Cryopreservation of Precision-cut Tissue Slices for Application in Drug Metabolism Research

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Cryopreservatie van weefselslices voor toepassing in het metabolisme onderzoek van medicijnen
(met een samenvatting in het Nederlands)

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Contents

List of abbreviations

ALT	alanine aminotransferase
AP	alkaline phosphatase
AST	aspartate-aminotransferase
BSA	bovine serum albumine
CDNB	1-chloro-2,4-dinitrobenzene
CPA	cryoprotectant agent
CYP	cytochrome P450
DNPSG	*S*-(2,4-dinitrophenyl)glutathione
DMSO	dimethyl sulfoxide
DSC	differential scanning calorimeter
FCS	fetal calf serum
γ-GT	gamma-glutamyl transpeptidase
GSH	glutathione
GST	glutathione-*s*-transferase
7-HC	7-hydroxycoumarin
7-HC-gluc	7-hydroxycoumarin glucuronide
7-HC-sulf	7-hydroxycoumarin sulfate
HES	hydroxyethyl starch
IIF	intracellular ice formation
KH	Krebs-Henseleit buffer
LDH	lactate dehydrogenase
MTT	3[4,5-dimethylthiazole-2-yl]2,5-diphenyltetrazoliumbromide
OH-T	hydroxyl metabolite of testosterone
PAH	*para*-amino hippurate
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
TEA	tetraethylammonium
UW	University of Wisconsin medium
VS4	vitrification solution 4
V_{crc}	*c*ritical cooling rate
V_{crw}	critical warming rate
WME	Williams medium E

Chapter 1

*General introduction**

*Based on:
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Introduction

Drug metabolism is an important item within drug safety research. Not only does it determine the remaining time of a drug in the body, it may also be cause of the formation of toxic metabolites by bio-activation. Therefore, to test the toxicity of new drugs, laboratory animal species should be selected that form approximately the same metabolites of a certain compound as humans to serve as an optimal model for human drug toxicity. *In vitro* research has been under growing interest for many disciplines within drug safety research, but pre-eminently comes in handy for serving the selection of adequately metabolizing animal species in late drug discovery and early development. In this phase, this is the only way to compare metabolism of a particular drug between animals and humans. Additionally, and not unimportantly, laboratory animal use can be reduced in this manner.

Tissue slices are a unique *in vitro* tool because they closely resemble the organ from which they are derived. Because no proteolytic enzymes are required for their preparation, cell to cell contacts are maintained in slices, as well as cell-matrix interactions and all cell types are present as in the whole organ *in vivo*. The invention of mechanical tissue slicers, like the Krumdieck tissue slicer, has facilitated slice preparation enormously (Krumdieck *et al.*, 1980). With this equipment, slices from various organs of various animal species are prepared under controlled conditions in cooled physiological buffer, so minimal trauma is inflicted and slices of consistent appearance are produced. Much effort has been put in the optimization of preparation and culturing techniques for predominantly liver slices but also for lung, kidney and intestinal slices. Amongst factors that are critical for maintaining optimal viability of slices are slice thickness, composition of the culture medium and the incubation system used. Under optimal conditions, tissue slices are kept viable for at least 24 h after preparation. Several informative reviews discuss practical considerations that determine tissue slice viability and the applicability of slices in pharmaco-toxicological settings (Gandolfi *et al.*, 1995; Parrish *et al.*, 1995; Bach *et al.*, 1996; Ekins, 1996b, Olinga *et al.*, 1997b; Lerche-Lagrand and Toutain, 2000; de Kanter *et al.*, 2002b).

Because of their characteristics, the choice of tissue slices as an *in vitro* tool for several applications in toxicology and drug metabolism research is self-evident. Many publications describe the use of precision-cut liver slices to study drug metabolism *in vitro* (see aforementioned reviews). Less focus has been put on the use of extra-hepatic tissue slices for drug metabolism

research. Some compounds, however, are metabolized extra-hepatically and studying metabolism with liver slices alone may give an incomplete view of *in vivo* metabolism. For example, Sandker *et al.* (1994) and Pahernik *et al.* (1995) compared *in vitro* metabolism of respectively Org344 and pimobendan by isolated hepatocytes with metabolism *in vivo* and found that *N*-gluronidation of these compounds was underestimated *in vitro*. It was suggested that these metabolites are mainly formed extra-hepatically (Sandker *et al.*, 1994). In a recent study by our laboratory we compared metabolism of a new drug in development between various *in vitro* systems of both hepatic and extra-hepatic origin. It was indeed found that the *N*-glucuronide of this drug, which was the main metabolite *in vivo*, was formed in small amounts by liver slices, while it was produced in relatively large amounts by small intestinal slices (de Graaf *et al.*, submitted). Others have shown as well that particularly kidney and intestinal slices but also lung slices are metabolizing several drugs (Fisher *et al.*, 1994; Vickers *et al.*, 1995; de Kanter *et al.*, 1999). More importantly, the relative contribution to drug metabolism by the different metabolizing organs not only differs between compounds but also between animal species (Vickers *et al.*, 1995; de Kanter *et al.*, 1999). Consequently, to rightfully predict *in vivo* metabolism for animal species selection, slices of both liver and extra-hepatic metabolizing organs may be required.

Cryopreservation of these tissue slices would greatly facilitate their use. Basically, by cryopreservation, living material is kept in a state at which biological processes are brought to a stand-still after cooling to liquid nitrogen temperatures (-196°C), so that storage for infinite years is theoretically possible (Mazur, 1984). When damage to cells is prevented during cooling and subsequent thawing, slices should remain viable and resume their functional activity when brought back to physiological temperatures (37°C). Cryopreservation of tissue slices has many advantages: In some cases, many more slices are produced from organs than can be used directly within an experiment. By cryopreservation, these left-over slices could be stored and thawed at any desired time-point. Except for these logistic advantages, another benefit of this approach is that the number of laboratory animals required is further reduced. Moreover, cryopreservation simplifies the use of organ slices of human origin, of which the supply is limited, irregular and unpredictable. Ideally, tissue slices of human and animal origin could be stored by cryopreservation, forming a tissue slice-bank from which slices can be derived and used for species comparison studies on drug metabolism and toxicity.

Application of several methods for the cryopreservation of liver slices, more or less successful, has been reported in the last decade (Fisher *et al.*, 1991; Wishnies *et al.*, 1991; Fisher *et al.*, 1993; de Kanter and Koster, 1995; Fisher *et al.*, 1996; Ekins, 1996a; Ekins *et al.*, 1996a; Ekins *et al.*, 1996b; Glöckner *et al.*, 1996; Glöckner *et al.*, 1998; Glöckner *et al.*, 1999; de Kanter *et al.*, 1998; Day *et al.*, 1998; Maas *et al.*, 2000a; Maas *et al.*, 2000b; de Graaf *et al.*, 2000b, Sohlenius-Sternbeck *et al.*, 2000; Glöckner *et al.*, 2001; Vanhulle *et al.*, 2001). Some cryopreservation methods exist for precision-cut kidney slices (Fisher *et al.*, 1993; Fisher *et al.*, 1996). To our knowledge, cryopreservation methods for other extra-hepatic tissue slices are not reported.

In this chapter, the basic principles and critical issues of cryopreservation are discussed. Furthermore, emphasis is put on viability and functionality end-points that can be used to determine the cryopreservation outcome. Finally, cryopreservation methods for tissue slices are reviewed.

Cryopreservation by prevention of intracellular ice formation

The basis of the development of most cryopreservation methods is the prevention of intracellular ice formation (IIF), which is thought to be incompatible with life. Cell dehydration by equilibrium freezing and vitrification both prevent IIF, yet have a completely different mechanistic principle.

Cell dehydration by equilibrium freezing: critical issues

When a system containing cells and surrounding medium is cooled below 0°C, the liquid initially stays unfrozen (supercooled). The formation of ice nuclei becomes more probable when the supercooling is persisting longer and lower temperatures are reached. Because nucleation is a statistical event, it is most probable that the first ice nucleus is formed in the relatively high volume of extracellular medium rather than in a cell. Because ice crystals subsequently grow from pure water in the extracellular medium, the solutes in the unfrozen medium become more concentrated. As a result of this, the chemical potential of supercooled water within the cell becomes higher than in the external medium and water flows out of the cell. When freezing is slow enough to allow sufficient exosmosis of water from the cell, the chemical potential of cellular water and unfrozen water

outside the cells remain in equilibrium and the cell completely dehydrates, without freezing intracellularly (Mazur, 1984). Numerous publications exists that report successful cryopreservation of isolated cell types and oligocellular systems using this approach, for example of red blood cells (e.g. Pegg, 1981), 1-8 cell mouse embryos (e.g. Whitingham *et al.*, 1972) and hepatocytes (e.g. Powis *et al.*, 1987; Condouris *et al.*, 1990; Diener *et al.*, 1993; Maas *et al.*, 2000a).

The curve relating viability of a certain type of cell after (slow) freezing to the freezing rate is generally bell-shaped: At supra- and suboptimal cooling rates, viability is relatively low (Mazur *et al.*, 1972). This suggests that survival is determined by two factors, reversibly depending on the cooling rate. At supra-optimal cooling rates, there is not enough time for intracellular water to diffuse out of the cell to equalize the chemical potential of the intra- and extracellular environment and ice crystals are formed intracellularly (Mazur, 1984). At sub-optimal rates, low viability is probably caused by concentrated solutes: Because cooling is very slow, cells are exposed to high intra- and extracellular salt concentrations or changes in pH during a relatively long time-period (Lovelock, 1953a; Mazur, 1970). Moreover, the more cells dehydrate, the smaller is the volume of the unfrozen fraction of liquid remaining within and surrounding the cells. Wiest and Streponkus (1978) and Mazur and Cole (1989) have postulated that cell death in the course of slow freezing is caused by this latter phenomenon. These problems can be circumvented by the addition of cryoprotectant agents (CPAs) that decrease the amount of liquid that can freeze, thereby lowering intracellular salt concentration and preventing an extreme volume decrease of cells (Lovelock, 1953b; Meryman *et al.*, 1977). However, only low CPA concentrations are tolerated during equilibrium slow freezing (Fahy, 1981), because the elevation of intracellular CPA concentrations due to cell dehydration may also contribute to cell damage by slow freezing (Fahy, 1986).

Which cooling rate is optimal for survival is dependent on the cell type. Important factors are the surface to volume ratio of the cell and the permeability of its membranes, both determining the amount of water that can leave the cell per unit of time. Red blood cells for example have, because of their disc-like shape, a high surface to volume ratio. Besides of this, their membranes are very permeable to water. Therefore, optimal cooling rates are higher for red blood cells than for most other cells (Mazur, 1984). The fact that different cell types have different optimal cooling rates has implications for the applicability of slow freezing methods for

cryopreservation of tissues and organs: tissues exist of multiple cell types that may require different cooling rates for survival and when a tissue is frozen with a certain cooling rate, survival of the different cell types may not be equally high. Furthermore, although for single cells extracellular ice formation during slow freezing is not thought to be deleterious, extracellular ice may cause severe damage in tissues, since it concentrates in the vascular or interstitial space (Pegg, 1987, Bischof *et al.*, 1997). For these reasons, slow freezing of organs and complex tissues (particularly when methods are used that are optimized with isolated cells) is usually not successful (e.g. Alink *et al.*, 1978; Taylor and Pegg, 1983).

Vitrification: critical issues

Vitrification is thought to be the most promising starting point for the cryopreservation of organs, since by this approach, cell damaging events like effects of concentrated salts and CPAs, IIF, intravascular and interstitial ice formation, all likely to occur during slow freezing of organs, are circumvented (Fahy and Hirsh, 1982). Essentially, vitrification is the solidification of a liquid not brought about by crystallization but by an extreme elevation in viscosity during cooling, so that the solution becomes a glass.

In principle, every solution (including water) can be vitrified, as long as the cooling rates are high enough. For vitrification to occur within ranges of cooling rates that are relevant for the cryopreservation of biological material, addition of CPAs is necessary. By assessing the critical cooling rate, i.e. that cooling rate that is required to prevent ice crystal formation, several compounds have been studied in their tendency to form a glass when mixed with aqueous solutions. The critical cooling rate of the CPA solution depends on the chemical properties of the CPA and on the solution that is used to dilute the CPA. For example, to avoid ice crystallization while cooling at a rate of 100-400°C/min (rates that are achievable with tissue slices), approximately 45% w/w glycerol (Boutron, 1993), 43% w/w DMSO (Baudot *et al.*, 2000), 35% w/w 1,2-propanediol (Boutron, 1993) or 30% w/w *l*/*d* 2,3 butanediol (Boutron, 1993) are required. The critical cooling rate of 30% w/w *l*/*d* 2,3 butanediol is lowered from 270 to 150°C/min when the cryoprotectant is dissolved in Eurocollins® (amongst others consisting of saline buffer and glucose) instead of water (Boutron, 1993). The critical cooling rate of a CPA can be reduced by applying pressure on the sample (Fahy and Hirsh, 1982).

Because high concentrations of CPAs are necessary to achieve vitrification, their toxicity is a major issue to be dealt with when vitrification protocols are designed (Fahy and Hirsh, 1982). CPA toxicity can be both chemical and osmotical of origin. One approach to reduce chemical toxicity of a cryoprotectant solution, without diminishing its glass forming tendency, is to combine different CPAs with different mechanisms of toxicity or that even antagonize each others toxic effects (Baxter and Lath, 1971; Fahy, 1984, Fahy *et al.*, 1987). Chemical toxicity can also be reduced by introducing the CPA at a low temperature. Protocols have been developed that elevate CPA concentrations stepwise at temperatures as low as –60°C (e.g. Elford and Walter, 1972, Kheirabadi and Fahy, 2000). However, decreasing the temperature of CPA introduction increases the probability of the occurrence of osmotic effects of CPA exposure. These effects occur, because the cell membrane is less permeable for most CPAs than for water. Consequently, when a cell is exposed to a certain CPA solution, it will first shrink, because of the higher osmolarity of the cryoprotectant solution, and eventually return to its normal volume when the CPA enters the cell. Reversibly, if cells permeated with CPAs are brought into CPA-free buffer solution for washing, water first enters the cell before the CPA gets out, resulting in (extensive) cell swelling and ultimately lysis (Levin and Miller, 1984; Pegg and Diaper, 1990). Osmotic damage may be prevented by introducing and removing the CPA stepwise, allowing the cells to regain their original volume before each additional step. To prevent cell swelling during CPA removal, washing solutions that are made hypertonic by addition of impermeable sugars (trehalose, sucrose) can be used (Pegg and Diaper, 1990).

Besides cryoprotectant toxicity, devitrification is another process that needs to be considered when designing a vitrification protocol for cryopreservation of biological material. Devitrification is the crystallization of water that occurs in samples that were originally vitrified upon cooling, when warming is too slow. The occurrence of devitrification can be explained by viewing two processes leading to ice crystal formation, i.e. nucleation and growth of ice crystals from those nuclei. These processes both occur during cooling, however, the formation of ice nuclei is favoured at relatively low temperatures. At these temperatures, the *growth* of ice crystals has almost come to a stand-still. So, during cooling the formation of ice crystals is less likely than on warming, when the temperature trace on which nucleation is favoured comes *before* the temperature trace of maximal ice-crystal growth (MacFarlane, 1987). This is illustrated by the difference between the critical cooling and warming rates (i.e. the warming

rate required to prevent devitrification during warming) of CPAs: while the critical cooling rates of 45% w/w glycerol, DMSO, 1,2-propanediol and *l*/*d*-2,3 butanediol solutions are 380, 14, 10 and 2.5 <°C/min (Boutron, 1993; Baudot *et al.*, 2000), the critical warming rates are much higher: 5.6 *10^8, 3300, 80 and 80°C/min respectively (Boutron and Mehl, 1990). Therefore, for successful cryopreservation by vitrification, emphasis should be put on attaining high enough warming rates to prevent IIF upon thawing in vitrified samples. Electromagnetic heating has been postulated as a manner to achieve this (Burdette, 1972).

Cells and tissues permeated with CPAs often have lower critical cooling and warming rates than bulk CPA solutions (Peridieu *et al.*, 1995; Takahashi and Hirsh, 1985). This phenomenon is probably triggered by the high protein content of the intracellular matrix, causing it to more or less behave as a cryoprotectant (Rapatz and Luyet, 1960). Moreover, in a tissue and within cells, liquid is compartmentalized. Compartmentalization of water is known to increase supercooling and inhibit crystal formation (Yamane *et al.*, 1992).

Viability determination of tissue slices

Cryopreservation of tissue slices is considered to be successful when they remain (functionally) viable for a sufficiently long period after thawing. A number of parameters have been used to determine the integrity of fresh and cryopreserved tissue slices. Roughly, commonly used parameters can be divided in 1) drug metabolism related parameters (metabolism of model substrates, activity of metabolic enzymes, enzyme content, Table 1a), 2) functionality parameters other than drug metabolism (urea synthesis, MTT reduction, protein synthesis, organic anion transport and gluconeogenesis, Table 1b) and 3) parameters that reflect the general cellular condition (histomorphological examination, potassium content, ATP content or energy charge, retention of cellular enzymes and GSH content, Table 1c).

