J. Pharm. Pharmac., 1969, **21**, 209–223

REVIEW

Cryoprotectants—a new class of drugs

ARMAND M. KAROW, JR.

Department of Pharmacology, Medical College of Georgia, Augusta, 30902, U.S.A.

Living mammalian tissue can survive at temperatures of −196°. Research in cryobiology (low temperature biology) offers new insights to the role of water in living systems as well as offering a method of suspended animation. Survival of frozen animal cells universally depends upon suitably controlling the cooling and warming rates and, in addition, almost always requires specific treatment of the cells with at least one chemical agent.

Techniques which permit cells to survive freezing and thawing are said to afford the cells with cryoprotection. Chemicals which aid in cryoprotection have been called *cryophylactic agents* (Huggins, 1964, 1965, 1966) and *solute moderators* (Karow & Webb, 1964, 1965a, b). The most preferable is the easily understood *cryoprotective agent* (or *cryoprotectant*) as suggested by symposium participants during the second annual (1965) meeting of the Society for Cryobiology.

PHYSIOLOGY OF FREEZING INJURY

The reasons that animal cells are usually killed by freezing have been adequately discussed in several recent articles (Karow & Webb, 1965b; Meryman, 1966; Robertson & Jacob, 1968) and will only be summarized here.

When water solidifies, it may either crystallize or vitrify. Vitrification, the formation of a glassy state, is achieved by ultra-rapid cooling rates such as may be obtained by allowing water vapour to condense on a surface cooled to −200°. When water molecules are cooled this rapidly, their thermal motion is arrested without sufficient time for organization of the molecules in a crystalline array. In 1937, Luyet predicted that if the water in biological systems could be vitrified, viability of the system could be maintained. Complete vitrification of biological systems may be impossible with currently available techniques. Cooling of biological systems, no matter how rapidly, has always yielded some crystals. At rates of 1 to 10°/min all of the ice is composed of hexagonal crystals. At faster rates the ice may form mixtures of hexagonal crystals with increasing proportions of cubic crystals or vitreous ice or both (Dowell, Moline & Rinfret, 1962; Luyet, Tanner & Rapatz, 1962; Bullivant, 1965). Despite this situation, as a rule, slower cooling rates (1°/min) are usually more conducive to survival than faster rates.

Microscopy of frozen cells or tissues reveals that relatively slow (1°/min or less) cooling produces only extracellular crystals which are relatively large in comparison to the cell. As the cooling rate increases, ice crystals become more numerous, very small, and uniformly distributed throughout the intra- and extracellular spaces. The small intracellular crystals formed during rapid cooling can cause extensive disruption to the cellular ultrastructure and cellular death. The membrane damage

is clearly demonstrated in the published electron micrographs of frozen liver (Trump, Young & others, 1965; Stowell, Young & others, 1965), frog erythrocytes (Rapatz & Luyet, 1961), and mammalian erythrocytes (Rapatz, Nath & Luyet, 1963).

Slow cooling may also result in death of the cells because the large extracellular crystals dehydrate the cells. During the formation of these crystals, the water within the cells is super-cooled and therefore at a higher vapour pressure than the ice (Mazur, 1960). This pressure gradient tends to force water from the cells and thus is incorporated into the growing crystals. As extracellular crystallization proceeds, the intracellular concentration of solutes rises (Lovelock, 1957). An increase in electrolyte concentration alters protein conformation and solubility. The precipitation of sparingly soluble buffer salts leads to pH changes. Biomolecules which normally do not interact because of their spatial separation are brought into close approximation. Also possible is the development of toxic levels of urea, dissolved gases and other metabolites.

In an important study, Meryman (1968) has further elucidated the influence of hypertonic salts on mammalian cells, human erythrocytes being the model. He found that, as expected, cell volumes decrease as the extracellular salt concentration increases, but a minimum volume is attained at four times isotonic saline. When the saline concentration is increased above five times isotonic, the cells begin to swell. This swelling was interpreted as representing damage to membrane integrity. The pressure gradient across the damaged membrane allows salt to enter the cells. When these hypertonic cells are then transferred to an isotonic medium, haemolysis occurs. Experimental evidence obtained by Meryman (1968) corroborates the concept that erythrocytes, after exposure to high extracellular salt concentrations, become more permeable to both sodium and potassium.

Perhaps equally as important is the effect of freezing on cellular "bound" water, i.e. water essential to the proper conformation of biological macromolecules. It is generally appreciated that the loss of "bound" water, results in irreversible denaturation of macromolecules (Klotz, 1958; Kauzmann, 1959; Richards, 1963). Hydrogen-bonded water will form hexagonal lattices similar to those of "typical" ice crystals, whereas hydrophobic interactions will cause water to form pentagonal lattices, similar to cubic ice and characteristic of clathrates. Recent crystallographic studies have provided better than 3 Å resolution of the tertiary structure of four proteins: myoglobin (Kendrew, 1962), egg-white lysozyme (Blake, Koenig & others, 1965), ribonuclease (Kartha, Bello & Harker, 1967), and oxyhaemoglobin (Perutz, Muirhead & others, 1968). The results clearly indicate that the exterior of these proteins is encased in layers of hexagonal water lattices while pentagonal, clathrate-like water structures tend to form interiorly. Calculations by Scheraga (1963) based on thermodynamic considerations indicate that even though H-bonds predominate over hydrophilic bonds as structural and stabilizing forces, the contributions of hydrophobic bonds is significant and may even predominate at specific temperatures.

DNA is greatly influenced by its water of hydration (Jacobson, 1953). Falk, Hartman, & Lord (1962, 1963a, b) have been able to study the location of individual water molecules in the rigid first hydration layer of the DNA helix. Additionally, Falk (1965) has shown that the ionic phosphate groups, located on the surface of nucleic acids, are important hydration sites that predictably interact in hexagonal lattice formation. Theoretical calculation (Sinanoglu, Abdulnur & Kestnor, 1964; Sinanoglu & Abdulnur, 1965) based on spectroscopic evidence indicates that the firmly

bound water associated with the purine and pyrimidine bases has clathrate characteristics and remains unchanged when the system is frozen.

The loss of bound water from macromolecules has been related (Karow & Webb, 1965b) to the observation that slow cooling, rather than fast, is generally more conducive to the survival of frozen cells. Even though during slow cooling some of the free intracellular water is incorporated into the extracellular ice crystals, the loss of bound water may be slow enough to allow sufficient time for simultaneous strengthening and growth of protective intracellular water lattices which surround proteins.

When the temperature is lowered quickly, natural groups of 4 to 6 water molecules tend to function as crystal nuclei and give rise to a fantastic number of minute intracellular as well as extracellular crystals. Time is insufficient for the growth of large extracellular crystals which would free cellular water and thereby inhibit intracellular freezing. Considerations of surface-to-volume ratios indicate that in the competition for water, the numerous and small incipient ice crystals of rapid cooling have a distinct thermodynamic advantage over the relatively bulky water lattices of proteins. Therefore during rapid cooling and freezing the water associated with proteins may be removed and subsequently incorporated in the tiny ice crystals. Even though intracellular ice itself is generally a lethal factor in rapid freezing, if protein and other cellular components are maintained structurally intact (Salt, 1959), intracellular ice formation should not be lethal. Salt (1959) has shown that the fat cells in the larvae of the goldenrod gall fly freeze intracellularly without subsequent harm to the cells of the fly. Also, x-ray diffraction demonstrates ice formation within viable human red blood cells during cooling to −196° at the rate compatible with their survival (Rinfret, 1963). A report by Sherman (1962) casts serious doubt about the intrinsic incompatability of intracellular ice with life. He showed by transplantation that slowly frozen (1°/min) parakeet tumours and mouse skin survived the presence of ice within the cytoplasm and nuclear karyoplasm. Rapid freezing (40°/s) was also employed and uniformly produced drastic decreases in survival rates. The morphologic changes studied by Sherman with light microscopy are in general agreement with the findings of Trump & others (1965).

