipids are disposed with a predominance of phosphatidyl choline and sphingomyelin in the outer leaflet and phosphatidyl-linositol, phosphatidylethanolamine, and phosphatidylserine (PS) in the inner leaflet. Loss of phospholipid asymmetry results in exposure of PS, which is an important apoptotic marker on the red cell surface. Exposure of PS on the outer cell surface promotes red cell removal from the circulation, whereas the surface glycoprotein CD47, which decreases during storage, inhibits phagocytosis. The phospholipid asymmetry is maintained by the ATP-dependent flipase (aminophospholipid translocase) activity. This activity counteracts phospholipid scrambling, which moves PS from the inner to the outer cell surface. Flipase activity decreases during storage but can be corrected by rejuvenation of the red cells. Phospholipid scrambling is normally low during storage but can be enhanced by photodynamic treatment for pathogen inactivation.

Although the macromolecules of the membrane are produced by red cell precursors, some of the components of the membrane are metabolically quite active. For example, cholesterol in the
vascular system by splenic or other macrophages. RBCs at the end of their normal lifespan for removal from the body. The process is marked by irreversible shape change, loss of membrane by microvesiculation, and exposure of negatively charged phospholipids on their surface. Oxidative damage also appears to play an important role. Presumably, apoptosis marks RBCs at the end of their normal lifespan for removal from the vascular system by splenic or other macrophages.

Eryptosis: programmed cell death in red cells

Although RBCs lack the nucleus and mitochondria associated with programmed cell death in most cell populations, they undergo a unique form of programmed cell death called eryptosis, which is associated with low pH, increased cell calcium, or energy loss with low ATP concentrations. The process is marked by irreversible shape change, loss of membrane by microvesiculation, and exposure of negatively charged phospholipids on their surface. Oxidative damage also appears to play an important role. Presumably, eryptosis marks RBCs at the end of their normal lifespan for removal from the vascular system by splenic or other macrophages.

Eryptosis is critically limiting in RBC storage because falling pH and ATP concentrations are a concomitant of closed system storage with the accumulation of acid breakdown products of glycolysis and acidic inhibition of glycolysis. The accumulation of hemoglobin-containing microvesicles in the supernatant appears to be a good marker of the process, as is increasing RBC calcium content with activation of calpain enzymes.

Summary

In the circulation, red cells metabolize glucose by the glycolytic pathway with its pentose and 2,3-DPG shunts. The energy gained provides ATP to maintain ion and glucose concentration gradients between the plasma and erythrocyte and to secure red cell deformability. It also secures NADH to keep hemoglobin in the reduced state and NADPH to protect SH groups on hemoglobin and membrane proteins. Finally, the production of 2,3-DPG is important for the optimal dissociation of oxygen from hemoglobin, whereas the rise and fall of non-hemoglobin-bound 2,3-DPG through the circulatory system induce repetitive changes in the membrane–cytoskeleton architecture, which could have implications for red cell flexibility and gas transport.

Red cell preservation in transfusion medicine

Liquid preservation

General considerations and principles

RBCs are the most commonly transfused blood components, and their use in a variety of physical circumstances and clinical conditions has shaped their development as products. As an example, the US military’s need to reduce the weight and breakage of blood bottles during shipment led to the development and adoption of plastic blood bags. This led to closed-system sterile component production and, fortuitously, exposing RBCs to the plasticizer diethylhexyl phthalate (DEHP), which reduced hemolysis during storage. Arguments over the removal of plasma between blood transfusions, led to the development of additive solutions. Regulatory agencies have also played a role, as the US Food and Drug Administration (FDA) recognizes that 4 million patients a year receive RBCs and that the product must be safe for all.

As a result of many competing forces, development and adoption of new RBC storage technology have been slow. Five major improvements occurred in the last 60 years: (1) the addition of phosphate, (2) the use of plastic bags, (3) the addition of adenine, (4) the development of additive solutions, and (5) the use of leukoreduction, which reduces hemolysis. The reasons for this slow progress have had to do with the perception of little need for change, limited investment in change, lack of understanding of RBC storage lesions, poor developmental strategies, and conservative regulatory stances. The major ongoing efforts, the development of advanced additive solutions and pathogen reduction methods, make slow progress for similar reasons.

For 60 years, it has been a societal goal that RBCs for transfusion should be available, safe, effective, and cheap. Making red cells readily available requires both the ability to take liquid units out of the refrigerator and administer them immediately to critically ill or injured patients and the ability to find rare units in frozen national and international inventories. Storage systems contribute to blood safety by isolating individual units in closed systems and reducing product breakdown and bacterial growth through cold storage. A major goal of storage systems is to maintain effectiveness by preserving the lifespan and function of fresh RBCs to the greatest extent possible. Keeping blood cheap requires controlling per-unit costs and not increasing health risks that will make new demands on other parts of the system. At the present time, the unit costs of RBCs remain low, and controlling those costs has been one factor in limiting progress on storage systems.

