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Cryobiology 46 (2003) 205–229

CRYOBIOLOGY

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Review

Protectants used in the cryopreservation of microorganisms☆

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Received 7 October 2002; accepted 7 May 2003

Abstract

The cryoprotective additives (CPAs) used in the frozen storage of microorganisms (viruses, bacteria, fungi, algae, and protozoa) include a variety of simple and more complex chemical compounds, but only a few of them have been used widely and with satisfactory results: these include dimethylsulfoxide (Me_2SO), glycerol, blood serum or serum albumin, skimmed milk, peptone, yeast extract, saccharose, glucose, methanol, polyvinylpyrrolidone (PVP), sorbitol, and malt extract. Pairwise comparisons of the cryoprotective activity of the more common CPAs used in cryomicrobiology, based on published experimental reports, indicate that the most successful CPAs have been Me_2SO, methanol, ethylene glycol, propylene glycol, and serum or serum albumin, while glycerol, polyethylene glycol, PVP, and sucrose are less successful, and other sugars, dextran, hydroxyethyl starch, sorbitol, and milk are the least effective. However, diols (as well as some other CPAs) are toxic for many microbes. Me_2SO might be regarded as the most universally useful CPA, although certain other CPAs can sometimes yield better recoveries with particular organisms. The best CPA, or combination of CPAs, and the optimum concentration for a particular cryosensitive microorganism has to be determined empirically. This review aims to provide a summary of the main experimental findings with a wide range of additives and organisms. A brief discussion of mechanisms of CPA action is also included.

Keywords: Cryopreservation; Cryoprotectants; Viruses; Bacteria; Fungi; Protozoa; Algae

A multitude of factors affect the effectiveness of cryopreservation in microorganisms, for example, species, strain, cell size and form, growth phase and rate, incubation temperature, growth medium composition, pH, osmolarity and aeration, cell water content, lipid content and composition of the cells, density at freezing, composition of the freezing medium, cooling rate, storage temperature and duration of storage, warming rate, and recovery medium [2,23,51,87,95,114,116]. One of the most important conditions is the composition of the medium used to suspend the organisms for freezing. Although a good survival of deep-frozen microbes (bacteria and microbial spores) has occasionally been observed without a protective additive, the presence of a suitable CPA usually increases the survival considerably. The discovery that glycerol and Me_2SO protect eukaryotic cells (including certain microbial cells) against freezing damage [137,200] marked the beginning of modern cryotechnology.

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0011-2240/03/$ - see front matter
doi:10.1016/S0011-2240(03)00046-4

This review will consider only those CPAs that have been more or less successfully applied in cryomicrobiology; additives that were unsuccessful or those used solely in the freeze-drying of microorganisms will be omitted.

Cryoprotective additives

CPAs can be classified in various ways, such as either low-MW or high-MW additives [181]. A more traditional division of CPAs [157] depends upon the rate of penetration: those that penetrate quickly, usually within 30 min, include methanol, ethanol, ethylene glycol (EG),[1] propylene glycol (PG), dimethylformamide, methylacetamide, and Me_2SO; glycerol which penetrates more slowly; and mono-, oligo-, and polysaccharides, mannitol, sorbitol, dextran, hydroxyethyl starch (HES), methyl cellulose, albumin, gelatin, other proteins, polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), polyethylene oxide (PEO), or polyvinyl alcohol which are all nonpenetrating or nonpermeating compounds that cause extracellular cryoprotection when present at concentrations of 10–40%. The permeability of some of these solutes (e.g., glycerol) depends markedly on temperature and cell type, and some penetrating CPAs might be regarded as low-permeable compounds under some circumstances. Moreover, some CPAs penetrate only the cell wall (CW) and not the cytoplasmic membrane (CM). Thus, three categories of additive might be distinguished [247]: (1) CPAs penetrating both CW and CM (Me_2SO, glycerol); (2) CPAs penetrating CW but not CM (mono- and disaccharides, amino acids, polymers with a low MW, e.g., PEG-1000); and (3) CPAs not penetrating even CW (polymers with a higher MW—proteins, polysaccharides, PEO, PEG-6000, dextran, HES, and PVP).

In the following review, CPAs are arranged according to their chemical structure (Table 1). Only the first three or so reports describing the use of a CPA in a particular microbial group are quoted in this review; a more complete bibliography up to 1995 can be found elsewhere [95].

Sulfoxides

Sulfoxides are oxidized thioethers containing one oxygen atom per molecule (the S–O group in the sulfoxide molecule is chemically almost inert) and they are soluble in water in contrast to the parent thioethers. Oxidation of sulfoxides results in sulfones with two oxygen atoms per molecule: dimethylsulfone lacks cryoprotective abilities [150].

Dimethylsulfoxide was introduced into cryobiology as a very effective, rapidly penetrating, and universal CPA. Interestingly, Me_2SO has also radioprotective properties for organisms. It was originally used to cryoprotect red blood cells (RBC) and spermatozoa [137]. Me_2SO has been applied to the cryopreservation of viruses [82,83, 162,259]; bacteria [7,77,182], including rickettsiae [69,94,133,255], mycoplasmas [208], chlamydiae [213], and cyanobacteria [12,50,263]); also fungi, including yeasts [22,43,76,96] and filamentous fungi [19,49,99]; algae [43,173,251,222]; and protozoa [37,56,57,258]. Only chemically pure grade Me_2SO should be used as a CPA [145]. The optimum Me_2SO concentration varies widely, from 1 to 32% (median ~10%). For the preservation of *Anaplasma marginale* in infected bovine RBC the concentration should be as high as 32% [133], whereas in *Dientamoeba fragilis* only 2.75% is required; there is no recovery at Me_2SO concentrations <2.2 or >3.5% [62]. *Entodinium simplex* and *Entodinium caudatum* require 3.9% [141,142], *Leptospira interrogans* 2.5% [192] and *Microcystis aeruginosa* 3.0% Me_2SO [263]. Me_2SO is a better protectant than glycerol or other CPAs for some viruses [180], *Spirillum volutans* [195], *L. interrogans* [192], *Escherichia coli* [223], and *Lactobacillus delbrueckii* [194], methanotrophic bacteria [81], the

[1] *Abbreviations used:* AFP, antifreeze protein; BSA, bovine serum albumin; CM, cytoplasmic membrane; CPA, cryoprotective agent/additive; CW, cell wall; EG, ethylene glycol; FCS, fetal calf serum; HES, hydroxyethyl starch; LN, liquid nitrogen; Me_2SO, dimethylsulfoxide; MW, molecular weight; PBS, phosphate-buffered saline pH 7; PEG, polyethylene glycol; PEO, polyethylene oxide; PG, propylene glycol; PVP, polyvinylpyrrolidone; RBC, red blood cells; saline, 0.85% NaCl in distilled water. Percentage concentrations are given as w/v for solid compounds and as v/v for compounds that are liquid at room temperature.