CHARACTERISTICS OF CRYOPROTECTANTS

Table 1 makes evident that a wide variety of chemicals offer cells cryoprotection. For many centuries man has been aware that living cells under certain (ill-defined) conditions could survive freezing and thawing. The use of chemical agents to prevent death of frozen tissues was first fully recognized by the general scientific community when Polge, Smith, & Parkes (1949) published their historic paper. These investigators discovered that Meyer's histological albumin solution allows avian sperm to survive freezing and thawing from −79°. Further investigation revealed that glycerol was the active cryoprotectant ingredient. In 1950, Smith reported that glycerol would also prevent haemolysis in blood slowly frozen to −79°. Subsequently many other cells were shown to be protected from freezing injury by glycerol.

Spurred by the encouraging reports on glycerol, Lovelock (1954a) found that a variety of other mono- and polyhydric alcohols, sugars, and amides protected human red cells during relatively slow cooling and warming. Workers in Luyet's laboratory thoroughly studied the cryoprotective ability of ethylene glycol (Luyet & Keane, 1952) which had previously been shown to protect chick embryonic heart tissue (Gonzales & Luyet, 1950). Keane (1953) later even demonstrated the protective

ability of urea and acetamide for the embryonic chick frozen heart tissue preparation. Bricka & Bessis (1955) observed that polyvinylpyrrolidone (PVP) as well as dextran, both macromolecular polymers, gave cryoprotection to frozen human erythrocytes.

Ten years after publication by Polge & others (1949), Lovelock & Bishop (1959) introduced perhaps the most versatile of all cryoprotectants, dimethylsulphoxide (DMSO). This substance is as effective as glycerol in preserving most tissues and has the added advantage of rapidly attaining osmotic equilibrium across cell membranes. Since the discovery of the cryoprotective ability of DMSO, numerous other compounds noted in Table 1 have been studied for cryoprotective action.

Table 1. *Selected research reports on cryoprotective ability of various compounds*

Compound	Cell or tissue	Reference
Acetamide	Erythrocytes, human	Lovelock (1954)
	Renal cells, human, cultured	Vos & Kaalen (1965)
	Sperm, bovine	Polge & Soltys (1960)
	Trypanosomes	Polge & Soltys (1960)
	Heart tissue, chick, embryo	Keane (1953)
L-Alanine	Bone marrow, mouse	Phan The Tran & Bender (1960b)
Albumin	Erythrocytes, human	Schreiner & others (1962)
Ammonium acetate	Erythrocytes, human	Meryman (1968)
Chloroform	Heart, dog	Connaughton & Lewis (1961)
Choline	Bone marrow, mouse	Bender & others (1960)
Dextran	Erythrocytes, human	Bricka & Bessis (1955)
		deVerdier & others (1965)
	Bone marrow, mouse	Bender & others (1960)
Diethylene glycol	Erythrocytes, human	Lovelock (1954)
	Renal cells, human, cultured	Vos & Kaalen (1965)
Dimethylacetamide	Erythrocytes	Nash (1962)
	Renal cells, human, cultured	Vos & Kaalen (1965)
Dimethylformamide	Erythrocytes, human	Nash (1962)
	Renal cells, human, cultured	Vos & Kaalen (1965)
Dimethylsulphone	*Renal cells, human, cultured	Vos & Kaalen (1965)
Dimethylsulphoxide	Blood, bovine	Rapatz & Luyet (1965)
	Erythrocytes, human	Huggins (1963)
		Lovelock & Bishop (1959)
	Leukocytes, human	Bouroncle (1967)
	Mitochondria, tomato	Dickinson & others (1967)
	Renal cells, human, cultured	Vos & Kaalen (1965)
	Sperm, bovine	Lovelock & Bishop (1959)
	Cornea, canine and human	O'Neill & others (1967)
	Uterus, guinea-pig	Farrant (1965)
	Heart, rat	Karow & others (1965)
Erythritol	Erythrocytes, human	Lovelock (1954)
	Bone marrow, mouse	Bender & others (1960)
Ethanol	Erythrocytes, human	Lovelock (1954)
	*Renal cells, human, cultured	Vos & Kaalen (1965)
Ethylene glycol	Erythrocytes, human	Lovelock (1954)
	Renal cells, human, cultured	Vos & Kaalen (1965)
	Bone marrow, mouse	Bender & others (1960)
	Skin, mouse, rat	Taylor & Gernstner (1955)
Formamide	Renal cells, human, cultured	Vos & Kaalen (1965)
	*Erythrocytes, human	Lovelock (1954)
		Nash (1962)
	*Heart tissue, chick, embryo	Keane (1953)
Glucose	Blood, bovine	Rapatz & Luyet (1965)
	Erythrocytes, human	Lovelock (1954)
		Strumia & others (1960)
	*Bone marrow, mouse	Bender & others (1960)
	Heart tissue, chick embryo	Luyet & Keane (1952)
	Renal cells, human, cultured	Vos & Kaalen (1965)
	Sperm, bovine	Polge & Soltys (1960)
	Trypanosomes	Polge & Soltys (1960)