Drug metabolism related parameters

The most obvious manner to test biotransformation capacity of a tissue slice is to measure metabolite formation of a model compound. Amongst the substrates that are often used for this purpose (Table 1a) is testosterone which is oxidized to several hydroxyl metabolites and androstenedione. Another regularly used model compound is 7-ethoxycoumarin (7-EC), which

is mainly *O*-deethylated to 7-hydroxycoumarin (7-HC) followed by *O*-glucuronidation and *O*-sulfation by liver slices, but mainly hydrolysed to 4-ethoxy-2-hydroxyphenyl propionic acid (EPPA) by extra-hepatic tissue slices (de Kanter *et al.*, 2002a). Other used model substrates are lidocaine (*N*-deethylation), naphtol (direct glucuronidation/sulphation) and chloro-dinitrobenzene (CDNB) (glutathione conjugation). These compounds are added to the intact slice and consequently metabolite formation not only depends on enzyme activity but also on cofactor content of the slice as well as transport of substrates into the slice and of metabolites out of the slice.

Specific activity of metabolic enzymes of slices is generally measured in slice homogenate after addition of cofactors or NADP regenerating system, circumventing the role of cofactor availability and transport. Enzyme activity assays used for slices include those for several CYPs (e.g. with 7-EC (ECOD-activity), 7-ethoxyresorufin (EROD-activity), 7-benzoxyresorufin (BROD-activity) or testosterone as substrate), *N*-acetyltransferases, gluthation-*s*-transferase (GST), glucuronyltransferases and sulfotrans-ferases (Table 1a).

Immunochemic assays are often used to measure the presence of several metabolic enzymes, without determining their activity (Table 1a).

Functionality parameters other than drug metabolism

In most terrestial vertebrates urea synthesis is the mechanism to dispose of NH_4 formed in the breakdown of amino acids and an exclusive property of liver tissue. Urea synthesis in the presence of ornithine sets off in the mitochondrial matrix of liver cells and is finalized in the cytosol and is a highly regulated, ATP-dependent cycle that involves several enzymes. The use of urea synthesis as a viability parameter for liver slices has been reported by several authors (Table 1b).

In the MTT (3[4,5-dimethylthiazole-2-yl]2,5-diphenyltetrazoliumbromide) test, tetrazolium is converted (at least partly) by succinate dehydrogenase, an enzyme linked to the inner mitochondrial matrix and involved in the citric acid cycle. Strongly light-absorbing formazan products are formed that precipitate in the cell. After extraction, the absorbance is assessed and used as a measure of slice functionality (Table 1b). Although MTT reduction is often used to determine mitochondrial function, there exist indications that MTT reduction is not solely converted by mitochondrial enzymes (Berridge and Tan, 1993).

Table 1a Drug metabolism related parameters used for viability determination of fresh and cryopreserved tissue slices

Parameter	References
Metabolism of model compounds	
- testosterone	de Kanter and Koster, 1995; Ekins, 1996c; Ekins *et al.*, 1996b; Olinga *et al.*, 1997a; de Kanter *et al.*, 1998; de Kanter *et al.*, 1999; de Graaf *et al.*, 2000b; Maas *et al.*, 2000a; Maas *et al.*, 2000b; Sohlenius-Sternbeck *et al.*, 2000; Glöckner *et al.*, 2001
- 7-EC, 7-HC	Barr *et al.*, 1991a; Barr *et al.*, 1991b; Wishnies *et al.*, 1991; Ekins, 1996a; Ekins, 1996c; Ekins *et al.* 1996a; Ekins *et al.* 1996b; Singh *et al.*, 1996; Olinga *et al.*, 1997a; Day *et al.*, 1998; de Kanter *et al.*, 1998; Glöckner *et al.*, 1998; Price, 1998b; de Kanter *et al.*, 1999; de Graaf *et al.*, 2000b; Sohlenius-Sternbeck *et al.*, 2000;
- lidocaine	Olinga *et al.*, 1997a; de Kanter *et al.*, 1998; de Kanter *et al.*, 1999;
- naphtol	Singh *et al*, 1996; Sohlenius-Sternbeck *et al.*, 2000
- CDNB	Ekins, 1996c; Maas *et al.*, 2000a; Maas *et al.*, 2000b
- paracetamol	Vanhulle *et al.*, 2001
Enzyme activity	
- CYPs	Gokhale *et al.*, 1995; Fisher *et al.*, 1995a; Steensma *et al.*, 1994; Drahusnuk *et al.*, 1996; Lake *et al.*, 1996; Müller *et al.*, 1996; Gokhale *et al.*, 1997; Müller *et al.*, 1998; Price *et al.*, 1998a; Vandenbrande *et al.*, 1998; Glöckner *et al.*, 1998;
- GsT	Toutain *et al.*, 1998; Maas *et al.*, 2000a; Maas *et al.*, 2000b;
- N-acetyl transferases	Gunawardana *et al.*, 1991
- glucuronyltransferases	Hashemi *et al.*, 1999; Vandenbrande *et al.*, 1998
- sulfotransferases	Hashemi *et al.*, 1999; Vandenbrande *et al.*, 1998
Enzyme content/expression	Steensma *et al.*, 1994; Drahusnuk *et al.*, 1996; Lake *et al.*, 1996; Price *et al.*, 1998a; Lupp *et al.*, 2001; Vanhulle *et al.*, 2001

The active transport of organic anions and cations by proximal tubuli cells within the kidney cortex is used as functionality parameter for kidney slices. Normally, the slice to medium ratio of radio-labelled model substrates such as tetraethylammonium (TEA) and *para*-amino hippurate (PAH) is used as a measure (Table 1b).

Protein synthesis is a coordinated and complicated interplay of several cellular macromolecules and takes place on the ribosomes associated to the RER. The synthesis starts with ATP-dependent activation of amino acids. The incorporation of ^{14}C-leucine into precipitable protein can be quantified as a measure for the capability of a slice to synthesize proteins and is used to determine functional activity of liver, kidney and lung slices (Table 1b).

Table 1b Functionality parameters used for viability determination of fresh and cryopreserved tissue slices

Parameter	References
Functionality parameters	
Urea synthesis	Fisher *et al.*, 1993; de Kanter and Koster, 1995; Day *et al.*, 1998; Maas *et al.*, 2000b
MTT reduction	Leeman *et al.*, 1995; Olinga *et al.*, 1997a; Obatomi *et al.*, 1998; Maas *et al.*, 2000a; Maas *et al.*, 2000b; Sohlenius Sternbeck *et al.*, 2000
Protein synthesis	Smith *et al.*, 1986; Fisher *et al.,* 1991; Fisher *et al.*, 1993; Fisher *et al.*, 1994; Fisher *et al.*, 1995a; Fisher *et al.*, 1995b; Ekins, 1996a; Fisher *et al.*, 1996; Glöckner *et al.*, 1998; Toutain *et al.*, 1998; Glöckner *et al.*, 1999
TEA transport	Fisher *et al.*, 1993
PAH transport	Fisher *et al.*, 1993
Gluconeogenesis	Fisher *et al.*, 1993

Gluconeogenesis is an *in vivo* characteristic of the liver and to lesser extend of the kidney cortex. Glucose is produced under consumption of ATP from various non-carbohydrate precursors via pyruvate. Gluconeogenesis is used as functionality parameter for liver slices (Table 1 b).

Parameters that reflect general viability of slices

Histomorphological examination of precision-cut tissue slices is reported by several authors to be a sensitive way to examine slice integrity (Table 1 c). To be able to study slices microscopically, thin longitudinal or cross sections are made that can be stained with standard staining solutions (H&E, toluidine blue) or specific or immunohistochemic stainings (e.g. glycogen (Lupp *et al.*, 2001); CYPs (Lupp *et al.*, 2001) Connexin32 (de Graaf *et al.*, 2000a)). Studying histomorphology is useful to determine cell or site specific toxicity of CPAs or damage by cryopreservation.

The retention or leakage of cellular enzymes with a relatively large molecular weight like alanine- or aspartate-aminotransferase (ALT, AST), lactate dehydrogenase (LDH), alkalic phosphatase (AP) and gamma-glutamyl transpeptidase (γGT) is often used to determine membrane integrity of cells in a slice (Table 1c). Since there is normally no active secretion of these molecules by cells and there is no evidence that cells benefit from the loss of these compounds, the release of these enzymes is considered to be a pathological manifestation (Danpure, 1984). LDH, AP, ALT and AST are relatively abundant in liver and are therefore used as

viability parameter for liver slices. For the same reason, LDH, AP and γGT retention can be used to determine the quality of kidney slices and LDH and AP for lung slices. AP is predominantly located in the mucosa cells of the intestines, hence can be of use to determine viability of the epithelium of intestinal slices.

ATP is the universal energy source of cells and loss of ATP has been directly associated with influx of extracellular sodium and calcium and efflux of intracellular potassium leading to cell swelling and eventually necrosis (e.g. Carini, 1999). It should be noticed, however, that directly after slice preparation ATP levels of slices are often low probably due to warm ischemia upon liver excision and low temperatures during slicing. After placing the slices in oxygenated, 37°C medium, ATP levels recover to physiological values within 1-4 h (Smith *et al.*, 1986; Smith *et al.*, 1987; Dogterom *et al.*, 1993; Toutain *et al.*, 1998; De Kanter *et al.*, 1999; Maas *et al.*, 2000a; Maas *et al.*, 2000b), due to a temperature dependent re-equilibrium phenomenon (Smith *et al.*, 1987). The major ATP consuming processes in a cell are protein synthesis and the regulation of cellular ion concentrations. Generally, ATP content is considered to be a very sensitive viability parameter and can be sensitively measured with luciferine-luciferase based assays (Gould and Subramani, 1988). The energy status of a cell can also be defined by the energy charge (½ADP + ATP)/(AMP+ADP+ATP) (Atkinson, 1968), which gives an indication of both energy consuming and producing processes in a cell.

Retention of cellular potassium is determined in slices (Table 1c) by several author as (sensitive) measure for membrane integrity but also for energy status, since Na^+/K^+ ATP-ases, directly responsible for intracellular ion regulation, are consuming a major part of cellular ATP. For this reason, it is not surprising that potassium content often correlates with cellular ATP content, showing a similar increase of initial low values upon culturing of the slices after preparation (Smith *et al.*, 1986; Smith *et al.*, 1987; Dogterom *et al.*, 1993; Maas *et al.*, 2000b).

Glutathione is measured as oxidized (GSSG) or reduced (GSH) form by several authors (Table 1c). The ratio of GSH/GSSG can be used to monitor oxidative stress.

Table 1c Parameters used for viability determination of fresh and cryopreserved tissue slices that reflect general viability

Parameter	References
Histomorphology	Smith *et al.*, 1985; Ruegg, 1987; Beamand *et al.*, 1993; Gokhale *et al.*, 1995; Ekins *et al.*, 1996a; Gokhale *et al.*, 1997; Leeman *et al.*, 1995; Singh *et al.*, 1996; Olinga *et al.*, 1997a; Toutain *et al.*, 1998; Hashemi *et al.*, 1999; De Graaf *et al.*, 2000a; de Graaf *et al.*, 2000b; Maas *et al.*, 2000a; Maas *et al.*, 2000b; Shigematsu *et al.*, 2000; Lupp *et al.*, 2001; Vanhulle *et al.*, 2001
Leakage of cellular enzymes	Smith *et al.*, 1985; Miller *et al.*, 1993; de Kanter and Koster, 1995; Fisher *et al.*, 1995a; Fisher *et al.*, 1995b; Olinga *et al.*, 1997a; Day *et al.*, 1998; de Kanter *et al.*, 1998; Obatomi *et al.*, 1998; Maas *et al.*, 2000a; Maas *et al.*, 2000b; Sohlenius-Sternbeck *et al.*, 2000; Glöckner *et al.*, 2001; Vanhulle *et al.*, 2001
K^+ content	Smith *et al.*, 1985; Smith *et al.*, 1986; Fisher *et al.,* 1991; Fisher *et al.*, 1993; Dogterom, 1993; Fisher *et al.*, 1995a; Fisher *et al.*, 1995b; Fisher *et al.*, 1996; Baumann *et al.*, 1996; Glöckner *et al.*, 1996; Müller *et al.*, 1996 Olinga *et al.*, 1997a; Sidelmann *et al.*, 1996; Glöckner *et al.*, 1998; Müller *et al.*, 1998; Price *et al.*, 1998a; Vandenbrande *et al.*, 1998; Glöckner *et al.*, 1999; de Graaf *et al.*, 2000b; Maas *et al.*, 2000b
ATP content	Smith *et al.*, 1986; Dogterom *et al.*, 1993; Miller *et al.*, 1993; Fisher *et al.*, 1995a; Sidelmann *et al.*, 1996; Singh *et al.*, 1996; Toutain *et al.*, 1998; De Kanter *et al.*, 1999; Martin *et al.*, 2000; Maas *et al.*, 2000a; Maas *et al.*, 2000b; Shigematsu *et al.*, 2000; Vanhulle *et al.*, 2001
Energy charge	Olinga *et al.*, 1997a
GSH content	Müller *et al.*, 1996; Singh *et al.*, 1996; Toutain *et al.*, 1998; Hashemi *et al.*, 1999; Müller *et al.*, 1998; Price *et al.*, 1998a; Maas *et al.*, 2000a; Maas *et al.*, 2000b; Glöckner *et al.*, 2001

Application of equilibrium slow freezing and vitrification for the cryopreservation of tissue slices

For tissue slices, with physical dimensions (8 mm x 200 μm) laying in between those of isolated cells and whole organs, a number of cryopreservation methods have been published in the past decade. Table 2a and 3a give an overview of existing cryopreservation methods that are mechanistically based on equilibrium slow freezing and vitrification respectively. Table 2b and 3b show the viability of slices after application of these methods.

Table 2a Slow freezing methods for cryopreservation of tissue slices

	Fisher *et al.* (1991)	Fisher *et al.* (1991)	Fisher *et al.* (1993)	Fisher *et al.* (1996)	Maas *et al.* (2000a)
Optimization					
Species, organ	pig liver	human liver	human kidney	dog liver	rat liver
Parameters optimized	CPA and CPA conc. (0.7,1.4, 2.8 or 4.2 M DMSO or 0.7, 1.4, 2.8 or 4.2 M glycerol or 1.4/1.4, 1.4/2.8 or 1.4/4.2 M DMSO/glycerol), CPA carrier solution (WM/10%FCS, KH, FCS, HS, NCS), cooling rate (0.5, 1, 2, 4 and 8 and 12°C/min)	cooling rate (0.5, 1, 2, 4, 8 or 12°C/min)	cooling rate (0.5, 1, 2, 4, 8 or 12°C/min)	cooling rate (0.5, 1, 2, 4, 8 or 12°C/min)	cooling rate: 0.5, 1 or 4°C/min, pre-incubation period (10, 20, 30 or 60 min), liver perfusion (yes or no), CPA (10% DMSO or 5% DMSO/4.1 % EG/10% PVP), CPA carrier solution (WME, WME/10% FCS, FCS, UW)
Ultimate method:					
CPA solution	1.4 M DMSO in FCS	1.4 M DMSO in FCS	1.4 M DMSO in FCS	1.4 M DMSO in FCS	10% DMSO in UW
CPA introduction	20 min, 25°C	20 min, 25°C	20 min, 25°C	20 min, 25°C	stepwise, 10 min 5%, on ice, then 15 min in cryovial 10% at 0°C
Cooling technique	computer controlled, slice basket	computer controlled, slice basket	computer controlled, slice basket	computer controlled, slice basket	computer controlled, cryovial
Cooling rate	12°C/min	1°C/min	12°C/min	0.5°C/min	1°C/min
Warming technique	basket in 37°C FCS	basket in 37°C FCS	basket in 37°C FCS	basket in 37°C FCS	vial in 37°C waterbath
Warming rate	“fast”	“fast”	“fast”	“fast”	“fast”
CPA outwashing	twice in 37°C FCS	twice in 37°C FCS	twice in 37°C FCS	twice in 37°C FCS	37°C WME/10% FCS
Remarks		variation between livers, perfused livers: better results			

WM= Waymouths medium, KH= Krebs-Henseleit buffer, FCS= fetal calf serum, HS= horse serum, NCS= newborn calf serum, EG=ethylene glycol, PVP= polyvinylpyrrolidone

Table 2b Viability of tissue slices after cryopreservation according to slow freezing methods