Table 1
Cryoprotectants used in microbiology

Compound	Formula	MW
Sulphoxides		
Dimethylsulfoxide	$(CH_3)_2SO$	78.13
Monohydric alcohols and derivatives		
Methanol	CH_3OH	32.04
Ethanol	C_2H_5OH	46.07
Polyvinyl alcohol	$[CH_2CHOH]_x$	$2–12 \times 10^4$
Diols and derivatives		
Ethylene glycol	$(CH_2)_2(OH)_2$	62.07
Propylene glycol	$CH_3CH_2CH(OH)_2$	76.09
Trimethylene glycol	$CH_2(CH_2OH)_2$	76.09
Diethylene glycol	$O(CH_2)_4(OH)_2$	106.12
Polyethylene glycol	$H[OCH_2CH_2]_xOH$	$2–400 \times 10^2$
Polypropylene glycol	$H[OCHCH_3CH_2]_x)OH$	$4–40 \times 10^2$
Polyethylene oxide	$(–CH_2CH_2O–)_x$	$3–80 \times 10^5$
Triols		
Glycerol	$(CH_2)_2CH(OH)_3$	92.09
Polyalcohols		
Mannitol, sorbitol, dulcitol	$C_6H_8(OH)_6$	182.17
Monosaccharides		
Glucose	$C_6H_{12}O_6$	180.16
Xylose	$C_5H_{10}O_5$	150.13
Disaccharides		
Sucrose	$C_{12}H_{22}O_{11}$	342.30
Lactose, maltose	$C_{12}H_{22}O_{11}\cdot H_2O$	360.31
Trehalose	$C_{12}H_{22}O_{11}\cdot 2H_2O$	378.33
Trisaccharides		
Raffinose	$C_{18}H_{32}O_{16}\cdot 5H_2O$	594.52
Polysaccharides		
Dextran, mannan	$[C_6H_{10}O_5]_x$	$1–200 \times 10^4$
Dextrin	$(C_6H_{10}O_5)\cdot xH_2O$	
Hydroxyethyl starch		
Ficoll		$7–40 \times 10^4$
Gum arabic (acacia)		25×10^5
Amides, *N*-alkylamides, imides		
Acetamide	NH_2COCH_3	59.07
Methylacetamide	$CH_3NHCOCH_3$	73.09
Dimethylformamide	$(CH_3)_2NCOH$	73.09
Dimethylacetamide	$(CH_3)_2NCOCH_3$	87.12
Succinimide	$NH(CO)_2(CH_2)_2$	99.09
Heterocyclic compounds		
Methylpyrrolidone	$CH_3N(CH_2)_3CO$	99.13
Polyvinylpyrrolidone	$[CHN(CH_2)_4CO]_x$	$3–36 \times 10^4$
Amino acids and carbonic acids		
Proline	$(CH_2)_3NHCHCOOH$	115.13
Glycine	CH_2NH_2COOH	75.07
Glutamic acid	$(CH_2)_2NH_2CH(COOH)_2$	147.13
Aminobutyric acid	$(CH_2)_3NH_2COOH$	103.12
Glutaric acid	$(CH_2)_3(COOH)_2$	132.12
Ammonium acetate	CH_3COONH_4	77.08
EDTA	$(CH_2)_2N_2(CH_2COOH)_4$	292.24
Proteins, peptides, polypeptides, and glycoproteins		
Blood serum, albumins		
Gelatin, peptones		

Table 1 (*continued*)

Compound	Formula	MW
Shell extract		
Glycoproteins, mucin		
Valinomycin	$C_{54}H_{90}N_6O_{18}$	1111.33
Gramicidin	$C_{60}H_{92}N_{12}O_{10}$	1141.46
Complex substrates		
Yeast extract		
Malt extract		
Skimmed milk		
Honey		
Nonionic surfactants		
Tween 80		1309.68
Triton, macrocyclon		

yeasts *Lipomyces starkeyi*, *Saccharomyces exiguus*, and *Candida bogoriensis* [165]; filamentous fungi *Neurospora crassa*, *Sclerospora sorghi*, certain *Pezizales*, *Volvariella volvacea*, and other basidiomycetes [6,32,73,100,132,233], algae *Enteromorpha intestinalis* [118], *Chlamydomonas reinhardtii* [151], and *Porphyra yezoensis* [120], marine microalgae [30,31], and protozoa *Trichomonas vaginalis* [138,167,169], *Tritrichomonas foetus* [167], *Toxoplasma gondii* [61], *Leishmania tropica* [29], *Babesia* spp. [46,88,177], *Naegleria* and *Acanthamoeba* spp. [105]. However, Me_2SO can be toxic to some biological systems: for example, 40% Me_2SO decreased the titre of T4 bacteriophage to 6% [254]. A growth-inhibiting activity of 10% Me_2SO was observed with a number of aerobic bacteria (*Staphylococcus*, *Micrococcus*, *Pseudomonas*, *Streptococcus*, *Lactococcus*, *Corynebacterium*, and *E. coli*), but not in anaerobes [70]. However, many bacteria tolerate very high Me_2SO concentrations without visible toxic effects and some (*Acinetobacter*, *Corynebacterium*, *Bacillus*, and *Streptomyces*) are even capable of multiplication in a growth medium containing 20–45% Me_2SO [67]. A few bacteria, e.g., *Treponema pallidum* [183] or *Chlamydia* spp. [205] and many fungi do not usually tolerate high concentrations of Me_2SO. Infectivity of *A. marginale* frozen in 4 M Me_2SO and held at 25 °C after thawing was destroyed after 96 h [134]. Me_2SO was less toxic than glycerol for *L. interrogans* and *Trypanosoma* spp. [192,258]. No marked toxicity of Me_2SO to filamentous fungi [233] or yeasts [96] was described, although the proportion of respiratory-deficient 'petite' mutants of yeasts increased during incubation at 30 °C with 9% or more Me_2SO, but only slightly when yeasts were exposed to 10% Me_2SO for 14 days at 4 °C; at the same time, the lethal effect of Me_2SO on the yeasts was very low, even at 40% concentration [96,271]. Me_2SO is less toxic at 0–5 °C than at higher temperatures and samples to be frozen with Me_2SO should be kept at a low temperature. With *T. pallidum*, and many other organisms, the toxic effect of Me_2SO could be abolished by including 10% or more blood serum but neither bovine serum albumin (BSA) nor gelatin were protective [183]. Toxicity of Me_2SO for some algae (*Chlorella* and *Crypthecodinium*) was detected at concentrations >2.5% [107,173,227]; on the other hand, the toxic effect on marine microalgae (*Chaetoceras*, *Nannochloris*, *Rhodomonas*, *Isochrysis*, *Nannochloropsis*, and *Tetraselmis*) was observed only at much higher Me_2SO concentrations (20–30%); these phytoplanktonic species also tolerated incubation in 20% Me_2SO at room temperature without any apparent loss of viability [30,31]. A slight toxic effect of Me_2SO (in terms of motility, replication, infectivity, or ultrastructure) was reported in some protozoa when they were exposed at room temperature or at 4 °C for 30–60 min: *Babesia* [44,45,193], *Trypanosoma* [58,209], *Toxoplasma* [164], *Tetrahymena* [188], *Trichomonas* [163,168], *Giardia* [160], or *Naegleria* [20]. Unlike *Naegleria*, most *Acanthamoeba* strains were markedly susceptible to 1% Me_2SO [156]. A toxic effect of 3–4 M Me_2SO for *Babesia rodhaini* was only seen after incubation at 38 °C for 1–4 h, but not at 4 °C [45]. In *L. tropica*, the toxic effect of 1.5 M Me_2SO

in growth medium was less detrimental than that of 1 M glycerol [29]. In conclusion, it is advisable to avoid concentrations of $Me_2SO > 15\%$, to shorten the period of exposure of cells to Me_2SO before freezing and after thawing, and to maintain the microbial suspensions during these intervals at a low temperature, preferably in an ice bath, to prevent possible adverse effects of Me_2SO.

Alcohols and derivatives

While polyhydric alcohols, especially glycerol but also glycols and sugar alcohols, have commonly been used as CPAs, the use of monovalent alcohols is comparatively infrequent, probably due to their toxicity for many biological systems as is well known. However, methanol and to a lesser extent ethanol, can be surprisingly effective, with a low toxicity, for some prokaryotic and eukaryotic cells.

Methanol is as effective a CPA as Me_2SO or glycerol for some cryosensitive strains of *S. cerevisiae* [115,131] and it seems to be the CPA of choice for the liquid nitrogen (LN) refrigeration of certain cyanobacteria and algae [12,41,116,175] and protozoa [116,202]. In these applications, it has been used at concentrations 2–10% (median 5%). For instance, 10% methanol was the only effective CPA for the LN refrigeration of *Euglena gracilis* [175] and was very effective for the cryoprotection of the anaerobic bacteria *Chloroflexus* [140], *Methylomonas*, *Methylococcus*, and *Methylocystis* spp. It was equally effective as PVP, but superior to Me_2SO, glycerol, and HES [81]. However it was ineffective in cryoprotecting diatoms [153]. Methanol has a very high rate of permeability, markedly surpassing that of Me_2SO as demonstrated in algal cells recently [41,246]. It is toxic for marine microalgae at the concentrations >5%, but *Tetraselmis chuii* tolerates >20% [30]. The cryoprotective action of 5% methanol was comparable to Me_2SO in *Nannochloris atomus* and *Nannochloropsis gaditana*, but unlike Me_2SO, it did not protect *Rhodomonas baltica*, *Isochrysis galbana*, *Chaetoceras gracilis*, and *T. chuii* [30]. Methanol was less toxic than either Me_2SO or glycerol to *E. gracilis* [174].