Table 1. *continued*

Compound	Cell or tissue	Reference
Glycerol	Blood, bovine	Rapatz & Luyet (1965)
	Erythrocytes, human	Lovelock (1954)
		Jones & others (1957)
		Lovelock & Bishop (1959)
		Pert & others (1965)
	Sperm, bovine	Polge & Soltys (1960)
	Tryanosomes	Polge & Soltys (1960)
	HeLa and L cells	Scherer & Hoogasian (1954)
	Renal cells, human, cultured	Vos & Kaalen (1965)
	Bone marrow, mouse	Bender & others (1960)
	Bone marrow, rabbit	Richards & Persidsky (1961)
	Bone marrow, human	Lochte & others (1959)
	Skin, mouse, rat	Taylor & Gerstner (1955)
	Cornea, rabbit	Capella & others (1965)
	Heart, hamster	Smith (1957)
	Heart, mouse, embryonic	Conway & others (1957)
	Kidney, dog	Halasz & others (1967)
	Mitochondria, rat, liver	Greiff & others (1961)
Glycine	Bone marrow, mouse	Phan The Tran & Bender (1960b)
Hydroxyethyl starch	Erythrocytes, human	Doebbler & Rinfret (1965)
Inositol	Bone marrow, mouse	Bender & others (1960)
	Renal cells, human, cultured	Vos & Kaalen (1965)
Lactose	Blood, human	Rinfret & Doebbler (1960)
	Erythrocytes, human	Strumia & others (1960)
		Rinfret (1963)
	Bone marrow, mouse	Bender & others (1960)
Magnesium chloride	Blood	Rapatz, personal communication
	Heart, rat	Karow & others (1967)
		Barner (1968)
Magnesium sulphate	Heart; rat, guinea-pig, rabbit, dog	Karow & others (1967)
Maltose	Blood, human	Rinfret & Doebbler (1960)
Mannitol	Bone marrow, mouse	Bender & others (1960)
	Renal cells, human, cultured	Vos & Kaalen (1965)
Mannose	Renal cells, human, cultured	Vos & Kaalen (1965)
Methanol	Erythrocytes, human	Lovelock (1954)
	*Bone marrow, mouse	Bender & others (1960)
	*Sperm, bovine	Polge & Soltys (1960)
	*Trypanosomes	Polge & Soltys (1960)
	Renal cells, human, cultured	Vos & Kaalen (1965)
Methylacetamide	Erythrocytes	Nash (1962)
Methylformamide	Erythrocytes, human	Nash (1962)
Methylurea	*Heart tissue, chick, embryo	Keane (1953)
Monoacetin	Erythrocytes, human	Lovelock (1954)
	Renal cells, human, cultured	Vos & Kaalen (1965)
Phenol	*Renal cells, human, cultured	Vos & Kaalen (1965)
Polyethylene glycol	*Erythrocytes, human	Lovelock (1954)
Polyoxyethylene	Erythrocytes, human	Doebbler & Rinfret (1965)
Polyvinylpyrrolidone	Erythrocytes, human	Bricka & Bessis (1955)
		Rinfret (1963)
		Doebbler & Rinfret (1965)
	Bone marrow, human	Persidsky & others (1962, 1965)
	Cartilage, human	Braid & others (1966)
L-Proline	Bone marrow, mouse	Phan The Tran & Bender (1960b)
Propionamide	*Heart tissue, chick, embryo	Keane (1953)
Propylene glycol	Erythrocytes, human	Lovelock (1954)
	Renal cells, human, cultured	Vos & Kaalen (1965)
Pyridine *N*-oxide	Erythrocytes, human	Nash (1961)
	Renal cells, human, cultured	Vos & Kaalen (1965)
Resorcinol	*Renal cells, human, cultured	Vos & Kaalen (1965)
Ribitol	Bone marrow, mouse	Bender & others (1960)
Ribose	Renal cells, human, cultured	Vos & Kaalen (1965)
L-Serine	*Bone marrow, mouse	Phan The Tran & Bender (1960b)
Sodium bromide	Bone marrow, mouse	Phan The Tran & Bender (1960a)
Sodium chloride	Erythrocytes, human	Doebbler & Rinfret (1965)
	*Renal cells, human, cultured	Vos & Kaalen (1965)
	Heart tissue, chick, embryo	Luyet & Keane (1952)

Table 1. *continued*

Compound	Cell or tissue	Reference
Sodium iodide	Bone marrow, mouse	Phan The Tran & Bender (1960a)
Sodium nitrate	Bone marrow, mouse	Phan The Tran & Bender (1960a)
Sodium nitrite	*Bone marrow, mouse	Phan The Tran & Bender (1960a)
Sodium sulphate	Bone marrow, mouse	Phan The Tran & Bender (1960a)
Sorbitol	Bone marrow, mouse	Bender & others (1960)
	Renal cells, human, cultured	Vos & Kaalen (1965)
Sucrose	Blood, bovine	Rapatz & Luyet (1965)
	Blood, human	Rinfret & Doebbler (1960)
	*Erythrocytes, human	Lovelock (1954)
	Bone marrow, mouse	Bender & others (1960)
	Skin, rat	Berggren & others (1966)
	Sperm, bovine	Polge & Soltys (1960)
	Trypanosomes	Polge & Soltys (1960)
Triethylene glycol	Erythrocytes, human	Lovelock (1954)
Urea	*Erythrocytes, human	Nash (1962)
		Doebbler & Rinfret (1965)
	*Renal cells, human, cultured	Vos & Kaalen (1965)
	Heart tissue, chick, embryo	Keane (1953)
L-Valine	*Bone marrow, mouse	Phan The Tran & Bender (1960b)
Xylose	Erythrocytes, human	Lovelock (1954)
	Sperm, bovine	Polge & Soltys (1960)
	Trypanosomes	Polge & Soltys (1960)

* Compound when used under conditions reported in the reference provides less than 40% survival.

Attempts to classify cryoprotectants on the basis of their cellular site of action have been unsuccessful. Some of these agents readily penetrate most cells (glycerol, DMSO) but others such as PVP and dextran must remain extracellular if they do not enter by pinocytosis. Sherman (1963) has presented evidence which even indicates that extracellular glycerol confers cryoprotection to nonfertilized mouse eggs or bull sperm while intracellular glycerol is actually toxic to these cells.

The cryoprotective ability of any given compound varies widely from tissue to tissue. It is well known that glycerol is almost ineffective as a cryoprotectant for bovine erythrocytes, but is a good agent for human red cells (Lovelock & Bishop, 1959). Similarly, human bone marrow frozen to $-80°$ in 15% glycerol has 58% survival, while mouse marrow subjected to the same conditions has only 12% survival (Ferrebee, Billen & others, 1957). The wide differences in tissues and their response to any given cryoprotectant is probably due to an interrelation between physical factors and biological variability. Glycerol is impermeable to some cells (Lovelock & Bishop, 1959) but in others may be absorbed by active transport (Zieve & Solomon, 1967). The rate of passive diffusion of a cryoprotectant across cell membranes is related to the solubility of the compound, its electrical charge, viscosity, and the temperature of administration.

The importance of hydrogen bonding between water and cryoprotectants was probably first recognized by Keane (1953). He believed the freezing of "freezable" water could be prevented by bonding the water to protective agents. Nash (1962) has observed that cryoprotection seems to be related to hydrogen bond acceptor capacity (i.e. the presence of lone-pair electrons on the cryoprotectant rather than electron deficient sites). By the use of the parachor, Nash (1966) has been able to develop a "protection coefficient" (Q) for organic non-electrolyte compounds. All the known protective substances for erythrocytes studied by Nash have a Q

value equal to unity or greater, ranging up to 3·5. It will be interesting to observe if Q can be used to predict new cryoprotectants.

Various investigators have noted a similarity in the types of compounds which afford cryoprotection and those which protect cells from drying and radiation or which protect proteins from thermal denaturation. Webb (1965) believes that the reason glycerol, DMSO, and cyclic polyhydroxy molecules, such as sugars, protect bacteria and viruses from dehydration injury is related to the ability of these compounds to form hydrogen bonds. It is commonly known that radiation damage to proteins in aqueous solutions is due to radiolysis of water with subsequent free radical formation. Glycerol and DMSO protect bacteria, yeast, tissue culture cells, mice, and rats from radiation damage (Vos & Kaalen, 1962; Ashwood-Smith, 1962, 1967). Thrombin is readily inactivated by 50° heat, but the enzyme can be stabilized by glycerol (Milstone, 1942) and by various sugars or their derivatives (e.g. xylose, glucose, methylglucosides, methylgalactopyranosides, sucrose) (Seegers, 1944). Glycerol proved to be the most effective agent. Prothrombin, also thermolabile, is stabilized by some of these polyhydroxy compounds.