Author	Species, organ	Incubation period	Method of	Viability parameter (% fresh slice value)	Remarks
Fisher *et al.* (1991)	pig liver	4 h	Fisher *et al.* (1991), method for pig liver	K^+ content (80 %), protein synth. (85%)	
	human liver	4 h	Fisher *et al.* (1991), method for human liver	K^+ content (60%), protein synth. (66%)	
Fisher *et al.* (1993)	human liver	4 h	Fisher *et al.* (1991), method for human liver	K^+ content (80%), protein synth. (77%), gluconeogenesis (82%), urea synth. (112%)	
	human kidney	4 h	Fisher *et al.* (1993)	K^+ content (78%), protein synth. (70%), PAH transport (83%), TEA transport (78%)	
Fisher *et al.* (1996)	dog liver	4 h	Fisher *et al.* (1996)	K^+ content (72%), protein synth. (75%)	
	dog kidney	4 h	Fisher *et al.* (1993), method for human kidney	K^+ content (67%), protein synth. (65%)	
Maas *et al.* (2000a)	rat liver	0, 2 h	Maas *et al.* (2000a)	2 h: MTT conversion (80%), CDNB conj. (15%), testost. metab. (60%), GsT activity (30%), GSH content (40%), LDH leakage (0-2 h: 85% of total LDH), ATP content (15%), histomorphology (no living cells). At 0 h: only ATP content and CDNB conj. different from fresh	
Maas *et al.* (2000b)	rat liver	0, 4, 8 h	Maas *et al.* (2000a)	ATP content (4 h, 8 h:<5%), K^+ content (4 h, 8 h: <5%), GSH content (4 h: 30%, 8 h: 20%), LDH leakage (after 4 h 90% leaked out, after 8 h: 90%), GsT activity (4 h, 8 h: 10-20%), CDNB conj. (4 h, 8 h: 10%), testost. metab. (4 h: comparable to fresh values, 8 h: 50%), urea synth. (0-3 h: 50%, 4-7 h: 20%, 8-11 h: 20%), histomorphology: 4 and 8 h: almost no living cells. 0 h: 50 (ATP content, K^+ content) to 100% (testost. metab.) of fresh values	method compared with rapid freezing method: rapid freezing better

Fisher *et al.* (1991; 1993; 1996) have reported the successful cryo-preservation of human liver and kidney slices as well as dog and pig liver slices by a classical equilibrium freezing approach after permeating the slices with 10% DMSO in Fetal Calf Serum (FCS) and optimal freezing rates varying between 0.5 (dog liver slices) and 12°C/min (human kidney slices). Viability and functionality were all more than 60% of fresh values, as

measured by potassium retention, protein synthesis, gluconeogenesis (human liver slices), urea synthesis (human liver slices), organic anion (PAH) transport (human kidney slices) and cation (TEA) accumulation (human kidney slices). However, the authors reported that rat liver slices could not be successfully cryopreserved using this approach, with a viability of approximately 10-25% compared to fresh slices (Fisher *et al.*, 1991). Besides this, viability of cryopreserved human liver slices depended more than that of fresh slices on the liver condition, determined by the duration of warm ischemia and the source from which the liver was derived (surgical waste or transplantation liver) (Fisher *et al.*, 1991; Fisher *et al.*, 2001). Maas *et al.* (2000a and 2000b) also showed that rat liver slices rapidly degenerate after thawing when they are slowly frozen (1°C/min), with particularly ATP content, CDNB conjugation and slice histomorphology showing marked differences compared with fresh slices. This is in contrast with rat hepatocytes that were frozen relatively succesfully with this method (Maas *et al.*, 2000a).

Vitrification of (human) liver slices was first reported by Wishnies *et al.* (1991) using a high molarity solution of 1,2-propanediol (4.7 M) in FCS which was introduced to the slices according to a stepwise protocol. Slices were subsequently vitrified by placing the stainless steel grids on which they were positioned directly into liquid nitrogen. In this manner the estimated cooling rate was approximately 5000°C/min and slices had a glassy appearance after reaching liquid nitrogen temperatures that is typical for vitrified tissue. Slices were reported to maintain both phase I and phase II biotransformation to some extent, but results varied considerably and seemed to be dependent on the donor liver from which slices were derived and on storage time in liquid nitrogen (with unclearified higher activity of vitrified and thawed slices after longer storage periods).

Ekins (1996a), using approximately the same method, found that the coupled metabolism of 7-EC to 7-HC and its conjugates was well maintained in vitrified and thawed rat liver slices. However, when the slices were incubated with 7-HC instead of 7-EC, conjugation was significantly lower (approximately 50% of fresh slices). Protein synthesis was only 3-35% of fresh slice levels. Vitrified dog liver slices had a very low conjugation activity (Ekins *et al.*, 1996b). In a later study, Ekins *et al.* (1996a) proposed the use of 'antifreeze' proteins for optimal vitrification of liver slices. Allthough results were improved using these proteins, biotransformation in thawed slices was still considerably lower than in fresh slices.

Table 3a Vitrification methods for tissue slices

	Wishnies *et al.* (1991)	Ekins *et al.* (1996a)
optimization		
Species, organ	human liver	rat liver
Parameters optimized	not shown	4.7 M 1,2 propanediol in FCS with or without antifreeze proteins
ultimate method:		
CPA solution	4.7M 1,2 propanediol in FCS	4.7M 1,2 propanediol in FCS with antifreeze proteins
CPA introduction	stepwise 3x5 min, 0°C	stepwise 3x8 min, 0°C
Cooling technique	direct exposure to liquid N_2 on tray	direct exposure to liquid N_2 on cryocassette
Cooling rate	5000°C/min.	5000°C/min.
Warming technique	tray direct in 37°C FCS	cryocassette direct in 37°C FCS
Warming rate	5000°C/min.	5000°C/min.
CPA outwashing	15 min. in 0°C FCS	15 min. in 0°C FCS
Remarks	unexplained variation between donors and different storage periods	

Table 3b Viability of tissue slices after cryopreservation according to vitrification protocols

Author	Species, organ	Incubation period	Method of	Viability parameter (% fresh slice value)	Remarks
Wishnies *et al.* (1991)	human liver	0-6 h	Wishnies *et al.* (1991)	total 7-EC metab., 7-HC conj. (results very variable)	
Ekins, (1996a)	rat liver	4 h	Wishnies *et al.* (1991)	7-EC *O*-deethylation (>200%), 7-HC conj. (7-EC as substrate: 100%), direct 7-HC conj. (50%), protein synth. (3-35%)	modifications from original method: cryocassette instead of tray, CPA pre-inc.: 3 x 8 instead of 3 x 5 min, results worse after 24 h storage
Ekins *et al.* (1996a)	rat liver	4 h	Ekins *et al.* (1996a)	metabolism 7-EC to 7-HC and conjugates retained, direct 7-HC conj. (25-40%) minor mitochondrial swelling	
Ekins *et al.* (1996b)	dog liver	1 h, 20 min. pre-inc.	Wishnies *et al.* (1991)	7-EC *O*-deethylation (>800%), 7-HC conj. (7-EC as substrate: <20%)	modifications from original method: cryocassette instead of tray, CPA pre-inc.: 3 x 8 instead of 3 x 5 min

Rapid freezing as an approach for cryopreservation of tissue slices

De Kanter and Koster (1995) have shown for the first time that rapid freezing (250°C/min) after pre-incubation with a low (12%) concentration of DMSO maintains viability of rat liver slices better than slow freezing (0.6, 1.5 or 50 °C/min) (Table 4a and b). This is a remarkable result because isolated rat hepatocytes only survive if cooling rates are very low (1°C/min) (Powis *et al.*, 1987; Condouris *et al.*, 1990; Diener *et al.*, 1993; Maas *et al.*, 2000a).

De Kanter and Koster found that after cryopreservation by rapid freezing and thawing, slices of rat and monkey maintained testosterone metabolism, ALT retention and urea synthesis at levels >66% of fresh slice values between 0 and 3 h post-thawing (Table 4b). The preference for rapid freezing for cryopreservation of rat liver slices has been confirmed by Glöckner *et al.* (1996) and Maas *et al.* (2000b). More recently, de Kanter *et al.* (1998) showed that human liver slices could also be successfully cryopreserved with this rapid freezing method, maintaining urea synthesis, ALT retention and phase I biotransformation of lidocaine, testosterone and 7-EC on levels of >66% of fresh slices. However, marked differences between conjugation of 7-HC after 7-EC hydroxylation of cryopreserved and fresh slices were observed. Particularly sulfate conjugation was decreased. Recently, Maas *et al.* (2000b) examining viability of rapidly frozen rat liver slices after thawing and culturing for 4 to 8 h, have shown that the outcome of rapid freezing depends on the parameter that is used to determine success. While testosterone metabolism, G*s*T-activity in the slice homogenate in the presence of GSH, and urea synthesis were well maintained in fast frozen slices for 4 and even 8 h of culturing, other parameters like ATP, GSH and potassium (K^+) content and slice histomorphology were decreased to levels around 20% of fresh slice values (see also chapter 2 of this thesis). Vanhulle *et al.* (2001) also found that P450-dependent drug metabolism and slice metabolic enzyme content were not different from fresh slices in rapidly frozen rat liver slices permeated with 10% DMSO, while other parameters (glycogen content, ATP content and phase II biotransformation), showed a progressive decrease compared to fresh slices. Rapidly deterioriating slice viability was also reported by Martin *et al.* (2000), however, they used UW/10% DMSO as CPA medium, which has been shown by Maas *et al.* (2000b) to be inadequate for rapid freezing of liver slices.

Table 4a Rapid freezing methods for tissue slices

	de Kanter and Koster (1995)	Glöckner *et al.* (1996)	Day *et al.* (1998)	de Graaf *et al.* (2000b)	Glöckner *et al.* (2001)
Optimization					
Species, organ	rat liver	rat liver	rat liver	rat liver	rat liver
Parameters optimized	CPA conc. (0, 6, 8, 12 or 16% v/v), temp. and duration of CPA pre-incubation (2, 20 or 37°C; 0, 30 or 60 min), cooling rate (0.6, 1.5, 50 or 250 °C/min)	slice thickness (0.25 and 0.5 mm), storage prior to cryopreservation (yes or no) cooling rate (1.5 or >250°C/min)	cooling and warming rate[b] (250 or 1000°C/min), CPA conc. (4.5, 7, 10, 15 or 22% v/v)	CPA conc. (0, 12, 18 or 30% v/v), CPA carrier solution (WME, FCS or UW), storage prior to cryopreserv. (15 or 75 min), slice thickness (8-23 cellayers)	CPA conc. (10, 20 or 30% v/v)
Ultimate method					
CPA solution	12% (v/v) DMSO in WME	10% (v/v) DMSO in SS	10% (v/v) DMSO in WME	18% (v/v) DMSO in WME	30% DMSO in SS
CPA introduction	in flasks on ice, 30 min	in flask on ice, 15 min	in 40 ml CPA sol. on ice, 30 min	in flasks on ice, 30 min	in flask on ice, 15 min
Cooling technique	5 slices + 1 ml CPA solution in cryovial, direct in liq. N_2	4-6 slices in cryovial without medium, direct in liq. N_2	5 slices between aluminium plates, direct in liq. N_2	5 slices + 0.5 ml CPA solution in cryovial, direct in liq. N_2	4 slices in cryovial without medium, direct in liq. N_2
Cooling rate	250°C/min	>250°C/min[a]	ca 1000°C/min[a]	250°C/min[a]	>250°C/min[a]
Warming technique	cryovial in 37°C waterbath	cryovial in 37°C waterbath	aluminium plate with slices direct in 37°C waterbath	cryovial in 37^{0}C waterbath	cryovial in 37°C waterbath, + 2 ml 37°C WME
Warming rate	200°C/90 s	'fast'	200°C/15 s	'fast'	'fast'
CPA outwashing	not	3x in SS, 25°C	1x, ice cold HBSS	briefly in WME, 10% FCS	by addition of 2 ml 37°C WME
Remarks	viability decreased when slices were stored at –80°C	storage on ice prior to freezing decreased viability		viability decreased by storage prior to freezing, thin slices (10 cellayers) better than thick slices (>14 cellayers)	prelim. experim. showed washing 1x in 37°C WME better than 3x in ice-cold SS

[a] not actually measured
[b] cooling and warming rates were varied simultaneously
SS = Sacks solution, HBSS = Hanks balanced salt solution

Table 4b Viability of tissue slices after cryopreservation according to rapid freezing methods

Author	Species, organ	Incubation	Method of	Viability parameter % fresh slice value)	Remarks
de Kanter and Koster (1995)	rat liver	3 h	de Kanter and Koster (1995)	urea synth.(85%), ALT retained (78%), testost.metab. (116%)	
	monkey liver	3 h	de Kanter and Koster (1995)	ALT retained (85-87%), urea synthesis (82-106%), testost. metab. (60-138%)	fast freezing superior to slow freezing,
Glöckner *et al.* (1996)	rat liver	2h	Glöckner *et al.* (1996)	K^+ content (71%), GSH (95%), ECOD activity (173%)	incubation at 30°C
de Kanter *et al.* (1998)	human liver	3 h	de Kanter and Koster (1995)	urea synth.(78%), testost. metab.(88%), 7-HC conjug. (7-EC as substr.: 40-100%), ALT retained (66%), total 7-EC metab.(88%), lidocaine metab.(84%)	sometimes no sulfate conjugates found
Glöckner *et al.* (1998)	rat liver	2, 24 h	Glöckner *et al.* (1996)	K^+ content (2 h: 40%, 24 h: 20%), ECOD and EROD activity in slice homogenate (2 h: 99 en 119%), ECOD in intact slice (2 h: 150%), protein synth.: measurable, inducibility P450 mRNA: measurable	incubation at 30°C, one human liver done but no fresh values given!
Day *et al.* (1998)	rat liver	3 h, 1 h pre-inc.	Day *et al.* (1998)	urea synth. (85%), 7-HC-sulfation (7-EC as substrate) (90%). ALT, LDH retained (70%, 60%)	
	human liver	3 h, 1 h pre-inc.	Day *et al.* (1998)	urea synth. (35 and 70%, 2 livers), 7-EC metab.(55%, 1 liver), LDH retained, ALT retained (75%, 1 liver)	
Glöckner *et al.* (1999)	human liver	2, 6 h	Glöckner *et al.* (1996)	K^+ content (2 h: 70%, 6 h: no fresh values given), protein synth. (2 h: 33%), P450-1A1 mRNA inducible	
Maas *et al.* (2000b)	rat liver	0, 4, 8 h	de Kanter and Koster (1995)	ATP content (4 h: 20%, 8 h: 10%), K^+ content (4 h: 20%, 8 h: 20%), GSH content (4 h: 40%, 8 h 30%), LDH leakage (after 4 h 60% leaked out, after 8 h: 80%), GsT activity (4 h, 8h: 65%), CDNB conj.(4 h: 20%, 8 h: 10%), testost. metab. (4, 8 h: comparable to fresh values), urea synth.(0-3 h: unchanged, 4-7 h: 50%, 8-11 h: 40%, histomorphology (4 and 8 h: some viable foci). 0 h: values unchanged, except CDNB conj.	compared with slow freezing method, fast freezing method better, WME better than UW as freezing medium

Table 4b, continued

Author	Species, organ	Incubation	Method of	Viability parameter (% fresh slice value)	Remarks
de Graaf *et al.* (2000b)	rat liver	4-5 h	de Graaf *et al.* (2000b)	histomorphology (62%), K^+ content (66%), testost. metab. (77%), 7-HC conj.(67-79%)	
Martin *et al.* (2000)	rat liver	3, 24 h	de Kanter and Koster, (1995)	MTT reduction (24 h: <15%), ATP content (24h: <5%), antipyrine hydroxylation (3 and sulfation (3h: 30-50%, 24h:<20%)	UW, with 50% FCS, 10% DMSO used instead of WME/12% DMSO
Sohlenius-Sternbeck *et al.* (2000)	rat liver	2-3 h (1 h pre-incub.)	de Graaf *et al.* (2000)	MTT reduction (86%), testost. metab. (75-100%), ropivacaine metab. (80-94%), 7-HC conj.(80-100%), naphtol conj.(74%)	
Glöckner *et al.* (2001)	rat liver	2, 24 h	Glöckner *et al.* (2001)	GSH content (4 h: 50%, 24 h: 40%), LDH leakage (0-24 h: 3 x as much in cryo slices than in fresh slices), testost. metab. (2 and 24 h: comparable to fresh), ECOD inducibility: comparable to fresh, MUG and PNP conj.: measurable up to 24 h	fresh slices were also exposed to 30% DMSO, washing steps of original protocol omitted
Vanhulle *et al.* (2001)	rat liver	4h, 24 h	Glöckner *et al.* (1996)	paracetamol conj. (4 h: 20-25% , 24 h: <10%), midazolam metab.(4 h: no difference, 24 h: 40-100%), LDH leakage (sign. higher in cryopreserved slices), ATP content (4 h: 35% , 24 h:<15%), glycogen content (4 h: 25%, 24 h: 15%), enzyme content (4, 24 h: not sign. different), histomorphology (some viable foci)	

Several attempts have led to a significant improvement of rapid freezing methods. Day *et al.* (1998) proposed a novel technique to enhance cooling and warming rates during cryopreservation and thawing, using aluminium plates with a high thermal conductivity. Results obtained with this device were significantly better than with the cryovials, originally used by de Kanter and Koster (1995). De Graaf *et al.* (2000b) showed that increasing the DMSO concentration used for pre-incubation of the slices prior to freezing, to 18%, improved viability of slices significantly (as measured by potassium content, phase I and II biotransformation and by examining slice histomorphology). Slices cryopreserved by this improved method remained viable and metabolically active up to 24 h post-thawing as reported in a later study (chapter 7). Sohlenius-Sternbeck and co-workers as well found

that the use of higher DMSO concentrations (18%) enhanced viability as measured by parameters that are sensitive for cryopreservation damage, as did Glöckner *et al.* (2001), who found better results after application of 20 and 30% DMSO than with 10% DMSO.