A short history of RBC storage systems

Rous and Turner developed the first red cell storage solution in 1916, a simple mixture of citrate and glucose. It was used initially to store rabbit RBCs for heterophil agglutination testing for syphilis, but when the cells appeared to be intact for four weeks later, they were reinfused back into the donor rabbits, raising the hematocrit without increasing the reticulocyte count or bile in the urine, which suggested that the reinfused cells were circulating. A year later, Rous’s postdoctoral fellow, Oswald H. Robertson, used this Rous–Turner solution to build the first successful blood bank in the Harvard Medical Unit attached to the British Expeditionary Force in France.

The major problem with the Rous–Turner solution was that it could not be heat sterilized, as the sugar caramelized, so there was a risk of bacterial contamination from the open mixing of the ingredients and adding the solution to the bottles. In 1943, Louit and Mollison solved this problem by lowering the pH of the solution to 5.0 to make acid–citrate–dextrose (ACD) solution.

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In exploring why RBCs seemed to do better in the lower volume of the anticoagulant solution, it became clear that the cells passively lose phosphate. This loss could be prevented by adding phosphate to...
the solution.\textsuperscript{47} Citrate–phosphate–dextrose (CPD) in a 1:7 volume ratio, 63 ml for 450 ml of whole blood, stored red cells slightly better than the older ACD.\textsuperscript{48} CPD became the standard anticoagulant in the United States, but ACD persisted in Europe, and both were used for 21-day storage with recovery of 70–80% of the RBC 24 hours after reinfusion of the stored cells back into their original donor.

At about the same time, Gabrio and her colleagues recognized that nucleotides were important for red cell metabolism, but almost a decade passed before researchers determined that adenine was the critical intermediate.\textsuperscript{49–51} In 1968, Shields formulated a mixture of CPD and adenine, CPDA-1, and showed that it markedly improved whole blood storage.\textsuperscript{52} However, during the subsequent 11 years that the FDA debated the safety of adenine, plastic bags revolutionized blood banking, and this excellent whole blood storage solution turned out to work less well with the packed RBCs left behind when plasma was removed.\textsuperscript{53} Beutler and West showed that the higher the hematocrit of RBC in concentrators, the lower the red cell ATP concentrations and the in vivo recovery.\textsuperscript{54} The obvious answer was to add back more volume and nutrients in the form of an “additive solution,” but the initial attempt to do this with a solution of bicarbonate, adenine, glucose, and phosphate (BAGP) did not work.\textsuperscript{55} CPDA-1 was finally licensed as a five-week storage solution in 1979, but only after Hogman had developed a simple additive solution of saline, adenine, and glucose (SAG) that also worked for five-week storage but without the adverse effects associated with the high pH.\textsuperscript{56} SAG went on to immediately work in Sweden.

The use of SAG was associated with 1% hemolysis by the end of five weeks of storage, so mannitol was subsequently added (SAG-M) as a “membrane stabilizer.”\textsuperscript{57} This reduced the hemolysis by more than 50%. Drawing whole blood into CPD, making component products, and storing the RBC concentrate with an additional 100 ml of SAG-M (CPD/SAG-M) are the standard RBC storage system in Europe. Minimal variants of this basic solution, additive solution-1 (AS-1) and additive solution-5 (AS-5), are widely used in the United States. Also used is a more substantial variant using citrate and phosphate in the place of mannitol (AS-3 or SAG-CP). All of the variants appear to be equivalent and provide about 82% recovery with about 0.4% hemolysis after six weeks of storage.\textsuperscript{58}

The most important limits of the first generation of additive solutions are the loss of membrane with resulting loss of deformability and viability that occur with prolonged storage. The progressive loss of 2,3-DPG may also be important in some situations, although the clinical impact is not well understood. More advanced additive solutions have been developed and licensed, but are not yet widely available.\textsuperscript{59} They work by providing phosphate and buffering and are capable of 7–8 weeks storage. They are considered to be attractive more for their ability to improve recovery, reduce membrane loss, and improve 2,3-DGP and/or ATP concentrations for all stored RBC than for their ability to extend storage further.

**Collection and separation procedures**

The volume of whole blood removed for storage, processing, and transfusion was historically 450 ml, a pint, in Western countries. For some newer collection systems, this amount has been increased to 500 ml, a half-liter, to increase the collection with each donation and to offset the losses associated with filtration leukoreduction. Products derived from both collection volumes, whether leukoreduced or not, are considered one unit. The interdonor differences in hematocrit and platelet count are sufficiently great that the yields of red cells, platelets, and plasma in components prepared from units of either whole blood collection volume show considerable overlap. Some have argued for a more standard definition of a unit, perhaps based on grams of hemoglobin, but it would make blood collection more difficult and wasteful.\textsuperscript{59}

As noted above, the volume of anticoagulant–nutrient solution is normally one-seventh the volume of the collected blood, 63 ml for a 450 ml whole blood collection and 70 ml for 500 ml. This volume ratio has been a standard for more than 50 years and was used with the anticoagulant–nutrient solutions ACD-A, CPD, and CPDA-1. There has long been a question of whether the first few drops of blood to enter the collection system are injured by their sudden immersion in the acid anticoagulant, but the red cells seem to tolerate this process with minimal hemolysis or loss of viability.\textsuperscript{60,61} At the end of collection, venous blood with a pH of about 7.35 has been mixed with anticoagulant–nutrient solution with a pH of 5.0–5.6, with a resulting pH of about 7.05 in the mixture. The high buffer capacity of the hemoglobin molecule limits the effects of the acidic primary collection solution.