Perhaps the mechanism of cryoprotection afforded by various chemicals is related in some manner to the pseudo-toxic effect on cardiac muscle observed independently in the laboratories of Karow (Karow & Webb, 1965a; Karow, Carrier & Clower, 1968), Robertson & Jacob (1968), and Almond, Anido & others (1966). When mammalian heart muscle is treated with glycerol, DMSO, dextran, or magnesium—all of which are cryoprotectants—the muscle ceases to contract. If the cryoprotectant is flushed from the tissue within a given period of time, contractions resume. The observations of Farrant (1964) on the reduction of contractility of smooth muscle (guinea-pig uterus and intestine) subjected to various cryoprotectants (DMSO, glycerol, methyl formamide, methyl acetamide, dimethyl acetamide) may be related to the pseudo-toxic phenomenon. Also perhaps related is the reversible reduction of conduction velocity in the DMSO-treated isolated frog sciatic nerve (Sams, 1967). In contrast to this pseudo-toxic effect, true toxicity is manifested by irreversible cardiac arrest when the muscle is incubated with the cryoprotectant for an excessive period of time or in toxic concentrations.

This pseudo-toxic effect may be a reflection of the interaction of the cryoprotectant with cellular macromolecules, especially proteins, or with the water of hydration surrounding these macromolecules. DMSO is readily bound to proteins of tissue and of plasma by a mole-for-mole replacement of water (Gerhards & Gibian, 1967). Rammler (1967; Rammler & Zaffaroni, 1967) has presented evidence that DMSO produces conformational changes in enzymes. These changes are usually reversible when the DMSO is removed. Of course, DMSO can interact with organized water lattices. It is well known that magnesium, an essential constituent of enzymes, probably plays a role in the conformation of these proteins and is also capable of inducing the formation of water lattices by promoting hydrogen bond formation (Kavanau, 1964). In excess, magnesium inhibits several enzyme systems including ATPase (Liebecq, 1953; Noda, Kuby & Lardy, 1954; Webb, Dodds & others, 1966). Dextran, by virtue of its numerous hydroxyl groups, can form hydrogen bonds and adsorb to proteins. Ordinarily, dextran does not penetrate the interstitial spaces, but in the isolated perfused hearts, capillary integrity fails and presumably even dextran of 70,000 *M* is capable of entering the tissue compartment. Certainly, therefore, dextran might influence membrane phenomena of cells.

The true toxicity of an agent must be considered when selecting a cryoprotectant for a specific tissue. As intimated earlier, the effectiveness and the toxicity of compound varies with tissues within an animal and even for the same type of tissue taken from different species. Two types of toxic phenomena are recognized, the intrinsic toxicity of the compound and non-specific toxic phenomena associated with cryoprotectants in general.

The non-specific cryoprotectant toxic effects on tissues are related to concentration, temperature of administration, and duration of exposure. As expected, when the concentration of cryoprotectant increases, toxic manifestation increases. An increase in cryoprotectant exposure time before freezing, and presumably also after thawing, increases the probability of toxic reactions at a given cryoprotectant concentration (Karow, Carrier & Clower, 1968). Cryoprotectant toxicity is decreased at any given concentration as the cryoprotectant is administered at cooler temperatures. This temperature effect has been demonstrated for DMSO on rat hearts (Karow & Webb, 1965a), rat skin (Holst, Feigl & others, 1966), dog cornea (O'Neill, Mueller & Trevor-Roper, 1967), guinea-pig uterus (Farrant, Walter & Armstrong, 1967), and human leukocytes (Bouroncle, 1967), as well as for glycerol on rat hearts (Karow & Webb, 1965a).

A few preliminary investigations have been made into the efficacy of cryoprotectant combinations. Diamond (1964) obtained 30 to 40% viability in cultures of *Entamoeba invadens* treated with 0·24M glucose plus 2·1M DMSO, slowly cooled, and rapidly warmed from liquid nitrogen temperatures. Strumia, Colwell & Strumia (1960) found that dextrose (0·5 to 1·2M), lactose (0·1 to 0·4M), or a mixture of lactose and dextrose (0·3 to 1·0M) allowed 90% or greater survival of rabbit and human erythrocytes cooled at a rate of approximately 100°/s to −65° followed by an equally rapid warming and thawing. Djerassi & Roy (1963) found that rat platelets treated with DMSO and sugar (dextrose, xylose, sucrose) could be frozen by immersion in liquid nitrogen yet upon thawing retain their morphologic integrity and ability to circulate in thrombocytopenic animals. The use of 5% dextrose plus 5% DMSO in plasma permitted a circulating yield of thawed platelets as high as 70 to 87% of the number observed when fresh platelets were injected. The use of either a sugar or DMSO alone provided slight or no cryoprotection. Other workers (Capella, Kaufman & Robbins, 1965) have found that 0·3M sucrose plus 1·4M DMSO provided better cryoprotection for frozen cornea from rabbit and man than either agent alone. Sucrose (0·3M) alone or in combination with DMSO (1·4M) or with glycerol (1·4M) provided frozen rat skin (Berggren, Ferraro & Price, 1966) with cryoprotection superior to that of DMSO or glycerol. Considering the ability of DMSO to assist the penetrability of compounds which are usually biologically impermeable, Robertson & Jacob (1968) have suggested that the combination of DMSO with other cryoprotectants may give cryoprotective synergism. They provide excellent experimental evidence with DMSO plus sucrose which supports this inviting idea. In my laboratory we (Karow, Webb & Stapp, 1965) have found that 6% dextran (*M* 70,000) in saline and 1·8M DMSO provide good cryoprotection to the rat frozen isolated heart when used individually but no protection when used in combination.

MECHANISMS OF CRYOPROTECTANT ACTION

Several attempts have been made to explain how chemicals are capable of offering cryoprotection. Such theories should account for the specificity of cryoprotectants,

i.e. why specific agents are protective and other similar compounds offer no protection (Doebbler & Rinfret, 1962). One of the first theories was proposed by Luyet and associates (Gonzales & Luyet, 1950; Luyet & Keane, 1952, 1953; Luyet & Gehenio, 1952a, b). They observed that one mm^3 fragments of chick embryo heart could survive rapid thawing after direct immersion into liquid nitrogen (−195°) if the tissue was treated before freezing with hypertonic solutions of ethylene glycol (5·3M), glycerol (3·5 to 7·5M), glucose (1·5M), or sodium chloride (1·0M). The evidence presented by these investigators indicates that partial osmotic dehydration of the tissue is of paramount importance in the survival of the rapidly frozen embryonic chick heart tissue. Other characteristics of the chemicals which they believe contribute to cryoprotection are "easy penetration into the tissue, relatively low toxicity, efficiency in binding water (as expressed in the colligative properties) and low eutectic temperature". These workers observed that air dehydration did not provide cryoprotection to the chick tissue but did protect "vinegar eels" (*Anguillula aceti*) frozen at −77° without the benefit of chemical protection (Gehenio & Luyet, 1951). Luyet & Gehenio (1940) have also provided an extensive review of other animals which survive freezing after partial dehydration.

Unfortunately the dehydration theory does not take into account the specificity of cryoprotectants. Additionally, Lovelock (1953b), on the basis of experimental evidence obtained with human erythrocytes, discredits the importance of dehydration. He found that glycerol provides excellent cryoprotection when the cells are immersed in a cold bath at −35° even though these red cells are only transiently dehydrated by suspension in glycerol (2·4M). They are rapidly restored to their original degree of hydration before freezing as the glycerol permeates the cell. If the red cells are treated with copper they are rendered impermeable to glycerol. Even though the copper-treated erythrocytes dehydrate when exposed to glycerol, the glycerol offers them no cryoprotection. In comparing the results presented by Lovelock (1953b), by Luyet & Gehenio (1952a), and by Luyet & Keane (1953) one must consider the important differences of tissue, rates of cooling, and the fact that some chemicals, including glycerol, apparently provide certain tissues with cryoprotection even though the agent is unable to permeate the cells (Bricka & Bessis, 1955; Sherman, 1963). Certainly it is premature to disregard the possible importance of partial dehydration as a means of cryoprotection.

