2 Biology of Cell Survival in the Cold: The Basis for Biopreservation of Tissues and Organs

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CONTENTS

2.1 INTRODUCTION

Transplantation science calls for effective methods of preservation since it is unavoidable that donor cells, tissues, and organs are required to withstand a period of ischemia and hypoxia as part of any transplantation protocol. Historically, interest in isolated organ preservation was recorded at the beginning of the present century when Carrel was the first to explore techniques for preserving tissues for the purposes of transplantation.[1–3] Carrel was awarded the Nobel Prize in 1912[4] and in subsequent studies in the 1930s investigating normothermic organ perfusion with serum or synthetic perfusates, he laid down the basic requirements for artificial perfusion technology relating to organ preservation and cardiopulmonary bypass.[5] It was recognized at that time that the viability of perfused tissues depends on a variety of factors that remain pertinent today. These included the need for the perfusion fluid (blood or synthetic solutions) to be free of emboli (gaseous or particulate), including agglutinated corpuscles if blood was used. Physical and chemical characteristics of the perfusion medium were also recognized to be of crucial importance. These include temperature, osmotic pressure, pH, oxygenation, and chemical composition.[5]

During the next two decades perfusion techniques were improved and invariably combined with hypothermia for effective preservation.[4] While the protective effects of low temperatures have been known and explored by mankind since the dawn of civilization and began to be documented as early as the seventeenth century,[6] the scientific basis for cell death following ischemia and its amelioration by hypothermia has only begun to be understood during the past fifty years. The modern era of low temperature cell preservation, which began in the 1950s, involves both hypothermic preservation at temperatures above 0°C and also cryopreservation at subzero temperatures utilizing freezing or vitrification techniques. The fundamental basis of low-temperature preservation is to use cold as a physical means of depressing function in a reversible way, i.e., to achieve a state of "suspended animation." The principles and mechanistic basis of cryopreservation that currently permit a wide variety of single cells and some simple tissues to be stored indefinitely at deep subzero temperatures will be discussed in other chapters. At present, most complex multicellular tissues and organs cannot be cryopreserved without incurring intolerable levels of cryoinjury (see References 7–9) and effective methods of preservation rely upon hypothermic storage at temperatures above the freezing point. The purpose of this chapter is to summarize the basic principles upon which mammalian cells can be safely held in suspended animation in the cold.

The cells of poikilothermic animals have adapted through evolutionary processes to survival in the cold,[10] but for the cells of euthermic animals, including man, cold is a stress that may be tolerated depending upon the degree and duration.[11] Some mammals have adapted to these cold stresses through the process of hibernation, and emerging clues for the mechanisms involved may, in the future, provide benefits for interventional strategies of hypothermic preservation of tissues harvested from nonhibernators.[12–14] What then is the scientific basis for the longstanding use of hypothermia as the cornerstone of virtually all effective methods of multicellular tissue and organ preservation? In this chapter, I will outline the basis for cell survival in the cold by considering the cell in relation to its environment, the known effects of ischemia and anoxia, the influence of hypothermia on ischemic events, and, finally, the strategies of interventional control of the extracellular environment to optimize preservation.

2.2 THE CELL IN RELATION TO ITS ENVIRONMENT

2.2.1 Differences between the Extracellular Environment and the Intracellular Milieu, and Their Control

A cell is defined morphologically by its limiting envelope, the plasma membrane, which separates it from its neighbors in a tissue or from its fluid environment. Within the body, the extracellular fluid bathing cells is maintained within closely regulated limits by a variety of control mechanisms and cells maintain a different, but even more constant intracellular milieu. Figure 2.1 shows schematically the contrasting balance of ions between the intracellular and extracellular spaces of a typical mammalian cell, with the extracellular complement of ions representing the typical composition of a balanced salt solution such as Ringer's or Krebs' etc. buffer solutions for *in vitro* cell maintenance.

Cells are able to maintain a stable internal environment very different from that which surrounds them by means of their membrane pumps, and principally by the sodium pump mediated by Na^+-K^+ ATPase. As illustrated in Figure 2.1, this is an energy-consuming process that extrudes sodium and accumulates potassium in the ratio of 3:2 (i.e., the pump is electrogenic and contributes to the cell membrane electrical potential). Each ion tends to diffuse back down its concentration gradient, but because potassium diffuses out more rapidly than sodium can diffuse in, the net loss of cations from the cell charges the cell membrane (negative internally). The voltage gradient also influences the distribution of anions, and Cl^-, which diffuses freely in response to the membrane potential, is excluded. Therefore, the sodium pump in effect extrudes NaCl and, because the membrane is highly permeable to water, an osmotic quota of water (180 molecules/solute molecule) leaves the cell with the Na^+ and Cl^-. Thus, the sodium pump controls cell volume as well as ion distribution and the maintenance of the intracellular environment is dependent upon metabolic generation of high-energy phosphates.

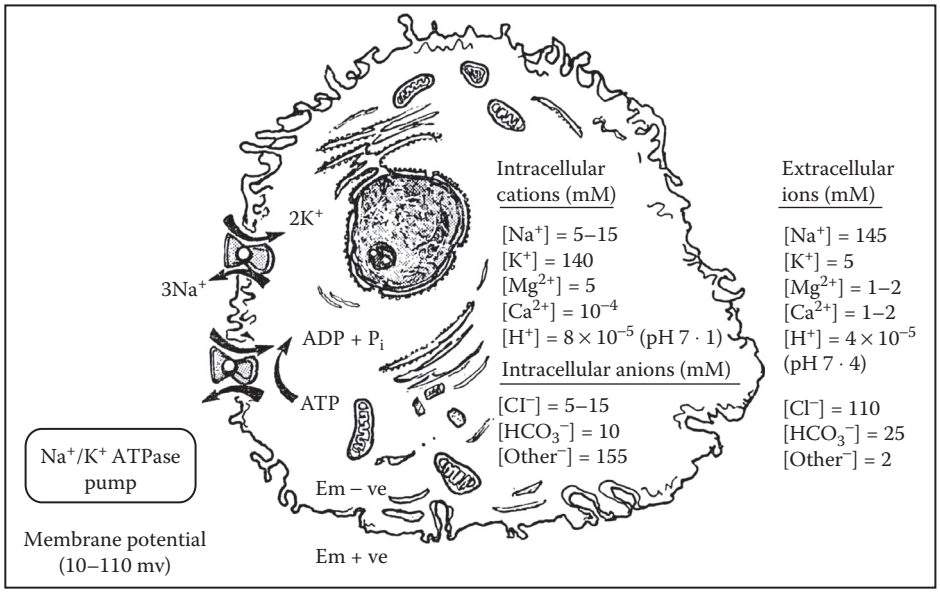

FIGURE 2.1 Schematic diagram of a typical mammalian cell showing the contrasting balance of ions between the intracellular and extracellular compartments. The cell expends energy in the form of ATP to fuel the membrane pumps that maintain the ionic gradients as an integral component of cell homeostasis. (See text for details).

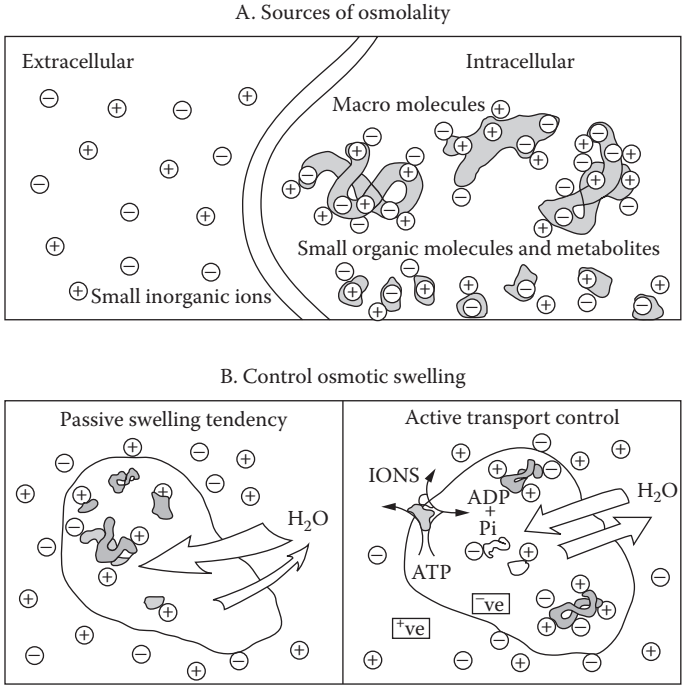

FIGURE 2.2 The cell in relation to its environment: schematic representation of the sources of osmolality inside and outside of cells and the role of active transport processes in controlling cellular hydration and cell volume. (See text for details).

Apart from small inorganic ions, the cell contains a variety of small organic molecules and metabolites as well as macromolecules that, unlike the inorganic ions, are not free to traverse the cell membrane, but are confined within the intracellular compartment. This has important implications for the control of cellular hydration and volume, as depicted in Figure 2.2. Osmolality is a colligative property and as such, depends upon the actual number of particles present in solution rather than on the size or nature of the molecules.[15] Hence, intracellular macromolecules themselves contribute very little to the osmolality of the cell interior since they are present in relatively small numbers compared with the number of small molecules and ions. However, macromolecules are invariably highly charged, attracting a large number of small counter-ions that contribute significantly to intracellular osmolality. In addition, a cell contains a high concentration of small organic molecules as products of metabolic processes and these impermeable metabolites are also associated with a complement of counter-ions (Figure 2.2). In contrast, the osmolality of the extracellular fluid is due principally to small inorganic ions that leak slowly across the plasma membrane. The distribution of these ions is governed both by the presence of the impermeant charged molecules inside the cell and by the active ion pumps associated with the plasma membrane.

These factors give rise to a greater concentration of inorganic ions inside the cell than outside at equilibrium, a phenomenon known as the Donnan effect.[16] Details of these essential cellular processes can be found in any standard physiology textbook[17] and have been discussed in relation to reduced hypothermic preservation by Pegg.[18,19]

2.2.1.1 Osmotic Cell Swelling

In essence, if the active ion transport processes are inhibited as a consequence of either energy deprivation and/or temperature reduction, then the impermeant solutes and colloidal material inside the cell give rise to water uptake. This in turn reduces the concentration of salt below that of the

extracellular fluid and more salt penetrates making the cell hyperosmolar, thus inducing more and more water uptake until the cell eventually bursts, an event known as membrane rupture. This colloid osmotic swelling is a very significant factor in the cell's relationship to its environment since the semipermeability of cell membranes vis-à-vis ions is only effectively maintained by metabolic processes that pump ions in or out of the cell. Where these processes fail the effective semipermeability is lost and the result is colloid osmotic swelling and lysis. Obviously, this is a very important phenomenon that has to be taken into consideration when designing methods of hypothermic cell preservation as discussed below.

Similar considerations apply to the composition of interstitial fluid surrounding cells and the plasma within capillaries. Because of the low permeability of capillaries to proteins, the interstitial fluid normally has a low concentration of these plasma constituents, which are predominantly serum albumin (67%; MW = 61,000) and serum globulins (30%) with much higher molecular mass (~100,000 Daltons). The interstitial fluid can be considered as an ultrafiltrate of plasma with the Gibbs-Donnan equilibrium applying to the distribution of ions.[17] The circulation is therefore responsible for maintaining a remarkably constant composition of the extracellular fluid with the osmolality being controlled at about 300 m osmol/kg to equate with that of the cytosol within cells as described above: cells will tolerate a perturbation of osmolality of only 10% without harm.

2.2.2 Essential Maintenance Processes

Under normothermic physiological conditions, control of a cell's peculiar internal environment with respect to its surroundings is a function of both the properties of the plasma membrane and of the cell's energy processes within the cell. The membranes are bilayer lipid-rich structures with associated embedded proteins that invariably function as transmembrane transporters of ions and small molecules, e.g., via specific channels. It is mentioned above that the plasma membrane provides a selective barrier to the diffusion of ions and other solutes, the distribution of which is regulated by active metabolic pumps that require a constant supply of energy. Adenosine triphosphate is the principal molecular store of chemical energy in the cell, and the biochemical pathways involved in the catabolism of nutrient substrates into a form of energy useful for driving biosynthetic reactions and other energy-requiring processes — such as control of the intracellular milieu — are well known and described in any standard biochemistry text.

Figure 2.3 shows a simplified outline of the three principal stages of catabolism that lead from basic nutrients through progressive oxidations to waste products with the yield of large quantities of ATP. The three major pathways involved in the oxidation of glucose are glycolysis, the citric acid cycle (Krebs cycle) and the electron transport chain. The glycolytic pathway is the only process by which cells can obtain energy in the absence of oxygen (anaerobically) and although of limited capacity this pathway is important for helping to sustain homeostasis in ischemic/anoxic cells.

2.2.3 Essential Role of the Circulation

In summarizing the fundamental processes of normal cellular function and homeostasis as a prelude to discussing the nature of ischemic injury and its prevention, it is important to emphasize the essential role of the circulation. The foregoing discussion has outlined in elementary terms the well-known basics of how a cell is sustained for normal function and the composition of the regulation of the interstitial fluid is an important function of the circulation. Other essential functions include the provision of an internal heat transfer system that closely regulates the temperature of tissues and organs. The circulation transports metabolites of which the fuels for respiration (glucose, fatty acids, and ketone bodies) are most important in the short term. For most tissues, respiration is predominantly aerobic with high demands for oxygen (e.g., 300 mL O_2 min/g dry weight of kidney renal cortex). The solubility of oxygen in tissue fluid is only about 24 mL/kg such that inhibition of aerobic energy production is very rapid following the onset of ischemia. Other

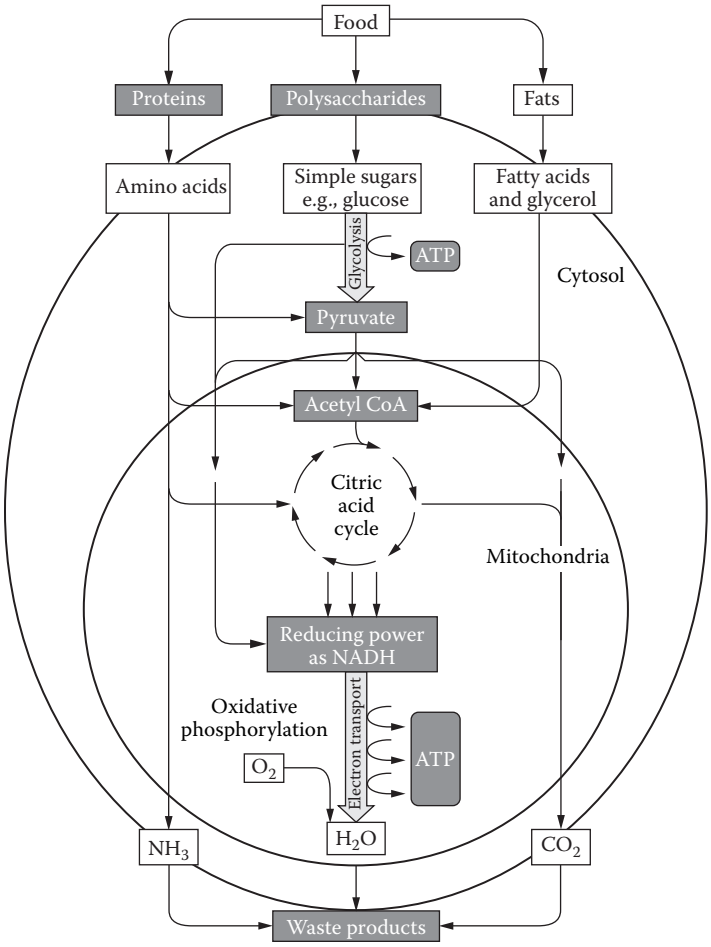

FIGURE 2.3 A simplified diagram showing the principal stages of catabolism that produces ATP used to drive biosynthetic reactions and other energy-requiring processes in the cell such as control of the intracellular milieu. Under hypoxic or hypothermic conditions, glycolysis is the only process by which cells can generate energy in an attempt to sustain homeostasis during ischemia (adapted from [235]).

necessary metabolites, besides those required for energy production, including amino acids, vitamins, glutathione, coenzymes, and many other factors, are also supplied to cells via the circulation. The catabolic products of cellular metabolism are removed by the vascular system. In light of the crucial role of the circulation in sustaining the normal environment and metabolic needs of cells, it is possible to appreciate the devastating consequences of a disruption to the vascular supply of a tissue or organ. A brief consideration will now be given to the effects of ischemia as a preface to a discussion of the positive and negative aspects of hypothermia as a protective modality against ischemic injury.