Whole blood in anticoagulant–nutrient solution was licensed for storage for three or five weeks, but has now been largely replaced by separately stored blood components. Making components from whole blood not only serves more patients but also improves the storage of the individual blood elements. Red cells are best stored cold, platelets at room temperature, and plasma frozen. Removing the white blood cells (WBCs) also improves RBC storage by removing a cell population with high-energy requirements and potential for damaging red cells by released enzymes.\textsuperscript{62} Schemes for separating whole blood into components are based on centrifugation. The standard “platelet-rich plasma” method used in the United States involves performing a low-speed “soft” spin to sediment the red cells against the pole of the bag opposite the connections to the satellite bags and then squeezing off the supernatant platelet-rich plasma from the top of the bag into the first satellite bag. This, typically, 500 ml of whole blood with a hematocrit (Hct) of 42%, consisting of 210 ml of red cells and 290 ml of plasma, is collected into 70 ml of anticoagulant–nutrient solution increasing the volume in the bag to 570 ml and reducing the Hct to 36%. Removing most of the supernatant plasma will increase the Hct in the bag to 80–90%, consisting of the original 210 ml of red cells and the remaining 22–45 ml of plasma/anticoagulant–nutrient solution mixture. Of the original 290 ml of plasma and 70 ml of anticoagulant–nutrient solution, about 90% is removed in this initial separation process. In Europe, an initial hard spin is generally preferred in order to harvest buffy coat for the production of platelets and increase the volume of the separated plasma. As noted above, the red cells do better if most of the plasma volume and anticoagulant–nutrient solution are replaced.

Recently, separation of whole blood into plasma and RBC by using a hollow-fiber filtration system has been described.\textsuperscript{63} Red cell parameters were similar to those obtained when routine centrifugation methods were used, and the filter did not cause hemolysis. Levels of plasma factor VIII and factor XI were slightly reduced with this prototype; however, there was no evidence of activation of the coagulation or complement systems, and this prototype has not been widely adopted. Currently, routine centrifugation methods are used.
critical nutrients for RBC during prolonged storage: dextrose, phosphate, and adenine. The recognition that RBC concentrates occasionally ran out of glucose led to development of CP2D, with twice the amount of glucose in CPD, and a newer-licensed CPDA-2 with a third more glucose than CPDA-1.\textsuperscript{64,65} The molar contents of the four licensed additive–nutrient solutions are shown in Table 9.1. Note that all of the anticoagulant–nutrient solutions are acidic, reducing the pH of the stored blood below 7.2 and leading to a burst of ATP production at the expense of the rapid depletion of 2,3-DPG as large amounts of 3-phosphoglycerate enter the glycolytic pathway.

The amounts of the nutrients in the additive nutrient solutions are critical depending on the intended storage times. There is enough glucose in whole blood to keep the red cells healthy for four days. Whole blood, stored in citrate alone for up to four days, was considered the safest form of storage before autoclavable solutions containing sugar were developed. The high glucose content of CP2D was necessary because it was first used with an additive solution that did not contain sufficient glucose, adenine–saline. Phosphate exists at 1–1.5 mmoles/L in plasma, but it needs to be present at higher concentrations in the nutrient solution to prevent diffusive loss against the high concentrations in the red cells when 2,3-DPG breaks down. Adenine is only needed for storage beyond 3 weeks and is an ingredient of all of the additive solutions.

In emergencies in remote locations, it may be necessary to collect fresh whole blood before new supplies of fully tested components are available. Under these circumstances, drawing whole blood into the primary collection bag containing the anticoagulant–nutrient solution and holding the whole blood at room temperature for up to 24 hours is associated with good short-term preservation of function of the blood components.\textsuperscript{56} The national blood services of Finland and Israel collect all of their whole blood on one day and process it on the next, holding it at 20°C overnight in the anticoagulant–nutrient solution before separating it into components.\textsuperscript{67,68} The Council of Europe Committee of Experts on Quality Assurance in Blood Transfusion Services has since 2005 advised that whole blood rapidly cooled after collection to 20–24°C can keep at this temperature up to 24 hours after collection before separation into red blood cells, platelets, and plasma.\textsuperscript{69} A large international study has examined the metabolic trade-offs involved in the warm overnight hold of whole blood before processing.\textsuperscript{70} The warm hold did reduce the initial storage pH and lead to a burst of ATP synthesis, but the differences are lost in the larger individual donor differences by six weeks.

Additive solutions

First generation

The first widely used additive solution, SAG, represented an attempt to replace the volume and sugar lost with plasma removal and add the adenine necessary for storage beyond three weeks.\textsuperscript{71} It was made with normal saline with 4.5% weight/volume glucose and 2 mmol/L of adenine. Stored RBC had good viability for five weeks but 1% hemolysis. In screening a large number of compounds for additives that would reduce the hemolysis, mannitol was identified as a compound that both reduced hemolysis and had an excellent safety record with intravenous (IV) infusion. The addition of 30 mmol mannitol reduced hemolysis and increased the osmolarity of the solution further.\textsuperscript{72} The solutions are made acidic to a pH of about 5.6 with hydrochloric acid to allow the glucose to be heat sterilized, but the solutions have essentially no buffer capacity so they do not make the RBC concentrates much more acidic than they were when separated from plasma. SAG-M and its close relatives, AS-1 and AS-3, are the most widely used additive solutions.