Ethanol, in contrast to glycerol, demonstrated a significant cryoprotective effect when *S. cerevisiae* was cooled rapidly but not when cooling was slow ($3\,^{\circ}C\,min^{-1}$) [131]. Ethanol has been used in cryomicrobiology at concentrations of 2–10% (median 9%). Ethanol was much more toxic and less protective than methanol for *Chlorella*; the microbial toxicity of alcohols generally increases with chain length, while protective ability decreases [176].

Polyvinyl alcohol was less effective than glycerol in protecting frozen *T. foetus* [128]. Satisfactory protection of refrigerated *Pseudoperonospora humuli* and *Plasmopara viticola* sporangia was reported when 10% polyvinyl ethanol was used in a mixture with 10% glycerol; also for the successful cryopreservation of plasmid-bearing *Alcaligenes eutrophus* [11,249].

Ethylene glycol (1,2-ethanediol) has been used as a CPA at concentrations 2–40% (median 10%) for the freezing of microorganisms of certain groups, namely the myxomycete *Physarella oblonga* [74], yeast [147], actinomycetes [117], rumen fungi [216], algae [119], and protozoa [139,218,227]. *Aspergillus flavus* spores survived a rapid cooling very well (94%) in the presence of 40% EG, compared to 4% survival in the control [147]. EG at a concentration of 4 or 10% was more effective than Me_2SO or PG for the cryoprotection of the anaerobic rumen fungus *Piromyces communis* [216] or, in combination with 5% proline, the alga *Eisenia bicyclis* [119]. On the other hand, EG was ineffective for the freeze storage of *Sclerospora* spores [132] and inferior to Me_2SO or glycerol for *Trichomonas vaginalis* [169], *Tetrahymena pyriformis* [228,229], and *Plasmodium chabaudi* [178]. Very good cryoprotection of *Leucocytozoon smithi* sporozoites suspended in 10% foetal calf serum (FCS) was observed in the presence of 2.5–5% of either EG, PG, or trimethylene glycol; the effect was comparable to that of Me_2SO or glycerol [234]. Unfortunately, EG is extremely toxic to some protozoans [227]. A general problem of diols is that they act as solvents for some microbial polysaccharides [181], leading to toxicity.

Propylene glycol (1,2-propanediol) has been used in cryomicrobiology at concentrations 5–10% (median 5%). It protected *S. cerevisiae* [198], *Zoophthora radicans* [217] and the alga *E. bicyclis* [119] very well. In combination with 10% Me_2SO, 5% PG was also very effective in freezing *P. yezoensis* [122].

Ten percentage of PG was effective for *L. smithi* sporozoites [234]. *Actinomyces noursei* was well protected, comparable to glycerol or even better, with 5% of either PG, diethylene glycol or PEG-2,000: it was less well protected by EG [117]. PG has been used in combination with Ficoll and dimethylacetamide for freezing *Ichthyophthirius multifiliis* [65]. Trimethylene glycol (1,3-propanediol) was tested for cryopreservation of *Leucocytozoon* protozoa [234].

Diethylene glycol (2,2-oxydiethanol; 10%) was protective for frozen *Enterobacter aerogenes* [204].

Polyethylene glycol has been used in cryomicrobiology at concentrations 5–45% (median 10%). PEG is available with MWs ranging between 200 and 40,000. The best results in repeated freezing and thawing of *A. noursei* were with MW 1500–3000 [117]. PEG-6000, in combination with 10% Me_2SO, was very effective for freezing the alga *P. yezoensis* [122]. PEG was as effective as Me_2SO, dimethylacetamide, dimethylformamide, PVP, glucose, sucrose, and albumins in protecting *E. aerogenes* rapidly frozen in LN [182,204], and 10% PEG was even better than 5% Me_2SO or 10% glycerol for the cryopreservation of nine mushroom species [187]. However, PEG-4000 and PEG-20,000 were clearly inferior to other additives (Me_2SO, glycerol, and sorbitol) in protecting yeast cultures during repeated freezing and thawing [165]. *Theileria parva* sporozoites were preserved with 5% PEG as well as with 5% Me_2SO, though less well than with 7.5% glycerol [113].

Polyethylene oxide (polyoxyethylene) has been reported to be an effective CPA in cryobiology [60,207]. It has been used in cryomicrobiology at concentrations 5–15% (median 10%). PEO-400 (5–15%) cryoprotected T4 phage as well as 5% Me_2SO, but glycerol and sucrose were harmful [254]. PEO-400 and PEO-4000 were as effective as glycerol in protecting *Staphylococcus aureus*, *Serratia marcescens*, *Shigella sonnei*, *Salmonella typhi*, and *E. coli* [253,273].

Glycerol (1,2,3-propanetriol), together with Me_2SO, has been the most widely used CPA in microbiology. The cryoprotective effect of glycerol was discovered much earlier than is usually stated [200]: Keith [110] observed that an addition of 5–42% glycerol to suspensions of *E. coli* in water permitted long-term survival of this bacterium at −20 °C. Undiluted or 50% glycerol was adopted for routine preservation of pathogenic prokaryotes and viruses at temperatures between 4 and −20 °C long before the 1950s [71,190,237]. Later, glycerol was applied at concentrations of 2–55% (median 10%), for the freezing of diverse viruses [35,162,241]; bacteria [85,90,93,204] including rickettsiae [244] and mycoplasmas [208]; myxomycetes [48], filamentous fungi [21,49,98], yeasts [43,171,250,267]; algae [43,173,251]; and protozoa [72,127,128,232]. Certain filamentous fungi survived freezing better when protected with Me_2SO than with glycerol. Glycerol also had a small or no protective effect for the bacterial genera *Methylomonas*, *Methylococcus*, and *Methylocystis* [81], *Spirillum* [195], *Anaplasma* [197] or the protozoan *T. vaginalis* [138]. On the other hand, glycerol was superior to Me_2SO for *T. parva* [113], *L. interrogans* [238] or the alga *Tetraselmis suecica* [68]. Glycerol toxicity has been observed in *Aegyptianella pullorum* [97], *Chlamydia* spp. [205], *Rhodospirillum rubrum* [90], *Staphylococcus*, *Micrococcus*, *Lactococcus*, *Streptococcus*, *Pseudomonas*, *Corynebacterium diphtheriae*, and *E. coli* [70], *Chlorella* [173], *T. pyriformis* [188], *Trypanosoma* spp. [258], *T. vaginalis* [149,168] or *T. foetus*, where the degree of toxicity was much greater in a citrate solution than in PBS [108,149]. Glycerol was significantly more toxic than Me_2SO to Newcastle disease virus [123], *Anaplasma phagocytophila* [69], *L. interrogans* [192], *Plasmodium* spp. [37], *L. tropica* [29], *Trypanosoma* spp. [258], *T. vaginalis* [138], *T. foetus* [45], *T. gondii* [61], *E. gracilis* [174], and *T. pyriformis* [228]. On the other hand, glycerol has been found to be less toxic than Me_2SO for *B. rodhaini* [45,46], *Trypanosoma congolense* and *Leishmania* [58], marine microalgae *Chlorella marina*, *Chaetoceras calcitrans*, and *Tetraselmis gracilis* [107] or the flagellate *Crypthecodinium cohnii* [227].

Mannitol and dulcitol were found to be inferior to glucose or glycerol for the freezing of *S. cerevisiae* [86], *E. bicyclis* [119], and *T. foetus* [128]. Inositol, at 5% concentration, had little or no cryoprotective effect for *S. cerevisiae* and *S. uvarum* [267].