The mechanism explaining the success of rapid freezing methods is not understood: the high freezing rates (100-1000°C/min.) will probably not result in cell dehydration by equilibrium freezing (Karlsson, 1993, Bischof *et al.*, 1997) and the cryoprotectant concentrations used (12-30%) are too low to achieve vitrification (Boutron and Kaufmann, 1978; Baudot *et al.*, 2000). Evidence exists, however, that water within cells/tissue vitrifies more readily than in a 'bulk' solution. It has been suggested previously that this may be caused by the fact that water within tissue behaves as if it were compartmentalized like in a hydrogel (Peridieu *et al.*, 1995). Fragmentation of a fluid in smaller droplets increases the tendency to supercool and therefore facilitates vitrification (Yamane *et al.*, 1992). Within a slice, water is compartmentalized in cells and interstitial space and for this reason de Kanter *et al.* (1995) have suggested vitrification within the slices as a possible explanation for the success of rapid freezing.

Aims and contents of this thesis

A major aim of the research described in this thesis was to develop cryopreservation methods for hepatic and extrahepatic tissue slices. Furthermore, the mechanism explaining the success of rapid freezing is studied. Finally, we aimed to evaluate the predictive value of *in vitro* metabolism by (cryopreserved) tissue slices for *in vivo* drug metabolism in comparison to other *in vitro* tools.

I The development of cryopreservation methods for hepatic and extrahepatic tissue slices

In chapter 2, a wide range of viability and functionality parameters is used to evaluate cryopreservation of rat liver slices by an equilibrium slow freezing cryopreservation method, originally developed for the cryopreservation of hepatocytes (Maas *et al.*, 2000a) and a rapid freezing method developed by de Kanter and Koster (1995). A full factorial statistical design is used to explain the differences in viability found between the two

methods including the factors cooling rate, cryopreservation medium, slice thickness and stepwise cryoprotectant pre-incubation as variables.

In chapter 3, an attempt is made to improve post-thaw viability and phase I and II biotransformation activity of rapidly frozen slices, by varying DMSO concentration and DMSO carrier medium, slice thickness and cold storage prior to cryopreservation.

In chapter 4, a first step towards cryopreservation of extra-hepatic tissue slices is made by using the rapid freezing method, originally developed for rat liver slices using 18% DMSO and a cooling and warming rate of approximately 200°C/min and by vitrification with high molarity mixtures of cryoprotectants, VM3 and VS4.

II Elucidation of the mechanism of cryopreservation by rapid freezing

In chapter 5 it is determined whether tissue vitrification, promoted by water partitioning in the tissue, could be the mechanism explaining high viability of rat liver slices, rapidly frozen after pre-incubation with 18% DMSO or VS4 (a 7.5M mixture of DMSO, 1,2-propanediol and formamide with weight ratio 21.5: 15: 12.4). Differential Scanning Calorimetry (DSC) is used to determine the extent to which crystallization or vitrification occurrs in CPA solutions (DMSO and VS4) and within liver slices impregnated with these solutions. Secondly, these events are related to survival of slices after thawing.

In chapter 6, an attempt is made to modify the susceptibility of rat liver slices to cryopreservation damage by pre-incubating them at 37°C prior to cryopreservation by rapid freezing or vitrification in order to enable them to regain physiological metabolism. In this manner, we tend to assess the importance of slice condition for cryopreservation outcome.

III Evaluation of the predictive value of drug metabolism by (cryopreserved) tissue slices for in vivo drug metabolism in comparison to other in vitro tools

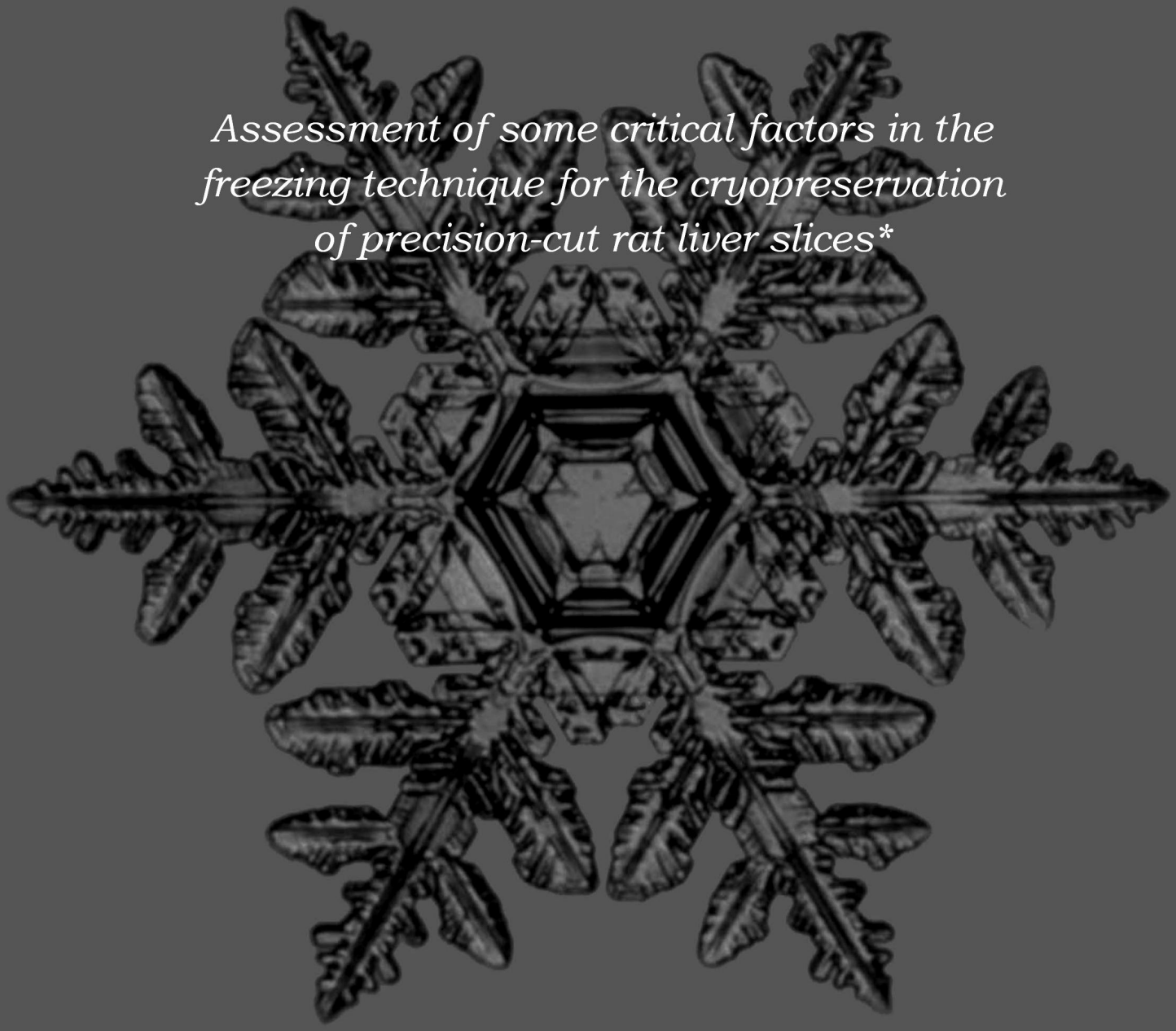
In chapter 7, various *in vitro* preparations are compared with respect to their ability to mimic *in vivo* metabolism. For this purpose, S9-liver homogenate, microsomes, cryopreserved hepatocytes, fresh and cryopreserved liver slices and slices of extra-hepatic metabolizing organs

(lung, kidney, intestines) are incubated with three pharmacologically active compounds, that are metabolized *in vivo* by a wide range of metabolic routes. Metabolites are identified and quantified with LC-MS/UV from the *in vitro* incubations and compared with metabolite patterns in faeces, urine and bile of dosed rats.

Finally, in chapter 8, the most important results and conclusions of this thesis as well as future perspectives of tissue slice cryopreservation for application in drug metabolism research are discussed.

Chapter 2

*Assessment of some critical factors in the freezing technique for the cryopreservation of precision-cut rat liver slices**



*Based on:
W.J.M. Maas, I.A.M. de Graaf, E.D. Schoen, H.J. Koster, J.J.M. van de Sandt and J.P. Groten. Assessment of some critical factors in the freezing technique for the cryopreservation of precision-cut rat liver slices.
Cryobiology **40**, 250-263 (2000)

Abstract

A number of studies on the cryopreservation of precision-cut liver slices using various techniques have been reported. However, the identification of important factors that determine cell viability following cryopreservation is difficult because of large differences between the various methods published.

The aim of the present study was to evaluate some important factors in the freezing process in an effort to find an optimized approach to the cryopreservation of precision-cut liver slices. A comparative study of a slow and fast freezing technique was carried out to establish any differences in tissue viability for a number of endpoints. Both freezing techniques aim at the prevention of intracellular ice formation (IIF) which is thought to be the main cause of cell death after cryopreservation. Subsequently, critical variables in the freezing process were studied more closely in order to explain the differences in viability found in the two methods in the first study. For this purpose, a full factorial experimental design was used with 16 experimental groups, allowing a number of variables to be studied at different levels in one single experiment.

It is demonstrated that ATP and K^+ content and histomorphology are sensitive parameters to evaluate slice viability after cryopreservation. Subsequently, it is shown that freezing rate and the cryopreservation medium largely determine the residual viability of liver slices after cryopreservation and subsequent culturing. It is concluded that a cryopreservation protocol with a fast freezing step and using Williams medium E as cryopreservation medium is the most promising approach to successful freezing of rat liver slices of those tested in this study.

Introduction

Precision-cut tissue slices are extensively used in studies on toxicity and metabolism of xenobiotics (Bach *et al.*, 1996; Olinga *et al.* 1997b). The use of slices for this purpose has several advantages over the use of isolated single cells. First, no digestive enzymes are required for slice preparation and, consequently, cell to cell contacts and normal tissue architecture remain intact. Secondly, slices can be used to study species differences, e.g in drug metabolism (Krumdieck *et al.*, 1980), since they can easily be prepared with the same method from tissue of numerous species. The use of human tissue in this field is of special interest, since it will help overcome difficulties with interspecies extrapolation. The supply of human tissue for *in vitro* research, however, is only limited and irregular. Successful cryopreservation and storage of tissue slices, without a marked loss of viability after thawing, would without doubt expand and facilitate experimental possibilities.

The main concern in cryopreservation is the formation of intracellular ice (IIF) both during freezing and thawing. IIF is thought to be the main cause of cellular damage and can be prevented or reduced using two different approaches. First, slow freezing techniques lead to cellular dehydration. Due to ice crystal formation in the extracellular medium, solutes concentrate in the nonfrozen phase. As a reaction to maintain osmotic equilibrium with their surroundings, cells dehydrate and do not freeze intracellularly (Mazur, 1984). Second, rapid freezing techniques, generally applied in the presence of high concentrations of cryoprotectant (CPA), result in the formation of amorphous instead of crystalline ice in the cellular fluid (vitrification) (Fahy, 1990).

For the cryopreservation of liver slices, both slow and fast freezing techniques have been described (for a recent overview see Glöckner *et al.*, 1998). Results, however, are highly variable and seem to be dependent on the animal species and the viability parameters used (Fisher *et al.*, 1991; Wishnies *et al.*, 1991; Fisher *et al.*, 1993; Ekins, 1996; Ekins *et al.*, 1996b). The identification of important factors that determine cell viability after cryopreservation is difficult due to numerous differences between the published methods, including the viability parameters determined, the equipment used for freezing and the incubation system for culturing slices.

For cryopreserving rat liver slices, the applicability of a slow freezing method was recently studied, showing that cryopreserved rat liver slices can be used for short term (ca 2 h) metabolism studies (Maas *et al.*, 2000a). Also

recently, a fast freezing technique was described (de Kanter and Koster, 1995; de Kanter *et al.*, 1998) reporting cryopreserved rat liver slices to be viable for 3 h after thawing. The reported time period of cell survival in these studies, although promising, may be too short for prolonged toxicological and metabolism research.

The aim of this study was to determine important factors in the freezing technique to find an optimized approach towards the cryopreservation of liver slices. For this purpose, two cryopreservation protocols that use a slow (Maas *et al.*, 2000a) and a fast freezing approach (de Kanter and Koster, 1995) were compared. The study was performed under identical experimental conditions (slices obtained from the same animal, cultured in the same incubation system, using the same medium), allowing the most direct comparison. A number of different parameters were measured to determine which cellular processes were most affected by cryopreservation. The two cryopreservation protocols studied differ not only in freezing rate but also in the cryopreservation medium, in the slice thickness and in the way the CPA is added to the slices. Therefore, in a second study, the variables in the two methods were looked at more closely to determine the importance of each variable in maintaining slice viability after cryopreservation. For this purpose, a set of experiments was performed according to a full factorial experimental design, allowing all variables to be studied in a single experiment.

Materials and methods

Chemicals.

Chemicals were obtained from the following suppliers; Gibco BRL (Paisley, Scotland): Phosphate Buffered Saline (PBS), William's Medium E (WME with Glutamax I) and gentamycin; PAA Laboratories GmbH (Linz, Austria): Fetal Calf Serum (FCS); Sigma Chemical Company (St. Louis, MO, USA): insulin (from bovine pancreas), dimethyl sulfoxide (DMSO), 1-chloro-2,4-dinitrobenzene (CDNB) and the blood urea nitrogen assay kit; Merck (Darmstadt, Germany): D-Glucose; Lamepro b.v. (Raamsdonksveer, The Netherlands): the University of Wisconsin solution (UW); Omnilabo (Breda, The Netherlands): ATP assay kit (Lumac biomass assay kit); Steraloids, Inc., (Wilton, NH, USA): testosterone and hydroxylated metabolites; Pierce (Oud-Beyerland, The Netherlands): the Coomassie Protein kit No.23200; Boehringer (Mannheim, Germany): the lactate dehydrogenase (LDH) kit.

Slice preparation

Male Wistar rats were sacrificed by decapitation. After excision, the liver was cut into lobules that were stored in washing medium (WME containing 10 % FCS) on ice. Slices of two different thicknesses were prepared from 8-mm biopsies in cold WME (pregassed with carbogen (95 % O_2, 5 % CO_2) for approx. 30 min), using a Krumdieck tissue slicer. Slices were washed in cold washing medium and stored in fresh washing medium on ice until use. The time between the sacrifice of the rat and the start of cryopreservation was ca. 2 h.

Culturing

Slices were cultured under carbogen atmosphere in 25-ml Erlenmeyer flasks (1 slice/flask) containing 5 ml culture medium consisting of WME with Glutamax I containing 0.1 µM insulin, 5 % FCS, 50 µg/ml gentamycin and 25 mM D-glucose, pregassed with carbogen. Flasks were tightly closed with a rubber stop.

Cryopreservation

The slow freezing technique (Maas *et al.*, 2000a) is further referred to as method A and the fast freezing technique (de Kanter and Koster, 1995) as method B and they are described in detail by the aforementioned authors and in Table 1. Method A involves computer-controlled slow freezing using a SyLab Icecube 1610 Computer Freezer. Slices were transferred to ice-cold UW solution containing 5% DMSO and were stored on ice for approximately 10 min. Slices were then transferred to cryovials (Costar, Cambridge, MA, USA) (5 slices/vial) containing 0.5 ml UW, 10% DMSO and slowly frozen at 0.5°C/min to -50°C followed by a 1°C/min freezing rate to -80°C, after which the vials were submerged in liquid nitrogen. Large temperature rise in the sample vial due to the release of heat during crystallization was prevented by injecting extra nitrogen into the freezing chamber. Total pre-incubation time at 0-2 °C was ca. 30 min.

With method B, slices were pre-incubated before freezing with 5 ml WME containing 12% DMSO in a 25-ml Erlenmeyer (maximum 5 slices/flask, under carbogen atmosphere) in a shaking waterbath (at ca. 100 rpm, at 2°C for 30 min). Slices were then transferred to 2-ml cryovials (4 or 5 slices/vial) together with 1 ml of the medium used for pre-incubation and frozen by direct immersion into liquid nitrogen.