Other widely used first-generation additive solution systems include CP2D/AS-3 in the United States and CPD/MAP (mannitol, adenine, and phosphate) in Japan.\textsuperscript{71} Table 9.2 provides a comparison of the ingredients and concentrations of the more common of these first-generation additive solutions.

Second generation

Second-generation additive solutions started with attempts to rebalance the final suspending solution and a search for additional nutrients for the packed RBC concentrates. BAGP was the result of the original attempt by Beutler to preserve both ATP and 2,3-DPG by raising the pH.\textsuperscript{52} However, raising the pH substantially above 7.2 led to high concentrations of 2,3-DPG at the expense of ATP and no improvement in storage function. The composition of a representative group of second-generation additive solutions is shown in Table 9.3.

The pH of RBC during storage is very important. Above pH 7.2, the bifunctional enzyme diphosphoglycerate mutase–phosphatase converts almost all 1,3-DPG into 2,3-DPG, depriving the cell of new ATP.\textsuperscript{27} Below a pH of about 6.4, the activities of the initial enzymes of glycolysis, hexosekinase and phosphofructokinase, are too low to support ATP production. In this narrow pH range between 7.2 and 6.4, hemoglobin, the mineral salts in the suspension, and bicarbonate all serve to buffer the protons produced by glycolysis. The approximately 60 g of hemoglobin present in a RBC can buffer about 8 mEq of protons in that pH range.\textsuperscript{72} However, conventional
first-generation acidic additive solutions, which result in an initial pH of 7.0, fail to take advantage of a quarter of that pH range and buffer capacity. Adding 20 mmol/L of phosphate delivers 2 mmol in the 100 ml of additive solution and buffers about 1 mmol of additional protons. Adding 20 mmol/L of bicarbonate again delivers 2 mmol to the final RBC suspension, which will be protonated to make carbonic acid, converted to CO₂ and water by red cell carbonic anhydrase, and buffer 2 mmol of protons as the CO₂ diffuses out of the plastic bag. Attention to formulation and pH balance in the design of second-generation RBC additive solutions can almost double the amount of ATP energy available to stored red cells by depressing diphosphoglycerate mutase activity while sustaining glycolysis.

Finding additional critical nutrients has been less successful. The only approved second-generation additive solutions are PAGGS-mannitol and AS-7. In PAGGS-mannitol, the initials stand for phosphate, adenine, glucose, guanosine, and saline. Guanosine was added because guanosine triphosphate was detected in red cells and known to decrease during storage. However, guanosine nucleotides play only a minimal role in critical events in RBC storage, inhibiting the primitive coagulation enzyme transglutaminase, and the solution worked only modestly better than first-generation additive solutions with a 74.6% 24-hour in vivo recovery after seven weeks in 10 units in the only published series.

As ingredients were added to advanced additive solutions, the salt concentration was generally reduced to maintain osmotic balance. Reducing the chloride concentration in the suspending solutions caused intracellular chloride to passively leave the red cells, but this can only occur if other anions countered the flow and phosphate, bicarbonate, and hydroxyl ions are the only available anions. Flu in the absence of these anions initially increases the intracellular pH, allowing for chloride shift phenomenon to maintain red cell 2,3-DPG.

The ErythroSol solutions were developed based on the ideas of Beutler, PAGGS-M, which is phosphate-adenine-glucose-guanosine-saline-mannitol, is licensed in Germany; PAGGG-M is phosphate-adenine-glucose-guanosine-sodium gluconate-mannitol, and it was developed by de Korte as a chloride-free variant of PAGGS-M; ErythroSol-1 and -2 were developed by Hogman; and AS-7 was developed by Hess and Greenwalt. All concentrations are in mmoles/L.

The experimental additive solution-81 (EAS-81) has now been licensed in the United States and Europe as AS-7. It was the end result of work that reexamined the use of bicarbonate as a buffer to prevent erythro-apoptosis by maintaining high concentrations of red cell ATP and limited hemolysis with mannitol and hypotonic conditions. Formulating the solutions to get the starting pH as close as possible to 7.2 preserves the 2,3-DPG concentrations for storage for at least eight weeks and excellent storage with recoveries of 84% at six weeks but failure at 7 weeks. Bicarbonate buffered AS-7 allows better storage, storage for 8 weeks, and storage after warm overnight hold. See Refs. 53, 64, 80, 81.