Sorbitol has been used in cryomicrobiology at concentrations 1–36% (median 9%). It was moderately cryoprotective for the alga *E. intestinalis* [118]

and as effective a CPA as 5% mannitol for *E. bicyclis* [119]. High survival rates of *Lipomyces starkeyi* and *Saccharomyces exiguus* in 10% sorbitol were observed even after 20 freeze–thaw cycles; the survival rate was similar to that of the same cultures suspended in 10% Me_2SO, but greater than with glycerol and PEG, although *Candida bogoriensis* was better protected with Me_2SO than with sorbitol [165]. Sorbitol (2 M) permitted the cryopreservation of cells of *S. cerevisiae* and *Schizosaccharomyces pombe* for electroporation [243] and 3.6% of sorbitol was combined with glycerol (17.5 or 19%) or Me_2SO to attain optimized cryoprotection for *Plasmodium berghei*, *P. falciparum*, *P. gallinaceum*, and *Babesia microti* [80,143,203,220]. A combination of 1 M sorbitol with 15% PVP-40,000 enhanced the survival rate of frozen protoplasts from sporidia of *Ustilago maydis* [63] and 0.5 M sorbitol was used in combination with 10% Me_2SO to cryoprotect the algal genera *Porphyra* and *Tetraselmis* [121,122, 248].

Saccharides and polysaccharides

Glucose has been used in cryomicrobiology at concentrations 1–18% (median 4%). Improved survival of certain bacterial cultures at −20 °C using glucose solutions was described very early [110]. Glucose was effective for T4 phage [240], *A. marginale* (in a mixture with sucrose [94]), *E. aerogenes* [204], yeasts [86,161,250], *Puccinia* spores [21], *P. berghei* in blood [101], *Babesia* spp. (in combination with PVP [46,92]), and *Entamoeba histolytica* [55]. Some strains of cryosensitive fungi like *Phytophthora palmivora*, *Entomophthora exitialis*, *Pythium sylvaticum*, and *Pseudophaeolus baudonii* were cryopreserved in a mixture of 10% Me_2SO and 8% glucose and this mixture was better than 10% Me_2SO alone [233]. Glucose (0.25 M) was toxic to the protozoan *T. pyriformis* at room temperature [188,228,229].

Xylose at a concentration of 5% plus 10% horse serum cryoprotected *Trypanosoma brucei* [202].

Sucrose, at concentrations 1–68% (median 10%), has quite frequently been used for the cryopreservation of microorganisms. The cryoprotective effect of this disaccharide was described by Keith [110] who observed a long-term survival of *Bacillus subtilis*, *B. megaterium*, *Proteus*, and *Micrococcus* spp. cultures when frozen with 10% sucrose at −10 °C. Sucrose was also cryoprotective at various concentrations for viruses [124,221,240], *E. coli* [25,60,211,265], *E. aerogenes* [204], *Lactococcus lactis* ssp. *lactis* [33], *L. delbrueckii* [194], *Methanococcus vannielii* [106], *Chlamydia* spp. [205], *Mycoplasma* spp. [109], *A. marginale* (in combination with glucose [94]), *B. rodhaini* [46]. However it was ineffective for many other microbes including some cryosensitive organisms, such as the cyanobacterium *Spirulina platensis* [245]. Exceptionally, 5% sucrose was reported to protect concentrated starter strains of *L. lactis* ssp. *lactis* better than 10% glycerol when stored at −20 to −70 °C [33]. Sucrose (0.25 M) was toxic to *T. pyriformis* at room temperature [188,229].

Lactose at concentrations 1–10% (median 8%) provided a better protection than glycerol in starter cultures of *L. lactis* ssp. *lactis* stored at −20 to −70 °C [33]. Lactose was also effective for the freezing of *E. coli* [60], *L. delbrueckii* [194], *S. cerevisiae*, but was less effective for *Streptomyces tenebrarius* [43] and ineffective for the cryosensitive cyanobacterium *S. platensis* [245]. A mixture of 5% lactose with 10% glycerol yielded very good results (better than glycerol alone or Me_2SO) with *S. cerevisiae*, *Pseudomonas aureofaciens*, and *S. tenebrarius* [43].

Maltose in combination with 10% glycerol was cryoprotective for *Scenedesmus* spp. algae [43].

Trehalose is a natural CPA, present in plant and yeast cells, and the only disaccharide that has two water molecules in its crystal. It has been used at concentrations of 5–19% (median 10%) as a CPA for certain viruses [84], *S. cerevisiae* [38,59], psychrophilic yeasts [15], *Lactobacillus bulgaricus* [53] and a mycorrhizal fungus [54], although the results with eukaryotic organisms were not very impressive except in the last study. The high internal pool of trehalose in many yeasts (up to 8% w/w) might play a role in protecting the cells during freezing and especially desiccation (it is probably a 'xeroprotectant' rather than a cryoprotectant) and against heat stress [38,64]. The trehalose content of yeast correlated well with viability after drying: when the yeast was grown

anaerobically, its trehalose content and cryoresistance decreased [75]. However, although trehalose is found in high concentrations in the cryoresistant *S. cerevisiae* strains, no direct links between cryoresistance and trehalose content should be made because the cryotolerance was greatly reduced in yeast grown under partially aerobic conditions; these cells were characterized by normal (high) levels of trehalose [75,130].

Raffinose (5%) in combination with 10% glycerol cryoprotected algae *Scenedesmus quadricauda*, *S. brasiliensis*, and *Chlorella vulgaris* [43]. No other trisaccharide has been tested as CPA.

Dextran has been used at concentrations 5–15% (median 9%). It was moderately protective for frozen *E. coli* [5,60,231]. Dextran (5%, MW ca. 500,000) increased the survival of *Pseudomonas* F8 from 2% (control) to 78% when it was deep-frozen in saline [3]. Dextran (5%) was moderately cryoprotective for the alga *E. intestinalis* [118], and in combination with 10% Me_2SO was effective for *P. yezoensis* [122]. Rapid freezing of protozoa in a mixture of dextran and sorbitol has been suggested [125]. The degree of polymerization of dextran can affect its cryoprotective effectivity: in *Pseudomonas* F8, the optimum MW for cryoprotection was 250–1000 kDa while those with MW 20–100 kDa were noncryoprotective [4]. Dextrans are usually nontoxic to microorganisms [4]. A highly cryoprotective dextran-like polysaccharide was detected in *E. coli* [18].

Extracellular polysaccharides produced by the yeasts *S. cerevisiae* and *Hansenula capsulata* (mannan and glucomannan) have been used with partial success to enhance the survival of several yeast strains frozen in LN with 10% Me_2SO or glycerol [17].

Inulin (fructosan) and glycogen (structurally similar to amylopectin) are water-soluble natural CPAs. No significant studies of cryoprotection of microorganisms have been carried out with these compounds. However, a glycogen-like or polyglucose reserve material, accumulated in *E. coli* cells at a variety of growth conditions, protected the cells from freeze–thaw damage [25].

Hydroxyethyl starch (2.5–25%, median 10%) has been successfully used alone or in combination with 50% serum or 3.4% BSA in LN storage of *P. berghei* and *T. parva* sporozoites [91,113,126], *S. cerevisiae* [115] and the methanotrophic bacteria *Methylomonas* and *Methylococcus* spp. [81]; HES was more effective than Me_2SO or glycerol.

Methylcellulose (1%) protected *Micrococcus luteus* and *Staphylococcus epidermidis* at −14 °C better than 15% glycerol [212].

Ficoll, a nonionic synthetic polymer of sucrose, was used at concentrations 5–7.5% (median 6%) as a CPA and was as effective as Me_2SO or PVP for the rapid LN refrigeration of RBCs infected with *A. phagocytophila* [197], and 5% Ficoll in combination with 10% Me_2SO was very effective in freezing *P. yezoensis* [122]. A combination of 10% Me_2SO with 6% Ficoll 400 was the most effective of four media that were tested for LN refrigeration of yeast cultures [230]; however, the results with a medium containing Me_2SO without Ficoll as an appropriate control were not presented, and the actual cryoprotective role of Ficoll has remained uncertain. Ficoll was also used in combination with PG and dimethylacetamide for the freezing of *I. multifiliis* [65].

Gum arabic (gum acacia; 2–10%), a branched polymer consisting of galactose, rhamnose, arabinose, and glucuronic acid, was better than glycerol, Me_2SO, or lactose in cryoprotecting T2 bacteriophage [162] and has also been successfully used for the cyanobacterium *S. platensis* [245].