The cryoprotectant theory offered by Lovelock (1953a, b; 1954a, b) has perhaps received the widest acceptance. In a series of experiments with samples of frozen mammalian erythrocytes, Lovelock (1953a, b) related the rise of ionic concentration with the extent of haemolysis. He demonstrated that the degree of haemolysis produced by freezing erythrocytes is equivalent to the haemolysis of red cells exposed to hypertonic saline at a concentration comparable to that found in frozen normal saline. In the presence of glycerol, the development of damaging concentrations of electrolytes was observed to be greatly reduced. Lovelock (1953b) concluded from his experimental results that the mechanism of protection seems to lie primarily in the colligative properties of the non-electrolyte cryoprotective solute which theoretically lowers the effective concentration of salt in equilibrium with ice at any given temperature below freezing. Thus by adding a cryoprotectant to the cells to be frozen, Lovelock (1953b) believed that the increase in salt concentration during freezing would not reach a biologically damaging level before the temperature was so low that the reaction rate of the injurious processes would be negligible. He

predicted that the ideal cryoprotectant should have a low molecular weight, be non-toxic in excessive concentrations, be highly soluble in aqueous electrolyte solutions, and possess the ability to permeate cells (Lovelock, 1953b, 1954a; Lovelock & Bishop, 1959). Experimental observations on the erythrocyte cryoprotective ability of 15 neutral organic solutes seemed to substantiate these ideas (Lovelock, 1954a).

A major triumph of this theory was its use to predict the cryoprotective capacity of DMSO (Lovelock & Bishop, 1959). Additional independent support for the Lovelock hypothesis is the observation that 10% (v/v) DMSO, a standard protective dose for most tissues, imperfectly protects the smooth muscle of the guinea-pig; but the muscle receives excellent protection from the gradual administration of 55% (v/v) DMSO. In the later instance the organ does not freeze even when cooled to −70°. In fact, uteri treated with high DMSO concentrations could be warmed slowly and still function well after thawing. Also, Meryman (1968) has been able to demonstrate that 4M ammonium acetate, a penetrating, relatively non-toxic salt with no apparent eutectic point, will protect human erythrocytes which have been frozen by slow cooling to −70°, and then thawed. Conversely ammonium chloride, with a eutectic point at −15·8°, is useless as a cryoprotectant for red cells.

Other observations have also been made which tend to discredit the colligative theory. Ethylene glycol (*M* 62) and E500M, a mixture of polyethylene glycols (*M* 635, range: 300 to 1,450), are both cryoprotectants for erythrocytes. One would predict from the colligative theory that ethylene glycol would be 10 times as effective as an equal weight of E500M, but Sloviter (1962) showed that both compounds were maximally effective at 40 g/100 ml. In other laboratories, equal cryoprotection was observed to be conferred to erythrocytes by a 10% (w/v) solution of glycerol or of PVP (Doebbler & Rinfret, 1965) and to mammalian cardiac muscle by 1200 mM DMSO, 66 mM Mg^{++}, or 0·86 mM dextran (Karow & others, 1968).

The colligative theory fails to explain other observations such as the cryoprotection afforded by non-penetrating compounds (see Table 1) such as sucrose, PVP, and dextran. Persidsky & Richards (1962) demonstrated that bone marrow cells are protected by PVP (*M* 30,000) during freezing and thawing. Although these cells are capable of absorbing PVP by pinocytosis, cryoprotection is not dependent upon intracellular PVP. The cells are protected even when the pinocytotic process is inhibited. Finally, the colligative theory offers no explanation of specificity; all low molecular weight solutes, regardless of their cryoprotective ability, reduce electrolyte concentration in equilibrium with ice during freezing. Hydrogen bonding could provide an explanation for molecular specificity in cryoprotection (Doebbler & Rinfret, 1962), but in no way does Lovelock implicate hydrogen bonding in his original theory.

Almost all solutes in water have the capability of retarding ice crystal growth. Furthermore, x-ray diffraction studies made in the Union Carbide Linde Laboratories (Dowell, Moline & Rinfret, 1962; Doebbler & Rinfret, 1965) indicate the presence of vitreous ice in rapidly frozen solutions of known cryoprotectants, but only hexagonal crystals in frozen solutions of structurally similar (but nonprotective) compounds. The growth rates of ice in super-cooled aqueous solutions of a variety of solutes have been positively correlated with the mole-equivalent of potential hydrogen-bonding sites provided by the solutes (Doebbler, 1966). The more hydrogen-bonding sites which a solute is capable of providing, the greater the reduction of the rate of ice crystal growth. Doebbler & Rinfret (1962) have shown that multiple hydrogen-bonding sites per molecule markedly increase protective capacity.

Doebbler (1966) suggested that cryoprotectants act by establishing hydrogen bonds with water, reducing the amount of water available for crystallization and thereby promoting the formation of vitreous ice. Thus cellular sites which would be functionally altered by the loss of bound water are protected since the formation of crystalline ice is avoided. Even though the presence of vitreous ice is yet to be resolved definitely (Luyet, Tanner & Rapatz, 1962), the theory of Dobbler has a great deal of merit.

Other theories place major emphasis on the alteration of protein conformation by ice. One of these theories perhaps may be said to have originated with published thoughts of Luyet & Gehenio (1940, 1952a, b) on the relation between dehydration and freezing injury. In essence, these workers contended that death from freezing was ultimately the result of the irreversible loss of cellular water essential to life, the "bound water". Later, Meryman (1956) noted that since glycerol could bind to water, it would limit the amount of water available for crystallization and thereby reduce the extent of cellular dehydration during freezing. In 1962, Doebbler & Rinfret (1962) re-emphasized the importance of hydrogen binding groups on cryoprotective compounds. They stated that "H-bonding sites would serve not only in primarily binding water, but also in forming and stabilizing an extended region of oriented water around each molecule. Disruption of the hydrated structure of the red-cell membrane could be a possible mechanism of freezing injury in addition to the chemical action of salt or the mechanical action of ice. H-bonding protective solutes may act in part to stabilize the surface hydration of the cell". Independently, Karow & Webb (1965b) arrived at a similar conclusion, i.e. that cryoprotectants, by virtue of their ability to create hydrogen bonds with water, stabilize the hydration lattice surrounding proteins and thereby reduce the probability of protein denaturation by desiccation during freezing. In a sense, cryoprotectants, according to this concept, "prefreeze" the cell to prevent the loss of water which serves to maintain structural conformation.

Although there is much evidence to support the concept that protein denaturation, via desiccation, is an important factor in freeze-injury, there is apparently no definitive experimental evidence to support the idea that hydration lattices of macromolecules are maintained intact during freeze-survival. However, Massaro (1967) has presented evidence which may be interpreted as suggesting that cryoprotectants are capable of preventing protein denaturation by preventing disturbance of the shell of water which hydrates proteins. Lactic dehydrogenase is a protein which is hybridized by freezing (Chilson, Costello & Kaplan, 1965) and also by the hydrogen-bond disrupting compound urea (Massaro, 1967). Cryoprotectants such as DMSO and polyhydroxy compounds not only prevent freeze-induced hybridization but also urea-induced hybridization. Also Doebbler & Rinfret (1962, 1965) have reported that urea decreases the amount of cryoprotection afforded by cryoprotective agents. Of course similar results are also predicted by the theories of Levitt (1962, 1966) and of Heber & Santarius (1964).