| Table 9.3 Composition and Properties of Some of the Second-Generation RBC Additive Solutions |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|
| BAGPM                          | PAGGS-M        | PAGGG-M        | ErythroSol-1   | ErythroSol-2   | AS-7           |
| NaCl                           | 72             | 72             | 72             | 72             | 72             |
| NaGluconate                    |                |                |                |                |                |
| Bicarbonate                    | 115            | 115            | 115            | 115            | 115            |
| Phosphate                      | 1              | 32             | 32             | 32             | 32             |
| Adenine                        | 1              | 2              | 2              | 2              | 2              |
| Glucose                        | 55             | 52             | 52             | 52             | 52             |
| Mannitol                       | 27             | 55             | 55             | 55             | 55             |
| Guanosine                      |                | 1.5            |                | 1.5            |                |
| Na₃Citrate                     |                |                | 25             | 21             |                |
| pH of solution                 | 6.3            | 6.3            | 7.4            | 7.4            | 8.8            |
| pH of RBC suspension           | 6.9            | 6.9            | 7.2            | 7.2            | 7.2            |
| Volume                         | 100            | 110            | 110            | 110            | 110            |

BAGPM is bicarbonate-adenine-glucose-phosphate-mannitol, the original additive solution developed by Beutler; PAGGS-M, which is phosphate-adenine-glucose-guanosine-saline-mannitol, is licensed in Germany; PAGGG-M is phosphate-adenine-glucose-guanosine-sodium gluconate-mannitol, and it was developed by de Korte as a chloride-free variant of PAGGS-M; ErythroSol-1 and -2 were developed by Hogman; and AS-7 was developed by Hess and Greenwalt. All concentrations are in mmoles/L.
Finally, increasing the volume of these advanced additive solutions increases the buffering capacity and allows greater storage time. With 300 ml of additive solutions, Hess and his colleagues were able to achieve storage times of 10–12 weeks, but the higher volume and lower storage hematocrit probably make such solutions inappropriate for clinical use, especially in infants and massively transfused trauma patients.  

Additional factors influencing RBC quality

Temperature and time lapses during collection and component preparation

Whole blood is collected at body temperature and must be maintained at room temperature if platelets are to be prepared from the collection. By present US regulations, whole blood must be separated into its components in eight hours if platelets and fresh frozen plasma are to be manufactured. If only RBC and frozen plasma are to be made, the whole blood can be held on ice for up to 24 hours. The problems with holding blood at room temperature are twofold. Red cells metabolize glucose at higher rates when warm, producing more lactate and protons that reduce the pH and further slow metabolism. In addition, labile coagulation factors are lost. Attempts to validate the 24-hour warm hold for six-week RBC storage using first-generation additive solutions have not been successful, but in Israel and Finland, where this system is used, RBC are stored for only five weeks because of the efficiency of their national blood services and short supply lines. AS-7 is now licensed in Europe for eight weeks of storage after an overnight hold. As noted above, fresh whole blood is occasionally collected in emergencies on the battlefield or in isolated locations such as Pacific Island nations and stored at room temperature for short periods. Based on the above experience and experimental data, blood probably maintains reasonable functionality for 24–72 hours if maintained between 19 and 25 °C. However, such use can only be recommended in the most urgent of situations.

Temperature and time lapses during storage

RBCs that have been allowed to rewarm to greater than 10 °C during storage are considered unfit for transfusion and are destroyed by FDA regulation. This has led to the wide use of the “30-minute rule,” meaning that units are discarded if they have been off ice for more than 30 minutes. Experimental work has shown that glucose is metabolized about 10 times as fast at 25 °C as at 4 °C and that RBCs stored at 25 °C lose viability 10 times as fast so that a day of storage at room temperature would reduce the in vivo recovery equivalent to 10 days of 4 °C storage. Thus, the regulation is very conservative with regard to temperature effects on metabolism, but it is also intended to prevent bacterial overgrowth in contaminated units.

Storage containers

Polyvinyl chloride (PVC) bags plasticized with DEHP are the standard RBC storage containers. The presence of DEHP reduces hemolysis by fourfold during storage by intercalating into the red cell membrane. Other plasticizers, such as butyryl-n-triethyl citrate, work almost as well, but they are more expensive (~5% more for a multibag set with leukocyte filter) and have an unusual smell when initially unwrapped.

Although questions about the safety of DEHP have been raised, they are based on very limited animal testing and must be balanced against the obvious safety value of being able to visually inspect the contents of blood bags and reduce RBC losses by extending shelf life. The general trend in Europe to avoid the slowly degradeable plasticizer, DEHP, has resulted in the introduction of butyryl-n-triethyl citrate plasticized multibag systems in Sweden, Spain, and Norway, but the use of PVC bags with DEHP remains prevalent in Europe and almost universal in the rest of the world.

Leukocyte reduction

Leukocyte reduction improves RBC storage by removing a highly metabolically active blood component that would make the bag more acidic sooner. WBCs also secrete cytokines, and when they die after exposure to the cold, they release proteolytic, glycolytic, and lipolytic enzymes that damage the red cell surface. On the other hand, some red cells are damaged and many red cells are lost in the leukocyte reduction filters (typically, 15–35 ml of blood is lost in the filters depending on its size and whether whole blood or concentrated red cells are being leukoreduced). Red cells from donors with sickle cell trait may also clot filters. Leukocyte reduction improves the red cell 24-hour in vivo recovery by several percent according to the best estimates. (See also Chapter 24.)