Amides and imides

Acetamide, dimethylacetamide, and dimethylformamide at concentration of 10% were found to be almost as effective as glycerol and Me_2SO in protecting frozen suspensions of *E. aerogenes*, but formamide (MW 45.04) was much less protective [182]. Acetamide was introduced into cryobiology by Lovelock [136]. It was used at 0.5 or 2% in skimmed milk for freezing the lactic streptococci *L. lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*, and *L. lactis* ssp. *diacetylactis* [77]. A protective effect of acetamide for frozen *T. brucei* was not confirmed [202]. Dimethylacetamide was used in combination with Ficoll and PG for freezing *I. multifiliis* [65].

Succinimide (1.3%) has cryoprotective properties for *Lactobacillus leichmannii* [104].

Heterocyclic compounds

N-Methylpyrrolidone showed a cryoprotective activity similar to glycerol, Me_2SO, dimethylacetamide, dimethylformamide, acetamide, PEG, PVP, and serum albumins in *E. aerogenes* frozen rapidly in LN [182].

Polyvinylpyrrolidone has been frequently used, both in general cryobiology [60,181,182,206] and in cryomicrobiology at concentrations 2–20% (median 10%). PVP was cryoprotective for *E. aerogenes* [182] and the additive of choice (superior to glycerol and Me_2SO, although the effect was not very distinctive) for the Gram-negative anaerobes *Fusobacterium nucleatum* and *Selenomonas sputigena* in a medium containing tryptone and yeast extract [79]. Very good cryoprotective activity was described with 10% PVP-40 (as good as methanol, and superior to Me_2SO, glycerol or HES) when used to protect the methanotrophic bacteria *Methylomonas*, *Methylococcus*, and *Methylocystis* [81]. The protective effect of PVP in *Pseudomonas* F8 (closely related to *P. fluorescens*) and *E. coli* increased with MW to reach maximum cryoprotection at ca. 90 kDa [4,257]. PVP (5%) combined with 3% L-glutamic acid was cryoprotective for *Campylobacter pylori* [189]; 7.5–20% PVP either alone or in combination with 7.5% Ficoll provided excellent protection for *Anaplasma* spp. in infected RBC stored in LN [197,239], and 5% PVP-30 in combination with 10% Me_2SO was very effective for freezing *P. yezoensis* [122]. PVP was inferior to Me_2SO and glycerol for cryoprotecting fungi, especially *Oomycota* [233], but 15% PVP-40 increased the survival of frozen *U. maydis* protoplasts [63]. PVP has been used for the cryopreservation of algae [9,118,173,176] and protozoa [125]: for example, it was as effective as Me_2SO for *T. gondii* cysts stored in LN [102]. For the LN preservation of *T. parva* sporozoites and *Babesia* spp. in infected RBC, 10–20% PVP scored as the best CPA [89,92,113,193,239,256]. The advantage of PVP is its very low toxicity at room temperature for a majority of microorganisms, including fastidious protozoa like *T. pyriformis* for which glycerol, Me_2SO, glucose and sucrose are all toxic, and for fungal protoplasts [63,188]. However, undialyzed PVP was toxic to *Pseudomonas* [4].

Amino acids and carbonic acids

Glutamic acid or sodium glutamate at concentrations of 1–5%, usually in combination with other compounds like glycerol or milk, were effective in cryoprotecting the algal genera *Scenedesmus*, *Chlorella*, *Nitzschia*, and *Phaeodactylum* [43,251].

L-Proline is a natural CPA: it was found to protect the cyanobacterium *S. platensis* [245] and the algae *E. intestinalis* and *E. bicyclis* [118,119].

Ammonium acetate at 0.1 M protected T4 bacteriophage during freezing in 0.1 M sodium bromide and was more effective than sucrose, glycerol, Me_2SO, or glucose at the same molar concentrations [240].

Peptides, proteins, and glycoproteins

Serum albumins have been used as CPAs at concentrations of 0.1–4% for a long time, especially for viruses and rickettsiae [14,82]. Human serum albumin was as protective as Me_2SO for measles virus frozen at −65 °C [83]. With *E. aerogenes*, human albumin and ovalbumin were comparable to Me_2SO and glycerol in their cryoprotective effects [182]. Serum albumin was found to be moderately protective for freezing *E. coli* [60] and *Mycobacterium leprae* [129]. The cryoprotective effect of 0.5% BSA for *L. interrogans* was confirmed and was greater than that of 5% Me_2SO, but lower than that of 10% glycerol [238]. The cyanobacterium *S. platensis* was successfully frozen in the presence of 2–4% of either ovalbumin, BSA, casein hydrolysate, or gelatin [245]; skimmed milk and casein were ineffective. The optimized cryopreservation protocol for *T. gondii* combined 4% BSA with 12.5% Me_2SO [13].

Inactivated blood sera from various vertebrate species (calf, horse, sheep, human, rabbit, and chicken) have been incorporated into freezing media, usually at 10–20% concentration and often combined with other CPAs, for the refrigeration of viruses [82,83,162,259], some bacteria [127,154,224,238] including chlamydiae [205,213], mycoplasmas [109,185] and cyanobacteria [245], yeasts [96], filamentous fungi [146], and protozoa [10,127,201]. In addition to a cryoprotective effect, the blood

serum or serum albumin might protect the cells against possible toxic effects of glycerol, Me_2SO, or other CPAs during the freeze–thaw treatment. Defibrinated blood itself is a CPA mainly because it contains serum. Especially some blood protozoa (trypanosomes and intraerythrocytic parasites) used to be cryopreserved in plain defibrinated blood prior to the advent of CPAs [36].

Gelatin is a weak to moderate CPA [60,181, 242], and it is mostly used to supplement other, more specific CPAs. The protective activity of gelatin at a concentration of 0.5–15% (median 2%) has been proved for *E. coli* and the cyanobacterium *S. platensis* [245].

Various kinds of peptone (protein hydrolysates containing peptides, amino acids, and inorganic salts, but devoid of lipids and sugars) protect microorganisms during freezing and thawing when used at a concentration of 0.4–20% (median 0.75%). For cryoprotective purposes, those peptones with a low salt content and ash values $<10\%$ w/w (such as peptone bacteriological, peptone mycological, and proteose peptone) are usually best. Peptones are often used as a diluent for microbes that are to be frozen; they offer additional cryoprotection with other CPAs, but their actual protective effect has rarely been examined [47,229,262,266]. Bacteriological peptone (10%) significantly increased the survival of frozen T4 bacteriophage (two distinct peptide fractions were responsible for the effect), *Pseudomonas* sp. and *S. cerevisiae* [47,241].

A prawn shell extract (a protein with a low MW [66] extracted from the surface of *Metapenaeus eusis*) was markedly cryoprotective for *Vibrio cholerae*, *Pseudomonas aeruginosa*, *E. coli*, and *S. marcescens* in the presence of Mg^{2+} (the divalent cation was important for the protective activity); the protective effect of this extract on *V. cholerae* frozen at $-20\,^{\circ}C$ for 72 h was ca. 100 times higher than that of glycerol, Me_2SO, and bovine serum or BSA [225].

Natural antifreeze proteins (AFP) and glycoproteins occur in many species of fish, insect, or plant and prevent their body fluids from freezing [261]. The natural AFPs have rarely been used in cryopreservation of microorganisms. A chimeric AFP expressed in *S. cerevisiae* fused with a chemically synthesized gene was found to increase twofold the survival of the yeast after rapid cooling in LN [152]. Extracellular glycoproteins produced by *Rhodosporidium toruloides* and *L. starkeyi* yeasts (mannose and glucose prevailed in the hydrolysate; the protein constituted ca. 6%) were used to enhance the survival of several yeast strains frozen in LN with 10% Me_2SO or glycerol [17]. In further studies, the cryoprotective effect of exoglycoproteins from *R. toruloides* and *Dipodascus australiensis* (mannose and glutamic acid were prominent components in the latter) was confirmed in a number of psychrophilic yeasts [15,16].