2.3 A SYNOPSIS OF ISCHEMIC AND HYPOXIC INJURY

Interest in the pathophysiology of ischemia is not limited to just the transplantation community, since an understanding of the detrimental effects of a reduced blood supply to tissues is also of crucial importance in the treatment of cardiovascular diseases and neuropathological complications of acute cerebral vascular occlusions, clinically considered stroke. Consequently, there now exists a vast literature on the subject of ischemia and reperfusion injury and, although not yet complete,

a considerable understanding of the nature of ischemic injury has emerged. This can only be outlined here in basic terms, but more complete accounts of the phenomena and mechanisms of ischemic injury in relation to the isolation of organs for transplantation have been published in the past, and a selection is cited here for reference.[18–20] More recent accounts that focus on cellular and molecular mechanisms are to be found in the literature relating to ischemia/reperfusion injury in the heart and tissues of the central nervous system that are most sensitive to an ischemic insult.[21–26]

2.3.1 The Ischemic Cascade

Excision of a tissue for transplantation means that ischemia is total and inevitable even though the period may be brief. An immediate consequence of cessation of blood supply to an organ is deprivation of the supply of oxygen to the tissues, but anoxia (total deprivation) or hypoxia (partial deprivation) is only one of the many consequences of a lack of blood supply. A scheme for the multifactorial cascade of events that ensue following the initiation of ischemia is depicted in Figure 2.4. The pivotal event is ATP depletion, which occurs within the first few minutes of oxygen deprivation. This early event leads immediately to a shift from aerobic to anaerobic metabolism (Pasteur effect), which very quickly becomes self-limiting with the production of lactate and protons. Cell depolarization also occurs very early in the cascade leading to a breakdown of ion homeostasis and a concatenation of other intracellular and membrane-associated events that eventually culminate in necrosis and cell death. A rise in the intracellular concentration of protons and calcium is at the center of many of the mechanisms now recognized to be contributory to cell death as a result of ischemia.[23,27]

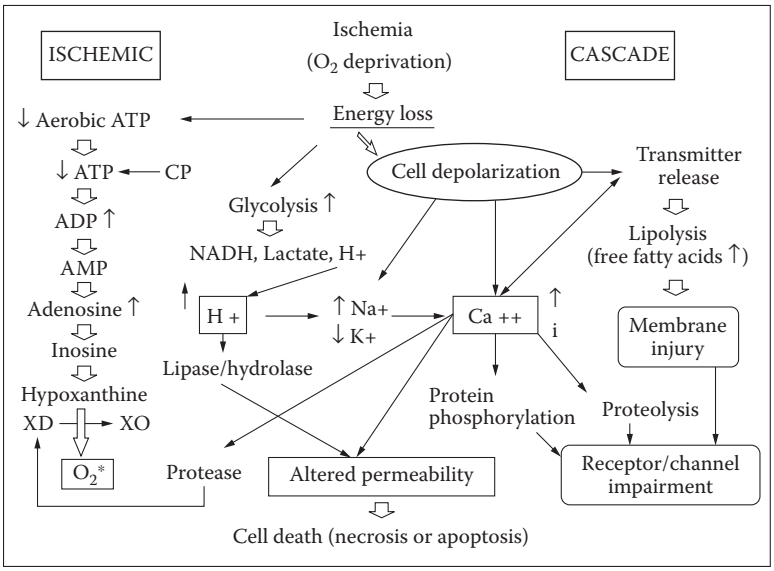

FIGURE 2.4 A scheme for the principal metabolic and ionic changes that proceed after the initiation of ischemia. The pivotal event is ATP depletion, which occurs within 1–2 minutes of oxygen deprivation. This early event leads immediately to a shift from aerobic to anaerobic metabolism, which very quickly becomes self-limiting with the production of lactate and H^+. Cell depolarization also occurs very early in the cascade leading to a breakdown of ion homeostasis and a concatenation of other intracellular and membrane-associated events that eventually culminate in cell death. A rise in the intracellular concentration of protons and calcium is at the center of many of the mechanisms now recognized to be contributory to cell injury as a result of ischemia.

It is well known that different cells have different susceptibilities to ischemic injury and this is due largely to a higher metabolic activity in the more sensitive tissues requiring a greater ongoing production of ATP. The progression from early onset reversible changes to subsequent irreversible injury is both time and temperature dependent. Moreover, a precise distinction between reversible and irreversible injury is difficult to specify. While some intermediate events such as ionic shifts may or may not be readily reversible, structural alterations in organelles, especially the mitochondria, are generally regarded as early signs of irreversible damage and the rupture of cell membranes is unequivocally fatal for the cell. Reduced temperatures can delay the progression towards a state beyond which cells are unable to recover normal function, and this is the basis of hypothermic preservation as discussed below. Under warm ischemic conditions, the time available during which cells will sustain only reversible changes is very restrictive for clinical procedures. For example, those tissues most sensitive to ischemia (e.g., heart and brain) are irreversibly damaged within a few minutes such that hypothermia is often needed as an adjunct to protect these organs *in vivo* during complex surgeries requiring lengthy periods of circulatory arrest.[28] The ischemic tolerance of the brain is known to be only 6 minutes at 37°C, but is extended to nearly 60 minutes when body temperature is reduced to 17°C (see Reference 28). During global warm cerebral ischemia, the high-energy phosphate stores of creatine phosphate are depleted within one minute; glucose and glycogen stores within 4 minutes; and ATP reserves within 5 to 7 minutes.[29] In contrast, some transplantable visceral organs such as the kidneys are able to tolerate much longer periods of warm ischemia. Detrimental changes occur first and become most severe in the proximal convoluted tubules. Gross ischemic changes in some tubules are observed after 30 minutes, with total necrosis of the majority of tubules after 60 minutes. Nevertheless, 90% of experimental animals survived with kidneys that suffered 30 minutes of warm ischemia and 75% survived after 60 minutes of ischemia, showing that much of the damage is recoverable despite evidence of permanent histological injury.[30]

The heart is highly intolerant of ischemia and continues to challenge researchers to devise ways of extending and improving methods of myocardial preservation. The heart is an obligate aerobic organ and the myocardium is exquisitely sensitive and dependent upon a continuous and adequate supply of oxygen for maintenance of normal contractile function. For the purposes of this discussion, it is appropriate to focus on the heart to summarize the effects of ischemia (depicted schematically in Figure 2.4), which must be alleviated in order to prolong preservation.

Under aerobic conditions, mitochondrial oxidative phosphorylation provides the primary energy source for the myocardium, which is able to use a variety of substrates such as glucose, free fatty acids, lactate, pyruvate, acetate, ketone bodies, and amino acids.[31] Myocardial oxygen reserve is exhausted within 8 seconds following the onset of global ischemia, and aerobic production of ATP ceases as the tissue oxygen tension falls below 5 torr.[32] During the first phase of ischemia, the myocardium depends upon energy production from glycogenolysis and anaerobic glycolysis. However, this pathway is an intrinsically inefficient way of maintaining myocardial ATP and is ultimately inhibited during prolonged ischemia by the accumulation of glycolytic metabolic degradation products (NADH, lactate and H^+).[33] ATP stores are temporarily buffered by the pool of creatine phosphate (CP), but this declines rapidly and is essentially depleted within 20 minutes of the onset of global ischemia.[34] During the initial 5–10 minutes of ischemia, ATP levels do not drop significantly, but decline to 50–60% of pre-ischemic levels during 30 minutes of interrupted coronary circulation.[34]

An important consequence of the increased glycolysis and ATP hydrolysis is the accumulation of protons in the cytoplasm, giving rise to a progressive development of intracellular acidosis.[35,36] Apart from contributing to metabolic block of the residual glycolytic energy production by inhibiting key enzymes (e.g., phosphofructokinase), protons also contribute to the activation of lysosomal hydrolases[37] and lipoprotein lipase.[38] This, coupled with proton-induced Ca^{2+} shifts and pH-induced membrane conformational changes, contributes significantly to increased membrane permeability.

As ischemia progresses, transmembrane ionic gradients are dissipated resulting in the loss of intracellular K^+ and accumulation of Na^+ and Cl^- due to the metabolic inhibition of Na^+-K^+ ATPase.[33] It is described in detail above that this in turn leads to osmotic cell swelling.[39] A decrease in intracellular pH also facilitates Na^+-K^+ exchange, exacerbating intracellular Na^+ overload.[40] This in turn contributes to an increase in cytosolic-free Ca^{2+} by facilitating extracellular Ca^{2+} uptake via the Na^+-Ca^{2+} exchange mechanism.[40,41] Other mechanisms, including inhibition of the energy-dependent sarcoplasm reticulum uptake of Ca^{2+} in myocardium[42] and excitatory amino acid stimulation of neurotransmitter receptors/channels in neuronal tissue,[27] further contribute to a massive rise in intracellular Ca^{2+}. The rise of $[Ca^{2+}]_i$ is further known to activate Ca-dependent phospholipases, phospholipase A_2, and phospholipase C,[43–45] as well as proteolytic enzymes, which are also responsible for membrane injury. These enzymes do not require oxygen or energy and so may function during or after ischemia. Hydrolysis of the membrane phospholipids releases free fatty acids (FFAs), which have detergent properties that can destroy the lipid portions of all membranes. The major FFA is arachidonic acid, which can be metabolized to free radicals, prostaglandins, and leukotrienes, which can produce further changes in membrane permeability and ion distribution.[46] It is further indicated in Figure 2.4 that increasing concentrations of intracellular calcium also activate enzymes that can convert xanthine dehydrogenase to xanthine oxidase, which is known to enhance superoxide formation from hypoxanthine, especially during reperfusion.[47]

It is now understood that calcium serves as a key messenger and modulator of intracellular signaling reactions, which affect certain enzymes, the membrane permeability, and transmitter release. Cells maintain an enormous gradient of calcium concentration across the plasma membrane (10,000:1) and the influx potential is controlled by both voltage-sensitive channels and receptor-controlled channels. Calcium efflux is via a Ca^{2+} translocase and a Na^+-Ca^{2+} exchange mechanism, both of which require energy. Also, calcium uptake into cellular organelles is energy dependent. During ischemia, Ca^{2+} enters the cell through both types of channels and, coupled with a reduced uptake by organelles, accumulates in the cytosol. This intracellular overload appears to be one of the common pathways leading to irreversible cell damage by the mechanisms summarized above.[27]

2.3.2 Structural Changes

As mitochondrial oxidative phosphorylation is the first casualty of ischemia, it is not surprising that structured alterations in the mitochondrion are regarded as early and sensitive indicators of ischemia and have been the subject of extensive study.[48] Detectable changes do not occur immediately, and first changes, manifest by disappearance of glycogen granules, lysis of cristae, and swelling, have been demonstrated after 30 to 40 minutes of ischemia in the human myocardium during cardiac surgery. At the later stages of ischemia, severe ultrastructural damage becomes evident and is characterized by extensive mitochondrial damage, pyknotic nuclei, cellular swelling, myofibrillar disruption, and the appearance of contracture bands.[49] After 40 minutes of normothermic ischemia, irreversible changes take place and reperfusion at this stage leads to explosive cell swelling, deposition of calcium phosphate, and intense ischemic contracture resembling rigor mortis.

2.3.3 Vascular Injury During and Subsequent to Ischemia

In addition to the injury sustained by parenchymal cells, it is now well established that ischemic organs are subject to further modes of injury relating to vascular effects. These are collectively referred to as the "no-reflow" phenomenon and "reperfusion injury," which has itself been shown to be a distinct phenomenon characterized by ultrastructural, functional, and metabolic alterations.

2.3.4 No-Reflow

A well established concern in isolated organ preservation for transplantation is that blood flow can fail to return in an organ that has suffered a period of ischemia.[50] Clearly, this is of great importance in determining the fate of the transplanted organ, the health and viability of which depends critically upon the patency of its vascular network. Various mechanisms have been proposed to account for this phenomenon, which is also of crucial importance for the outcome of hypothermically stored organs. Contributory factors include ischemically induced vascular collapse; osmotic swelling of vascular endothelium leading to increased vascular resistance and vessel occlusion; and erythrocyte clumping producing blockage of capillaries and the formation of infarcts. The increased rigidity of red cells due to ATP depletion is considered to be a principal cause of reduced deformability and the most significant component of the no-reflow phenomenon. Weed et al. proposed that ATP normally chelates intracellular calcium and, when ATP is no longer available, calcium binds to membrane proteins, rendering the membrane more rigid.[51] Additionally, it is known that the capillaries become increasingly leaky to protein after more than 30 minutes of ischemia, which would lead to loss of the oncotic pressure that retains fluid in the capillaries, and, hence, to an increase in the hematocrit within the vessels. This, in turn, would increase viscosity dramatically and lead to stagnation.[52] No reflow during rewarming and reintroduction of blood into cold ischemic organs is now known to involve a network of complex interactions between vascular endothelium, blood components, and free radicals that is referred to as reperfusion injury (see References 23, 53, 54).

2.3.5 Reperfusion Injury

The concept of reperfusion injury comes from the well-known fact that making a tissue hypoxic does not necessarily produce injury, but after reperfusion such tissues show marked and occasionally severe damage. Several possible interacting mechanisms of reperfusion injury are often described and include the following:[23,53,54]

1. Cell-derived free radicals (the oxygen paradox)
2. Actions and products of inflammatory cells in the blood, especially neutrophils and platelets
3. Effects of intracellular calcium accumulation (the calcium paradox)
4. Loss of membrane phospholipids

A complete understanding of the various proposed mechanisms of reperfusion injury is far from clear and remains under intensive investigation. While a detailed discussion of the various, seemingly disjointed hypotheses is beyond the scope of this article, it is appropriate for subsequent discussion of the effects of hypothermia to include a few salient comments regarding the role of free radicals.

During the past decade, oxygen-derived free radicals (ODFR) have been the focus of attention as mediators of various tissue injuries and particularly microvascular injury.[23,25] A free radical is a molecule with an odd, unpaired electron in its outer shell (denoted by a dot, thus $R^{\bullet}$), and this chemically "unsatisfied" electron renders the molecule highly unstable and reactive. Free radicals are inherently damaging since this high reactivity can precipitate chain reactions that produce increasingly reactive and toxic free radicals. Reactive species derived from oxygen are generated because oxygen normally undergoes tetravalent reduction to water by accepting four electrons simultaneously in the mitochondrial cytochrome oxidase system. However, as much as 2% of cellular oxygen undergoes univalent reduction, accepting one electron at a time and creating a superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide, and eventually a hydroxyl free radical ($OH^{\bullet}$), thus the following equation:

$$O_2 \xrightarrow{e^-} O_2^{-\bullet} \xrightarrow{e^- + 2H^+} H_2O_2 \xrightarrow{e^- + 2H^+} H_2O + OH^{\bullet}$$

The hydroxyl free radical is the most reactive of all and will oxidize any organic molecule almost instantaneously. The small quantities of free oxygen radicals produced during a cell's normal metabolism are detoxified by the enzyme superoxide dismutase (SOD):

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

This protective enzyme occurs in all aerobic tissue but is found in substantial quantities only inside the cell. ODFRs are also detoxified by the naturally occurring enzymes catalase and peroxidase. During ischemia, the production of hypoxanthine is greatly increased as a result of the catabolism of ATP as shown on the left of Figure 2.4. In the absence of oxygen, the enzyme xanthine dehydrogenase (type XD) is converted to xanthine oxidase (type XO), which converts hypoxanthine to xanthine and this reaction also involves calcium. During reperfusion when the ischemic tissue is again exposed to oxygen, xanthine oxidase catalyzes the generation of superoxide radicals in the following reaction:

$$\text{xanthine} + H_2O + 2O_2 \xrightarrow{XO} \text{uric acid} + 2O_2^{-\bullet} + 2H^+$$

In tissues such as the myocardium, defense mechanisms against superoxide-mediated ischemic injury are well developed in the form of scavenging enzymes. These may be antioxidants, such as glutathione peroxidase and catalase, or chain breaking antioxidants, such as SOD, ascorbate (vitamin C), and α-tocopherol (from vitamin E). The emerging role of ODFR in ischemic injury has raised the question as to whether or not injury ascribed to ischemia is in fact reperfusion injury that is initiated by ischemia, but precipitated by reperfusion. This has led to the concept of scavenging free radicals during both ischemia and reperfusion. The role of drugs like allopurinol, which inhibits xanthine-oxidase in preventing superoxide-mediated injury, is therefore readily apparent. Also, compounds that chelate those transition metals like iron — which are known to catalyze the formation of free radicals and thereby contribute to tissue injury by initiating and propagating lipid peroxidation — have been shown to ameliorate tissue damage.[55] For example, desferroxamine inhibits the Haber-Weiss reaction or more efficient Fenton reaction in which highly reactive hydroxyl radicals are generated when H_2O_2 accepts an electron from a reduced metal ion such as Fe^{2+}[25].