Table 1 Schematic overview of the two cryopreservation methods used in Study I

	Method A (Maas *et al.*, 2000a)	Method B (de Kanter and Koster, 1995)
Wet weight slices (mg)	16 - 18	19 - 21
Slice thickness (µm) *	± 200-250	± 250-300
Addition of CPA	stepwise addition	immediate addition
Freezing rate	0.5°C/min	± 250°C/min
Cryopreservation medium	UW	WME
CPA concentration	10% DMSO	12% DMSO

*) In a calibration study (results not shown), series of slices were cut at different settings of the Krumdieck tissue slicer. Wet weight was correlated to slice thickness, that was determined morphometrically. In further studies, wet weight of the slices was used to set slice thickness.

Study I

In this study, methods A and B were compared under standardized experimental conditions. A number of viability parameters were studied to determine which parameters were most sensitive to cryopreservation. The viability parameters studied were ATP and potassium (K^+)-levels, histomorphology, total glutathione (GSH) levels, leakage of lactate dehydrogenase (LDH), total glutathione-*S*-transferase (GST) activity, urea formation and the metabolism of CDNB and testosterone.

Experimental design

The experimental design of study I is presented in Table 2. Three separate experiments were performed. All parameters were determined at three time points after slice preparation and thawing and incubation (0, 4 and 8 h) in three slices. Leakage of LDH was determined during two successive periods of 4 h after isolation or thawing of the slices (three per group).

Viability parameters

Histomorphology. One half of a slice was fixed in 70 % ethanol at 4°C for at least 24 h. After dehydration, slices were vertically embedded in paraffin and cross sections (5-µm thickness) were stained with hematoxylin and eosin. Histomorphological quality of the slices was scored by estimating the percentage of viable cells in the slice cross section by two investigators. For this purpose, both nuclear appearance and cytoplasmatic staining were taken into account and compared to cell appearance in fixed samples from fresh liver. Slice thickness was evaluated by counting the number of cell layers.

Table 2 Experimental setup of Study I

		Sampling/incubation time (h)		
Experiment	Viability parameter	0	4	8
I	GSH, GST activity	0	4	8
	LDH activity		0-4	4-8
	Urea synthesis	0-3	4-7	8-11
II	ATP, K^+ and histomorphology	0	4	8
	Testosterone hydroxylation	0-1	4-5	8-9
III	CDNB metabolism	0-0.15	4-4.15	8-8.15
	Histomorphology	0	4	8

Note. This study consisted of three independent experiments

ATP content. One half of a slice was quickly rinsed in physiological saline and immersed in cold 70% ethanol in potassium-free HPLC water (Baker, Deventer, The Netherlands), containing 2 mM EDTA (pH 10.9). The slice was homogenized using a Branford sonifier (50 % duty cycle, 5 s). ATP was determined using the luciferin, luciferase assay on a Lumac Biocounter M500 (Singh *et al.*, 1996).

Potassium content. In the slice homogenate, potassium content was measured using a Beckman 2 electrolyte analyser (Beckman Instruments-Netherlands division BV, Mijdrecht, The Netherlands).

GST activity. GST activity in the slice homogenate was determined on a Varian Cary IE spectrophotometer using 1 mM CDNB and 1 mM GSH as substrates. *S*-(2,4-Dinitrophenyl)glutathione formation (DNPSG) was measured for 3 min at 25°C (detection wavelength: 340 nm).

Metabolism of testosterone. To determine the metabolism of testosterone, slices were incubated for 60 min with 250 µM testosterone in 12-well plates (Costar, Cambridge, MA, USA; 2 ml/well) on a gyratory shaker in a humidified incubator at 37°C, 40% O_2, 5% CO_2 at ca. 80 rpm. Testosterone metabolites were determined by HPLC on a Hypersil ODS column (Chrompack, The Netherlands) according to van 't Klooster *et al.* (1993).

Metabolism of CDNB. For determining CDNB metabolism, slices were incubated for 15 min with 25 µM CDNB in 12-well plates (2 ml/well) on a gyratory shaker in a humidified incubator at 37°C, 40% O_2, 5% CO_2 at ca. 80 rpm. DNPSG was quantified by HPLC using a Zorbax ODS column according to van Iersel *et al.* (1996).

GSH content. GSH was determined using a Shimadzu RF1501 spectrofluorimeter after reacting with o-phtalaldehyde according to Hissin and Hilf (1976).

LDH activity: LDH activity in the medium and the slice homogenate was measured with a BM/Hitachi 911 using a commercially available kit (Boehringer, Mannheim, Germany).

Total protein. To an aliquot of the slice homogenate, 2 M NaOH (25 %v/v) was added to dissolve the protein. The protein solution was diluted (1:5 minimally) with PBS. Subsequently, protein content was measured by a Cobas-Bio centrifugal spectrophotometer with a Coomassie Protein kit using bovine serum albumin (BSA) as a standard.

Statistical analysis.

The data were analyzed by pairwise comparisons of mean values for the methods A and B, respectively. Comparisons were carried out separately for fresh and cryopreserved slices at each of the time points. Statistical significance was assessed by t-tests using log transformed data. Standard deviations were estimated from pooled data from all groups of slices that were treated in the same way.

Study II

The aim of the second set of experiments was to specify the variables in methods A and B that could explain differences in slice viability found in Study I.

Experimental design

The two cryopreservation methods compared in study I differ not only in freezing rate but also in the cryopreservation medium, in the slice thickness and in the mode of addition of the CPA (see Table 1). A set of two experiments was carried out according to a full two-level factorial design. In this full factorial design, each of the variables in the two cryopreservation methods was studied at all levels of the other variables as illustrated in Table 3. The design contained four factors representing the main variables in methods A and B. Furthermore, the factor 'experiment' was introduced to establish the reproducibility of the effects.

Viability parameters

Four viability parameters were selected from study I based on their sensitivity and ability to distinguish between the two cryopreservation methods compared. ATP, K^+, histomorphology and GST activity were all determined in the same slice. Parameters were measured as described for Study I.

Table 3 Experimental design of Study II

	Factors (variables)			
Combination	Medium	CPA addition	Freezing speed	Slice thickness*
1	UW	stepwise	fast	thick
2	UW	immediate	fast	thick
3	UW	stepwise	fast	thin
4	UW	immediate	fast	thin
5	WME	stepwise	fast	thick
6	WME	immediate	fast	thick
7	WME	stepwise	fast	thin
8	WME	immediate	fast	thin
9	UW	stepwise	slow	thick
10	UW	immediate	slow	thick
11	UW	stepwise	slow	thin
12	UW	immediate	slow	thin
13	WME	stepwise	slow	thick
14	WME	immediate	slow	thick
15	WME	stepwise	slow	thin
16	WME	immediate	slow	thin

Note. This study consisted of two experiments. In both experiments, four slices were treated according to each combination. Directly after thawing and after 4 h of additional culturing, two slices were sampled for the determination of slice viability. In addition to the the four factors that represent the main variables between method A and B, the factor 'experiment' was introduced (see materials and methods).

* The actual difference in thickness between 'thin' and 'thick' slices was determined at the end of the experiments by counting the number of cell layers in the slides that were made for histomorphological examination. The average number of cell layers in the slice in two experiments was 13.4 for thick slices and 10.7 for thin slices.

Statistical analysis

Data obtained directly after thawing or after an additional culture period of 4 h were analyzed separately. Statistical analyses were carried out on the natural logarithm of the biochemical parameters and the square root of thickness (number of cell layers). For histology scores, the empirical logistic transformation was used, defined as: transformed score = log [(score + 0.5)/ (100 - score + 0.5)] (Cox, 1970). Statistical analyses were based on the mean transformed data from two slices for each parameter (ATP, K^+, histomorphology and GST activity). The outcome of each of the combinations (illustrated in Table 3) is dependent on effects that are caused by the four handling factors, the factor 'experiment' and the random variation. From the 32 values (mean of two slices) for each parameter (16 possible combinations in two experiments), a total of 31 effects can be calculated (namely, 5 main effects, 10 two-factor interactions, 10 three-factor interactions, 5 four-factor interactions, and 1 five-factor interaction). To illustrate this: the main effect of medium, for example, is calculated as the mean of all observations made with the UW medium minus the mean of

the observations with the WME medium. Other main effects are calculated analogously. Two-factor interactions occur in those cases were the observed effect can not be explained solely by the main effects. The interaction between medium and freezing, for example, is calculated as the difference between the main effect of medium at slow freezing and the main effect of medium at fast freezing. This value is divided by 2, causing all interactions to have the same standard error as the main effects. The remaining interactions are calculated in a way analogous to the calculation of the two-factor interactions. The design and the calculation of the effects is described by Box *et al.* (1978). Statistical significance of the 31 effects on each viability parameter was determined by calculating the standard error of the effects with the PSE(45) (Pseudo Standard Error) method as described by Schoen and Kaul (2000) and using this standard error to judge the effects. Effects were considered significant ($\alpha = 0.05$) if they were larger then 2.35 times their standard error.

Results

Study I

Histomorphological examination

Directly after preparation of the slices, the average percentage of viable cells in the slice was scored 85 ± 5%. After 4 and 8 h in culture, this percentage was slightly decreased to 74 ± 4 and 69 ± 5 %, respectively. Immediately after thawing, the histomorphogical views of slices frozen according to methods A and B closely resembled each other. Compared to freshly prepared slices, a slight increase in the number of pyknotic nuclei was observed and cytoplasmic staining was slightly more eosinophilic. The percentage of viable cells per slice was 63 ± 12%. Differences between slices frozen according to methods A and B were more pronounced after an additional 4 h of culturing, where the percentage of viable cells per slice ranged from 0 to 5 % and 5 to 50 % for methods A and B respectively (Fig. 1a and b). After 8 h of culturing, the percentage of viable cells frozen according to method B was decreased, ranging from 5 to 25 %. In some slices, viable cells appeared in small groups in the venal area and at the edges of the slice. Many dead cells had fragmented nuclei. In slices frozen according to method A, only a few viable cells were observed randomly distributed over the slice. Affected cells showed mostly pyknotic nuclei.

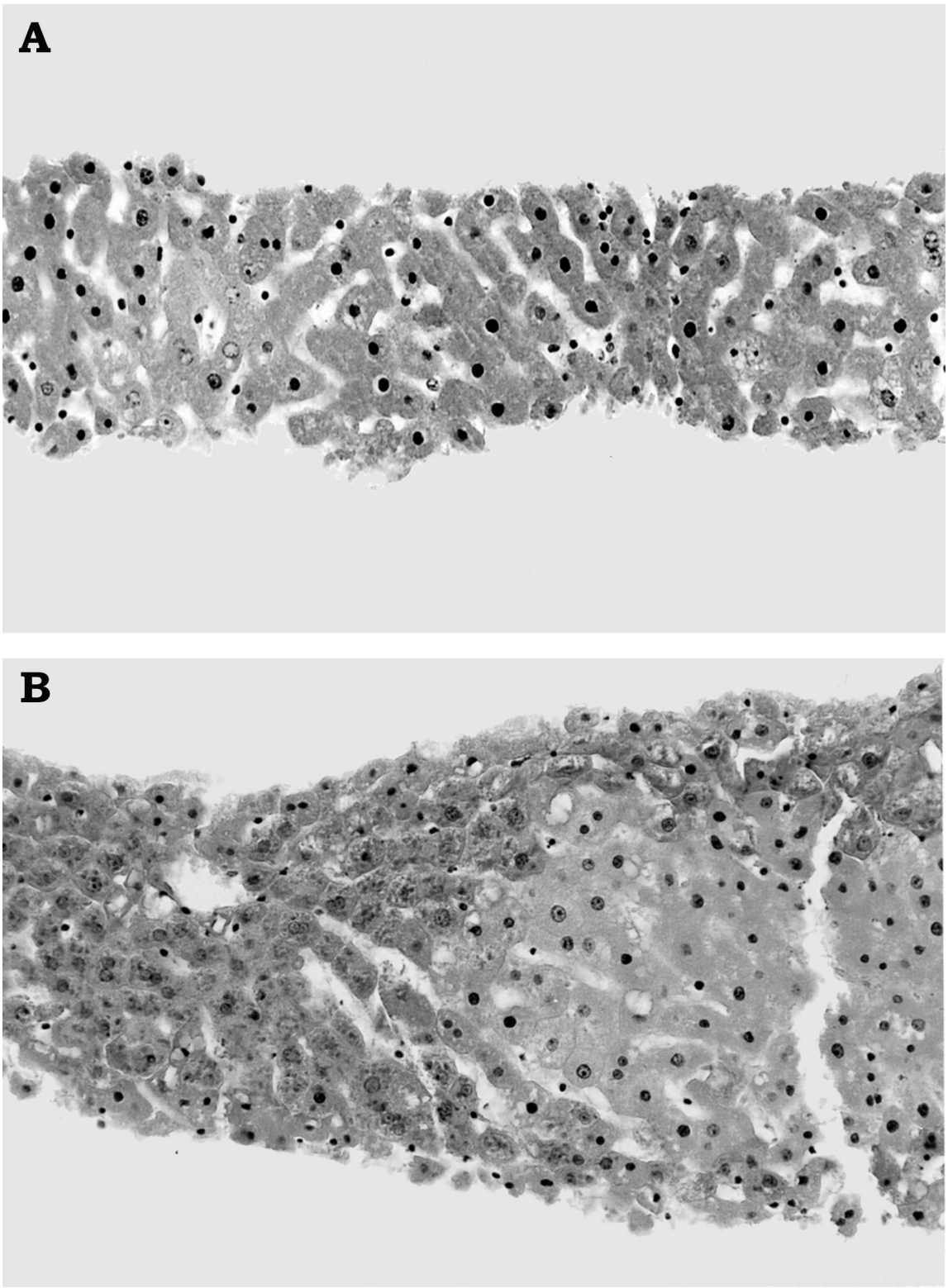


Fig. 1 Histomorphological view of cryopreserved rat liver slices, 4 h after thawing and subsequent culturing (**A**) Method A, (**B**) Method B.

Data obtained from fresh and cryopreserved rat liver slices are presented in Fig. 2. For fresh slices, slice thickness was the only difference between the two methods. Data from cryopreserved slices are discussed below.

ATP, K^+ and GSH content

After cryopreserved slices were thawed and subsequent cultured for 4 and 8 h, ATP and potassium levels in slices cryopreserved according to method B were significantly higher than those levels in slices cryopreserved according to method A (Fig. 2a and b). No difference was observed in GSH content (Fig 2c). Potassium content in slices cryopreserved according to method B was ca. 20% of that measured in fresh slices, both after 4 and 8 h in culture.

LDH leakage

LDH leakage from slices cryopreserved according to method A was significantly higher than leakage from slices frozen according to method B (Fig. 2d). Cryopreservation according to method A and additional culturing for 4 h, resulted in 90 % leakage of total LDH. For slices frozen according to method B, leakage of LDH increased from 60 to 80% of total LDH after 4 and 8 h in culture, respectively.

GST activity

Total GST activity in the homogenate of slices frozen according to methods A and B significantly differed at all time points (Fig. 2e). In slices cryopreserved according to method A, GST activity declined to 15% of values of fresh slices after 4 h and remained at the same level after 8 h in culture. In slices frozen according to method B, respectively 67% (after 4 h) and 58% (after 8 h) of total GST activity remained.

Metabolism of CDNB

In cryopreserved slices, CDNB metabolism decreased with time with only limited formation of DNPSG left after 8 h (Fig. 2f). DNPSG formation in slices cryopreserved according to method B was only after 4 h of culturing significantly higher than DNPSG formation in slices frozen according to method A.