Levitt (1962, 1966) has proposed, in contrast to the hypothesis that cryoprotectants act on cellular water, that these agents attach directly to proteins. As water is removed from proteins during freezing, glycerol might attach by hydrogen bonds to the hydrophilic sites on proteins and presumably serve to maintain protein conformation. The glycerol would thereby substitute for the bound water and prevent denaturation by forming a protective coat around protein molecules.

Heber & Santarius (1964) also suggested that organic hydroxy-compounds such as

sugars are cryoprotectants by virtue of their ability to retain water or substitute for water in structures sensitive to dehydration. They noted that monosaccharides have been shown to form unstable, hydrogen bonded complexes with proteins in aqueous solutions (Giles & McKay, 1962). Since water firmly bound to disaccharides prevents them from reacting with proteins in the manner of monosaccharides, Heber & Santarius (1962) proposed that during freezing, a portion of the water bound to disaccharides may be lost and thereby permit disaccharide-protein interaction. They also postulated that even sugars larger than disaccharides, presumably polymeric sugars, might provide protection in a manner similar to the disaccharides.

The theories of Levitt (1962, 1966) and of Heber & Santarius (1964) both suggest that protection results from the stability of the hydrogen bonds established between the cryoprotectant and proteins. These hydrogen bonds do not rupture during the freezing process. Just as in the theories previously discussed, there is no good experimental evidence to support the concepts of these authors. As with other theories, a serious objection is that they cannot explain in an entirely satisfying and convincing manner the mechanism by which exogenously administered high molecular weight compounds (e.g. PVP, dextran) serve as cryoprotectants. Nevertheless, in consideration of the apparent importance of water to protein conformation, it may be expected that evidence will be found to support at least one of the theories that relate chemical cryoprotection to an active and direct maintenance of protein conformation during freezing.

In contrast to these theories, Shikama (1963) has suggested, on the basis of experimental data he obtained from freezing myosin A, myosin B, and catalase, that cryoprotectants stabilize the clathrate-like icebergs on native proteins rather than directly modify ice crystal formation. He observed that although cryoprotectants do reduce the extent of denaturation, they are incapable of altering either the time course or the critical temperature (−10 to −80°) of freeze-induced denaturation. Based on Claussen's (1951) report that some clathrates have a cubic pentagonal rather than a hexagonal framework, Shikama assumes that clathrates around non-polar side chains of amino-acids will also be cubic. Since cubic clathrates have a very low thermodynamic stability, Shikama proposes that their structure might be altered when exposed to hexagonal ice. He noted that several laboratories have reported electron diffraction studies of ice which indicate that only hexagonal crystals form in pure water frozen at temperatures warmer than −80°. Vitreous ice which has been formed by rapid cooling to −180° will transform to cubic ice when warmed to −140°. When further warmed to −80°, the transition from cubic to hexagonal crystals is complete. Shikama believes that the cubic structure of clathrates would be kept stable in the presence of cubic ice. In his experiments, enzymatic inactivation was maximal in the range of −10 to −80° which corresponds to a high yield of hexagonal ice crystals. When he quickly cooled the enzymes to temperatures below this range, the inactivation was minimal. When the enzymes were warmed to any given temperature in the critical range above −80°, the extent of inactivation which occurred coincided with the inactivation obtained by cooling the enzyme directly to that critical temperature.

For Shikama's theory to be seriously considered, its assumptions must be better grounded. All the proteins of known tertiary structure are folded in such a way that clathrate-forming hydrophobic groups are oriented toward the interior of the molecule (Kendrew, 1962; Blake, Koenig & others, 1965; Kartha, Bello & Harker, 1967).

Hydrogen bonding groups compatable with hexagonal ice are found on the "surface" of proteins. Thus hexagonal ice probably might not interfere at all with clathrate-like structures. In any case, by no means do all clathrate hydrates attain a cubic configuration. This is especially true of clathrates around polyfunctional guests (guests with nonpolar groups as well as ionic and hydrogen-bonding sites) such as would be expected of proteins (Frank, 1967, personal communication). Finally, his theory does not explain the well known observation that slow cooling, conducive to hexagonal crystal formation, increases the survival rates of most frozen cells.

Lohmann, Fowler & others (1964) have proposed that glycerol's cryoprotective ability depends upon the chelation of metal ions that are important and necessary to biological activity. They demonstrated by electron spin resonance studies that glycerol forms a complex with certain metal ions including Cu^{++}, and Fe^{+++}. The existence of such complexes in the biological environment would severely limit the ability of glycerol to penetrate cell membranes. The data presented by Lohmann's group (1964), suggests that glycerol would offer cryoprotection when used in concentrations that are lower than those known to be necessary. Perhaps future investigations from Lohmann's laboratory will elucidate further their proposal.

Some of the objections to the discussed theories are obviated if one is willing to concede that it is not necessary to insist upon a single unified comprehensive mechanism of chemical cryoprotection. It may well be true that some—or even all—cryoprotectants operate through more than one of the discussed mechanisms. It cannot be doubted, however, that much more research is required in order to convert the theories of cryoprotection to actual fact.

SUMMARY

Cryoprotectants are a new class of drugs which specifically act to maintain the viability of frozen animal cells. The chemical spectrum of these agents includes certain polyhydroxy alcohols, sugars, inorganic cations, amino-acids, and macromolecules as well as some sulphoxides and amides. All of the cryoprotectants are water soluble. They seem to act by physically or chemically modifying the cellular water before freezing. There is substantial evidence which indicates that cryoprotectants directly interact with the hydration shell of biologically important macromolecules and thereby influencing macromolecular conformation. It is conceivable that future studies on cryoprotectants will not only serve to elucidate their mechanism of action, but also contribute to the design of other types of drugs which generally act by altering the receptor hydration shell. Perhaps even more important will be new insights into the role of water in biological systems.

REFERENCES

ALMOND, C. H., ANIDO, H., SEABER, A. & MACKENZIE, J. W. (1966). *Dis. Chest.*, **50**, 78–80.
ASHWOOD-SMITH, M. J. (1962). *Cryobiology*, **1**, 242–243.
ASHWOOD-SMITH, M. J. (1967). *Ann. N.Y. Acad. Sci.*, **141**, 45–62.
BARNER, H. B. (1968). In *Organ Perfusion and Preservation*, Editor: Norman, J. C., pp. 717–730. New York: Appleton.
BENDER, M. A., PHAN THE TRAN, & SMITH, L. H. (1960). *J. appl. Physiol.*, **15**, 520–524.
BERGGREN, R. B., FERRARO, J. & PRICE, B. (1966). *Cryobiology*, **3**, 272–274.
BLAKE, C. C. F., KOENIG, D. F., MAIR, G. A., NORTH, A. C. T., PHILLIPS, D. C. & SARMA, V. R. (1965). *Nature, Lond.*, **206**, 757–761.
BOURONCLE, B. A. (1967). *Cryobiology*, **3**, 445–455.
BRAID, H. L., HAGERTY, R. F. & LEE, W. H., JR. (1966). *Transplantation*, **4**, 94–99.
BRICKA, M. & BESSIS, M. (1955). *Compt. r. Séanc. soc. biol.*, **149**, 875–877.