Washing

Washing RBC in saline to remove the plasma causes small losses of red cells in the bag transfers as well as loss of the supporting nutrients, glucose, phosphate, and adenine. Because the cells are used soon after washing, generally within 6–24 hours, only the glucose loss is physiologically important. There is still a small amount of glucose inside the washed RBCs, but it is metabolized quickly, especially if they are not promptly refrigerated. In the absence of glucose, metabolism stops and the cells are very susceptible to oxidative stress and erythro-apoptotic changes.

Irradiation

During storage at 4 °C, red cells lose potassium. This potassium collects in the supernatant fluid in the closed storage bag at a rate of about 1 mEq/day until equilibrium is reached between intracellular and extracellular concentrations, usually at about 60–70 mEq/L depending on the storage hematocrit (Table 9.4). Gamma irradiation in doses of 2500 cGy, given to prevent graft-versus-host disease, damages the red cell membranes and increases this rate of potassium loss to approximately 1.5 mEq/day; however, the reinfused red cells have normal in vivo survival. The current FDA regulation that irradiated RBC expire 28 days after irradiation limits the potential maximum potassium concentration by limiting the period of potassium loss. Nevertheless, care must be taken in all situations when large volumes of older, high-potassium RBC are used to prime cardiopulmonary bypass, dialysis, or apheresis circuits or infused into the central circulation and then administered at high flow rates.

Table 9.4 Effect of Storage Duration of Characteristics of RBC Concentrates in AS-1

<table>
<thead>
<tr>
<th>Duration</th>
<th>35-day</th>
<th>42-day</th>
<th>49-day</th>
<th>56-day</th>
</tr>
</thead>
<tbody>
<tr>
<td>(%) recovery</td>
<td>86</td>
<td>82</td>
<td>76</td>
<td>71</td>
</tr>
<tr>
<td>% hemolysis</td>
<td>0.28</td>
<td>0.32</td>
<td>0.51</td>
<td>0.68</td>
</tr>
<tr>
<td>ATP μM/g Hb</td>
<td>3.1</td>
<td>2.7</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>(K⁺) meq/L in plasma</td>
<td>45</td>
<td>50</td>
<td>52</td>
<td>60</td>
</tr>
</tbody>
</table>

Unpublished data obtained from the US FDA under the Freedom of Information Act.
Pathogen reduction

Pathogen reduction systems developed so far require additional manipulation of and stresses on the stored RBCs that are system specific. Examples from two such proposed systems are as follows. Diethylenamine (DEA) was proposed as a red cell–permeable nucleic acid crosslinker with broad pathogen killing potential and minimal direct red cell toxicity. However, the system required exposure of the RBCs to DEA for 20 hours at room temperature and extensive secondary washing to reduce the remaining amounts of this carcinogenic chemical. When the time- and temperature-related decrease in RBC pH was added to the effects of washing the RBC with large volumes of acidic solutions and storing them in an acidic additive solution, red cell recovery suffered. Riboflavin, a photoactive oxidizer, has also been proposed as a highly red cell–permeable molecule for pathogen reduction in conjunction with UV light. However, RBCs are so optically dense that the units must be diluted and transferred to large bags only a few millimeters thick for photo-treatment, and the photo-treatment must be done above a critical temperature. The bag transfers all involve losses of red cells, for photo-treatment, and the photo-treatment must be done above a critical temperature. The bag transfers all involve losses of red cells, and high concentrations and increases their in vivo recovery, probably by releasing ATP. This is the fundamental idea behind second-generation additive solutions.

Rejuvenation

Red cell loss of viability during storage is different than red cell senescence in the body. In the body, red cells undergo cumulative oxidative damage that leads to reduced enzyme activities and cross-linking of cytoskeletal components. These are essentially irreversible processes and are ultimately associated with macrophage clearance of old red cells possibly mediated by phosphatidyl serine exposure or neoantigen formation. The changes that lead to loss of viability during storage are largely reversible by a process called rejuvenation. Hogman showed that rejuvenating red cells at the end of six weeks of storage in SAG-M increased their 24-hour in vivo recovery from 77 to 89%. This rejuvenation is a strictly metabolic recharging of red cells at the end of their storage period. Such cells have a low pH as well as low ATP and 2,3-DPG concentrations. They can be rejuvenated by incubation in a high-pH solution of phosphate, inosine, pyruvate, and adenine (PIPA, Rejusol90, Cytosol Labs, Braintree, MA) for 2 hours. Such incubation increases their ATP and 2,3-DPG concentrations and increases their in vivo recovery, probably by allowing them to internalize negatively charged membrane phospholipids that would otherwise signal clearance by macrophages. Return of the normal distribution of phospholipids also prevents red cells from participating in plasma coagulation reactions. Rejuvenation does not reverse the oxidative damage to band 3 of the cell membrane, desialation of glycoproteins, or loss of membrane. However, the fact that RBCs can be rejuvenated at the end of storage by increasing their pH and ATP suggests that improved storage could be achieved by a method designed to maintain pH and ATP. This is the fundamental idea behind second-generation additive solutions.