The end result of prolonged ischemia is cell death mediated by the mechanisms outlined above, which characterize the process of necrosis, or pathological cell death. An alternative mode of cell death involving a programmed or regulated process involving *de novo* gene transcription, and referred to as apoptosis, is discussed briefly below in the context of hypothermic preservation.

The culmination of the cascade of interactive ischemic events is a tissue that is unable to resume its normal function upon restoration of its blood supply. The sequence of injurious processes advances at such a rate that irreversible damage is sustained by most organs within one hour of ischemia at 37°C, and at much shorter times in highly metabolic tissues such as cardiac muscle. The role of low temperatures in protecting against ischemic injury and providing a means for preserving tissues will be considered in the remainder of this chapter.

2.4 HYPOTHERMIA IN RELATION TO ISCHEMIC EVENTS

Cooling cells to varying degrees has proved to be the foundation of nearly all effective methods of viable tissue preservation. However, the consequences of cooling are not exclusively beneficial

in combating the effects of ischemia, and hypothermic protection is a compromise of the benefits and detriments of cooling as outlined in the ensuing discussion.

2.4.1 General Suppression of Reaction Rates

The fundamental basis of all biologic and chemical processes is molecular activity and mobility, which are governed by thermal energy. This means that as temperature is lowered molecular motion is slowed.[15] The removal of heat from a system slows down both physical and chemical processes in proportion to the loss of heat and therefore to the fall in temperature. Physical phenomena such as osmotic pressure depend solely on the rate of molecular motion so that the decrease in the rate of the process is proportional to the fractional change in absolute temperature. Many chemical reactions, however, depend upon an energy of activation, which is the minimum energy required for molecules to react. This results in a special relationship between the rate of reaction and temperature described originally by Arrhenius and outlined below (see[15]). Since the processes of deterioration associated with ischemia and anoxia are mediated by chemical reactions, it has proved well founded to attempt to prevent or attenuate these changes by cooling. Biochemical processes involve molecular interactions that are invariably catalyzed by enzymes in reactions that require energy input from cellular stores such at ATP or creatine phosphate. Cooling can affect all components of these reactions including the energy status of the substrate molecules, the stability of the enzyme protein, and the capacity of the cell to supply biological energy. The rate of biophysical processes such as diffusion of ions and osmosis declines linearly with temperature by approximately 3% per 10°C. It is apparent, therefore, that biophysical events are relatively only slightly affected by the comparatively modest temperature changes imposed during hypothermic storage of tissues for transplantation. It is only at much lower temperatures that the rate of biophysical processes becomes significantly important, especially at subzero temperatures when phase changes lead to both ice formation and solute concentration changes.[7,9,15]

By comparison, the rate of chemical reactions, including the biochemical processes that constitute metabolic activity, is slowed significantly more by a given degree of hypothermic exposure. It is well established that within the temperature range 0–42°C oxygen consumption in tissues such as kidney, liver, and heart decreases by at least 50% for each 10°C fall in temperature.[18,56] Oxygen consumption (VO_2) is a reasonable measure of metabolic activity since for practical purposes, tissue and cellular stores of oxygen do not exist and cells rely upon the circulation to deliver oxygen in quantities determined by the rate of O_2 consumption. The magnitude of decrease of VO_2 by cold storage is therefore regarded as an index of the degree of reduction of metabolic activity. For tissues having a high rate of metabolism, such as the brain VO_2 at 5°C has been estimated to be 6% of the normothermic rate.[28,57] The quantitative relationship between energy requirements for biochemical processes and temperature changes have been expressed mathematically in different ways:

1. The Arrhenius Relationship: Biochemical processes, in common with all chemical reactions, occur only between activated molecules the proportion of which in a given system is given by the Boltzman expression exp (-E/RT) where E is the activation energy, R is the gas constant, and T is the absolute temperature. According to the Arrhenius relationship, the logarithm of the reaction rate (k), is inversely proportional to the reciprocal of the absolute temperature:

$$-\log k = A(-E/2.3\ RT)$$

A graphical plot of log k against 1/T will yield a straight line with a slope of E/2.3R if the relationship represents a single rate-limiting step. However, many examples in the literature demonstrate discrete "breaks" in the linearity of Arrhenius plots as illustrated in Figure 2.5 and discussed below.[58,59]

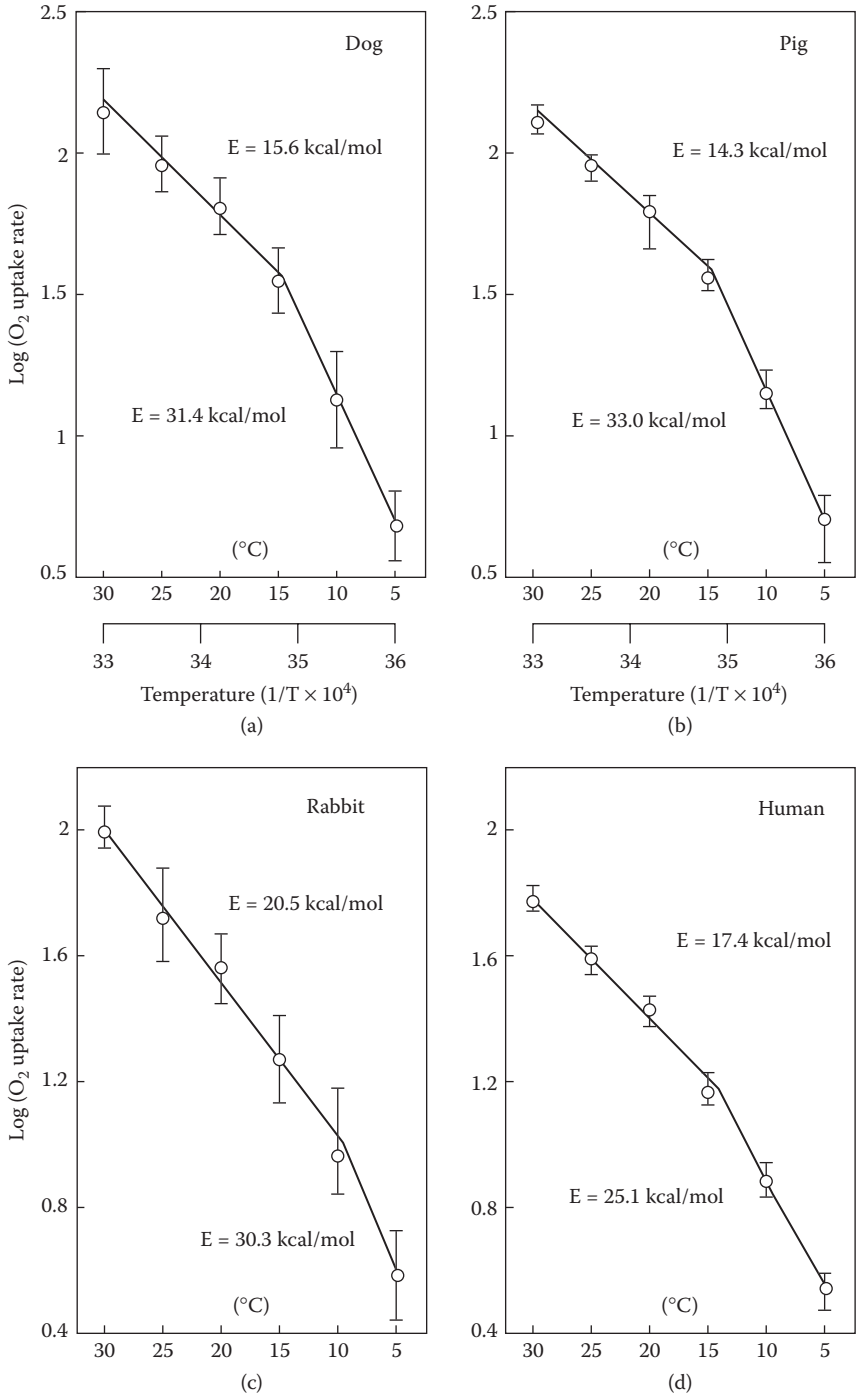

FIGURE 2.5 Arrhenius plots depicting the effect of temperature on ADP-stimulated respiration in mitochondria from four different species. (Adapted from Southard et al.[59]).

2. Van't Hoff Rule[60] relates the logarithm of a chemical reaction rate directly to temperature and is commonly expressed in the form of the respiratory quotient or temperature coefficient, Q_{10}, where Q_{10} is the ratio of reaction rates at two temperatures separated by 10°C. Accordingly,

$$Q_{10} = (K_2/K_1)\ 10^{(T2-T1)}.$$

For most reactions of biological interest Q_{10} has a value between 2 and 3, but some complex, energy-dependent reactions have a Q_{10} between 4 and 6, and are more likely to stop completely at low temperatures.[61] Both Q_{10} and Arrhenius plots have been used to quantitate changes in metabolic processes occurring in biologic systems, whether they are enzyme reactions in single cells or the oxygen consumption of the entire human body. The Q_{10} for whole-body oxygen consumption is approximately 2, indicating that, in general, metabolic rate is halved for each 10°C drop in temperature.[28] Some individual tissues, however, have been shown to exhibit a Q_{10} as high as 5 to 8, demonstrating the profound effect cooling can have on retarding reaction rates.[58]

The impact this cooling effect has on ischemic tolerance is amply illustrated by data from hypothermic preservation of mammalian kidneys. It is explained above that kidneys can tolerate only about 45 minutes of warm ischemia before incurring irreversible injury. However, tolerance is extended to 2 hours at 15 to 25°C,[62] 6 to 7 hours at 5 to 15°C[63] and 12 hours at 0°C without serious injury.[64] The same holds true for tissues that are exquisitely sensitive to ischemic injury such as the myocardium and neuronal tissue.[28,65] For example, based upon the estimate that VO_2 for the brain is 6% of the normothermic rate at 5°C, Bering postulated that the brain may tolerate ischemic periods for up to 3 hours at temperatures below 5°C.[57] This has proven to be consistent with our own recent demonstration of hypothermic protection of the heart, brain, and visceral organs during $3^1/_2$ hours of cardiac arrest and whole-body asanguineous perfusion at 7°C.[28,66,67] Importantly, it should be pointed out here that the ischemic tolerance of organs both *in situ* and *ex vivo* is not only a function of temperature reduction *per se*, but is also maximized by manipulation of the extracellular environment of the component cells in terms of the chemical composition of the perfusate. I will return to this important consideration in subsequent discussions below.

2.4.1.1 Metabolic Uncoupling

While it is clear that cooling has a profound effect upon biochemical reaction rates and that this in turn can slow degradative processes and reduce the rate of substrate and energy depletion, it is important to realize that not all reaction rates are affected to the same degree, or even in the same manner, by cooling. For example, Southard et al. studied the comparative effect of temperature on the rate of a membrane-bound enzyme catalyzed reaction and ADP stimulation of respiration in mitochondria isolated from the kidney cortex of four species commonly used for transplantation studies, including human.[59] Their findings are summarized in Figure 2.5, which shows that the Arrhenius plots appear discontinuous with "break" points at 15°C or higher for dog, pig, and human and at 10°C for rabbit. Dog and pig mitochondria showed the greatest increase in activation energy at temperatures below the break point. In general, an Arrhenius plot with a distinct "break" has been taken to indicate that the rate-limiting step has changed but the Arrhenius Law still holds true on either side of the break. Although the interpretation of discrete changes in the slope of Arrhenius plots has been contentious, the Lyons-Raison phase change hypothesis of chilling injury has received much attention and general acclaim.[68] This hypothesis states that at a certain critical temperature within the chilling injury range, the membrane lipids undergo a transition from a liquid-crystalline to a solid gel state.[69,70] As illustrated in Figure 2.5, the same phenomenon has been demonstrated in the cells of mammalian organs,[58,59] and the two main consequences of the transition thought to eventually result in cell injury are an increase in membrane permeability (discussed further in a subsequent section), and an increase in the activation energy of membrane-bound enzymes. While an increase in E in itself may not be damaging to a cell, it has been proposed that the damage is

probably responsible for the different behavior of soluble enzyme systems and membrane-associated enzyme systems. Thus, the result would be the accumulation or depletion of metabolites at the point of entry into mitochondria. Hence, the membrane phase transitions in subcellular membranes could cause metabolic imbalance and provide one component of injury sustained by homeothermic cells during cold exposure.

One interpretation of cold resistance in various cold-adapted species is that their cell membranes maintain a greater degree of fluidity at low temperatures compared to most normothermic, non-cold adapted species. Although the role of membrane fluidity and phase transitions in cold adaptation has not been accepted unequivocally, it has been demonstrated in mitochondria from a warm-blooded species (dog) that low levels of membrane lipid perturbers such as adamantine (cyclodecane) can abolish the discontinuity in the Arrhenius plot, suggesting that membrane fluidity had been increased during cold exposure.[59] Other lipid perturbers (e.g., Butylated hydroxytoluene) have been shown to protect homeothermic cells against cold shock, which occurs during rapid cooling from normal temperatures to 0°C.[71] Nevertheless, this potential mode of protection for mammalian organs during cold storage has not yet been widely investigated as an interventional strategy.

The data of Southard et al., illustrated in Figure 2.5, also emphasize that caution should be exercised when comparisons of cellular responses to cold are made between species. It can further be appreciated that if a single biochemical process — in this case ADP-stimulated mitochondrial respiration in the kidneys from four different euthermic species — is affected differently by cooling, that it is also likely that the different biochemical reactions within a given cell will not be affected to the same extent, or in the same way, by a reduction in temperature. Figure 2.6 shows schematically the large number of different, integrated chemical reactions that constitute common metabolic pathways within a cell. Each of these reactions will be affected in a different manner and to a different degree by cooling, and so the possibility exists for uncoupling reaction pathways and producing harmful consequences. This type of metabolic perturbation is largely beyond the influence of investigator intervention since it is not possible to select which processes will be depressed by cooling. It is nevertheless important for optimal preservation that any cold-induced dislocation of interconnected pathways is fully reversible or recoverable upon rewarming and reperfusion.

2.4.1.2 Optimum Temperature for Hypothermic Storage

Cooling prolongs *in vitro* survival because it slows metabolism, reduces the demand for oxygen and other metabolites, and conserves chemical energy. However, it does not affect all reactions to the same extent, and the net result of cooling on integrated metabolizing systems is complex, not entirely predictable, and not completely understood. The application of mathematical relationships such as the Arrhenius and the Van't Hoff rules to help quantitate, predict, and understand the mechanisms of hypothermic preservation are somewhat simplistic since they relate to temperature change as the only variable. Nevertheless, this has proved useful in practice because the complexities of cooling integrated tissues and organs do not permit convenient separation of the interacting variables that affect the outcome of hypothermic preservation. Moreover, the effect of hypothermia *per se* on transplanted tissues and organs is confounded by the effects of prior warm ischemia and hypoxia that will undoubtedly influence the susceptibility and response of component cells to cooling.