Mucin (a purified extract from bovine submaxillary gland or porcine stomach) contains more carbohydrates than glycoproteins, in the form of glycosidic esters of disaccharides (aminoglycids) bound to the polypeptide chain. Bacterial suspensions containing 5% mucin retained their viability when stored frozen for >2 years [272].

Ionophores gramicidin (a linear polypeptide complex consisting of glycine, alanine, leucine, valine, and tryptophan) and valinomycin (a cyclic peptide consisting of valine, hydroxyisovaleric acid, and lactic acid) increased the cryoresistance of *E. coli* to slow cooling and warming in the presence of EDTA [28]; they affect the potassium and sodium gradients in the cell.

Complex compounds

Yeast extract contains (w/w) ca. 11% total nitrogen, 3% phosphate, 12% ash, 1% salt, and vitamins (nicotinic acid, riboflavin, and other compounds). At concentrations 0.25–5% (median 0.5%), it was found to be as good as glycerol or Me_2SO, and superior to many other CPAs (sucrose, casein, egg albumin, glutamate, and apple juice) for the cryoprotection of lactic acid bacteria [7,104,235]. Yeast extract was included in the freezing medium as a supporting CPA for yeasts [96,230] and protozoa [229] with good results.

Malt extract usually contains (w/w) ca. 52% maltose, 20% glucose, 15% dextrin, 6% other carbohydrates, and 5% protein. It has been used at concentrations 0.5–20% (median 2.5%) with good results as a protective medium for preserving lactic acid bacteria in LN [104]. As a supporting CPA, malt extract was also used in yeasts [17,96,230] and filamentous fungi [260].

Skimmed milk (nonfat milk solids) at a concentration of 1–10% (median 10%) has often been used for the cryopreservation, but even more frequently in the freeze–drying, of many microorganisms, sometimes in combination with other CPAs [42,78]. Keith [110] described the cryoprotective effect of milk on *E. coli* when frozen at −20 °C. *Mycobacterium tuberculosis* suspended in milk remained 100% viable for at least one year after storage at −70 °C [112]. Skimmed milk was used for the cryopreservation of *L. interrogans* [224], mycoplasmas [109], *Pasteurella multocida* [264], and lactic acid bacteria [39,40,77,196]. Milk with glycerol was effective in the cryoprotection of phytopathogenic bacteria [172] and fungi [42,249]. An almost equal protective effect of 10% skimmed milk with 10% glycerol was found with frozen yeasts *S. cerevisiae*, *Debaryomyces hanseni*, and *Klyuveromyces marxianus* [219]. Semen samples diluted in milk, containing *T. foetus* and maintained at −79 °C, revealed viable trichomonads 4 months later [155]. Skimmed milk has also been useful in the cryopreservation of *Tetrahymena* [229]; for the long-term storage of human herpesvirus at −70 °C it was a better CPA than rabbit serum, egg yolk, allantoic fluid, or PBS [236].

Egg yolk has been favored for cryopreservation of the pathogenic rickettsiae *R. provazekii* and *R. typhi* [14].

Trypticase–soy broth (peptone soya) differs from other peptones considerably in that it contains also carbohydrates (ca. 14% w/w). This sort of peptone has been used at concentrations of 0.5–5% (median 1.75%) as a diluent in several cryopreservation studies; cryoprotective effects of this broth without any other specific additives were found in *S. cerevisiae* and *Streptomyces tenebrarius* [43], where it was comparable to 5% Me_2SO or glutamate.

Honey (10%) was better than glycerol in cryoprotecting *Acetobacter* and *Gluconobacter* spp. [87,269,270].

Spent growth medium (a filtrate of stationary culture) added to the freezing medium protected *E. coli* cells against death by repeated freezing and thawing; the filtrate was effective even at a 10^{-5} dilution and lost its influence when heated in the presence of alkali [191].

Surfactants

The nonionic detergents Tween 80 (polyoxyethylene sorbitan monooleate), Triton WR-1339 (Tyloxapol, alkyl aryl polyether alcohol) and Macrocyclon (PEG ether of octylphenol formaldehyde) protected *E. coli*, *E. aerogenes* and *B. subtilis* from freezing damage almost as effectively as glycerol but only at high rates of cooling and at low cell densities [24,25,27]. When *E. coli* suspended in saline was frozen rapidly and thawed slowly, the survival was only 3%, whereas it increased to 92% when 1% Tween 80 was added: this prevented damage to the CM [26]. Tween 80 enhanced the protective effect of glycerol for *Puccinia graminis* urediniospores stored in LN [49]. Tween 80 has sometimes been added to cooling media as a dispersing agent but its cryoprotective role has remained obscure [104,111,112].

Cations

Viruses are noncellular organisms and differ from other microbes in their requirements for the composition of the freezing medium. For instance, Mg^{2+} and Ca^{2+}, when added to PBS, play a major role in the cryopreservation of certain viruses [221], while they are usually harmful for eukaryotic microorganisms in that they can cause osmotic injury at the eutectic point. However, some halophilic microorganisms need Na^+, K^+, or Mg^{2+} for the best survival after freezing [31,52].

Mixtures of cryoprotectants

CPAs can interact with each other in mixtures, or with crucial cell molecules, thereby producing effects other than those that would occur with individual CPAs [215]. One compound in a mixture may dominate the other(s) or they may combine to produce additive or synergic effects: it has been observed that the protective effect of combinations of CPAs can be greater than one would expect if the action of each agent were simply additive. It is often advisable to combine the use of rapidly penetrating and nonpenetrating (or slowly penetrating) agents in the cryoprotection of microbial cells, such as 10% Me_2SO or glycerol or methanol with

5% glucose or sucrose, lactose, maltose, raffinose, sorbitol, methyl cellulose, PEG-6000, and PVP. Even three CPAs may be combined, for example Me_2SO with glucose and PEG [43,55,116, 202,215,233,247]. A mixture of 10% Me_2SO with 8% glucose was superior to either Me_2SO or glycerol alone for the cryoprotection of cryosensitive fungi *Entomophthora exitialis*, *Pythium sylvaticum*, and *Pseudophaeolus baudonii* [233]. The optimum combination for amoebae (*Acanthamoeba castellani*, *Naegleria australiensis*, and *N. fowleri*) was 12% Me_2SO with 4–10% glucose [105], or 90% FCS with 10% Me_2SO [156]. Glycerol (10%) combined with 5% lactose, maltose, or raffinose has been used in the cryopreservation of yeasts (*S. cerevisiae*), bacteria (*P. aureofaciens* and *S. tenebrarius*), and algae (*Scenedesmus* spp., *C. vulgaris*, and *Anacystis nidulans*). With algae, 5% sodium glutamate was preferred to the saccharides [43]. A twofold better recovery of *Scenedesmus subspicatus* was observed when a mixture of sucrose, PVP and methanol was used as the CPA combination instead of sucrose alone [8]. A glycerol/sorbitol mixture was successful for the cryopreservation of *P. falciparum* [143], and 10% polyvinyl ethanol with 10% glycerol was effective for plasmid-bearing *Alcaligenes eutrophus* [11].

Frequency of use of particular cryoprotectants

By far the most generally and widely used CPAs in microbiology are Me_2SO and glycerol. The numbers of nonreview papers (i.e., those related to original experiments) dealing with particular CPAs (Table 2) show the frequency of their use (in decreasing order): Me_2SO 314, glycerol 308, blood serum or serum albumin or defibrinated blood 238, skimmed milk 61, sucrose 44, peptone 38, yeast extract 36, glucose 32, PVP 29, methanol 25, trypticase soy 21, sorbitol 15, malt extract 13, dextran 13, and EG 10; remaining CPAs have been recorded in <10 microbiological papers. Although this statistics might be biased by customs in particular organism groups, it indicates considerable differences among microbial taxons in the use of particular CPAs. For instance, Me_2SO is used much less often than glycerol for the cryopreservation of fungi but more with algae and protozoa; methanol has been widely used for the preservation of algae whereas peptones, yeast extract, and malt extract have been avoided; skimmed milk is the preferred CPA for bacteria.