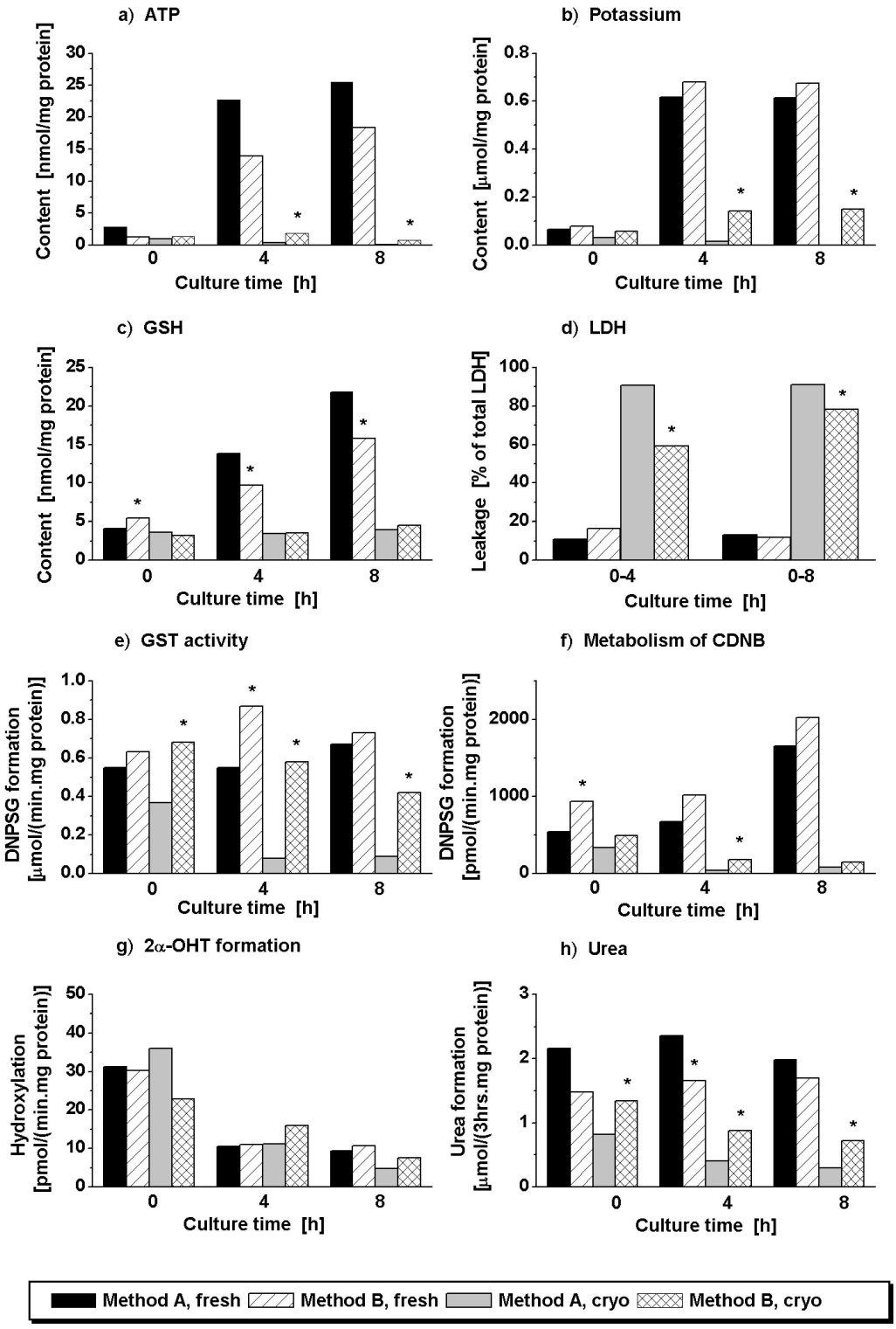


Fig. 2 Study I: viability parameters determined in fresh and cryopreserved rat liver slices, directly after preparation or thawing and after 4 or 8 h of culturing. Statistical analysis was carried out separately for fresh and cryopreserved slices at each of the time points. *Significantly different, $P < 0.05$.

Metabolism of testosterone

No significant difference in the formation of hydroxylated metabolites of testosterone (OHT) determined in this study (2 -, 6ß-, and 16 -OHT) was found between fresh and cryopreserved slices at any time point (both in slices cryopreserved according to methods A and B). The formation of 2 -OHT is shown in Fig. 2g.

Urea synthesis

Following cryopreservation, urea synthesis in slices cryopreserved according to method B was significantly higher than that in slices frozen according to method A at all time points (Fig. 2h): After 8 h in culture, urea synthesis in slices frozen according to method A and B was 17% and 44% of fresh values, respectively.

Study II

In this study, variables between the two cryopreservation methods (that is freezing rate, cryopreservation medium, slice thickness and the way of CPA addition) were studied more closely in order to explain the differences in slice viability found in study I.

Determination of critical factors (significant effects) directly after thawing.

The main effects of all five variables on the selected viability parameters directly after thawing are graphically presented in Fig. 3, left side. In slices cryopreserved according to method A, relatively high potassium levels were measured, resulting in a significant effect of the factor 'medium' ('WME/UW' in Fig. 3) on the parameter K^+. Furthermore, based on the same parameter, slice thickness was determined to decisive, with thick slices containing slightly higher potassium levels than thin slices. Finally, a significant difference was also seen between the two experiments ('II / I' in Fig. 3) on the parameters ATP and GST activity.

Determination of critical factors (significant effects) after 4 h of culturing

The main effects of all five variables on the selected viability parameters after 4 h of culturing are graphically presented in Fig. 3, right side. The choice of the cryopreservation medium appeared to significantly affect the outcome of the viability parameters histomorphology, GST activity and potassium content. Slices cryopreserved in WME showed better histomorphology and higher GST activity and potassium levels, after 4 h of culturing.

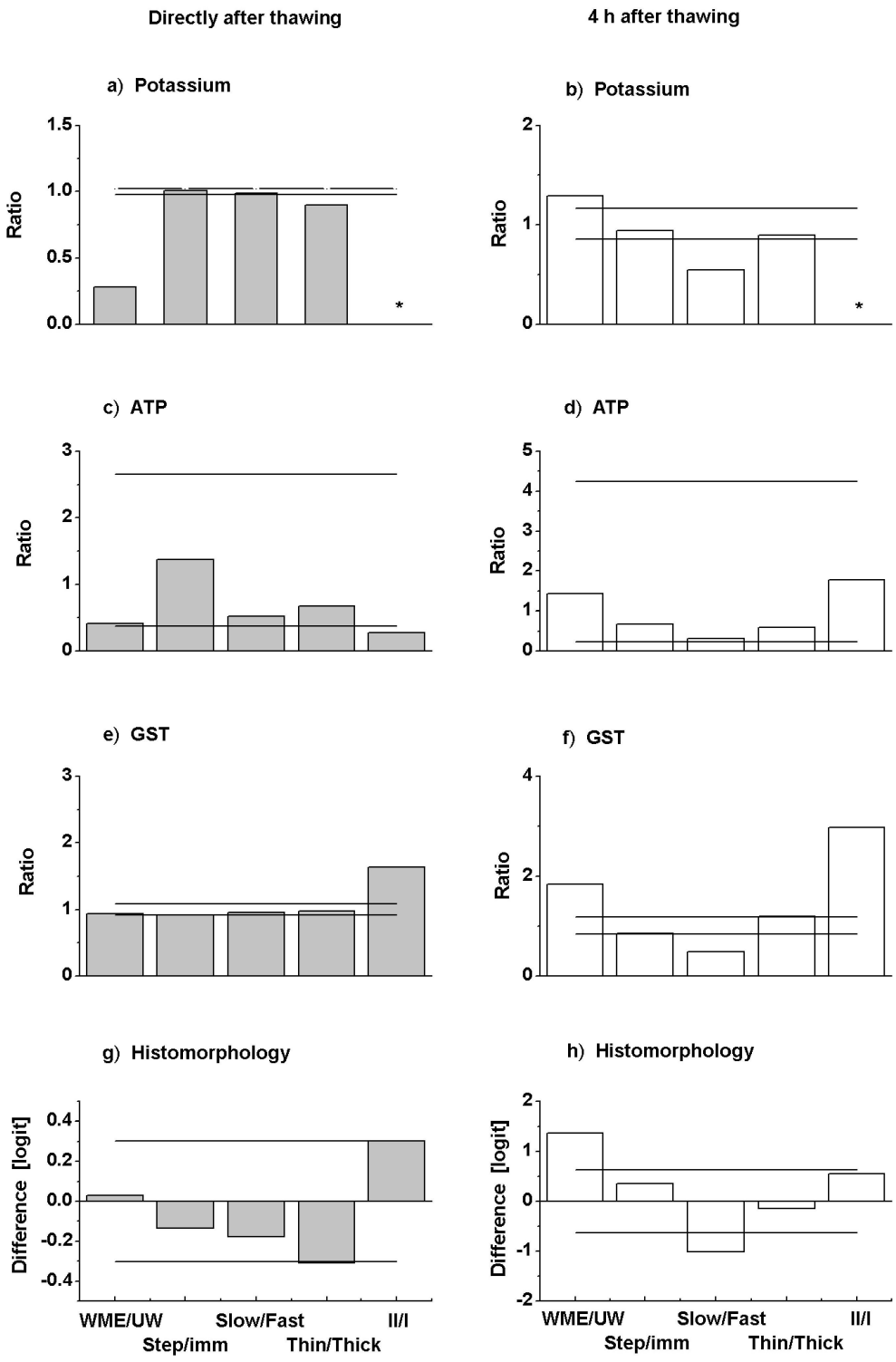


Fig. 3 Study II: main effects of experimental factors on viability parameters directly after thawing (left) and after 4 h of culturing (right). Boundaries between which the effects are nonsignificant ($\alpha = 0.05$) are given with horizontal lines. The Y-axis represents the ratio between geometric means taken at both levels of a factor. For histomorphology, effects are represented as differences on a logit scale. Step/imm, stepwise addition/immediate addition of CPA. *Potassium content was determined in one experiment only.

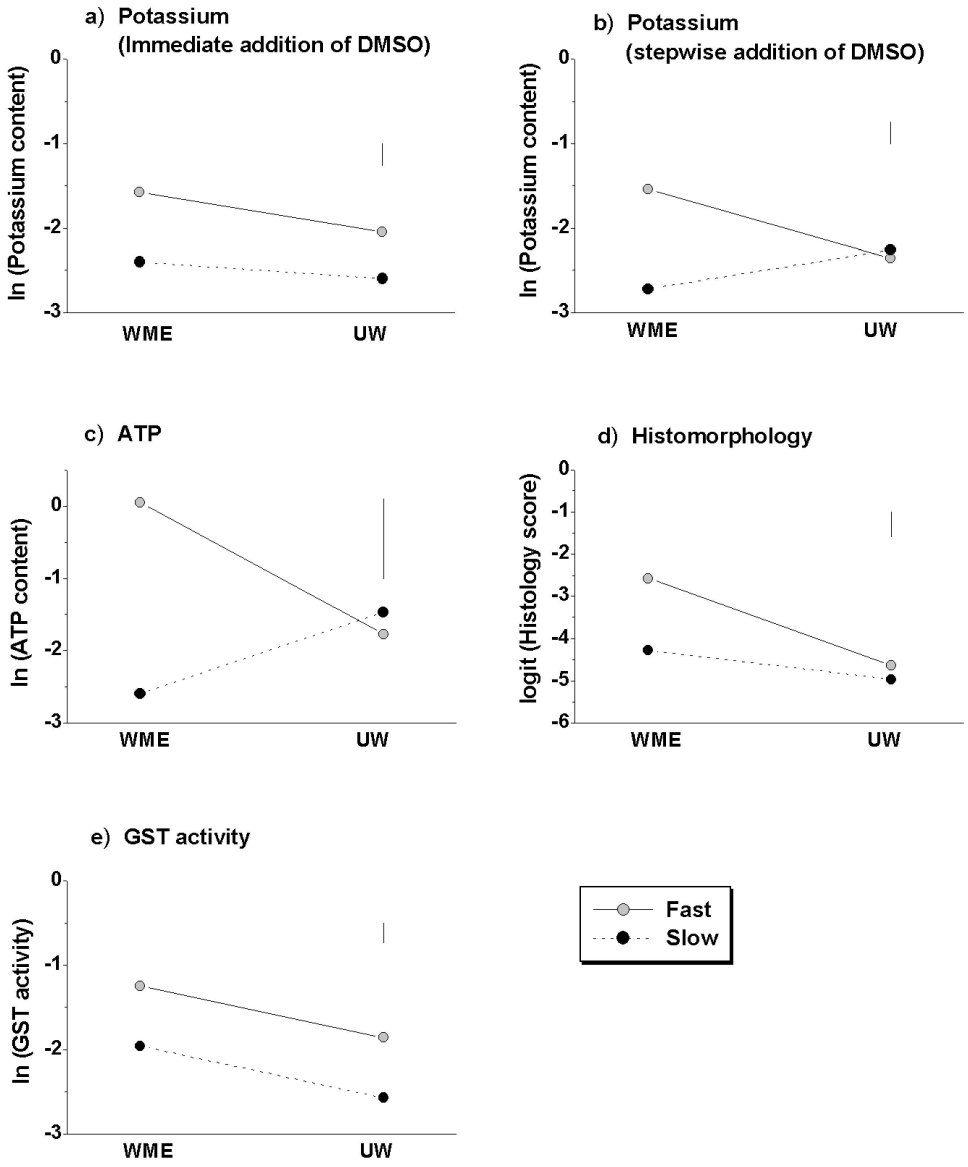


Fig. 4 Study II: joint effects (interactions) of experimental factors (variables) 4 h after thawing and subsequent culturing for various viability parameters. Vertical lines indicate the smallest difference between variables (fast vs slow freezing and WME vs UW as freezing medium) that is statistically significant (least significant difference: α =0.05).

Also, the freezing rate was determined to significantly affect the outcome of the same parameters. This finding was consistent in both experiments of Study II.

For ATP, the observed effect could not be explained solely by the effect of the main variables. However, a significant interaction was observed between freezing rate and medium. This interaction was also found on the parameters histomorphology and GST activity. For potassium content, a significant (three factor) interaction between freezing, medium and CPA addition was observed. These interactions are graphically presented in Fig.

4. The use of UW instead of WME caused a decrease in levels of all parameter in fast-frozen slices.

For all viability parameters determined, highest levels were measured in slices that had been fast frozen in WME, independent of slice thickness. The average potassium level was 0.21 ± 0.03 μmol/mg protein which was ca. 30% of the value measured in fresh slices. ATP levels in study II showed a large variation (as illustrated in Fig. 3), ranging from 2% to 14% in the first experiment and from 2% to 25% in the second experiment (both compared to fresh slices). Considering histomorphology, the average percentage of viable cells was 10 ± 9%. The highest number of viable cells scored in Study II was 35%. The average GST activity was 33 ± 15% and 53 ± 18% (both compared to fresh slices) in Experiments I and II, respectively.

Discussion

The aim of the present study was to evaluate some important factors in the freezing process in an effort to find an optimized approach for the cryopreservation of precision-cut liver slices. To meet this purpose, a comparative study was carried out using two freezing techniques for the cryopreservation of rat liver slices (de Kanter and Koster, 1995; Maas *et al.*, 2000a) to establish any differences in tissue viability for a number of endpoints. Subsequently, a second study was performed to identify the most important variables between the two cryopreservation methods that could explain the differences in slice viability after cryopreservation observed in the first study. The second study was performed according to a multifactorial experimental design, that has been shown to be an effective way to study main effects and interactions of several variables in toxicological research with different end-points (Groten *et al.*, 1991; Schoen *et al.*, 1996).

The comparative study between method A and B was performed under identical conditions. Directly after thawing, differences between the two methods were limited to urea synthesis and GST activity, with method B showing significantly higher levels for both parameters. After 4 and 8 h of culturing, viability of slices cryopreserved according to method B was significantly higher than that of slices frozen according to method A, based on all parameters with the exception of GSH content and testosterone hydroxylation.

A clear difference between the viability parameters was observed with respect to their sensitivity to cryopreservation. Urea synthesis and GST activity in the slice homogenate were relatively well maintained with method B, while the metabolism of testosterone was maintained by both methods. This latter finding is consistent with the report that membrane bound enzymes, such as the cytochrome P450 complex, are resistant to freezing stress (Ekins *et al.*, 1996b). However, a more pronounced loss of viability was apparent when ATP and K^+ content and histomorphology were considered.

In the second study we tried to specify the important variables that could explain the differences between the two cryopreservation techniques that were found in the first study. Directly after thawing, a significant medium effect was observed on the parameter K^+. Furthermore, a significant experiment effect was detected on GST activity and ATP content. These effects, however, were considered of less importance since they could be explained by the high concentration of potassium in the UW solution (that was not complete removed from the slice during washing), or biological variation.

After 4 h in culture, two variables were detected that could explain to a large extent the differences between the two cryopreservation methods, namely the freezing rate and the cryopreservation medium. Viability of slices that were rapidly frozen using WME as cryopreservation medium was highest compared to all other groups. This effect was observed on all parameters except ATP.

With slow freezing of rat liver tissue, it was recently shown that in the presence of DMSO, cellular dehydration continued below -20°C, until maximum dehydration was reached (Smith *et al.*, 1998). In the present study, the DMSO concentration was in the same range as used in Smith *et al.* (1998) and slow freezing was continued until -50°C. We therefore expect almost maximum cellular dehydration to occur, with minimal IIF. However, long term exposure (caused by the low freezing rate) of cellular membranes to concentrated intracellular solutes (resulting from cellular dehydration), may have caused cellular damage. With the fast freezing approach used here, we expect intracellular ice to form, since the freezing rate does not allow for cellular dehydration and both freezing rate and CPA concentration are too low to allow for vitrification to occur. However, our results indicate that fast freezing is still favourable over slow freezing for the cryopreservation of rat liver slices. We can speculate that the ice crystals formed at least in some of the cells, are small and therefore cellular damage

is limited. It can be hypothesized that more rapid freezing and thawing (ice crystals tend to be small at high cooling rates (Mazur, 1984)) might contribute to higher survival rates. Only recently, it was demonstrated that a significant increase in liver slice viability could be achieved when aluminium plates instead of cryovials were used for freezing. Due to the much higher thermal conductivity of aluminium, higher freezing rates could be reached (Day *et al.*, 1998). Preliminary data using small stainless steel grids and higher CPA concentrations, confirm these observations (Maas *et al.*, de Graaf *et al.*, unpublished results).