Using tissue culture cells as an experimental model, Kruuv et al. have examined the effects of *pure hypothermia* on cell viability in the absence of any prior hypoxia.[72,73] They showed that the Arrhenius plot of inactivation (killing) rates of cells exposed to reduced temperatures changes slope at approximately 7 to 8°C, implying that there are distinct mechanisms of hypothermic inactivation above and below this transition temperature. In the range of 8 to 25°C, the activation energy from the Arrhenius plot for control cells is about 15 kcal/mol, which falls within the range of temperature

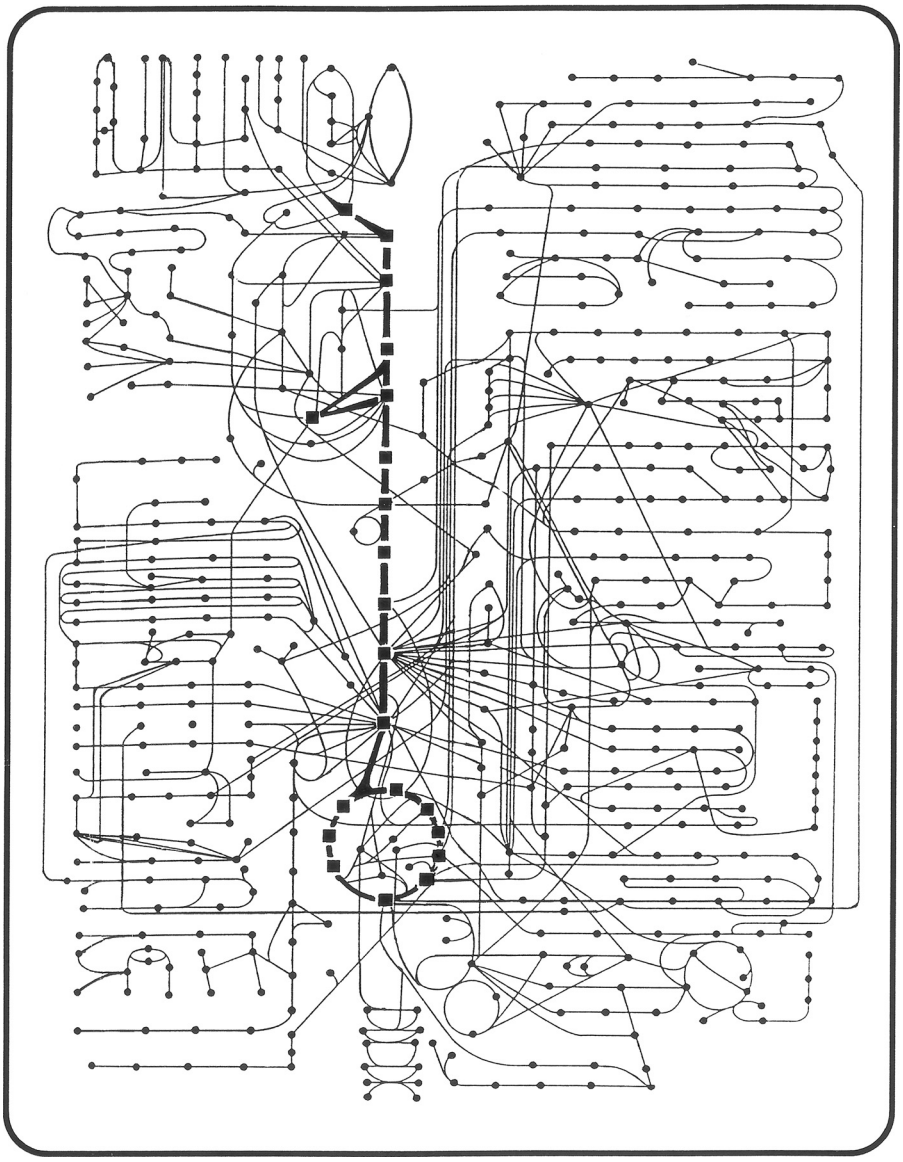

FIGURE 2.6 Schematic diagram illustrating the complexity of integrated biochemical pathways in a typical cell. About 500 common metabolic reactions are shown with each metabolite represented as a filled circle. The centrally placed reactions of the glycolytic pathway and TCA cycle are shown as bold. A typical mammalian cell synthesizes more than 10,000 different proteins, a major proportion of which are enzymes. (Adapted from Alberts et al.[235]).

coefficients of metabolic processes (10–30 kcal/mol) and much lower than that for protein denaturation. Below 8°C, the magnitude of the apparent activation energy is large (–61 kcal/mol). These values have been interpreted to suggest that unbalanced metabolism is probably the rate-limiting step for hypothermic inactivation in the higher temperature range, and membrane lipid phase transition or cold denaturation of a critical protein is likely to be responsible for the strong temperature dependence in the lower range. It is apparent, therefore, that the optimum temperature for hypothermic storage will depend upon a variety of factors involving the interaction of hypothermia, the nature of the cell, and the chemical composition of its environment. This is well illustrated

by reference to the voluminous literature relating to the role of hypothermia in protecting the ischemic heart and the associated design of cardioplegic solution. Although the ability of hypothermia to reduce myocardial energy demand is well established, the importance of its contribution, the optimum temperature, and the possibility that some cellular injury may be induced by the cooling have been debated and widely reported (for example, see citation 74). A summary statement will suffice for the purpose of the present discussion.

The first point is that because electromechanical work accounts for approximately 85% of the oxygen demand of the myocardium, chemical arrest of the heart in diastole, with agents such as potassium or magnesium, is an important addition to hypothermia to achieve optimum preservation. Although hypothermia can abolish organized myocardial contractions, energy-consuming ventricular fibrillation can persist even at low temperatures. It is well established that irrespective of the temperature, the myocardium is best preserved during global ischemia by the combination of chemical and hypothermic arrest. With respect to temperature, the general consensus from a variety of experimental and clinical observations is that maximal myocardial preservation during ischemic arrest is best achieved in the range of 10 to 20°C. For example, Tyers et al. showed that metabolic recovery was best when the myocardium was kept at 10 to 15°C with rapid reperfusion recovery of high energy phosphates and glycogen, compared with metabolic deterioration at 4°C.[75] Shragge et al.[76] progressively lowered the myocardial temperature *in vitro* to 0.5°C and found no significant decrease in the concentration of ATP or the glycogen stores in the nonanoxic hearts. These findings, confirmed by others, led to the conclusion that hypothermia in the absence of ischemia is not harmful to the myocardium. Such conclusions do not, however, account for the rate of cooling that in other cellular systems is known to influence cold-induced injury by way of an ill-defined mechanism termed "thermal shock" (see below).

The second point to note is that it has been suggested that the safe period of ischemia can be increased by adding oxygen to the cardioplegic medium in order to satisfy the small but continued metabolic demands of the cold arrested heart.[77] The oxygen consumption of the ischemic myocardium at 15°C is 0.27 mL O_2/min/100g tissue. Nonoxygenated crystalloid (asanguineous salt solution) cardioplegic solution administered at 10°C contains 0.86 ml O_2/100 ml of solution,[78] and so a prohibitively large volume of cardioplegic solution would have to be injected into the coronary circulation to avoid a myocardial oxygen debt occurring within a few minutes. Moreover, it has been shown that enhanced myocardial protection can be achieved by using oxygenated media in the form of either sanguineous or asanguineous solutions.[79,80] In principle, the advantage of delivering oxygen (and possibly other crucial metabolites) to the arrested heart is the maintenance of cell respiration and oxidative phosphorylation during global ischemia at temperatures that permit significant metabolism to proceed (10–20°C). This is in contrast to the strategy of tissue-suspended animation at lower temperatures (ice storage 0–4°C) where the objective is to reversibly inhibit all cellular function. The former strategy of metabolic support during hypothermic storage at ~10°C may demand continuous perfusion to supply essential substrates and remove toxic catabolic products. The practical implications of this are discussed below and elsewhere in this book (see Chapter 9).

Although tissue energy requirements are minimal at deep hypothermic temperatures, there are suggestions that constant supply of oxygen along with adenosine as a precursor to the ATP will result in superior ATP levels[81–86] and minimum oxidative and metabolic stress in preserved tissues. However, there is no clear consensus among the research community about the need for oxygen supply during hypothermia. It has been assumed that low concentrations of molecular oxygen, such as that dissolved in organ preservation solutions, is sufficient to support the generation of free radicals during prolonged storage.[87,88] Therefore, it is recognized that hypothermia may set the stage for a progressive development of tissue injury as a result of reactions and processes that occur during hypothermia, but that fuel changes that proceed for a considerable time after normal conditions of temperature and oxygen tension are resumed. Others have shown that a moderated oxygen tension is beneficial during hypothermic preservation, which suggests that oxidative stress

can lead to adaptation in tissues and increased production of antioxidants. It has been shown in experimentation that rats that were gradually exposed to oxygen increased their production of antioxidants in lungs.[89] Nevertheless, numerous investigations have suggested that oxygen supply is essential during hypothermic preservation of livers.[90–92] Recent studies on survival transplantation[93] of rat livers from donors with nonbeating hearts suggest that the saturation of UW solution with atmospheric air is a primary requirement for the preservation and restoration of ATP levels and mitochondrial functions.[94,95] Previous studies by Stubenitsky et al. have also shown that the oxygenated hypothermic preservation of warm ischemic kidney slices can restore normal tissue ATP levels.[96] More investigation is required to arrive at a consensus on optimum oxygen tension requirements that can provide superior graft function and prevent oxidative damage.

There is strong evidence, therefore, that optimum preservation of tissues and organs by using low temperatures requires careful consideration of the storage temperature in relation to other important factors including the characteristics of the cell, its environment, and the strategy adopted to effect maximum protection. The effect of hypothermic storage temperature *per se* has not been studied extensively in a wide variety of systems but the available evidence to verify this as a general effect extends beyond tissue culture cells and heart preservation outlined above. For example, in kidney preservation studies, Hardie et al. found 5°C to be superior to storage at 0.5°C and Pegg et al. showed significantly better preservation at 10°C compared with storage at 5°C.[97] Even in whole-body protection during hypothermia, we have demonstrated improved outcome in a canine model when the nadir temperature during several hours of hypothermic cardiac arrest was 7°C compared with 1.5°C.[28]

2.4.2 Effect upon Energy Metabolism

Under normal circumstances the supply of energy-rich compounds to fuel a cell's requirement for homeostatic control is continuously replenished by oxidative phosphorylation in the mitochondria. During cooling, however, there is a progressive exhaustion of chemical energy reserves in a cell despite the general suppressive effect of cooling on metabolism. Studies that clearly demonstrate the rapid depletion of adenine nucleotides during cold storage of organs at 0 to 2°C are suggestive that mitochondrial function is severely impaired by hypothermia.[98] However, it has been demonstrated in the liver, for example, that the same tissues stored at 8 to 10°C can reestablish ATP reserves if an adequate supply of oxygen is maintained by continuous perfusion as discussed above.[94,95,99] Moreover, it has also been established during hypothermic kidney preservation that the balance between glycolysis and complete oxidation of fatty acids at l0°C is controlled by the oxygen tension. Pegg et al. showed that glycolysis provided the principal source of energy at l0°C when the pO_2 =150 mm Hg, but that oxidation of caprylic acid provided the main fuel when pO_2 was raised to 650 mm Hg.[88] Furthermore, it had previously been demonstrated by Huang et al. using well-oxygenated kidney cortex slices that the preferred substrate for energy metabolism was also markedly influenced by temperature. Under normothermic conditions glucose, amino acids, ketone bodies, and fatty acids were all utilized, but only short-chain fatty acids and ketone bodies were consumed at 10°C.[100] Clearly, the effect of cooling on metabolism is complex and should not be regarded as causing a simple uniform retardation of all biochemical reactions.

The effects of cooling on mitochondrial processes are especially important for the outcome of cell preservation since it is essential for cell viability that the energy status is either maintained during storage or readily reestablished during rewarming. The crucial importance of this is readily appreciated in regard to the hypothermic storage of myocardial tissue, which is widely recognized to have special demands for its preservation compared with other organs.[101] One fundamental difference between the heart and other transplanted organs that is reflected in the tolerance to cold ischemia is that the heart, as a contractile organ, must be able to sustain 90% of its function very soon after rewarming and reperfusion in order to be life sustaining. The heart, therefore, has much greater energy demands upon reperfusion for immediate mechanical work and adequate contractile

function. The myocardium is known to be predisposed to ischemic contracture during prolonged cold storage: this is a progressive increase in myocardial stiffness with concomitant reduction of compliance and ventricular volume. The depletion of high-energy phosphate reserves causes the actin and myosin to interact, resulting in a progressive and eventually irreversible contracture of the heart. So, the basis of the problem is energy deprivation and dysregulation of intracellular homeostasis such that the onset of contracture occurs when ATP falls to less than 80% of normal values. In the early stages contracture does not necessarily imply a dead myocardium and strategies to delay the onset of ischemic contracture and promote the retention and repletion of high-energy phosphates are crucial for adequate methods of prolonged myocardial preservation.[101]

The suppression of oxidative phosphorylation at low temperatures is indicative of a mitochondrial defect. As illustrated in Figure 2.5, oxygen consumption by isolated mitochondria decreases with falling temperature, usually with a change in the rate at about 15°C.[59] More specifically, research has indicated that the enzymes responsible for translocating adenine nucleotides (AN translocase) across the mitochondrial membrane become ineffective at temperatures below the transition point of the Arrhenius plot. Also, the enzymes responsible for transporting NADH across the mitochondrial membrane via the malate-aspartate shuttle are ineffective at low temperatures. For example, it is known that the adenine nucleotide translocase of rat liver demonstrates an abrupt decrease in activity at 18°C, and although it is not rate-limiting at 37°C it could be limiting at low temperatures.[102] It is now believed that the failure of aerobic metabolism during hypothermia is principally due to the inactivation of mitochondrial transport enzymes, despite the fact that it has also been demonstrated in some tissues that adenine nucleotides can be synthesized at the temperatures used for hypothermic storage, providing the appropriate substrates are present. Therefore, it is clear that the once-believed notion that it is the absolute concentrations of residual high energy phosphates that might dictate survival, and that their complete exhaustion results in loss of viability, is not tenable. A great deal of evidence has now established the importance of the ability of hypothermically-stored cells to resynthesize energy-rich compounds during rewarming, which will be dependent upon the status of the adenine nucleotide pool remaining at the end of storage. As ATP and adenosine diphosphate (ADP) reserves are depleted during ischemia, the accumulating adenosine monophosphate (AMP) is dephosphorylated by 5'-nucleotidase enzymes to adenosine and other freely diffusable metabolites. Hence, these nucleosides are readily lost from the cell and no longer available for resynthesis of ATP. Moreover, as explained above, the synthesis of ATP depends upon active translocating processes from the cytosol into the mitochondria and vice versa, involving highly temperature-dependent enzymes.[102] The extent of degradation of ATP (and CP) to the diffusable breakdown products shown in Figure 2.4 is also important for another reason besides the depletion of ATP precursors that are crucial for subsequent immediate repletion of high-energy compounds. The second important aspect of this deamination pathway relates to the probable exacerbation of reperfusion injury by the generation of free radicals during and after cold storage as described above.

The complexity of low-temperature effects on mitochondrial respiration is not limited to the impairment of translocase enzymes. Recent studies have shown that other enzymes that control reactions of the tricarboxylic acid (TCA) cycle and the electron transport chain are affected differently by cold storage. In mitochondria isolated from hearts stored at 4°C for 12 and 24 hours, the rate-limiting enzyme in the TCA cycle, citrate synthetase, has been shown to be more susceptible to cold storage than the rate-limiting enzyme in the electron transport chain, cytochrome c oxidase.[103] Such observations highlight the multifactorial nature of mitochondrial dysfunction after prolonged hypothermic storage.