BULLIVANT, S. (1965). *Lab. Invest.*, **14**, 1178–1195.
CAPELLA, J. A., KAUFMAN, H. E. & ROBBINS, J. E. (1965). *Cryobiology*, **2**, 116–121.
CHILSON, O. P., COSTELLO, L. A. & KAPLAN, N. O. (1965). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **24** (Suppl. 15), S55–S65.
CLAUSSEN, W. F. (1951). *J. chem. Phys.*, **19**, 259–260.
CONNAUGHTON, P. J. & LEWIS, F. J. (1961). *Surg. Forum*, **12**, 177–179.
CONWAY, H., GRIFFITH, B. H., SHANNON, J. E. & FINDLEY, A. (1957). *Ibid.*, **8**, 596–599.
DEVERDIER, C. H., ERICKSSON, A., HJELM, M. & HOGMAN, C. F. (1965). *Biblthca haemat.*, **23**, 702–708.
DIAMOND, L. W. (1964). *Cryobiology*, **1**, 95–102.
DICKINSON, D. B., MISCH, M. J. & DRURY, R. E. (1967). *Science, N.Y.*, **156**, 1738–1739.
DJERASSI, I. & ROY, A. (1963). *Blood*, **22**, 703–707.
DOEBBLER, G. F. (1966). *Cryobiology*, **3**, 2–11.
DOEBBLER, G. F. & RINFRET, A. P. (1962). *Biochim. biophys. Acta*, **58**, 449–458.
DOEBBLER, G. F. & RINFRET, A. P. (1965). *Cryobiology*, **1**, 205–211.
DOWELL, L. G., MOLINE, S. W. & RINFRET, A. P. (1962). *Biochim. biophys. Acta*, **59**, 158–167.
FALK, M. (1965). *Can. J. Chem.*, **43**, 314–318.
FALK, M., HARTMAN, K. A. & LORD, R. C. (1962). *J. Am. chem. Soc.*, **84**, 3843–3846.
FALK, M., HARTMAN, K. A. & LORD, R. C. (1963a). *Ibid.*, **85**, 387–391.
FALK, M., HARTMAN, K. A. & LORD, R. C. (1963b). *Ibid.*, **85**, 391–394.
FARRANT, J. (1964). *J. Pharm. Pharmac.*, **16**, 472–483.
FARRANT, J. (1965). *Nature, Lond.*, **205**, 1284–1287.
FARRANT, J., WALTER, C. A. & ARMSTRONG, J. A. (1967). *Proc. R. Soc.*, B, **168**, 293–310.
FERREBEE, J. W., BILLEN, I. M., URSO, W. C., THOMAS, E. D. & CONGDON, C. C. (1957). *Blood*, **12**, 1096–1100.
GEHENIO, P. M. & LUYET, B. J. (1951). *Biodynamica*, **7**, 41–52.
GERHARDS, E. & GIBIAN, H. (1967). *Ann. N.Y. Acad. Sci.*, **141**, 65–76.
GONZALES, F & LUYET, B. J. (1950). *Biodynamica*, **7**, 1–10.
GILES, C. H. & MCKAY, R. B. (1962). *J. biol. Chem.*, **237**, 3388–3392.
GRIEFF, D., MYERS, M. & PRIVITERA, C. A. (1961). *Biochim. biophys. Acta*, **50**, 233–242.
HALASZ, N. A., ROSENFIELD, H. A., ARLOFF, M. J. & SEIFERT, L. H. (1967). *Surgery*, **61**, 417–421.
HEBER, U. W. & SANTARIUS, K. A. (1964). *Plant Physiol.*, **39**, 712–719.
HECHTER, O. (1965). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **24** (Suppl. 15), S91-S102.
HOLST, H., FEIGL, P., BROWN, A & LEHR, H. B. (1966). *Cryobiology*, **2**, 315.
HUGGINS, C. E. (1963). *Transfusion*, **3**, 483–493.
HUGGINS, C. E. (1964). *Ann. Surg.*, **160**, 643–649.
HUGGINS, C. E. (1965). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **24** (Suppl. 15), S190–S195.
HUGGINS, C. E. (1966). *Monographs Surg. Sci.*, **3**, 133–173.
JACOBSON, B. (1953). *Nature, Lond.*, **172**, 666–667.
JONES, N. C. H., MOLLISON, P. L. & ROBINSON, M. A. (1957). *Proc. R. Soc.* B, **147**, 476–497.
KARTHA, G., BELLO, J & HARKER, D. (1967). *Nature, Lond.*, **213**, 862–865.
KAROW, A. M., JR., CARRIER, O., JR. & CLOWER, B. R. (1968). *J. Pharm. Pharmac.*, **20**, 297–301.
KAROW, A. M., JR., UNAL, M. O., CARRIER, O., JR., DANIEL, C. R., WEBB, W. R. & HOLLAND W. C. (1967). *Cryobiology*, **3**, 335–340.
KAROW, A. M., JR. & WEBB, W. R. (1964). *Surg. Gynec. Obstet.*, **119**, 609–620.
KAROW, A. M., JR. & WEBB, W. R. (1965a). *Cryobiology*, **1**, 270–273.
KAROW, A. M., JR. & WEBB, W. R. (1965b). *Ibid.*, **2**, 99–108.
KAROW, A. M., JR., WEBB, W. R. & STAPP, J. E. (1965). *Arch. Surg.* **91**, 572–574.
KAUZMANN, W. (1959). *Adv. Protein Chem.*, **14**, 1–63.
KAVANAU, J. L. (1964). *Water and Solute-Water Interactions.* San Francisco: Holden-Day. p. 165.
KEANE, J. F., JR. (1953). *Biodynamica*, **7**, 157–169.
KENDREW, J. C. (1962). *Brookhaven Symp. Biol.*, **15**, 216–226.
KLOTZ, I. M. (1958). *Science, N.Y.*, **128**, 815–822.
KNORPP, C. T., MERCHANT, W. R., GIKAS, P. W., SPENCER, H. H. & THOMPSON, N. W. (1967). *Ibid.*, **157**, 1312–1313.
LEVITT, J. (1962). *J. theoret. Biol.*, **3**, 355–391.
LEVITT, J. (1966). In *Cryobiology*, Editor: Meryman, H. T., pp. 487–494. London: Academic Press.
LIEBECQ, C. (1953). *Biochem. J.*, 54: xxii–xxiii.
LOCHTE, H. L., FERREBEE, J. W. & THOMAS, E. C. (1959). *J. Lab. clin. Med.*, **53**, 117–126.
LOHMANN, W., FOWLER, C. F., MOSS, A. J., JR. & PERKINS, W. J. (1964). *Experientia*, **20**, 290.
LOVELOCK, J. E. (1953a). *Biochim. biophys. Acta*, **10**, 414–426.
LOVELOCK, J. E. (1953b). *Ibid.*, **11**, 28–36.
LOVELOCK, J. E. (1954a). *Biochem. J.*, **56**, 265–270.
LOVELOCK, J. E. (1954b). *Nature, Lond.*, **173**, 659–661.
LOVELOCK, J. E. (1957). *Proc. R. Soc.* B, **147**, 427–433.
LOVELOCK, J. E. & BISHOP, M. W. H. (1959). *Nature, Lond.*, **183**, 1394–1395.
LUYET, B. J. (1937). *Biodynamica*, **1** (No. 29), 1–14.
LUYET, B. J. & GEHENIO, P. M. (1940). *Life and Death at Low Temperatures*, Normandy: Biodynamica, pp. 1–341.
LUYET, B. J. & GEHENIO, P. M. (1952a). *Biodynamica*, **7**, 107–118.