A pairwise mutual comparison of the effectivness of the more common CPAs used in microbiology is shown in Table 3. The data are somewhat biased in that peptones, sera, and similar complex additives are frequently included in freezing media, sometimes as a part of the original inoculum, but may not be explicitly listed in published reports. In this pairwise comparison, a cryoprotective index is calculated as the percentage of cases in which particular CPA A gives better viability results after freezing than the CPA B out of all comparisons between A and B; thus when the index for a given CPA is $>50\%$, that CPA is more often successful than unsuccessful when compared with the other CPA. The CPAs with the highest total cryoprotective score are Me_2SO, methanol, diols (EG and PG), serum or serum albumin; glycerol, PEG, PVP, and sucrose are less successful; other sugars, including trehalose and the polymers dextran and HES, sorbitol and milk are relatively the least effective. However, it is always very important to take the toxicity of individual CPAs for particular microorganisms into consideration.

Equilibration

It is advisable to leave microbial suspension in contact with permeable CPAs for the time that is required to 'equilibrate' intracellular solutes before freezing [2,23,51,87,95,105,114,116,171,186, 226,228]. This is typically 10–60 min at 0–10 °C. Me_2SO or methanol, as quickly penetrating CPAs, do not need long equilibration periods; usually 15 min at 4 °C is enough [141,142,150,274]. The equilibration temperature and period for glycerol should be higher and longer (1–4 h) with some cell types; for example the optimum equilibration time for *Pleurotus* strains was 1–2 h [144]. Glycerol was less effective for the cryopreservation of *T. vaginalis* if equilibrated at 0–5 °C rather than 37 °C; the survival rate increased gradually with prolonged

Table 2
Frequency of use of individual additives in cryomicrobiology, based on the number of nonreview papers dealing with the particular CPA used alone (numerator) or in combination with another additive (denominator)

Compound	Viruses	Bacteria	Fungi	Algae	Protozoa
Me_2SO	14/6	42/12	31/18	42/17	76/56
Methanol	—	2/0	2/1	17/2	0/1
Ethanol	—	—	1/0	1/0	—
Polyvinyl alcohol	—	0/1	1/0	—	1/0
Ethylene glycol	—	1/0	3/1	1/1	2/1
Propylene glycol	—	1/0	2/0	1/1	0/2
Trimethylene glycol	—	—	—	—	1/0
Diethylene glycol	—	2/0	—	—	—
Polyethylene glycol	—	3/0	2/0	0/1	0/1
Polyethylene oxide	1/0	3/0	—	—	—
Glycerol	7/6	63/23	79/5	17/4	56/48
Mannitol and dulcitol	—	—	1/0	1/0	—
Inositol	—	0/1	1/0	—	—
Sorbitol	1/0	—	1/1	2/7	0/3
Glucose	1/0	2/9	5/3	—	0/12
Xylose	—	—	—	—	0/2
Sucrose	6/0	14/7	4/5	4/2	0/2
Lactose	—	2/3	1/1	—	—
Maltose	—	—	—	0/1	0/8
Trehalose	1/0	1/2	3/1	—	—
Raffinose	—	—	—	0/1	—
Dextran	1/0	7/1	1/0	1/1	0/1
Hydroxyethyl starch	—	1/0	1/0	—	0/3
Methyl cellulose	—	1/0	—	—	—
Ficoll	—	1/0	0/1	0/1	0/1
Gum arabic (acacia)	1/0	1/0	—	—	—
Acetamide	—	1/1	—	—	0/1
Dimethylformamide	—	1/0	—	—	—
Dimethylacetamide	—	1/0	—	—	0/1
Succinimide	—	1/0	—	—	—
Methylpyrrolidone	—	1/0	—	—	—
Polyvinylpyrrolidone	1/0	7/1	1/0	5/2	0/12
Proline	—	—	—	2/1	—
Glutamic acid	—	3/1	—	1/1	—
Ammonium acetate	1/0	—	—	—	—
Citrate	1/0	—	—	—	—
Blood (defibrinated)	—	5/16	—	—	19/51
Blood serum	12/10	11/11	1/9	1/0	10/59
Serum albumins	7/0	6/2	—	—	1/7
Gelatin	—	4/1	2/1	—	—
Peptone	3/0	8/7	1/14	0/1	1/3
Trypticase Soy	—	0/8	1/1	0/1	0/10
Shell extract	—	2/0	—	—	—
Glycoproteins	—	2/0	1/2	—	—
Mucin	—	1/0	—	—	—
Valinomycin	—	1/0	—	—	—
Gramicidin	—	1/0	—	—	—
Yeast extract	—	3/8	1/14	—	0/10
Malt extract	—	2/0	1/10	—	—
Skimmed milk	3/1	32/11	2/6	3/0	0/3
Egg yolk	1/0	1/0	—	1/0	—
Honey	—	2/0	—	—	—

Table 2 (*continued*)

Compound	Viruses	Bacteria	Fungi	Algae	Protozoa
Tween 80	—	3/1	0/1	—	—
Triton	—	1/0	—	—	—
Macrocyclon	—	1/0	—	—	—
Mg^{2+}, Ca^{2+}	3/0	1/0	—	—	—
Na^{+}, K^{+}	1/0	1/0	—	—	—

equilibration at 25 °C, and the optimum equilibration was ca. 100 min at 37 °C [168]. Cryoprotection of sporocysts of *Eimeria tenella* with 7.5% glycerol gradually improved with the equilibration time, increasing from 15 min to 19 h [186]. Also 7.5% Me_2SO protected the sporocysts better when equilibrated for 1–19 h than for 15 min at room temperature. This exceptionally long equilibration time is due to the generally low permeability of the sporocysts. *T. vaginalis* [168] and *P. chabaudi* [178] had to be equilibrated at temperatures above 20 °C to enable glycerol to penetrate and cryoprotect, while the optimum equilibration temperature was 0 °C for Me_2SO. However, there was no pronounced effect of equilibration time on the survival rate of *T. parva* sporozoites in medium containing 7.5% glycerol [113].

Many cells, especially eukaryotic ones, are quite sensitive to osmotic shock. Therefore permeating CPAs like Me_2SO should be added to, and removed from, the suspensions gradually to minimize osmotic stress, for example by adding drop by drop at about 4 °C. Osmotic stress may be reduced in some organisms by using hypertonic solutions such as 1.75% NaCl or sorbitol solutions during recovery after thawing. The survival of frozen *E. coli* was increased by adding sucrose to the diluting fluid after thawing [148]. Resuscitation of frozen, glycerol-preserved sporozoites of *T. parva* required the presence of glycerol in the recovery medium [113]. The survival of intraerythrocytic parasites following cryopreservation with permeable CPAs has been shown to be inversely related to the extent of haemolysis [178]. RBC lysis may occur during inappropriate recovery for example by placing the RBC directly into an isotonic environment. The best survival of frozen *P. chabaudi* and *P. falciparum* was observed when the frozen RBC infected with trophozoites were diluted after thawing with equal volumes of hyperosomotic solutions −15% glucose in PBS [178,179] or 3.5% NaCl [143].

Many CPAs are toxic to cells at normal temperatures, and should be removed by centrifugation or dilution after thawing. For instance, the Me_2SO concentration should be lowered to $<0.35\%$ in suspensions of most eukaryotic cells.