It is clear from the foregoing discussion that the best methods of hypothermic preservation might depend upon the maintenance of high energy reserves, the prevention of ATP precursor depletion, and some level of continued metabolism, which, in turn, will be dependent upon the oxygen tension and storage temperature. Such considerations have led Pegg to suggest that future advances in organ preservation might be achieved by studying higher temperatures where

translocase enzymes are more active and to use higher oxygen tensions.[97] Also, the provision of purine or nucleoside precursors for adenine nucleotide repletion, and the use of pharmacological inhibitors of 5'-nucleotidase, such as allopurinol, are regarded as important components of modern-day preservation solutions and may prove to be advantageous for strategies designed to avert an energy crisis in hypothermically stored cells.[97,104,105]

2.4.3 Effect upon Ion Transport and Cell Swelling

We were reminded earlier that intracellular ionic composition and volume regulation of a cell is maintained by a "pump-leak" mechanism in which membrane-bound enzymes transport various ions and solutes to counter the passive diffusion driven by chemical potential gradients. These active pumps are inhibited by hypothermia both by its direct effect on enzyme activity and by the depletion of high energy reserves as mitochondrial energy transduction fails. The effect of applied hypothermia is therefore similar to that produced by anoxia, but the mechanism is different. This has important implications for its reversibility: even if an adequate reserve of ATP is maintained, the membrane pumps are unable to utilize ATP at low temperatures. When the temperature returns to normal the pumps are again able to use ATP and quickly restore the cell's ionic gradients. In the temperature range commonly used for preservation (0°–10°C) the activity of Na^+K^+-ATPase is essentially abolished; for example it is documented that for many cells the activity of Na^+-K^+-ATPase at 5°C is only about 1% of its normal level at physiological temperature.[106]

The resultant passive redistribution of ions and water across the cell membrane and the concomitant change in the membrane potential has been demonstrated to be rapidly and fully reversible in the short term.[107] The ready reversibility of all parameters upon rewarming was due to the retention of the necessary substrates and high energy compounds during hypothermic preservation so that the activity of the ATPase enzymes was quickly restored. While these radical changes in ion and water fluxes as a result of cooling are reversible in the short term, a gradual accumulation of detrimental effects eventually become irreversible in a way similar to the progression of warm ischemic events. Changes in the distributions of sodium and potassium may not cause irreversible alterations, but perturbation of normal transmembrane sodium gradients can adversely affect many secondary transport systems, such as those for glucose and amino acids as well as electrical events in excitable tissues. The various cation transport systems in cells are interrelated and energy dependent, such that all are influenced by temperature changes.[106]

However, cation fluxes are not all affected in the same way during cooling; for example, a disparate effect of temperature on the permeability of dog erythrocytes to cations has been reported.[108] Sodium flux was shown to increase during cooling from 37°C to 20°C and then decrease during subsequent cooling. Also, potassium flux exhibited a minimum at 12°C and then increased during further cooling, whereas water transport decreased in accordance with a typical Arrhenius relationship. This suggests that sodium, potassium, and water are transported across the red cell membrane by independent mechanisms that are affected differently by temperature changes, thus providing further illustration of the complexity of interdependence between homeostatic processes in cooled cells.

2.4.3.1 Divalent Cation Transport

Cells are known to have interrelated cation transport systems depending on energy supply, and are thereby affected by reduced temperatures. Moreover, it is recognized that changes in the distribution of divalent cations (Ca^{2+}, Mg^{2+}) as a consequence of ischemia, hypoxia, and even cooling are especially important in cellular injury. The belief that calcium, in particular, is a mediator of cell death is based upon accumulated evidence over several decades from observations in a wide variety of tissues and pertains to cell death from a variety of causes. During the past decade, the recognition of the central role of calcium-mediated effects in the death of cells of ischemically-sensitive tissues

such as heart and brain has led to intense scrutiny of the effects of perturbations in divalent cation homeostasis.[27,109] With the advent of the excitotoxic hypothesis of neuronal cell death, emphasis has shifted away from the traditional ideas of Ca-mediated injury being caused by influx of calcium into energy-compromised cells via voltage-sensitive channels, toward mechanisms involving agonist-operated calcium channels gated by excitatory amino acid receptors.[21,27,109,110]

As shown in Figure 2.1, cytosolic calcium concentration is 10,000-fold lower than extracellular concentrations, and it is now postulated that enhanced or unbalanced calcium influx across the plasma membrane represents a final common pathway in cell death mediated by various conditions that share the tendency to induce an abnormal membrane permeability for calcium. For example, excessive intracellular accumulation of calcium has been implicated as playing a pivotal role in ischemia-induced neuronal death and the evolving knowledge of the mechanisms involved include the following: alteration of electron transport in the respiratory chain, which leads to swelling and destruction of mitochondria; the release of additional excitotoxic neurotransmitters; activation of deleterious intracellular enzymes such as lipases and proteases, which break down cellular protein and lipid structures; and the formation of potentially harmful oxygen and hydroxyl free radicals and cellular depolarization.[109] Massive calcium accumulations also occur as a result of the so-called calcium paradox induced in cardiac cells when hearts are perfused successively with a calcium-free medium followed by a solution containing calcium. Calcium loading ensues because the calcium free perfusion dramatically increases membrane permeability to calcium. Calcium functions as a second messenger in the regulation of numerous biochemical and physiological processes, often by activating regulatory proteins such as Calmodulin, which, in turn, can activate many intracellular enzymes including protein kinases.[109] It has been proposed that the significant protective effect of mild hypothermia against ischemic brain injury might be mediated in part by inhibition of calcium-induced effects and the prevention of inactivation of important Ca-dependent enzyme systems such as the kinases.[109,110] However, it is generally recognized that cooling has no beneficial effect in preventing inhibition of the (Ca^{2+} Mg^{2+})-ATPase pump mechanism (and may even accentuate it), which is generally the most important factor in controlling intracellular Ca^{2+} concentrations. The effects of cooling on calcium homeostasis results not only from the inhibition of transmembrane pumping, but also from the inhibition of calcium sequestration by endoplasmic reticulum and mitochondria. All three pathways are involved in control of free calcium-ion concentrations, and all are sensitive to ATP depletion. Thus a reduced supply of ATP in cells stored hypothermically can result in increasing intracellular free calcium concentrations via redistribution from internal compartments, even if the external calcium concentration is lowered by perfusing with a low-calcium solution or calcium-channel-blocking drugs are added to the extracellular medium.

Calcium antagonists acting at the site of the plasma membrane will presumably be ineffective at preventing increased concentration of ionic calcium mobilized from intracellular stores. It has been discovered that the rapid influx of calcium responsible for the calcium paradox occurs via channels that are not affected by slow channel blockers, and at temperatures below 27°C changes in the cell membrane decrease slow channel currents, obviating the need for calcium channel blockers.[111]

2.4.3.2 Proton Activity Changes

The scheme depicted in Figure 2.4 for the principal events of the ischemic cascade shows that elevation of the concentration of protons — i.e., increasing acidity — is regarded as a contributory central event in the process of cellular injury ensuing from O_2 deprivation and energy depletion. Moreover, reduced temperatures are also known to influence pH regulation, which is another important homeostatic mechanism for cell survival. It has frequently been reported that hydrogen ion concentration increases in a variety of mammalian cells during hypothermic storage such that tissue pH has been recorded to fall to 6.5 to 6.8 within a few hours of cold storage.[112,113] Acidity is widely recognized as a hazard for cells with the accumulation of protons contributing to a variety

of deleterious processes including metabolic block of glycolysis and structural damage. Destabilization of lysozomes releasing harmful proteases and catalysis of oxidative stress by mobilization of free heavy metals have been implicated as mechanisms of cellular tissue injury during acidosis.

Figure 2.1 depicts that under physiological conditions (37°C) cells actively regulate pH within narrow limits with intracellular pH being maintained at a lower value (pH = 7.0 ± 0.3)[114] than the extracellular fluid (pH = 7.4). The structure/function relationships of biomacromolecules, especially proteins, are governed by their tertiary and quaternary structure, which, in turn, rely upon the maintenance of charged moieties within the molecule. Pertubation of pH homeostasis in conjunction with the altered ionic environment during hypothermia can serve to markedly alter the structure/activity relationship of molecules such as enzymes that rely on electrochemical neutrality to maintain their net charged state, relative to the neutral point of water.[115] In aqueous solutions such as the intracellular fluid, the concentrations of protons (H^+) and hydroxyl ions (OH^-) are determined by the ionization constant of water (pKw), which increases as temperature decreases (see Figure 2.7). Intracellular electrochemical neutrality (equal concentrates of H^+ and OH^-) is therefore maintained at reduced temperatures only if pH rises in concert with pKw to maintain a constant OH^-/H^+ ratio, i.e., the quantity of protons needed for neutrality falls as temperature decreases. It is important to understand that a given pH value is not a measure of electrochemical neutrality unless it is related to a specific temperature, because neutrality is also dependent upon the concentration of hydroxyl ions. Hence it is only at 37°C that an extracellular pH of 7.4 yields a "neutral" intracellular pH that is optimal for physiological function. In other words, if pH does not rise as temperature is lowered there will be a relative excess of protons despite an apparently "normal" pH value. The disturbance of intracellular neutrality by the accumulation of H^+ during hypothermic ischemia not only has a profound effect upon macromolecular structure and poisons the active sites of enzymes, but also causes metabolites to lose their charged state, and therefore are able to diffuse down concentration gradients as nonionized lipophilic molecules. The depletion of important metabolites for the regeneration of high-energy phosphates during reperfusion is therefore exacerbated by relative acidity. The Donnan equilibrium responsible for maintenance of transcellular ion gradients and cellular water content is also dependent upon electrochemical neutrality within the cell.

At the systemic level, a failure to understand that it is the OH^-/H^+ ratio that is the critical determinant of protein structure and enzyme function, rather than the pH value *per se*, has been responsible for much misunderstanding and controversy about acid-base management during hypothermia.[116,117]

2.4.4 Acid-Base Regulation During Hypothermia

It has been established in ectothermic (cold-blooded animals), and in the blood of warm-blooded animals cooled in a closed system that does not permit gas exchange, that pH rises in parallel with the neutral point of water (pN) during cooling in the range 0°C to 40°C (the Rosenthal relationship[118]). Figure 2.7 shows that the rate of change of pH with temperature is –0.0157 pH units/C° and is referred to as alpha-stat pH regulation in recognition of the fact that both intracellular pH and blood pH buffering is dominated by the degree of ionization (α) of the imidazole moieties of proteins (See References 117, 119).

On the basis of both *in vitro* and *in vivo* experiments it has been generally accepted that acid-base regulations in nearly all vertebrates are consistent with primary regulation of α-imidazole resulting in a stable OH^-/H^+ ratio and the observed change in blood pH with temperature (Figure 2.7). Figure 2.7 also shows that intracellular pH is close to neutrality and closely parallels the rise in pN, whereas in ectotherms and mammalian blood *in vivo*, body fluid pH is maintained at higher levels, i.e., it is more alkaline, than intracellular pH. The purpose of maintaining an alkalotic extracellular milieu may be to provide the cell with a proton-sink for the acidic products of its metabolism. Although this pH management strategy is the most prevalent in the animal kingdom,[120] an alternative process has evolved in hibernating mammals: their metabolism and metabolic function

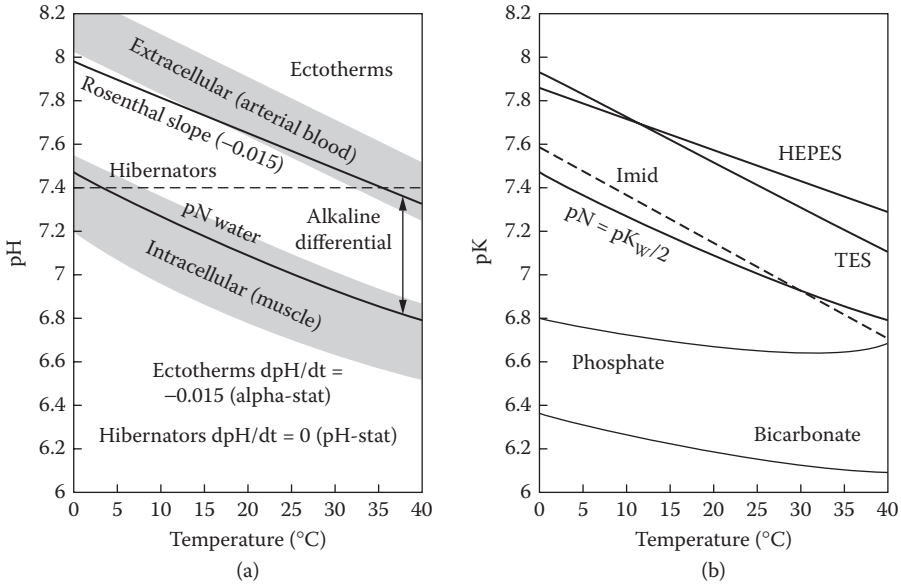

FIGURE 2.7 The relationships between pH and pK as a function of temperature relative to acid-base control in biological systems during hypothermia. Shaded areas show the range of pH values reported for both the blood and intracellular fluid of ectotherms (cold-blooded animals) as a function of body temperature. The blood of warm-blooded mammals including man falls within this range when cooled without gas exchange. The pH change of blood during cooling parallels that of the neutral point of water (pN) and the temperature coefficient is given by the Rosenthal slope. In contrast, hibernators do not maintain a constant degree of alkalinity between the extracellular and intracellular compartments, but instead maintain a constant pH of 7.4 (pH-stat versus α-stat regulation). The importance of buffer capacity relative to preserving electrochemical neutrality during cooling is discussed in the text. In contrast to the physiological buffers — phosphate and bicarbonate that do not retain their relative buffer capacity during cooling — the imidazole (Imid) group of histidine and synthetic buffers, such as HEPES* and TES,** are effective buffers over the entire temperature range due to the fact that their temperature coefficients (dpK/dt) parallel that of water (pK_w = dissociation constant of water). (Adapted and extended from various sources including References 119, 236.) *Note:* *N-2(Hydroxyethylpiperazine)N-2-ethanesulfonic acid **N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

continue at a body temperature as low as 5°C and they maintain arterial pH at 7.4, irrespective of systemic temperature (pH stat regulation).