In addition to the freezing rate, the cryopreservation medium was found to be an important factor determining the success of cryopreservation: the use of WME resulted in higher viability than the use of UW, which is successfully used in cold storage of tissue. This finding is unexpected, since we believed the effect of the UW solution to be beneficial, by preventing cold-induced cell swelling (Southard and Belzer, 1993) leaving less unbound water in the cells to crystallize.

No effect of CPA addition or slice thickness was observed in the present study. Due to the much lower cell membrane permeability of DMSO than of water, the rapid introduction of CPA in the medium can cause an osmotic shock leading to cell damage (Levin and Miller, 1981). In the present study, no significant beneficial effect of stepwise addition of the CPA on slice viability was observed. Most likely, the CPA concentration used was low enough to prevent osmotic damage.

With respect to slice thickness, this study showed no major effect on viability of the slices after cryopreservation. The actual difference between 'thin' and 'thick' slices was statistically significant (average difference of ca. 3 cell layers). Theoretically, slice thickness could be an important factor in the success of cryopreservation. Thermal gradients may occur in thick slices, leading to differences in freezing rate between the edges and middle of the slice. Furthermore, penetration of CPA in thick slices could be insufficient. Indeed, in some slices that had been fast frozen in WME, viable cells were observed in groups located around venal area or at the edges of the slice. This indicates that CPA penetration or freezing rates may have been insufficient in cells located deeper in the tissue, and that the use of thin slices is favourable. The reason why slice thickness was not found to be a determining factor is most likely due to the fact that the difference in slice thickness was too small to cause a clear effect on viability.

In summary, this study shows that hydroxylation of testosterone, urea synthesis and GST activity were relatively insensitive to cryopreservation

stress. ATP and K^+ content and histomorphology were more clearly affected and can therefore be considered more sensitive parameters for evaluating slice viability after cryopreservation. Freezing rate and the cryopreservation medium were demonstrated to be major determinants of the residual viability of liver slices after cryopreservation and subsequent culturing.

Acknowledgements
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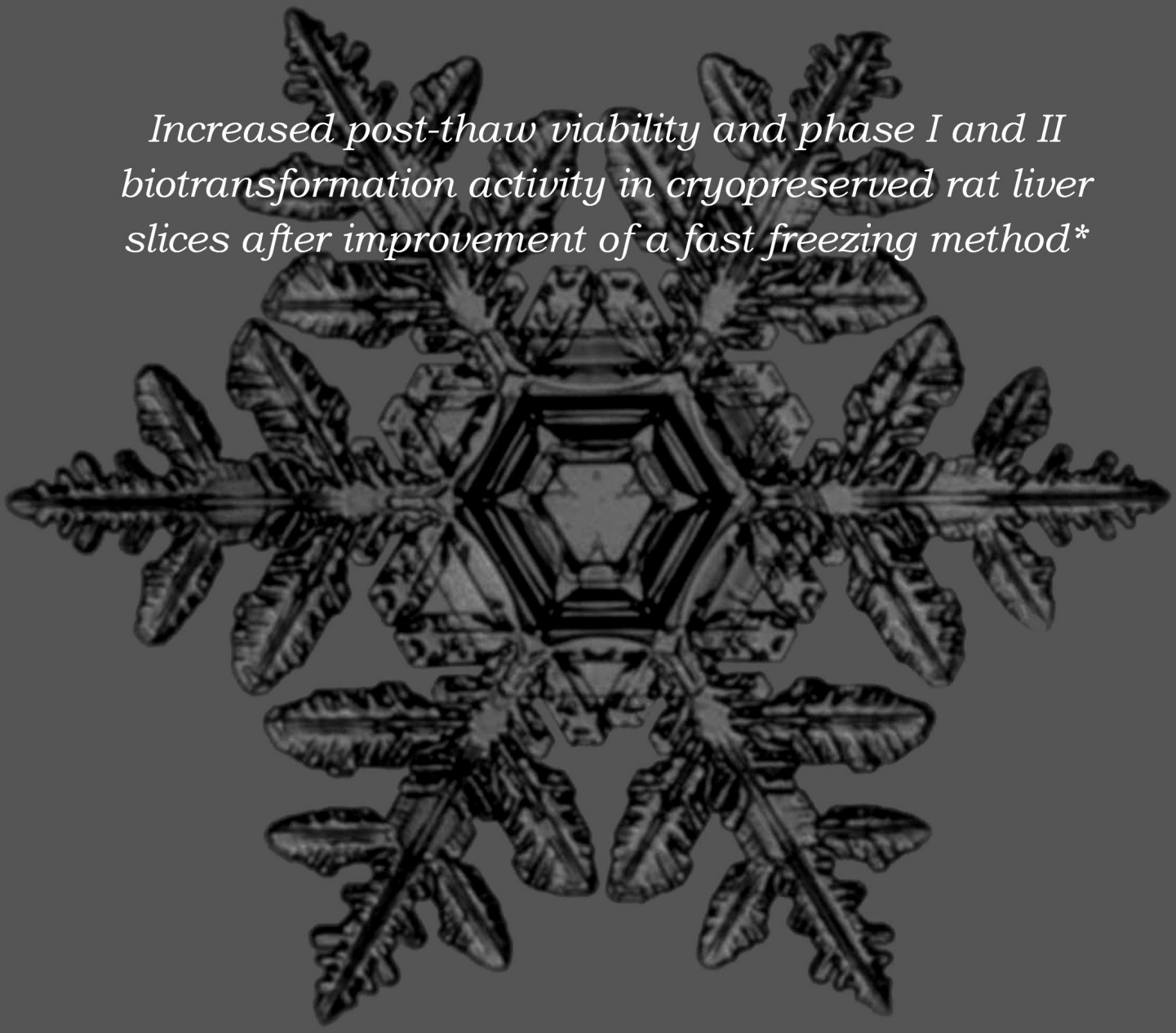
Chapter 3

*Increased post-thaw viability and phase I and II biotransformation activity in cryopreserved rat liver slices after improvement of a fast freezing method**



*Based on:
I.A.M. de Graaf, D. van der Voort, J.H.F.G. Brits and H.J. Koster.
Increased viability and phase I and II biotransformation activity in cryopreserved rat liver slices after improvement of a fast freezing method.
Drug Metabolism and Disposition **28 (9)**, 1100-1106 (2000)

Abstract

An existing cryopreservation method for liver slices applies 12% dimethylsulfoxide and rapid freezing. We previously found that cells in rat liver slices, cryopreserved in this manner, deteriorated rapidly upon culturing. To improve this cryopreservation method, we varied the dimethylsulphoxide concentration (0, 12, 18 and 30%), the cryopreservation medium (Williams Medium E, Fetal Calf Serum and University of Wisconsin medium), slice thickness and the storage period at 4°C during slice preparation prior to cryopreservation. After thawing, slices were cultured for 4 h at 37°C before their viability was evaluated by their potassium content and the number of intact cells determined histomorphologically. The biotransformation capacity of liver slices cryopreserved by the improved method was assessed by testosterone oxidation, hydroxycoumarine sulfation and glucuronidation.

Best results were obtained with 18% dimethylsulphoxide in Williams medium E: the potassium content of cryopreserved slices was higher than 65% and the number of intact cells was higher than 60% of that in fresh slices; with 12% dimethylsulphoxide, potassium content was less than 40% and the number of intact cells was less than 30%. Results did not differ between the three cryopreservation media. Viability of thin slices (8-10 cell layers) was better maintained than that of thicker slices (>14 cell layers). Storage at 4°C of slices before cryopreservation decreased viability after cryopreservation. Both oxidative and conjugation activities were better than 60% of fresh values.

Although results varied, slices cryopreserved with this improved method and cultured for 4 h retained viability between 50-80% and biotransformation activity between 60-90% of fresh slices.

Introduction

Precision-cut tissue slices are extensively used as *in vitro* tool for studies on toxicity and biotransformation of xenobiotics (Bach *et al.*, 1996; Olinga *et al.* 1997b). A major advantage of tissue slices over primary cell cultures is that cell-to-cell contacts and normal tissue micro-architecture remain intact. In addition, tissue slices are prepared according to a rapid, reproducible and relatively simple method (Krumdieck *et al.*, 1980). Cryopreservation of liver slices can extend their use, for slices from animals and man could then be used in a single experiment, facilitating species comparison and decreasing the number of laboratory animals required.

A number of cryopreservation methods for liver slices has been described in recent years. Roughly, methods can be divided in those that use (computer-controlled) slow freezing (Fisher *et al.*, 1991; Fisher *et al.*, 1993; Fisher *et al.*, 1996), those that use vitrification protocols requiring high concentrations of cryoprotectants (CPAs) (Wishnies *et al.*, 1991; Ekins, 1996; Ekins *et al.*, 1996a) and those that apply rapid freezing with low concentrations of DMSO (de Kanter and Koster, 1995; Glöckner *et al.*, 1996; Glöckner *et al.*, 1998; de Kanter *et al.*, 1998). Success of reported methods is variable and seems dependent on animal species (Fisher *et al.*, 1991) and viability parameters (Maas *et al.*, 2000b). Particularly, phase I biotransformation is well preserved by all methods, while phase II biotransformation capacity is reported to be preserved to much lesser extent or not at all (Wishnies *et al.*, 1991; Ekins *et al.*, 1996a and b; de Kanter *et al.*, 1998; Maas *et al.*, 2000b). No publications have reported preservation of intact histomorphology of thawed liver slices.

The rapid-freezing method for cryopreservation, developed in our laboratory (de Kanter and Koster, 1995; de Kanter *et al.*, 1998), has the advantage of using relatively low and nontoxic concentrations of CPA (12% DMSO). Moreover, the method is relatively simple: slices are put in cryovials with medium and directly submersed in liquid nitrogen. Using this method, slices apparently maintained functional activity (testosterone oxidation, urea synthesis) and cell membrane integrity (alanine aminotransferase retention) close to levels of fresh slices in the early post-thaw stage (0-3 h after thawing). Others, who used rapid-freezing methods have found similar results (Glöckner *et al.*, 1996; Glöckner *et al.*, 1998)

Recently, we compared the usefulness of this method, which presumably minimizes intracellular ice crystal formation (IIF) by intracellular vitrification (Kanter and Koster, 1995) to another method that was designed to

prevent IIF by dehydrating cells by slow freezing (Maas *et al.*, 2000b). Although the rapid-freezing method better maintained phase I biotransformation activity and urea synthesis, cell integrity as revealed by histomorphological examination was severely affected by either method. Moreover, cryopreserved slices had low contents of intracellular potassium (potassium) and ATP and were incapable to perform glutathione (GSH) conjugation of chlorodinitrobenzene due to cofactor (GSH) loss.

In the present study, we attempted to improve results on slice viability after cryopreservation by rapid-freezing. Two approaches were used for this purpose: One approach was to try to inhibit ice-crystal formation and thus stimulate vitrification of intracellular and extracellular water. To achieve this, we varied the concentration of DMSO, a well-known glass former (Boutron and Kaufmann, 1978; Hey and Macfarlane, 1996; Baudot *et al.*, 1999). Additionally, WME was replaced as CPA carrier medium by University of Wisconsin Medium (UW) and Fetal Calf serum (FCS), media that contain hydroxyethyl starch (HES) and bovine serum albumine (BSA) respectively, that bind water and thus stimulate water vitrification extracellularly (Körber *et al.*, 1982; Takahashi and Hirsh, 1985).

The other approach was to alter the susceptibility of slices for cryopreservation damage. To that end, slice thickness was varied. Slice thickness may be of importance, because ice crystal formation is more likely to occur in the cryopreservation medium surrounding the slices during freezing than in the tissue itself, where vitrification is favoured (Tahahashi and Hirsh, 1985; Peridieu *et al.*, 1995). In very thin slices, therefore, relatively more cells might suffer from ice-crystal formation on the outside of the cell than in thicker slices (de Kanter and Koster, 1995). In very thick slices, on the other hand, CPA diffusion to the inner cell layers may be impeded causing a suboptimal cryopreservation condition in these cells.

In tissues, kept at subphysiological temperatures, intracellular ion concentrations change and cells swell (Belzer and Southard, 1988). Inevitably, slices are kept for some time in buffer at 4°C before they enter the cryopreservation procedure. Therefore, the duration of this step was tested for its effect on the cryopreservation result.

As endpoints for optimization of viability, potassium retention and slice histomorphology were used, parameters that have been shown to be highly sensitive to cryopreservation damage (Maas *et al.*, 2000b). Slices, cryopreserved by an improved protocol, were tested on their phase I (testosterone oxidation) and II biotransformation capacity (hydroxycoumarine sulfation and glucuronidation) subsequently.

Materials and methods

Materials

7-Hydroxycoumarine glucuronide and sulfate were generous gifts of dr. P. Olinga, Center for Pharmacy, University of Groningen, The Netherlands; Krebs Henseleit buffer, BSA, insulin and gentamycine were obtained from Sigma, Axel, The Netherlands; WME+glutamax, FCS and phosphate-buffered saline (PBS) were from Gibco BRL, Breda, The Netherlands; DMSO (>99.9% pure), cryovials were from Greiner, Alphen a/d Rijn, The Netherlands; Coomassie Protein kit No. 23200, was from Pierce, Oud-Beyerland, The Netherlands; testosterone and 11β-Hydroxytestosterone were from Fluka, Buchs, Switserland; hydroxytestosterone metabolites were from Steraloids Inc., Wilton NH, USA; 7-hydroxycoumarine was from Merck-Schuchart, Hohenbrunn, Germany; hematoxylin and eosin were from Sigma, St. Louis, MO, USA. glucose, HPLC-water and all other chemicals were from Baker, Deventer, The Netherlands.

Preparation of slices

Male rats (Wistar, obtained from Harlan CPB, Horst, The Netherlands) had free access to food and water. They were anesthesized with 65% CO_2, 35% O_2 before the livers were removed. Tissue cores (8 mm) were prepared from the freshly excised liver, using an electrical drill (Metabo BSE 5010) with a tissue coring tool (Alabama R&D, Munford, AL, USA). Slices were prepared subsequently using a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA), filled with oxygenated (95% O_2, 5% CO_2) ice-cold Krebs Henseleit buffer, supplemented with $NaHCO_3$ (25 mM) and $CaCl_2$ (2.5 mM). To determine optimal slice-thickness for cryopreservation, the slicer was set to produce slices of various thickness. Thickness of slices generated in this manner was evaluated by microscopically counting the number of cell layers in paraffin cross-sections.

After preparation, slices were washed and kept on ice in WME with 10% FCS until use. To determine the influence of this cold storage period on slice viability after cryopreservation, the length of time between slice preparation and the start of the cryopreservation method was varied in some experiments.

Cryopreservation and thawing of slices

Before freezing, slices were pre-incubated in a shaking water bath (110 times/min) for 30 min in a 25-ml Erlenmeyer flask (6 slices/Erlenmeyer) on

ice. Each Erlenmeyer flask contained 5 ml oxygenated medium (WME, UW or FCS) supplemented with CPA. As CPA, various concentrations of DMSO were used. Subsequently, slices were put in 2-ml cryovials with 0.5 ml pre-incubation medium. Hereafter, the cryovials were directly submersed in liquid nitrogen. Following this procedure, slices were cooled at approximately 250°C/min to -196°C (de Kanter and Koster, 1995). After storing the cryopreserved slices in liquid nitrogen for 30 min[1] or more, slices were thawed by placing the cryovials in a 37°C waterbath. After visible ice was vanished, slices were washed shortly with WME, 10% FCS.

To determine possible adverse effects of pre-incubation with DMSO on the slices, some slices were pre-incubated with DMSO, but not cryopreserved. The cryopreservation medium was washed away immediately at the end of pre-incubation whereafter slices were incubated for viability testing.

Incubation of slices

Fresh and cryopreserved slices were incubated for 4 h at 37°C, floating in 5 ml medium in a 25-ml Erlenmeyer flask (1 slice/Erlenmeyer), placed in a waterbath shaking back and forth (110 times/min), under humid carbogen (95% O_2, 5%CO_2). This incubation system maintains viability of fresh liver slices for at least 24 h (Olinga *et al.*, 1997a). WME+glutamax was used as incubation medium, supplemented with FCS (5%), 0.1 µM insulin, 50 mg/l gentamycine and D-glucose (25 mM). After 4 h incubation, tests on viability and biotransformation capacity were performed.

Potassium content

To determine potassium content, slices were washed in a physiological salt solution (37°C) prepared with potassium-free HPLC-water. Subsequently, the slice was cut in two equal parts. One part was used for histomorphological examination (see below). The other part was immersed in 70% ethanol in HPLC water, containing 2 mM EDTA (pH 10.9), for potassium and protein determination. For this purpose, the slice was homogenized using a Branford sonifier (50% duty cycle, 5 s). Potassium content was measured in the homogenate using a Beckman 2 electrolyte analyser (Beckman Instruments, Mijdrecht, The Netherlands).

[1] Theoretically, it is expected that freezing and thawing of slices determine viability after cryopreservation and not the storage period in liquid nitrogen (Mazur, 1984). We compared viability of slices stored for 30 min with slices from the same liver that were stored for approximately 48 h. Indeed, no differences were found.