Frozen storage of RBCs

RBCs can be frozen, and in the frozen state they are stable for long periods. Valeri has reported on RBCs stored for 37 years. Four methods of freezing RBCs have been extensively tested, two have been developed for practical use, and one remains in common use.91

During red cell freezing, water turns to ice and the salt concentration of the remaining intracellular water increases, drawing in more water and expanding the cell. Under normal circumstances, this leads to cell rupture. Rupture can be prevented by freezing the cells so rapidly that water does not have time to enter, or by diluting the total cellular water with a cryoprotectant so that not enough water enters to rupture the cell. Although several cryoprotectants can work, glycerol is the standard material used because of its cost and safety. The two systems for RBC cryopreservation that have been developed for clinical use both use glycerol. One system used a “low” concentration of about 20% and rapid cooling by plunge freezing in liquid nitrogen; the other uses a “high” concentration of about 40% and slow cooling in −80 °C freezers. The low-glycerol frozen RBCs must be maintained in the vapor phase of liquid nitrogen, whereas the high-glycerol frozen RBCs are stable at temperatures below −65 °C. The high cost of maintaining liquid nitrogen freezers and the difficulty of transporting products frozen in liquid nitrogen both limit the utility of the low-glycerol system. High-glycerol frozen RBCs can be transported on dry ice. When the RBCs are thawed, the glycerol must be removed promptly to prevent it from poisoning the red cell metabolism and to protect the recipient as the glycerol-loaded red cells would swell and rupture if placed directly into the bloodstream. Thus, once thawed, the RBCs must be deglycerolized by washing in a set of graded salt solutions. In the past, the glycerolization and deglycerolization of RBC for freezing were open manual processes, so the thawed RBCs had to be used within 24 hours or discarded.

The recent partial automation of a closed system for glycerolizing and deglycerolizing RBCs now allows them to be kept post thaw in the liquid state for two weeks.92 RBCs collected in any of the standard licensed systems can be frozen up to six days after collection and stored for up to 10 years. The deglycerolized RBCs are then stored in AS-3. Net losses of red cells in the freeze–thaw–wash process are of the order of 5–15%, and the 24-hour in vivo recovery of infused red cells is about 78% after two weeks.93 Frozen RBCs entail substantial costs for processing and storage, probably four times that of a standard liquid unit, so only for the rarest blood units is there an advantage for frozen RBCs.

Oxygen delivery

RBCs have many functions, but the most important are bulk oxygen transport and microvascular perfusion for the delivery of oxygen and removal of CO₂. There is evidence that RBC metabolism and storage lesions affect both of these aspects of oxygen delivery. Bulk oxygen transport involves the loading of oxygen onto RBC hemoglobin in the lungs and its distribution to the arterioles of critical organs, including the heart, brain, liver, kidneys, gut, and skeletal muscle. Bulk oxygen transport also depends on RBCs being dense, flexible discs that flow in the middle of the bloodstream surrounded by poorly oxygen-soluble plasma that limits premature oxygen off-loading. Loss of RBC density through swelling, their flexibility
through membrane loss, and their ability to slide smoothly back and forth over each other because of shape change have been discussed previously. Each of these storage lesions reduces the effectiveness of the laminar flow mechanism and increases the diffusive loss of arterial oxygen to nearby veins, creating shunt. The neural and endocrine mechanisms that maintain blood volume, pressure, and pulsatile flow are numerous and generally beyond the scope of this chapter. However, the heart is the critical organ first affected by limitation of bulk oxygen delivery. Oxygen extraction in the left ventricle is typically 70% at rest, twice that of the brain, making coronary artery vasodilatation, increased heart muscle perfusion, and increased oxygen extraction the primary mechanisms by which the heart adapts to an increasing workload. The first two of these response mechanisms are primarily driven by adrenergic activity, and the RBCs have adrenergic receptors whose stimulation appears to reduce RBC aggregation and increase deformability.93 These adrenergic pathways require ATP and GTP. Effects of RBC storage on RBC bulk viscosity have been described, and the restoration of stored RBC ATP content through “rejuvenation” largely restores bulk viscosity to prestorage levels.94 All this suggests that maintaining RBC energetics during storage to maintain ATP and GTP concentrations to limit membrane loss and change will have the greatest effects on bulk oxygen transport.

Since the discovery of the effect of 2,3-DPG on the oxygen affinity of hemoglobin, it has been common to discuss the oxygen delivery functionality of stored RBCs in terms of their 2,3-DPG content. This is an oversimplification for several reasons. Firstly, although it is clear that low 2,3-DPG can reduce oxygen delivery in animal models by as much as 12% at the critical point where low hemoglobin concentrations make oxygen delivery flow dependent, these concentrations are well below standard transfusion triggers.95 Secondly, 2,3-DPG works in red cells by binding and stabilizing deoxyhemoglobin. This moves the base of the oxy-hemoglobin dissociation curve to the right and increases the P50, but most oxygen transport occurs at the top of the binding curve and is relatively unaffected by DPG concentrations, as the arterial PO2 is much higher than the P50. However, 2,3-DPG plays an important role in red cell membrane oxygen transport and cytoskeleton architecture because of its interaction with attachment points between the cell membrane and the cytoskeleton, and the interaction of deoxyhemoglobin with band 3. These interactions, the critical importance of oxygen transport, and the retrospective data that suggest an association between adverse patient outcomes and increased storage time of banked RBCs drive continuing efforts to reduce 2,3-DPG depletion during storage with modern additive solutions. Until that is achieved, it has been recommended in Norway to use RBCs in SAGM, not older than 10 days, for the transfusion of intensive care ward patients.