A detailed discussion of these pH-regulatory phenomena is beyond the scope of this chapter, but the implications of the choice of strategy that will deliver optimum protection during clinical hypothermia — or hypothermic preservation of isolated organs for transplantation — has been addressed in numerous publications over the past few decades (for examples, see References 117, 120–123). Although these considerations have sometimes led to a controversial debate, there are persuasive arguments with supporting evidence in favor of the alpha-stat strategy. Again, examples from studies on exquisitely sensitive ischemic tissues such as heart and brain are of particular interest. It has been shown in numerous studies that the electrical stability, contractility, and hemodynamics of the heart is better preserved during hypothermia when the α-stat scheme is adopted, as opposed to constraining pH to 7.4.[117,123,124] Concerns for brain protection during hypothermic cardiopulmonary bypass have led to studies of the effect of pH and temperature on cerebral metabolism and cerebral blood flow with the latter being appropriately maintained during α-stat management.[125] In a direct comparison of the α-stat versus pH-stat management of dogs subjected to 60 minutes of cold (17°C) ischemic circulatory arrest, it was demonstrated that the α-stat strategy resulted in better protection of ischemic tissues than in those animals whose pH was maintained at 7.4 throughout. Improved protection was manifest as a better cardiac output, twice the cerebral

blood flow, lower peripheral resistance, and a significantly better postischemic ventricular performance on rewarming.[126]

Strategies of pH management for optimum hypothermic preservation of isolated organs have not yet been studied extensively, but if α-stat regulation proves to be generally beneficial to maintain a constant degree of alkalinity between the extracellular and intracellular compartments to preserve electrochemical neutrality during cooling, the question of appropriate pH buffers needs to be carefully considered. Figure 2.7 illustrates that the intrinsic physiological buffers phosphate and bicarbonate do not retain their relative buffering capacity during cooling, due to the temperature coefficients of their dissociation constants (dpK/dt). In sharp contrast, the imidazole group of the amino acid histidine retains effective buffer capacity over the entire temperature range because its dpK/dt is closely aligned with that of water. In synthetic systems such as manmade organ preservation media, or hypothermic blood substitutes, other buffers are available with equivalent or greater temperature coefficients to that of the pK_w for water. For example, the aminosulfonic acid buffers introduced by Good et al.[127] have been shown to possess superior buffer capacity and temperature coefficients for applications involving hypothermia.[127–130] For example, the zwitterionic aminosulfonic acid compound HEPES* has found widespread *in vitro* use as a biocompatible buffer and the close match of its pKa/dt with that of water and imidazole is illustrated in Figure 2.7. Moreover, the aminosulphonic acid buffers may be a better choice than imidazole/histidine for preservation solutions since imidazole has been criticized as being too reactive and unstable to be a satisfactory biological buffer and has been shown to uncouple electron transport from phosphorylation.[131] Moreover, we have recently shown unequivocally that the ampholyte HEPES provides superior buffering capacity and efficiency compared with histidine for pH regulation in the critical range of pH 7.0–7.8.[128] The efficacy of improved buffering capacity using aminosulfonic acid buffers such as Bicine and Tricine as additives to UW organ preservation solution has also been demonstrated to improve the metabolic status of hypothermically stored livers.[132] In this study Bicine and Tricine provided a higher buffer capacity and greater protection than histidine.[132] HEPES has been documented to be highly effective in combating the alterations in acid-base homeostasis of ischemic hearts.[133,134] We have demonstrated the effectiveness of a variety of these sulfonic acid buffers in cryobiological applications[130,135–137] and consideration of their use *in vivo* has recently been advocated.[67,138–140]

Control of acidosis in the cells of preserved organs will be impacted by the exchange of protons and buffer species between the intracellular and extracellular compartments. With respect to external buffering power and intracellular pH, Garlick et al. addressed the question of whether protons produced within ischemic cells are transported to the extracellular space. Such an export process would slow down as extracellular pH decreased. They hypothesized that if the external pH was maintained by increasing external buffering, the proton export could continue longer, thereby reducing the fall in pH_i. They provided some support for this hypothesis using hearts perfused with Krebs-Henseleit supplemented with HEPES.[134] Clearly, intracellular pH (pH_i) can be influenced by external buffers if there is exchange of intracellular buffer species such as phosphate and/or protons. Moreover, there is the possibility that external buffers can permeate into the intracellular space and thereby directly act as pH_i buffers. As far as I am aware, there have been very few specific studies to examine the permeation of buffer species into cells. However, relatively small organic compounds such as histidine (155 daltons) and the aminosulphonic acid buffers (approximately 160 to 230 daltons; e.g., BICINE = 163 daltons and HEPES = 238 daltons) might be expected to permeate during prolonged hypothermic exposure. There is recent evidence that much larger molecules such as the disaccharide trehalose (342 daltons) can permeate into cells as a result of hypothermia-induced phase changes in the plasma membrane.[141]

Many of the reported studies purporting to examine changes in pH_i have relied upon NMR, but Lareau et al. have cautioned about the interpretation of intracellular pH changes based upon

* N-2(Hydroxyethylpiperazine)N-2-ethanesulfonic acid

^{32}P NMR spectroscopy because inorganic phosphate leaks from cells during prolonged hypothermic storage. For this reason, it is not always possible to follow the time course of pH_i from the chemical shift of the Pi peak.[142]

2.4.5 Effect of Hypothermia on the Generation of Free Radicals

The emerging role of oxygen derived free radicals (ODFR) in tissue injury and its participation in reperfusion injury is mentioned above. Important questions that arise in the context of cold storage of tissues and organs include whether free-radical–mediated tissue injury proceeds during cold ischemia, and what effect temperature reduction has on the processes of free radical generation and the mechanisms of tissue injury. Fuller and Green and their colleagues have considered these questions; their work provides a basis for the summarizing statements recorded here.[143]

In essence, it is recognized that cooling increases the susceptibility of cells to produce free radicals and attenuates the natural defense mechanisms by which cells normally deal with the low-level free radical production in metabolism. Due to a lower activation energy, free radical reactions are depressed less by temperature reduction than by the enzymatic processes used to scavenge them. As outlined above, the highly reactive radicals can react in a nonenzymatic way with a variety of biomolecules, including membrane lipids, proteins, and nucleic acids in order to become energetically more stable species and, in the process, generate other radicals at the site of attack. A chain of such reactions can be established, especially when catalysts are present in the form of species capable of redox cycling, i.e., shuttling between oxidation states by accepting and donating electrons. Transition metals such as copper and iron — which are fundamentally important in cell metabolism as intrinsic components of enzyme systems like the cytochromes of the mitochondrial electron transport chain — are effective catalysts of such radical chain reactions. Loss of homeostatic control of transition metals during cold ischemia is therefore a potential hazard to cells because of the promotion of free radical production and subsequent tissue injury during either cold storage or reperfusion. It is the balance between production and removal of free radicals that is crucial to the cell such that a combination of excessive radical generation and hindrance of normal defense mechanisms can redirect the processes in favor of injury. There is evidence that some of the natural defense mechanisms against free radicals become depleted during cold storage, and that the addition of natural pharmacological scavengers including SOD, catalase, or mannitol to cold storage media may improve the viability of hypothermically stored organs.[143,144]

Although it is possible for the production of injurious free radicals to be enhanced during cold storage, it is important to appreciate that the resultant cell damage may not occur entirely at the low temperature. On the contrary, there is a growing body of evidence that reintroduction of oxygenation via a regular blood supply upon rewarming and reperfusion provides a powerful impetus for further oxidative stress. A principal pathway is the stimulation of enzymically driven radical reactions such as the xanthine/xanthine oxidase system involving the interaction of ATP catabolic products with molecular oxygen as discussed in an earlier section and depicted in Figure 2.4. Vascular endothelial cells are thought to be particularly vulnerable to the type of injury mediated by free radical generation due to this so called “respiratory burst” mechanism. Nevertheless, it is known that low concentrations of molecular oxygen such as what is dissolved in organ preservation solutions is sufficient to support the generation of free radicals during prolonged storage.[143] Therefore, it is recognized that cold exposure may set the stage for a progressive development of tissue injury as a result of reactions and processes that occur during hypothermia, but which fuel changes that proceed for considerable time after normal conditions of temperature and oxygen tension are resumed.

Unrestricted bursts of free radical activity are known to cause membrane damage to a variety of cellular components due to the hazards of lipid peroxidation. While cells employ a number of repair mechanisms to recover from this type of injury, cell survival depends upon whether slavage pathways are overwhelmed or whether a point of irreversible damage is reached during the

storage/reactivation process so that cell death becomes inevitable. During the transition from reversible cell injury to irreversible cell death, calcium is strongly implicated as an important link between free-radical-mediated changes and eventual cell necrosis. It is clear from the foregoing discussions that in tissues exposed to cold there is potential for unbalanced free radical reactivity and elevated intracellular free calcium levels, and subsequent warming and reperfusion is likely to greatly potentiate these adverse events.

Evidence linking cold ischemic damage and oxidative stress has come from studies involving a variety of tissues, most notably the heart, kidney, and liver.[143] In recent years, strategies aimed at circumventing oxidative stress and reperfusion injury under both hypothermic and normothermic conditions have understandably been focused upon the roles of antioxidants and calcium channel blockers. An example in which this approach has been adopted is the development of the "Carolina Rinse Solution" designed specifically to improve the preservation of cold-stored livers by protecting against reperfusion injury.[145,146] Other studies have indicated that antioxidant supplementation of existing cold storage media such as UW solution may be advantageous in preserving organs with increased oxidation stresses sustained from suboptimal donors in clinical practice.[147–149] Nevertheless, a clear picture for unequivocal benefits of using agents such as antioxidants and calcium channel blockers in a clinical setting has yet to be established. Some insights into the cellular and molecular mechanisms of cold storage injury with an emphasis on oxidative stress have recently been reviewed by Rauen and DeGroot.[150]

2.4.6 Structural Changes

The interrelationship between structure and function is fundamentally important for cellular homeostasis, which is governed by an intricate array of biochemical, physiological, and biophysical processes. Moreover, these processes are compartmentalized within the cell such that the structure of biological membranes and the cytoskeleton are integral components of cell viability and vitality.

The sensitivity of biological structures to temperature change is well known and the thermal denaturation of proteins in particular is well documented. Thus, most proteins, as well as nucleic acids and many polysaccharides, are able to exist in their biologically active states only within a limited temperature range that is characteristic of the macromolecule and its environmental conditions such as ionic strength and pH. While a great deal more is known about heat denaturation of proteins at elevated temperatures, there are well described examples of cold denaturation involving the spontaneous unfolding of proteins or dissociation of the multi-subunit structure into biologically inactive species, which may or may not reassemble on rewarming to normal temperatures.[151,152]

It was mentioned earlier in the discussion of metabolic uncoupling that membrane components are also affected by cooling, such that membrane properties relating to both the selective diffusion barrier to solutes and active regulation involving membrane-associated proteins (e.g., ion transporters) are altered by cooling. The effect of cooling on the thermophysical properties of membrane lipids is generally regarded as the fundamental basis of temperature effects upon membrane structure-function relationships.

In essence, it is well established that it is the fluid mobile lipid phase of membranes that supports their functional orientation and conformational movements, including that of integral or transmembrane proteins. During cooling, individual phospholipids undergo an abrupt change from a disordered fluid, or liquid crystalline state, to a highly ordered hexagonal lattice (gel state) at a specific transition temperature. Biological membranes comprise complex mixtures of lipids such that sharp transitions between the liquid crystalline and gel phase do not occur, but rather the membrane exhibits a phase transition temperature range. Moreover, compositional characteristics such as the cholesterol:phospholipid ratio in the membrane can serve to broaden or "smooth out" the phase transitions. In a past review of the effects of cooling on mammalian cells, Fuller provided a concise yet informative synopsis of these membrane-orientated effects of reduced temperatures.[153] It has been postulated on the basis of measurements in both model systems and intact cells that a phase

separation occurs within the plane of the membrane as cooling proceeds. Such cold-induced changes in the degree of membrane fluidity render it thermodynamically unfavorable for membrane proteins to remain in the gel phase, such that they may be redistributed laterally into regions of low-melting-point lipids that remain in the liquid crystalline phase. This process is thought to result in packing faults due to the development of lipid-rich and protein-rich microdomains in a membrane undergoing phase transitions. One consequence of this could be a change in membrane permeability and alteration in the solute barrier function of the membrane.[154] Phase separation of mammalian membranes have been demonstrated at temperatures in the region of 10°C and below,[155] and this correlates with the trend towards increased permeabilities to both ions and even large molecules such as proteins during cooling.[156] In addition to phase separations another form of cold-induced membrane damage includes the actual loss of membrane phospholipids, which is intuitively more deleterious than phase changes that may largely be reversible.

2.4.6.1 Thermal Shock

It has been discovered in some cells that the rate of cooling is also a determinant of injury. The phenomenon, which is not well understood, is referred to either as thermal shock, cold shock, or chilling injury. The phenomenon is distinct from the well-characterized effects of cooling rate in frozen cells (see Chapter 8). The pure effects of cooling, in the absence of solute concentration changes or ice formation, are thought to be related to the thermotropic properties of cell membranes involving phase transitions as discussed earlier.[157] It has been proposed that rapid cooling in the absence of freezing can cause mechanical stresses on membranes induced by differential thermal contraction.[18]

2.4.6.2 Stress Proteins

The change in the structure of a protein is a common response to stress. Cells accumulate incorrectly folded proteins as a consequence of stresses such as hypoxia or temperature change. Proteins that experience unfolding or conformational changes do not remain soluble and are transformed into denatured proteins. It is now well established that one of the defense mechanisms adopted by cells to counteract such effects of stress is to synthesize new proteins commonly referred to as "stress proteins" or "heat shock proteins" (HSP) because of their increased synthesis by many cell types after exposure to elevated temperatures.[158] The general role of this group of highly conserved stress proteins is believed to be homeostatic in that they protect the cell against the harmful consequences of traumas and help promote a quick return to normal cellular activities once the stress has terminated. The principal mammalian HSPs are classified into families on the basis of their molecular weights and sequence homologies, and include the 8 kD protein ubiquitin, small HSPs (20–28KD); HSP 60; HSP 70; and HSP 90.[159] The HSP 70 family is the most widely studied. These proteins appear to bind to denatured and unfolded proteins and prevent further aggregation and precipitation. Some HSP 70 proteins are always present in cells and have been assigned a role as "molecular chaperones" because they mediate the correct assembly and folding of proteins. The general protective function of "stress" proteins is also thought to be mediated by their chaperone-like ability to associate with other proteins in the cell and modify their function and fate.[160]

The induction of stress proteins in response to cold (cold shock proteins) has been identified in invertebrate species such as bacteria and yeast.[161,162] Until recently it was unknown whether similar families of cold shock proteins are induced in mammalian cells. However, the contention by some that "recovery of cells from cold temperatures does not result in synthesis of specific shock proteins"[153] will clearly need to be reevaluated in the light of recent evidence demonstrating that cold shock induces the synthesis of stress proteins in a variety of mammalian cells[163–165] and organs.[166] Moreover, the natural response to heat stress by synthesis of "shock proteins," a phenomenon referred to as thermotolerance, has been explored as a potential interventional strategy

to protect against induced ischemia and reperfusion injury. The idea is to precondition cells to better withstand hypothermia by previous exposure to a less severe stress, or sublethal dose of the same stress. It has been demonstrated in both cells and organ systems that "stress conditioning" can induce cold tolerance in mammalian systems.[167–169] Cytoprotection is achieved by way of a complex adaptation in cellular metabolism analogous to that seen in natural thermotolerance. A dominant metabolic change associated with hyperthermia-induced cytoprotection is the increased expression of heat-shock genes. Recent studies have confirmed in diverse animal models that the levels of heat shock proteins are increased manyfold in heat-shocked animals,[168,169] and that the stress conditioning led to significantly improved functional recovery and decreased cell death in various tissues after subsequent cold ischemia. It is particularly noteworthy that in each of these mammalian models a positive temporal association was demonstrated between the functional protection of stress conditioning and enhanced expression of inducible heat-shock proteins.

2.4.6.3 Cytoskeleton

Cold induced changes of cellular structural proteins that constitute cytoskeletal components such as microtubules have been known since the mid 1970s. This cold sensitivity appears to be mediated by depolymerization of component polypeptide units[170] and, in general, is readily reversible upon rewarming.[171] Nevertheless, the possibility exists for reassembly of the microtubules in a way that results in cellular abnormalities.[172]

2.4.6.4 Apoptosis vs. Necrosis in Cold-Induced Cell Death

Injured or dying cells exhibit characteristic changes in cell morphology and, as described above, the culmination of the deleterious events comprising the ischemic cascade is characterized by marked structural changes in cells and eventual cell death. It is now known that there are two distinct ways in which cells may die: necrosis — caused by a general failure of cellular homeostatic regulation following injury induced by a variety of deleterious stimuli including hypoxia, toxins, radiation, and temperature changes — and apoptosis, or "programmed cell death," which is a regulated process distinguishable from necrosis by numerous morphological biochemical and physiological features.[173,174] In regard to structural changes, apoptosis involves distinct morphologic features including blebbing of the plasma membrane (but not loss of integrity), cell shrinkage with the formation of apoptotic bodies, chromatin condensation, and internucleosomal DNA fragmentation in a specific nonrandom pattern. These characteristics are distinguishable from necrotic changes that involve degenerative processes such as cell and organelle swelling, rupture of plasma and lysosomal membranes, and a random pattern of DNA degradation.