LUYET, B. J. & GEHENIO, P. M. (1952b). *Science*, N.Y., **116**, 526.
LUYET, B. J. & KEANE, J. K., JR. (1952). *Biodynamica*, **7**, 119–131.
LUYET, B. J. & KEANE, J. K., JR. (1953). *Ibid.*, **7**, 141–155.
LUYET, B. J., TANNER, J. & RAPATZ, G. (1962). *Ibid.*, **9**, 21–46.
MASSARO, E. J. (1967). *Biochem. biophys. Acta*, **147**, 45–51.
MAZUR, P. (1960). *Ann. N.Y. Acad. Sci.*, **85**, 610–629.
MERYMAN, H. T. (1956). *Science, N.Y.*, **124**, 515–521.
MERYMAN, H. T. (1966). In *Cryobiology*. Editor: Meryman, H. T. pp. 1–44. London: Academic Press.
MERYMAN, H. T. (1968). *Nature, Lond.*, **218**, 333–336.
MILSTONE, H. (1942). *J. gen. Physiol.*, **25**, 679–687.
NASH, T. (1961). *Nature, Lond.*, **192**, 360–361.
NASH, T. (1962). *J. gen. Physiol.*, **46**, 167–175.
NASH, T. (1966). In *Cryobiology*. Editor: Meryman, H. T. pp. 179–211. London: Academic Press.
NODA, L., KUBY, S. A. & LARDY, H. A. (1954). *J. biol. Chem.*, **210**, 83–95.
O'NEILL, P., MUELLER, F. O. & TREVOR-ROPER, P. D. (1967). *Br. J. Ophthal.*, **51**, 13–30.
PERSIDSKY, M. & RICHARDS, V. (1962). *Nature, Lond.*, **196**, 585–586.
PERSIDSKY, M. D., RICHARDS, V. & LEEF, J. (1965). *Cryobiology*, **2**, 74–78.
PERT, J. H., MOORE, R. & SCHORK, P. K. (1965). *Biblthca haemat.*, **23**, 674–682.
PERUTZ, M. F., MUIRHEAD, H., COX, J. M. & GOAMAN, L. C. G. (1968). *Nature, Lond.*, **219**, 131–139.
PHAN THE TRAN & BENDER, M. A. (1960a). *Proc. Soc. exp. Biol. Med.*, **104**, 388–390.
PHAN THE TRAN & BENDER, M. A. (1960b). *Exp. Cell Res.*, **20**, 651–654.
POLGE, C., SMITH, A. U. & PARKES, A. S. (1949). *Nature, Lond.*, **164**, 666.
POLGE, C. & SOLTYS, M. A. (1960). In *Research in Freezing and Drying*. Editors, Parkes, A. S. & Smith, A. U. pp. 87–100. London: Blackwell.
POMERAT, C. M. & MOORHEAD, P. S. (1956). *Texas Rep. Biol. Med.*, **14**, 237–263.
RAMMLER, D. H. (1967). *Ann. N.Y. Acad. Sci.*, **141**, 291–299.
RAMMLER, D. H. & ZAFFARONI, A. (1967). *Ibid.*, **141**, 13–23.
RAPATZ, G. L. & LUYET, B. J. (1961). *Biodynamica*, **8**, 295–315.
RAPATZ, G. L. & LUYET, B. J. (1965). *Ibid.*, **9**, 333–350.
RAPATZ, G. L., NATH, J. & LUYET, B. J. (1963). *Ibid.*, **9**, 82–94.
RICHARDS, F. M. (1963). *Ann. Rev Biochem*, **32**, 269–300
RICHARDS, V & PERSIDSKY, M. (1961). *Surgery*, **50**, 288–297.
RINFRET, A. P. (1963). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **22**, 94–101.
RINFRET, A. P. & DOEBBLER, G. F. (1960). *Biodynamica*, **8**, 181–193.
ROBERTSON, R. D. & JACOB, S. W. (1968). *Adv. Surg.*, **3**, 75–159.
SALT, R. W. (1959). *Nature, Lond.*, **184**, 1426.
SAMS, W. M., JR. (1967). *Ann. N.Y. Acad. Sci.*, **141**, 242–247.
SCHERAGA, H. A. (1963). In *The Proteins*, Vol. 1. Editors: Neurath, H. and Bailey, K. pp. 477–594. New York: Academic Press.
SCHERER, W. F. & HOOGASIAN, A. C. (1954). *Proc. Soc. exp. Biol. Med.*, **87**, 480–487.
SCHREINER, H. R., ROBBINS, H. D., SAKAIDA, R. R., SHORT, A. J. & RINFRET, A. P. (1962). (abstr.) *Fedn Proc. Fedn Am. Socs exp. Biol.*, **21**, 68.
SEEGERS, W. H. (1944). *Arch. Biochem.*, **3**, 363–367.
SHERMAN, J. K. (1962). *Anat. Record*, **144**, 177–189.
SHERMAN, J. K. (1963). *J. cell. comp. Physiol.*, **61**, 67–84.
SHIKAMA, K. (1963). *Sci. Rep. Tôkohu Univ.*, Ser. IV (Biol.) **27**, 91–106.
SINANOGLU, O., ABDULNAR, S. & KESTOR, N. R. (1964). In *Electronic Aspects of Biochemistry*. Editor: Pullman, B. pp. 301–311. New York: Academic Press.
SINANOGLU, O. & ABDULNAR, S. (1965). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **24**, (Suppl. 15) S12–S23.
SLOVITER, H. A. (1962). *Nature. Lond.*, **193**, 884–885.
SMITH, A. U. (1950). *Lancet*, **2**, 910–911.
SMITH, A. U. (1957). *Proc. R. Soc.* B, **147**, 533–544.
STOWELL, R. E., YOUNG, D. E., ARNOLD, E. A. & TRUMP, B. F. (1965). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **24** (Suppl. 15), S115–S141.
STRUMIA, M. M., COLWELL, L. S. & STRUMIA, P. V. (1960). *J. Lab. clin. Med.*, **56**, 576–586.
TAYLOR, A. C. & GERSTNER, R. (1955). *J. cell. comp. Physiol.*, **46**, 477–502.
TRUMP, B. F., YOUNG, D. E., ARNOLD, E. A. & STOWELL, R. E. (1965). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **24** (Suppl. 15), S144–S167.
VOS, O. & KAALEN, M. C. A. C. (1962). *Int. J. Radiat. Biol.*, **5**, 609–621.
VOS, O. & KAALEN, M. C. A. C. (1965). *Cryobiology*, **1**, 249–260.
WEBB, S. J. (1965). *Bound Water in Biological Integrity*, pp. 120–124. Springfield: Thomas.
WEBB, W. R., DODDS, R. P., UNAL, M. O., KAROW, A. M., JR., COOK, W. A. & DANIEL, C. R. (1966). *Ann. Surg.*, **164**, 343–350.
ZIEVE, P. O. & SOLOMON, H. M. (1967). *Am. J. Physiol.*, **213**, 1275–1277.