However, the retrospective data suggesting that stored RBCs are dangerous are highly suspect. Such data are generally confounded by the common situations that sicker patients receive more transfused blood, sicker patients die at increased frequencies, and patients who receive more blood have a higher chance of receiving older blood.96 Specifically, the study most frequently quoted to suggest that longer stored RBC units affect the outcome in cardiac surgery97 is deeply flawed, with the excess numbers of patients receiving seven or more units in the arm receiving >14-day stored RBCs accounting for more than 60% of the excess mortality. There are now three large randomized trials, ARLIPI, RECESS, and ABLE,98 showing no effect of prolonged RBC storage on mortality in neonates, repeat cardiac surgery patients, or critically injured patients, respectively. This is in spite of the fact that 15–25% of the old red cells are removed from the circulation by the reticuloenothelial system within 24 hours after transfusion. Cells age in storage, but the storage changes seem compensated in vivo by still poorly defined mechanisms.

At the microvascular level, oxygen delivery is maximized by increasing capillary density and perfusion and limiting shunting. These processes are controlled in large part by endothelial cells, and more than a dozen mechanisms are involved with intermediates as diverse as the respiratory gases O2 and CO2, nitric oxide, proteins like the endothelins, and lipid endoperoxides such as leukotrienes. Endothelial cells have the mitochondrial-derived energy, synthetic capability, and connections to provide the feedback between tissue oxygen tensions and end-arteriole sphincters that control capillary perfusion. Here, the connections between RBC deformability and secretory capacity appear most strongly connected to oxygen delivery. Probably the most important for the microvascular flow of transfused RBCs is the loss of membrane, cell deformability, and ATP secretion that occurs with storage. Direct observation of the flow characteristics of fresh, stored, and stored rejuvenated RBCs suggests that ATP secretion in response to increased shear, by which red cells dilate small vessels to maintain their forward flow, is most important.99 Membrane deformability, which is regulated by the ATP-modulated cytoskeleton, is also important for the passage of red cells through capillaries that are half their diameter. Maintaining high ATP concentrations is therefore again a critical function of modern additive solutions. Stress shear also regulates endothelial NO production, and increased shear stress by old rigid red cells could therefore have a positive influence on microvascular flow of old blood.

Validation of RBC quality and in vivo recovery

RBC storage systems have historically been validated by demonstrating that the stored cells do not hemolyze during storage and that they circulate normally after reinfusion. Measures of normal circulation have included increments in recipient hemoglobin, and demonstrating that the stored cells do not hemolyze during storage and that they circulate normally after reinfusion. Measures of normal circulation have included increments in recipient hemoglobin, and determining the recovery and in vivo half life of the transfused red cells. Transfused red cells have been counted using differential agglutination, radioactive tracer labeling, and flow-cytometric differential counting.100

Chromium-51 labeling has been the standard method used in the United States for 50 years. There is a published standard method, but only a handful of laboratories perform the measurement, and a recent attempt to gather a decade’s experience identified only 900 measurements.96 Chromium-51 labeling persists in the United States because it is the only validated method compatible with autologous red cell reinfusion, and the infectious disease risks of allogeneic RBCs are considered unacceptable for storage system development work. The methods of red cell labeling have been the subject of an excellent review.101

When performing chromium-51 labeling, it is important to recognize that red cells from different donors may have very different 24-hour in vivo recovery values. In a typical study of a modern additive solution with an 84% mean recovery, individual units of donor RBCs have recoveries as high as 95% and as low as 65%.62 These differences in viability correlate with differences in the red cell ATP concentrations, but poorly (r2 = .4).
There have been attempts to measure the functionality of red cells, as opposed to their survival. Presumably, the functionality of red cells is related to their ability to deliver oxygen to tissues and to flow in the microcirculation. However, none of the red cells’ rigidity or microcirculatory flow measures is widely available or validated. The level of 2,3-DPG is just a surrogate marker for red cell function and is limited by the difficulty of performing the test. In a recent shared sample exercise, 12 of the world’s premier labs could not consistently measure 2,3-DPG.102

Finally, in countries that do not perform chromium-51 recovery measures, there has been a trend to using the ATP concentration as a red cell quality measure. Although the ATP is a poor surrogate for recovery in small clinical trials, its central role in the inhibition of erythro-apoptosis probably gives it special importance. Unfortunately, the measure is not very reproducible from laboratory to laboratory, so a wide margin of safety is required.102

Summary
RBC storage systems work remarkably well, making red cells for transfusion available, safe, effective, and cheap for the populations who can organize effective national blood systems. They are a product of slow and empiric development, and our present systems do not reflect our current understanding. They can be expected to improve in the future.

Key references
A full reference list for this chapter is available at: http://www.wiley.com/go/simon/transfusion