While many of the diverse stresses known to cause necrotic cell death have also been reported to induce apoptosis in a variety of cells, the role of low temperatures as a possible stimulus of programmed cell death has only recently begun to emerge.[175] The earliest reports appeared in 1990 when it was shown that cultured mammalian fibroblast cells at the transition from logarithmic to stationary growth were killed by brief exposures to 0°C and rewarming at 37°C.[176] The observed cell killing required only a few minutes of hypothermic exposure and the affected cells exhibited characteristics of apoptosis. Moreover, the kinetics of cell death served to distinguish cold-induced apoptosis from the lethal effects of longer-term cold storage (hours or day), and also showed characteristics inconsistent with direct chilling injury, or cold shock.[176] Most recently, we have reported that apoptosis can be detected in a significant proportion of both vascular smooth muscle and endothelial cells subjected to brief (30 to 120 seconds) cooling.[177] As shown in Figure 2.8 for both cell types, apoptosis was not found after exposure to hypothermia in the range +5 to 0°C, but was induced at lower temperatures with a maximum response occurring at –10°C.[177]

There is increasing evidence, therefore, that apoptosis may represent another manifestation of cold injury. However, it is clear that all cells cannot be as susceptible as those used in Nagle's

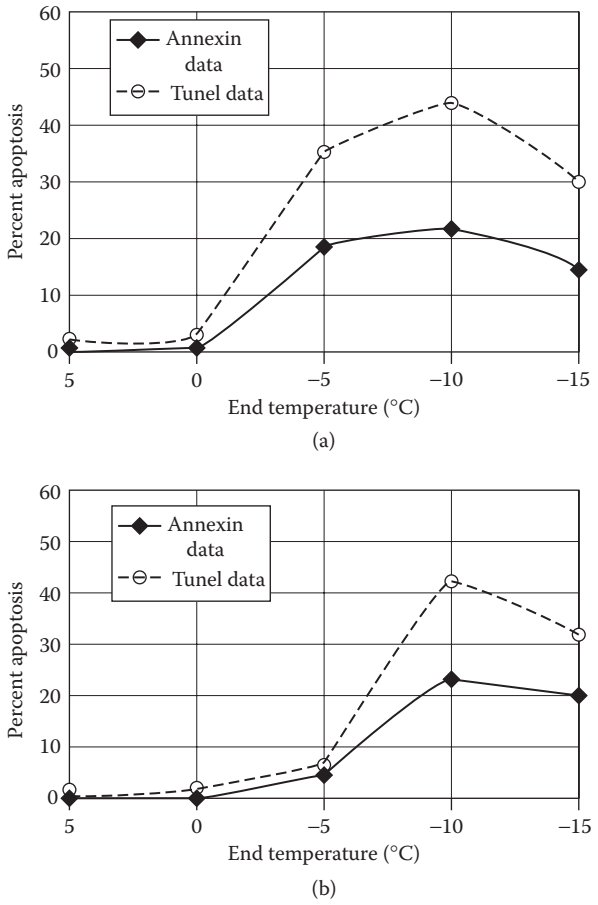

FIGURE 2.8 Extent of apoptosis detected in human coronary artery endothelial cells (a) and arterial smooth muscle cells (b) as a function of exposure temperature. (Data from Tatsutani et al.[177])

study or hypothermic preservation for practical lengths of time would not be possible, which is not the case. Susceptibility to cold-induced apoptosis is likely to depend critically upon the cell growth cycle as demonstrated in Nagle's study, and the temperature of exposure as demonstrated by Tatsutani's study. Recent studies in cultured hepatocytes and liver endothelial cells have indicated that reactive oxygen species might play a key role in mediating cold-induced apoptosis.[178]

It is becoming increasingly more evident that apoptosis plays an integral role in cell death induced by the rigors of both hypothermia and cryopreservation.[175,176,179–181] More specifically, apoptosis has been identified to be directly associated with delayed-onset cell death (DOCD). This is defined as death associated with cold exposure that is not apparent immediately upon rewarming, but extending over the postexposure recovery period.[181] Recent research into the causative apoptotic and necrotic pathways responsible for low-temperature-induced DOCD has identified the contribution of multiple apoptotic pathways, including receptor- and mitochondrial-induced apoptosis.[180,182] Investigations into these pathways, their progression, and their induction stressors has begun to facilitate new methods for improving preservation efficacy through the modulation of the cellular and molecular responses of a cell undergoing preservation (both hypothermic and cryopreservation).[28,67,137,175,180,183,184]

Incorporation of specific apoptotic protease inhibitors in preservation media has now been reported to markedly improve the survival of a variety of cells and tissues.[182,183,185,186] Furthermore, investigation into the modification of the carrier medium from that of standard

extracellular-type culture media — with or without cryoprotectants — to that of specifically designed intracellular-type preservation solutions, such as Hypothermosol[28,67,187,188] and Unisol,[189,190] have led to studies showing significant improvement in preservation efficacy.[181,189,191–196] In a series of independent studies based upon the HypoThermosol formulation, the hypothesis that cell preservation in "Intracellular-type" solution experience reduced hypothermic stress during cryopreservation was tested and yielded higher cell survivals.[181] Moreover, the new generation of solutions has been shown to provide better long-term hypothermic preservation compared to the industry standard (UW-ViaSpan). For example, human liver cells after just three days storage in UW at 4°C die within three days of return to physiological conditions. In marked contrast, cells stored in HypoThermosol solutions survive and propagate from three to five days later. We have demonstrated similar comparisons with a variety of other tissues and cell types stored in Unisol at 4°C.[189,190] Moreover, when experimental inhibitors of apoptosis were added to HypoThermosol, human liver cell lines survived up to eight days at 4°C, compared to only 24 hours for cells stored in ViaSpan.[197]

These and several ensuing studies have provided strong evidence in support of this concept, which has now opened the door to a new approach to modulating preservation injury by focusing on molecular mechanisms associated with cold-induced cell death. Even before this latest flurry of studies that elucidate the molecular mechanisms of cellular demise — and thereby identify ways of circumventing the problems — some of the principles were embodied in the rational design of the baseline media. More specifically, the designs of hypothermic blood substitutes such as HypoThermosol, and the newer formulation Unisol, both incorporate some components that possess recognized antioxidant activities and hence implied anti-apoptotic activity.[28,67,187,188] For example, reduced glutathione is a component of both formulations as a multifaceted molecule that is also known to fulfill a natural role in the regulation of apoptosis.[198]

Since apoptosis is proving to be a distinct and significant factor in limiting hypothermic storage of cells and organs, strategies aimed at preventing apoptotic cell death should be pursued. Adding anti-apoptotic compounds, such as LXR-015, to hypothermic storage media has been reported to improve hypothermic preservation of rat liver in EuroCollins solution.[199] A variety of low-molecular-weight compounds have now been identified and used to either enhance or inhibit programmed cell death such that apoptosis is now amenable to pharmacological intervention.[200]

Once a cell has been exposed to a death stimulus one of two main pathways becomes activated: the intrinsic mitochondrial pathway or the extrinsic death receptor pathway.[201] The progression of apoptosis can be halted at numerous checkpoints along the apoptotic pathways. Two key areas of research that have proven successful in extending the life of cells in culture involve the inhibition of mitochondrial dysfunction and the inhibition of caspases. Apoptosis may be inhibited through the genetic engineering of mammalian cells to over-express an anti-apoptotic gene (such as the Bcl-2 gene[202,203]), or through the addition of a chemical inhibitor to the environmental medium — whether it be a culture medium or a preservation solution. As an alternative to pharmacological intervention, it has been suggested that gene therapy could provide the means to modify grafts such that they become less susceptible to preservation injury associated with apoptosis.[202] This novel approach involves transfecting murine livers with a recombinant adenovirus vector encoding the Bcl-2 gene to reduce apoptosis during the preservation time (the Bcl-2 gene product has been shown to promote cell survival by inhibiting the process of apoptosis[203]). Grafts expressing Bcl-2 showed significant reduction of enzymes associated with liver damage compared with control grafts.[202] However, the genetic engineering approach is less amenable to practical intervention for the purpose of improving biopreservation.

2.5 INTERVENTIONAL CONTROL OF THE EXTRACELLULAR ENVIRONMENT TO OPTIMIZE PRESERVATION

This section outlines the cellular effects of hypothermia in relation to an ischemic or hypoxic insult. Hypothermia is the bedrock of all useful methods of preservation and this has proven to be most effectively applied by controlling the extracellular environment of cells directly — and the intracellular environment indirectly — during cold exposure. It is beyond the scope of this chapter to deal with the strategies that have been adopted to maximize the benefits of cooling for tissue and organ preservation, but some salient points will be offered as a prelude to discussion in ensuring chapters. Moreover, other authors have reviewed in detail the approaches that have been taken to design interventional techniques for hypothermic preservation.[20,204–206]

It is mentioned above that control of the extracellular environment of cells to optimize preservation may be based on one of two strategies: static cold storage, or flush preservation, and low-temperature continuous perfusion. A third technique, involving gaseous perfusion (retrograde oxygen persufflation), also exists, but it remains largely experimental.[204,206]

2.5.1 Flush Cold Storage

It will be apparent from the foregoing discussion that these different strategies call for different approaches to interventional control of the extracellular environment in order to optimize preservation. In principle, flush preservation is based on the premise that reducing temperature so that it is near — but not below — the ice point (0°C) precludes the need to support metabolism to any significant extent, and that the correct distribution of water and ions between the intracellular and extracellular compartments can be maintained by physical rather than metabolic means. Since during the period that metabolic pumps are inactivated the driving force for transmembrane ion flux is the difference in ionic balance between intracellular and extracellular fluid, and driving force for water uptake (cell swelling) is the impermeant intracellular anions, these changes can be prevented or restricted by manipulating the extracellular environment to abolish the chemical potential gradients. On this basis a variety of flush, or organ wash-out solutions, have been devised and evaluated for cold storage; these are often referred to as "intracellular-type" solutions due to their resemblance *in some respects* to intracellular fluid.[20,204,206]

The principal design elements of the intracellular-type flush solutions have been to adjust the ionic balance (notably of the monovalent cations) and to raise the osmolality by including an impermeant solute to balance the intracellular osmotic pressure responsible for water uptake. However, the most important factor for the efficacy of cold flush solutions appears to be the prevention of cellular edema by inclusion of impermeant solutes since it has been established that ionic imbalances, especially potassium depletion, are readily and rapidly reversible.[207]

Attention to the biophysical properties of intracellular-type flush solutions to restrict passive diffusional processes has unquestionably led to the development of techniques during the past few decades that have provided the basis of clinical organ preservation. Nevertheless, it is recognized that further optimization of cold flush solutions can be achieved by inclusion of biochemical and pharmacological components that will be effective in counteracting the deleterious effects of ischemia and reperfusion injury. To some extent, this approach has been incorporated in the design of the University of Wisconsin organ preservation solution (UW solution marketed as "ViaSpan"; Barr Labs) which has become the most widely used solution for cold flush preservation of kidneys, livers, and pancreata.[104,105,208] (See Chapter 8 for additional discussion on the rational design of hypothermic blood substitutes and storage media).

With due consideration for the effects of ischemia, hypoxia, hypothermia, and reperfusion injury on cells, coupled with the proven efficacy of various existing organ preservation solutions, a general consensus of the most important characteristics in the design of hypothermic storage

TABLE 2.1
Desirable Properties of a Hypothermic Preservation Solution or Blood Substitute

1. Minimizes hypothermically-induced cell swelling
2. Prevents expansion of the interstitial space (especially important during perfusion)
3. Restricts ionic imbalances
4. Prevents intracellular acidosis
5. Prevents injury from free radicals
6. Provides substrates for regeneration of high energy phosphate compounds during reperfusion

Based on Belzer and Southard[104]

solutions has emerged. These are summarized in Table 2.1 based upon the exposition of Belzer and Southard.[104]

2.5.2 Continuous Hypothermia Perfusion Preservation

The desirable properties of hypothermic solutions outlined in Table 2.1 are also applicable to controlling the extracellular environment by way of continuous perfusion techniques. In contrast to static cold storage, continuous perfusion is usually controlled at around 10°C and is based upon a different principle: it is generally assumed that a moderate degree of cooling will reduce metabolic needs but that continuous perfusion is required to support the suppressed metabolism and remove catabolic products. Because it is assumed that sufficient metabolic activity remains to actively regulate a near-normal cell volume and ionic gradients, the perfusates are generally acellular, isotonic, well-oxygenated solutions, having a composition that more closely resembles plasma than intracellular fluid. Such perfusates are therefore designated as "extracellular-type" solutions. They are perfused through the vascular bed of an organ at a pressure sufficient (typically 40 to 60 mm Hg) to achieve uniform tissue distribution. To balance this applied hydrostatic pressure and prevent interstitial edema, oncotic agents such as albumin or synthetic macromolecular colloids are incorporated into the perusates.[204] Substrate support of the remaining metabolism at ~10°C is also an important consideration and it has been shown in several organs that high energy adenine nucleotides can be synthesized during either hypothermic perfusion preservation,[20,95,206] or oxygenated hypothermic reperfusion[209] as discussed in an earlier section above.

In addition to the principal objective of supporting metabolism, continuous perfusion also provides other advantages over flush preservation. These include the washing out of accumulated lactate and protons, which removes the metabolic block on glycolysis. This is thought to be especially beneficial for organs that have suffered prior warm ischemia. Perfusion also facilitates the removal of erythrocytes from the microcirculation and helps to maintain vascular patency during prolonged storage. Continuous perfusion has been shown to provide the best means of achieving prolonged hypothermic preservation (e.g., 3 to 7 days for kidneys[208]), but concerns for damage to the vascular endothelium during prolonged perfusion may be a limiting factor.[210] Despite the apparent advantages of continuous perfusion over the flush techniques, static cold storage remains the method of choice at most clinical transplant centers. This is largely because of its simplicity and convenience over the complexity, expense, and risks of vascular damage associated with prolonged continuous perfusion technology. Nevertheless, in the context of utilizing expanded criteria donor organs to make available more organs for transplantation, machine perfusion technology is considered essential and there is currently a resurgence of clinical interest in these techniques.[211–213] Further discussion of the relative merits of continuous perfusion versus static cold storage is beyond the scope of this chapter but is addressed further in Chapter 9).

2.5.3 Approaches Toward Universal Tissue Preservation

Although it has been experimentally verified that cell metabolism continues at temperatures as low as 10°C and that adenine nucleotides can be resynthesized during hypothermic preservation if appropriate substrates are provided, it is considered unlikely that this level of metabolism can prevent transmembrane ion and water movements: this is due principally to the temperature sensitivity of the active pumps. Hence, some advocates of continuous perfusion have modified the perfusate accordingly by increasing both the K^+ concentration and the osmolality (see Reference 206) Similarly, modification of cold flush solutions can be considered to circumvent some of the identified limitations of that approach. For example, it is described earlier that the lack of support of metabolism during ice-storage can be addressed by raising the temperature of storage, and by incorporating biochemical substrates and raising the oxygen tension to promote adenine nucleotide repletion. Also, the use of pharmacological agents such as inhibitors of 5'-nucleotidase (e.g., allopurinol) has been advocated as a means of averting adenine nucleotide depletion.

Therefore it is apparent that optimum control of the intracellular and extracellular environment of cells during hypothermia depends upon the interaction of a variety of factors that include temperature, oxygen tension, acidity, osmotic pressure, and chemical composition of the perfusion fluid or wash-out solution.

As transplantation science progresses and the demand for effective methods of preservation of an increasing variety of tissues and organs escalates, so the search for a "universal" scheme of cold storage has become an important goal. The modern history of organ preservation since the early work of Belzer's group and Collins's group in the 1960s has shown that the original solutions devised for kidney preservation are ineffective for other visceral organs, such as liver and pancreas.[104,208] The introduction of the UW solution in the 1980s, which has proven to be universally effective for the intra-abdominal organs, has been heralded by its inventors as approaching a general organ preservation solution.[104] Moreover, the improvements of UW solution over other organ storage solutions, notably Collins's solution or its variants, have been ascribed to organ-specific differences that demand careful consideration of the choice of components selected to fulfill the objectives summarized in Table 2.1.[104] Nevertheless, UW solution has not proven to be as effective for the intrathoracic organs, heart, and lung as it has for intra-abdominal organs. This is due largely to special demands of highly metabolic tissues such as the myocardium as discussed above.[101]

It is now recognized that the successive phases of the transplantation procedure involving organ procurement, storage, transportation, reimplantation, and reperfusion may impose different requirements for optimum preservation at the different stages. This is illustrated by evidence that heart preservation with the intracellular-type solution, EuroCollins, was enhanced when the heart was initially arrested and subsequently flushed prior to reperfusion, with an extracellular-like cardioplegic solution.[214,215] It is clear, therefore, that any single formulation of preservation solution is unlikely to provide optimum protection during all the processing stages of a transplantation procedure or the interventional stages of complex surgeries, so a combination of appropriately designed solutions may prove necessary. In addition to the basic elements in the design of cold-storage solutions described above, it has been anticipated that inclusion of additional biochemical and pharmacological agents that might counteract the deleterious effects of cold ischemia and reperfusion injury would enhance the cytoprotection properties of preservation media. This strategy has been explored using either newly designed media that incorporate a cocktail of cytoprotective additives such as Carolina Rinse solution,[145,146] or by using existing preservation solutions such as UW, or EuroCollins solution, as a vehicle for the pharmacological additives. For example, the addition of aprotinin to UW and EuroCollins solutions has been shown to increase endothelial cell viability in hypoxic cold storage conditions and led to improved lung preservation.[216] The rationale for such studies was based upon the fact that endothelial cell damage, destabilization of mitochondria and cell membranes, and the release of proteolytic enzymes are known to be associated with organ preservation injury, and aprotinin has anti-proteolytic and membrane stabilizing properties.