Mechanisms of cryoprotective action

The differing permeability of CPAs in turn affects the mechanisms by which they exert their protective effects [23,157,159,210,211]. The agents may provide protection by being intracellular or extracellular [199]. All effective permeant CPAs are highly hydrophilic [60,148,181,199] due to the presence of chemical groups forming strong hydrogen bonds with water, especially hydroxyl, amide, sulfoxide, and to a lesser extent, carboxyl and amino groups. For this reason, many CPAs can also protect microorganisms and their proteins against drying, thermal destruction and radiation. The importance of strong hydrophilic bonds [60,148,181] is illustrated by a comparison of the cryoprotective properties of Me_2SO with those of dimethylsulfone which is less hydrophilic and is not protective. Similarly, the *d*-form and *l*-form isomers of 2,3-butanediol are strongly hydrophilic and protective whereas the *meso*-form is less hydrophilic and much less protective for RBC [150,199]. Permeable CPAs make the CM more plastic and they bind intracellular water colligatively which prevents excessive dehydration, reduces salt toxicity and prevents the formation of large ice crystals within the cell. Penetrating CPAs stimulate a fine crystalline (quasiamorphous) ice structure and they form a gel-type glass phase below the eutectic point, therefore preventing hyperosmotic injury ('solution effects') to the cells,

Table 3
Pairwise comparisons of cryoprotective effectiveness measured by survival rates after freezing with the more common CPAs used in cryomicrobiology, based on published experimental reports

Compound	Me_2SO	Met	EG	PG	PEG	Gly	Sor	Glu	Suc	Lac	Tre	Dex	HES	PVP	Serum	Milk
Me_2SO		6/5	6/2	0/3	2/1	45/14	4/0	6/0	10/1	2/0	2/0	3/1	2/2	13/7	1/1	2/0
Methanol			NC	0/1	1/0	6/4	NC	3/1	3/1	1/0	1/0	NC	1/0	4/1	NC	NC
EG				2/1	0/1	4/5	1/1	3/1	2/1	NC	NC	1/0	NC	1/0	NC	1/0
PG					NC	1/1	1/0	1/0	1/0	NC	NC	1/0	NC	1/0	NC	NC
PEG						1/3	0/1	NC	NC	1/0	1/0	NC	1/0	1/0	NC	NC
Glycerol							2/0	5/2	6/5	2/1	3/1	6/0	2/2	9/7	3/1	3/0
Sorbitol								1/1	1/1	NC	NC	1/1	NC	1/1	NC	1/0
Glucose									2/2	NC	NC	2/1	NC	1/2	NC	1/0
Sucrose										1/1	1/2	2/1	NC	1/2	0/3	1/2
Lactose											1/0	NC	NC	0/1	0/1	0/2
Trehalose												NC	NC	0/1	NC	NC
Dextran													NC	0/1	0/1	NC
HES														0/2	0/1	NC
PVP															0/1	NC
Serum																1/1
Overall score (%)	74	64	52	79	50	47	37	31	32	21	23	18	31	43	67	33

The figures show the number of papers with better scores for the CPA in a row (numerator) and the number of papers with better scores for the CPA in the corresponding column (denominator).

NC, no comparison.

Overall score (%) = the proportion of pairwise comparisons reporting a higher survival after freezing with a particular CPA out of the total number of comparisons with all other CPAs.

and surface lesions that are caused by NaCl [135–137,147,148,157,166]. Some additives increase membrane permeability which may be beneficial at slow cooling rates. According to the colligative theory [135,136,157], penetrating CPAs reduce the concentration of salt by decreasing, on a simple colligative basis, the amount of frozen water. Semi-permeable CPAs induce partial dehydration of cells prior to freezing, they concentrate between the CM and the CW as a buffer layer against the growing ice, and protect CM mechanically. On the other hand, nonpermeable CPAs adsorb on the microbial surface where they form a viscous layer, cause partial efflux of water from the cell, inhibit the growth of ice crystals by increasing solution viscosity, and keep the structure of ice amorphous in the close proximity of the cell. However, they do not interact directly with CW or CM [4,158,207]. Polymers also alter the properties of the solution during the actual cooling and warming process and may retain water in the liquid state at temperatures as low as −35 °C. A similar protective theory [147] is based on the difference in vapor pressure between solute and ice: penetrating CPAs act by diminishing the rate of migration of water by lowering the vapor pressure of the intracellular fluid colligatively and increasing the vapor pressure of the extracellular solid. Nonpenetrating agents protect mainly against extracellular ice formation.

Glycerol, Me_2SO, and many other CPAs decrease the freezing-point of water and biological fluids by colligative action (glycerol/water to a minimum of −46 °C and Me_2SO/water to −73 °C). Therefore they lessen the concentration of salts dissolved in solutions, in turn inhibiting osmotic shock [85,135,137,159,252]. They also prevent eutectic crystallization. Glycerol protected *T. brucei* suspended in Alsever solution with 0.94% NaCl, whereas it did not in the same medium without sodium chloride; on the other hand, nonpermeable additives (5–10% sucrose, glucose, or xylose) protected the trypanosomes in Alsever solution [202]. A similar effect was revealed in *E. aerogenes*: diethylene glycol was protective with saline whereas the nonpenetrating CPAs glucose, sucrose or PEG-10,000—all at 10%—strongly protected the cells suspended in water without NaCl [204]. Besides colligative effects, the cryoprotective action of Me_2SO may also be related to the capacity of this agent to protect the surface of cells from hyperosmotic stress. Dimethylsulfone, in contrast to Me_2SO, lacks cryoprotective abilities which might be due to its precipitation from the solution during freezing as a result of which its concentration does not increase at subzero temperatures [150].

Phase diagrams of the system CPA/NaCl/water have indicated that the protective activity of hydroxy compounds such as sugars (trehalose, sucrose, etc.) or glycerol might be caused by the ability of these agents to prevent injurious eutectic freezing of cell fluids by trapping salts (NaCl) in a highly viscous or glass-like phase [103]. For instance, a highly viscous trehalose–water 'syrup' (or 'glass'), formed at temperatures <0 °C, prevents eutectic transitions of NaCl/water [34,184]. Water molecules seem to be trapped between the trehalose molecules (0.35 g water/g trehalose remains unfrozen) and the crystallization of ice is inhibited. This behavior, also observed in aqueous solutions of sucrose, might be connected with the cryoprotective activity of trehalose in biological systems. Natural AFPs and glycoproteins have been shown to cause a noncolligative depression of freezing point at very low concentrations, to inhibit ice recrystallization, and to limit ion leakage mainly by suppressing binding to incipient ice crystals. AFPs could thus be described as a special class of crystal growth inhibitors. They are also capable of protecting the structural integrity of membranes [152]. The cryoprotective activity of PVP fractions of differing molecular mass was tested in *E. coli*: PVP 90 kDa gave the best protection and also had maximum viscosity and the lowest temperature of crystallization (−22 °C) of all the PVPs tested [257].

Additional mechanisms of CPA action have occasionally been proposed. It has been suggested that PEO molecules react with several biologically important metal ions during freezing [170]. The natural CPAs trehalose and L-proline stabilize cell membranes and reduce membrane changes during freezing [214]. Glycerol and Me_2SO protected membrane vesicles of *Mycobacterium phlei* from the effects of freezing; these vesicles contain the enzymes of the electron transport chain [1]. In

experiments with *E. coli*, glycerol reduced the damage to both CW and CM, while Tween 80 only prevented CM damage of the cells that were frozen in saline [26]. Both permeable (glycerol, Me_2SO) and nonpermeable (dextran, PVP, and sucrose) compounds protected the surface lipopolysaccharides of *E. coli* cells against freezing injury [211] and the effects of detergents such as 0.02% lauryl sulfate [231]. Measurements have shown that Me_2SO, glycerol, sucrose, HES, PVP, and dextran are surface active. Me_2SO and glycerol exert their influence on the aqueous side of the lipid monolayer, while PVP enters it but can easily be excluded by surface pressure [268]. Me_2SO, in contrast to glycerol, was found to interact with the CM and to stimulate ribonucleotide polymerase and transcription in cell systems. It also caused changes in secondary structure (increase in P-sheet and loss of random coil content) in the cells of *Bradyrhizobium japonicum* [274].

A theoretical quantitative evaluation of the effectiveness of a CPA has been suggested, involving a so-called protection coefficient Q, formulated as follows:

$$Q = V \times S,$$

where V is volatility and S is molar solubility [181]. The greater the Q value, the more effective should be the cryoprotective activity of a particular compound. According to this theory, volatility and solubility in water are two very important characteristics for penetrating CPAs. Hydrogen donor and acceptor groups obviously play a significant role in the potential of a substance to protect organisms against freeze–thaw damage. However, it is very difficult or sometimes impossible at present to predict the actual activity that a specific CPA will have because the exact nature of the cryoinjury and its prevention is often unknown. Therefore the best CPA and its optimum concentration for a particular cryosensitive microorganism still has to be determined empirically, by trial-and-error. Me_2SO (10%) might be generally regarded as the most universal CPA, although other cryoadditives can sometimes yield better recoveries in certain microorganisms, and combinations of CPAs might be valuable for particularly fastidious organisms.

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