TABLE 2.2
Biochemical and Pharmacological Additives* for Preservation Media

Classification	Examples
Anti-platelet aggregation/vasoactive agents	Prostacyclin, PGE1, Mg^{2+}
Calmodulin inhibitors	Chlorpromazine (CPZ), trifluoperazine
Calcium Channel Blockers	Nicardipine, nifedipine, verapamil, CPZ
Protease and phospholipase inhibitors	CPZ, verapamil, calpain antagonists (e.g.AK275)
Anti-oxidants/free radical scavengers	Glutathione, catalase, SOD, allopurinol, dimethylthiourea, vitamin-E (or Trolox), Tempol, magnesium ascorbyl phosphate, Lazaroids
Anti-apoptotic agents	LXR-015, cycloheximide, Z-VAD-FMK, Q-VD-OPH
Iron chelators	Desferroxamine
Membrane Stabilizers	CPZ, Dexamethosone, trehalose
"Cytoprotective" agents	PGE1, glycine
Metabolic Substrates:	
Sugars	glucose, fructose, ribose
Nucleotide precursors (HEP enhancers)	Adenine, Adenosine, Fructose diphosphate, Glyceraldehyde-3-phosphate
Oxygen-carriers	Perfluorocarbons, PEG-hemoglobin
Trophic Factors	Growth factors, nucleic acid derivatives, ribonucleotides, glycosaminoglycans

* Illustrative examples not intended to be a comprehensive list of potentially efficacious additives for preservation solutions.

A wide variety of classes of drugs and biochemical agents have been advocated as potential supplements for improved organ preservation such that complex cocktails of additives might be conceived for optimum organ preservation. Although far from exhaustive, Table 2.2 illustrates the variety of additives that have been suggested to be potential supplements for preservation solutions but it is outside the scope of this chapter to attempt a discussion or review of these strategies. Nevertheless, one important cautionary point that should be emphasized is the potential interaction of components that might be detrimental, rather than beneficial, to outcome. For example, it has been reported that allopurinol and trifluoperazine (see Table 2.2) improve the function of canine kidneys harvested from non-heart-beating donors when used independently as supplements for Belzer's Machine Preservation Medium. However, when used in combination, these additives unexpectedly proved to be detrimental to preservation outcome and are not recommended for combined use in machine perfusion preservation.[217] Optimization strategies for the design of preservation cocktails must not only take account of the possible interactions of additive components, but also their differential effectiveness at various stages of the procurement, preservation, reperfusion, and reimplantation paradigm. Some studies have indicated that drug administration during hypothermic storage has therapeutic benefits for resuscitating tissues after warm ischemia and is more effective than the same drug given *only* during reperfusion.[218] Yet other reports indicate that some drugs, particularly antioxidants, are effective only when present in the reperfusion buffer medium, not in the cold-storage solution.[219] Clearly, an unequivocal strategy for hypothermic solution design has not yet emerged and remains a complex issue impacted by many factors including organ-type, condition, pretreatment, storage interval, temperature, and reperfusion conditions.

2.5.4 Multi-Organ Protection and Total Body Cooling

Interest in general or universal tissue-preservation techniques is exemplified by the need for methods of protecting multiple vital organs, and even the whole body, for applications in modern-day surgery.

Multiple-organ harvesting for transplantation can be optimized by hypothermic perfusion of the whole cadaver, or donor organ blocks comprising several organs (see reference 220), to minimize warm ischemic injury. The ultimate challenge is perhaps protection of the entire body against the effects of global ischemia during periods of circulatory and/or cardiac arrest for "bloodless" surgery.[28,67,139,140]

Surgeons have developed skills that allow very complex, corrective, and life-saving operations to be performed, notably on the heart and brain. As we have reviewed previously,[28] many of these complicated, time-consuming procedures have the inherent need for temporary cessation of blood flow and demand protection of the patient against the deleterious effects of ischemia and anoxia. Although hypothermia is routinely used as an adjunctive protective modality for surgical procedures that require a period of cardiac arrest, there are restrictive time constraints (less than one hour at temperatures usually not lower than 18°C) on the interval of cold ischemia if neurological sequelae are to be avoided. It is well recognized that the window of opportunity for safe surgical intervention could be extended by using greater degrees of hypothermic metabolic suppression, but this becomes unacceptably dangerous, due principally to the effects of profound hypothermia on the blood leading to coagulopathies and irreversible microvascular blockage as discussed above and elsewhere.[28]

The experimental approach we have explored to avoid these complications is to employ a technique of asanguineous blood substitution using acellular synthetic solutions designed to protect the heart, brain, and visceral organs during several hours of bloodless perfusion. The concept of using ultraprofound hypothermia (<10°C) and complete blood replacement is appealing because deeper hypothermia can provide more effective suppression of metabolism, thereby extending the tolerance to ischemia and minimizing the demand for oxygen to levels that can be adequately supplied in a cold aqueous solution without the need for special oxygen-carrying molecules. Complete exsanguination ameliorates the complications associated with increased viscosity, coagulopathies, and erythrocyte clumping of cooled blood. Moreover, vascular purging can remove harmful catabolic products and formed elements that might participate in the ischemia and reperfusion injury cascades. Total exsanguination provides the opportunity to control directly the vascular and extracellular compartments with fluids designed to be protective under the conditions of ultraprofound hypothermia. Solutes can be added to maintain ionic and osmotic balance at the cellular and tissue levels; biochemical and pharmacological additives can help sustain tissue integrity in a variety of ways including efficient vascular flushing, membrane stabilization, free-radical scavenging, and providing substrates for the regeneration of high-energy compounds during rewarming and reperfusion. In essence, these are the principles that are embodied to a greater or lesser extent in the design of various solutions used for *ex vivo* organ preservation and we have adopted similar principles in the design of new hypothermic blood substitutes (HBS).

Our working hypothesis has been *that acellular solutions can be designed to act as universal tissue-preservation solutions during several hours of hypothermic whole-body washout involving cardiac arrest, with or without circulatory arrest.* On this basis, we have formulated and evaluated two new types of solutions designated "Purge" and "Maintenance" HBS that fulfill separate requirements during the asanguineous procedure.[28,66,67,139,140] The principal solution (e.g., HTS/M or Unisol-I in Table 2.3) is a hyperkalemic intracellular-type solution specifically designed to maintain cellular integrity during the hypothermic interval at the lowest temperature. The second solution (e.g., HTS/P or Unisol-E in Table 2.3) is designed to interface between the blood and the maintenance solution during both cooling and warming. This companion solution is therefore an extracellular-type flush solution designed to aid in purging the circulation of blood during cooling since the removal of erythrocytes from the microvasculature is an important objective during ultraprofound hypothermia. The "purge" solution is also designed to flush the system (vasculature and CPB circuit) of the hyperkalemic maintenance solution during warming and possibly help to flush out accumulated toxins and metabolic by-products that might promote oxidative stress and free radical injury upon reperfusion.

TABLE 2.3
Composition of New Hypothermic Blood Substitutes Compared with "Gold Standard" Organ Preservation Solutions

Components (mM l^{-1})	BMPS	UW	HTS/M	HTS/P	Unisol-I	Unisol-E
Ionic						
Na+	100.0	30.0	100.0	141.2	62.5	141.2
K+	25.0	125.0	42.5	3.0	70.0	6.0
Ca++	0.5	–	0.05	1.5	0.05	1.5
Mg++	5.0	5.0	5.0	1.0	15.0	5
Cl–	1.0	–	17.1	111.0	30.1	122.0
SO_4-	–	5.0	–	1.0	–	1.0
pH Buffers						
H_2PO_4-	25.0	25.0	10.0	1.2	2.5	1.2
$HPO_4$2-	–	–	–	–	–	–
HCO_3-	–	–	5.0	25.0	5.0	25.0
HEPES	10.0	–	25.0	25.0	35.0	25.0
Impermeants						
Lactobionate-	–	100.0	100.0	–	30.0	–
Raffinose	–	30.0	–	–	–	–
Sucrose	–	–	20.0	–	15.0	–
Mannitol	30.0	–	20.0	–	25.0	–
Glucose	10.0	–	5.0	5.0	5.0	5.0
Gluconate	85.0	–	–	–	70.0	–
Ribose	0.5	–	–	–	–	–
Colloids						
HES	5%	5%	–	–	–	–
Dextran 40	–	–	6%	6%	6%	6%
Pharmacologics						
Adenosine	–	5.0	2.0	1.0	2.0	1.0
Glutathione	3.0	3.0	3.0	3.0	3.0	3.0
Allopurinol	1.0	7.4	–	–	–	–
Dexamethasone*	–	8.0	–	–	–	–
Adenine	5.0	–	–	–	–	–
Insulin**	40.0	100.0	–	–	–	–
Osmolality (mOsm/Kg)	–	320	360	305	350	315#
pH (t°C)	–	7.4	7.6@	7.6@	7.6	7.5
[K+] [Cl-]	25	0	727	684	2100	732

BMPS: Belzer Machine Perfusion Solution (KPS1 from Organ Recovery Systems). **UW:** University of Wisconsin solution (ViaSpan from Barr Laboratories). **HTS/M:** HypoThermosol/Maintenance (BioLife Solutions). **HTS/P:** HypoThermosol-Purge (BioLife Solutions). **Unisol-I:** Unisol-Intracellular Base (Organ Recovery Systems). **Unisol-E:** Unisol-Extracellular base (Organ Recovery Systems). * = mg/l. ** = U/l. @ = at 25°C. $ = at 0°C

Based upon the principles that have emerged from isolated organ preservation studies, an attempt was made to incorporate the important characteristics in the formulation of the hypothermic blood substitute solutions, and components that might fulfill multiple roles were selected wherever possible. Conceptually, this strategy would maximize the intrinsic qualities of the solutions that, by design as universal tissue-preservation solutions, would inevitably be a hybrid of other hypothermic perfusates and storage-media.

The composition of the new hypothermic blood substitutes is listed in Table 2.3 and the rationale for their formulation is described elsewhere.[28,67,190,221] These solutions have been shown to protect the brain, heart, and visceral organs during 3.5 hours of cardiac arrest and global ischemia in an asanguineous canine model during controlled profound hypothermia at less than 10°C.[28,66,67] More recently, this approach has been applied experimentally to animal models (porcine and canine) of hemorrhagic shock.[139,140,222–224,225] This novel approach to clinical suspended animation (or *corporoplegia,* meaning literally "body paralysis"[226]) has been explored for resuscitation after traumatic hemorrhagic shock in preclinical models relevant to both civilian and military applications.[225,139,222,223] In exsanguinating cardiac arrest (CA) conventional resuscitation attempts are futile and result in 100% mortality.

In prior research studies our collaborators at the University of Pittsburgh introduced the use of cold aortic saline flush at the start of CA to rapidly induce protective hypothermia during prolonged CA (120 min) for hemostasis followed by resuscitation.[222,223] Using a canine model, they showed that a saline flush to a brain temperature of 10°C resulted in normal survival after 90 minutes, but not consistently after CA = 120 minutes. However, an additional study in which Unisol plus the antioxidant Tempol was evaluated as a comparative "optimized flush" showed a markedly improved outcome in regards to physiology, neurology, and brain histology after 120 minutes CA compared with the saline flush.

In separate studies, our collaborators at the Uniformed Services University of the Health Sciences have developed a porcine model of uncontrolled lethal hemorrhage in which a combination of the *Maintenance* and *Purge* solutions were used in a cardiopulmonary bypass (CPB) technique to effect profound hypothermia and prolonged cardiac arrest (60 minutes) with resuscitation after surgical repair of the vascular deficit induced to effect exsanguinations.[139,140] In the most recent study, after rewarming and discontinuation of CPB, pigs were recovered and monitored for 6 weeks for neurological deficits, cognitive function (learning new skills), and organ dysfunction. Detailed examination of brains was performed at 6 weeks. All the normothermic control animals died, whereas 90% of the HBS animals survived and were neurologically intact, displayed normal learning and memory capability, and had no long-term organ dysfunction. Profound hypothermia markedly diminished total body metabolic activity as evidenced by significantly lower buildup of lactic acid during the periods of hypothermia and rewarming. Histologic examination of brains from the HBS survivors after 6 weeks revealed no ischemic damage in any of the animals, in marked contrast to the brains from control animals, which all showed diffuse ischemic damage.[225]

Successful application of this technique to man would provide a greater than threefold extension of the current limits of less than one hour for "safe" arrest without a high risk of neurological complications. This novel approach to bloodless surgery would significantly broaden the window of opportunity for surgical intervention in a variety of currently inoperable cases, principally in the areas of cardiovascular surgery, neurosurgery, and emergency trauma surgery. This provides further evidence for the protective properties of solutions such as Unisol used for global tissue preservation during whole-body perfusion in which the microvasculature of the heart and brain are especially vulnerable to ischemic injury.[28] Moreover, the application of solution design for clinical suspended animation under conditions of ultraprofound hypothermia places the HBS solutions HypoThermosol and Unisol in a unique category as universal preservation media for all tissues in the body. In contrast, all other preservation media, including the most widely used commercial solutions such as UW-ViaSpan are established for specific organs, or groups of organs (e.g., UW for abdominal organs and Celsior, Cardiosol, or Custodiol for thoracic organs).[128,204] Moreover, the demonstrated efficacy of these synthetic, acellular, hypothermic blood-substitute solutions justifies their consideration for multiple organ harvesting from cadaveric and heart-beating donors.[227]

2.6 CONCLUDING COMMENTS

Biopreservation employing hypothermic temperatures and appropriately designed solutions has become a critical component of organ transplant therapy during the era of transplantation science. Hypothermic storage of organs is based upon reductions in metabolic demands and oxygen requirements as temperature is reduced. Four levels of hypothermia have been defined in the medical literature as mild (32° to 35°C), moderate (27° to 32°C), deep or profound (10° to 27°C), and ultraprofound (<10°C).[228] Profound hypothermia has been the most common hypothermic temperature range applied for tissue and organ preservation. Initially, hypothermia is usually applied by flushing organs with cold solution either just prior to removal from the donor or immediately after removal from the donor. The solution is left in the organ vasculature and surrounds the organ during hypothermic storage and transportation to the recipient. In some cases, the solution bathes the exterior of the organ and is actively perfused through the organ's vasculature during hypothermic storage and transport. Traditionally, different solutions are used for flushing and *static* hypothermic storage of organs than are used for *perfusion* hypothermic storage of organs as outlined above and discussed in Chapter 9.

For cell and tissue preservation the history of preservation strategies, particularly with regard to appropriate solution design, is not so well established. There have been very few studies aimed at optimizing solution design specifically for isolated cells, tissues, or engineered tissue constructs. In some cases hypothermic organ preservation solutions have been used, but in general biologists have tended to stick with what they know and have used tissue culture media, even for low-temperature exposure. For the reasons expounded in this chapter this is inappropriate and it is preferable to focus on the formulation of new solutions designed to protect cells and tissues during hypothermic exposure. Similarly for cryopreservation the role of the vehicle solution for cryoprotective additives (CPAs) as a determinant of cell survival has often been overlooked, or trivialized, by assuming that regular tissue culture medium will suffice. There is now substantial evidence that this is not the case and a strategy to design new solutions that take account of the biology of cell survival in the cold clearly leads to improved methods of biopreservation.[136,181,182,187–190,195,196,229–234,9,226]

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