Protein determination

To an aliquot of the slice homogenate, 2M NaOH (25% v/v) was added to dissolve the protein. Hereafter, the protein solution was diluted (1:5 minimally) with PBS. Subsequently, protein content was measured by a Cobas-Bio centrifugal spectrophotometer with a Coomassie Protein kit, BSA was used as a standard.

Histomorphological examination

After fixation with 70% ethanol at 4°C for at least 24 h, slices were dehydrated in a series of ethanol and xylene using a Shandon 2LE Processor. Subsequently, slices were vertically embedded in paraffin and cross sections (4 μm thickness) were made which were stained with haematoxylin and eosin (H&E). Upon microscopical examination, slice viability was determined by estimating the percentage of viable cells in the slice cross section. For determining viability, nuclear shape and staining and cytoplasmatic staining were taken into account.

Testosterone metabolism

Metabolism of testosterone was determined after 60 min of incubation with 250 μM testosterone in homogenate extracts by HPLC as earlier described (van 't Klooster *et al.*, 1993), but using a flow of 0.8 ml/min. 11β-Hydroxytestosterone was used as internal standard. Of all testosterone metabolites peak areas were determined, but only formation of 2α-hydroxytestosterone (an important hydroxy-testosterone metabolite in rats) was quantified (as production in picomoles per slice per min). The formation of this metabolite was linear throughout the 60 min incubation with the substrate, with R^2=0.999.

7-HC conjugation

Conjugation of 7-HC to 7-HC-glucuronide (7-HC-gluc) and 7-HC-sulfate (7-HC-sulf) was determined after incubation of 60 min with 100 μM 7-HC. The conjugates were determined in the slice homogenate by HPLC with an Inertsil ODS-3 (150x2.1 mm) column and a gradient of 100% water with 3 g/l ammonium acetate to 100% pure methanol in 30 min and 3 min isocratic at 100% methanol. Detection was at 325 nm (Walsh *et al.*, 1995). Formation of 7-HC-gluc and 7-HC-sulf was linear throughout the 60 min incubation with the substrate with R^2= 0.995 and 0.998 respectively.

Statistical analysis

For the variables DMSO concentration, cryopreservation medium and storage time, an analysis of variance (ANOVA) has been applied to the viability data, considering data of one experiment as one observation, followed by comparisons between data with the t-test. A Bonferroni correction of the *P* values was used where applicable. To study the effect of slice thickness, an ANOVA has been applied for the fresh and cryopreserved slices separately. Statistical significance of the relation (both with a linear and a quadratic fit) between viability and slice thickness was subsequently determined with the F-test. For all tests, $P<0.05$ was considered statistically significant.

Results

Histomorphology of precision-cut liver slices

Precision-cut liver slices always have damaged cells at the cutting edges (1-2 cellayers at each edge). These damaged cells are also taken into account with viability scoring. That means that 20-30% of the cells are always non-viable and, therefore, slices will get a maximal 'viability' score of 70-80%.

Effect of DMSO on the viability of fresh slices.

To discriminate the possible toxicity of DMSO used in the cryopreservation procedure from damage caused by cryopreservation itself, the effect of DMSO on the viability of fresh slices was determined. To that end, freshly prepared slices were subjected to the cryopreservation pre-incubation with DMSO and, without actual cryopreservation, cultured for 4 h at 37°C.

Exposure of slices to DMSO at concentrations up to 18% during pre-incubation (not followed by cryopreservation) did not cause adverse effects on slice potassium content or on slice histomorphology (Fig. 1). The use of 30% DMSO resulted in a statistically significant reduction in potassium content of fresh liver slices and a nonstatistically significant reduction in intact cells.

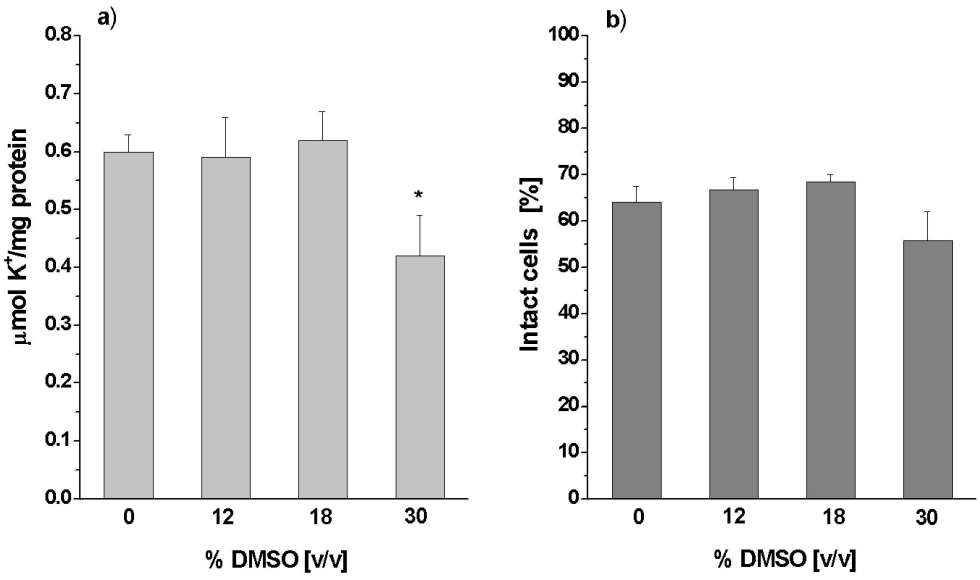


Fig. 1 Potassium content **[a]** and histomorphology **[b]** of fresh slices in culture for 4 h after pre-incubation with various concentrations of DMSO. Values are shown as mean of individual experiments (6-9 experiments, 3 slices per experiment) +SEM. *values are significantly lower ($p<0.05$) than those of slices that were pre-incubated without DMSO.

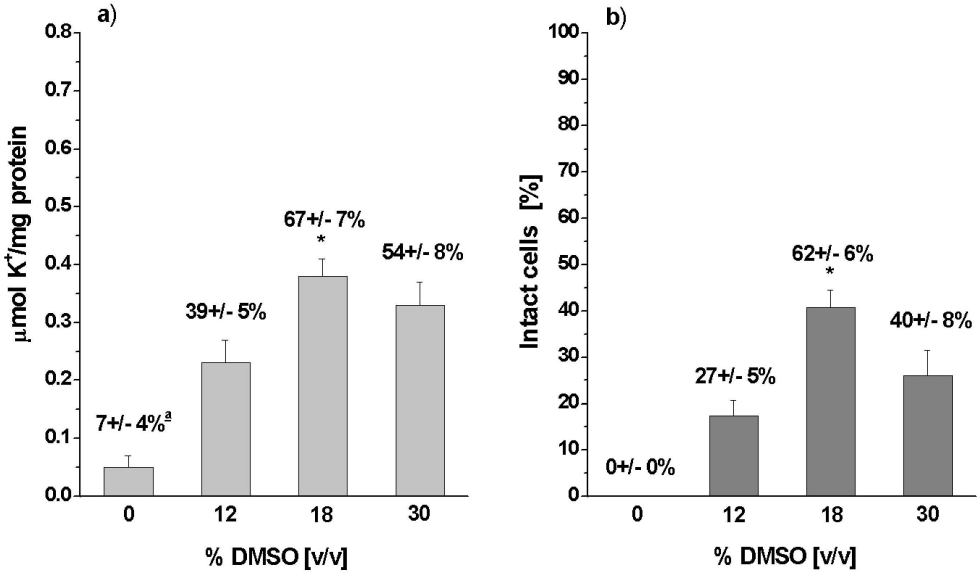


Fig. 2 Potassium content **[a]** and histomorphology **[b]** of cryopreserved slices pre-incubated with various concentrations of DMSO. Slices were cultured for 4 h after thawing. Values are shown as mean of individual experiments (4-10 experiments, 3 slices per experiment) +SEM. [a] % of value of fresh slices (i.e. slices that were not pre-incubated with DMSO or cryopreserved) + SEM. *values are significantly higher ($p<0.05$) than those obtained with 12% DMSO.

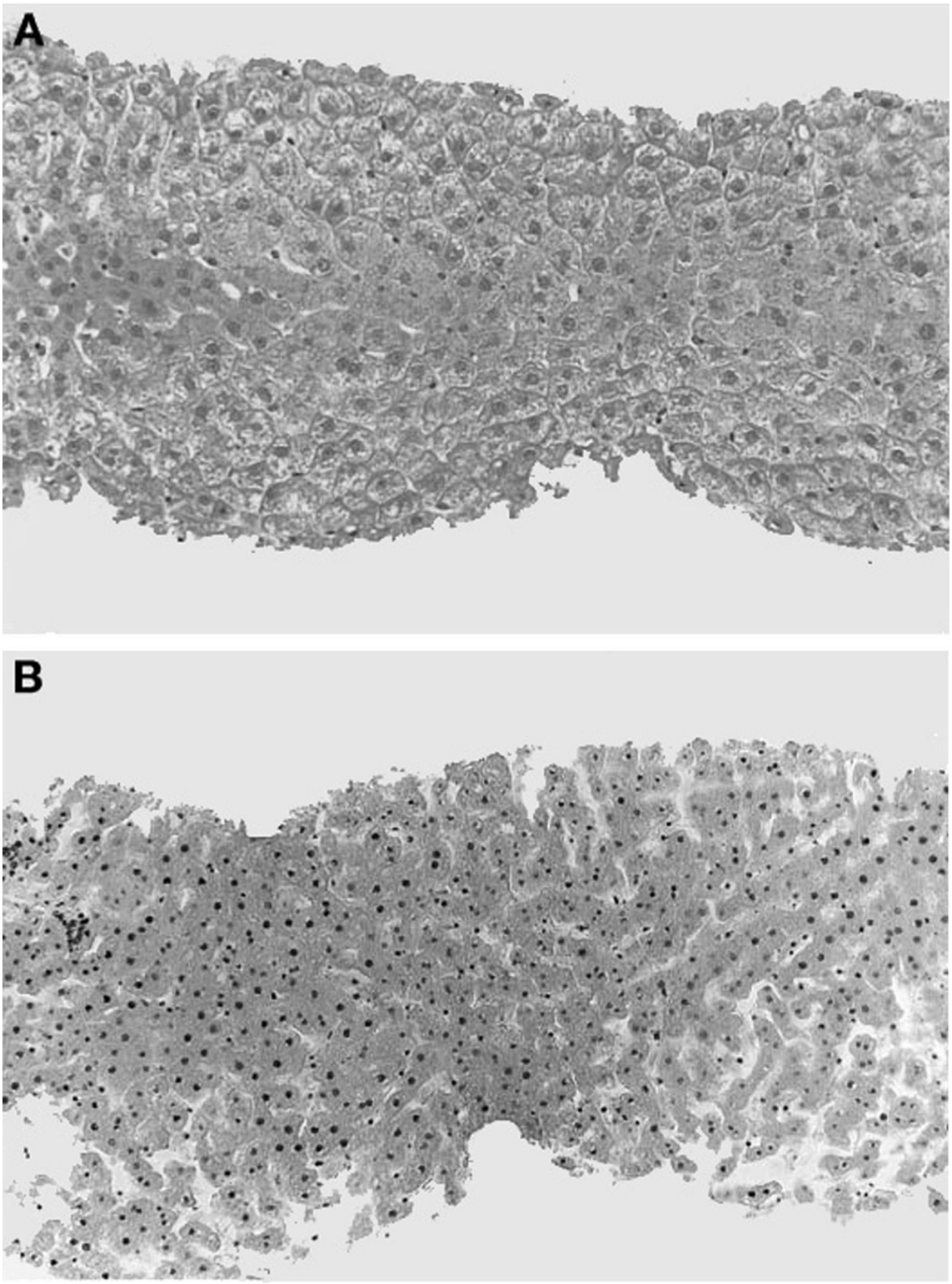


Fig. 3 Histomorphology of fresh and cryopreserved liver slices after 4 h in culture (H&E staining) **[A]** fresh slice (not cryopreserved, nor pre-incubated), **[B]** cryopreserved slice, pre-incubated without DMSO.

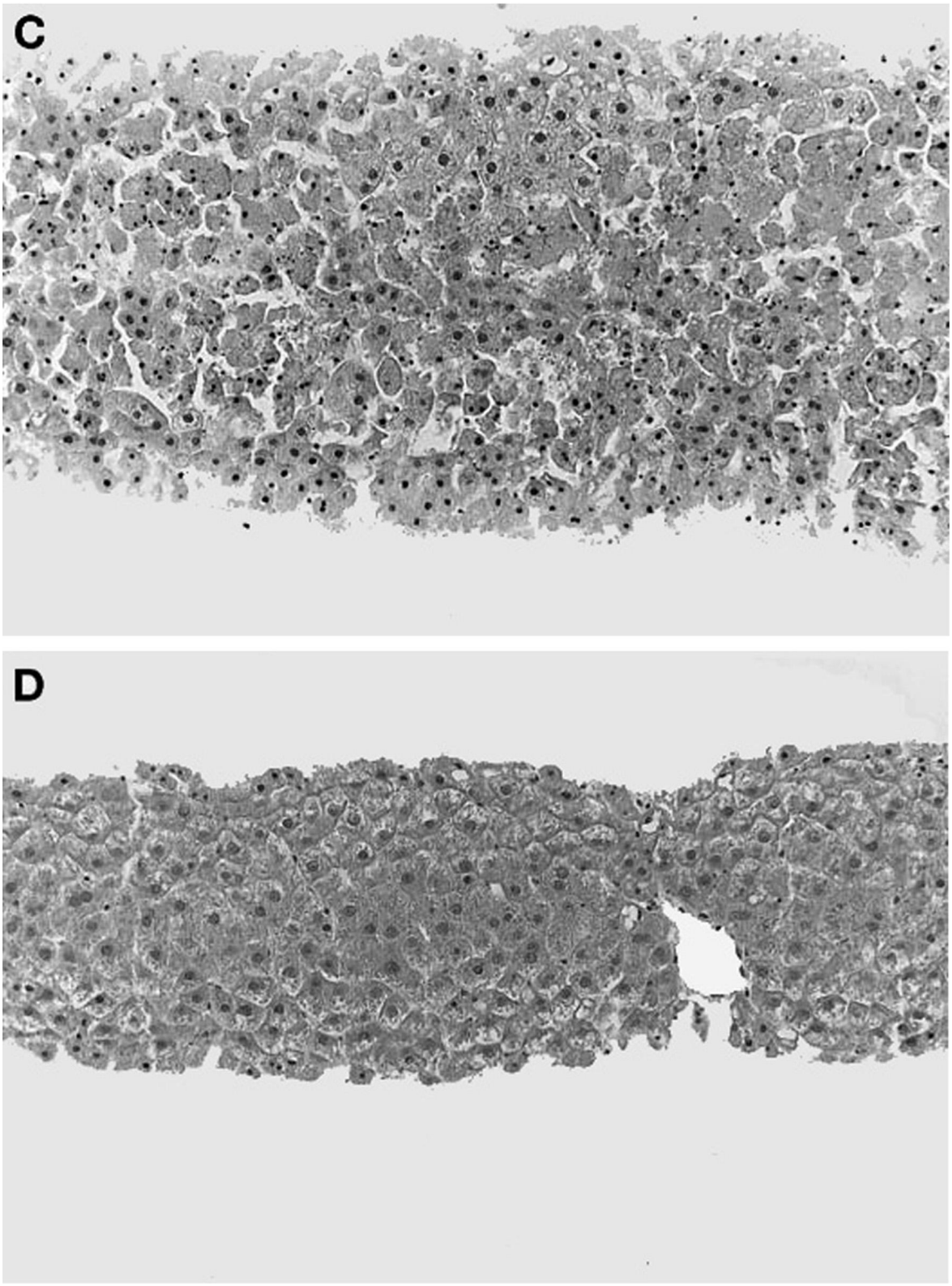


Fig. 3, continued [C] cryopreserved slice, pre-incubated with 12% DMSO, **[D]** cryopreserved slice, pre-incubated with 18% DMSO.

Cryoprotection by DMSO

Immediately after thawing, potassium concentration and the number of intact cells in cryopreserved slices were comparable to fresh slices, independent on DMSO concentration used (12-30%) (data not shown). After 4 h of culturing, however, 18% (v/v) DMSO better retained viability of slices than 12% and 30% (Fig. 2). The potassium content of slices cryopreserved with 18% DMSO was over 65% of the content of fresh slices (i.e. slices that were not pre-incubated with DMSO or cryopreserved), and higher than the results with 12 or 30% DMSO (40 and 55% respectively). Histomorphological examination gave similar results. The number of intact cells in slices cryopreserved with 18% DMSO was over 60% of that of fresh slices and with 12 and 30%, this was considerably lower (Fig. 2a). Without DMSO, only few cells were intact, and in most cells pyknosis and karyorhexis was observed (Fig. 3a), whereas with 12% DMSO, a number of cells with intact nuclei could still be seen. In slices cryopreserved with 18% DMSO, the aspect of the cells (Fig. 3d) is hardly distinguishable from that in a fresh slice (Fig. 3a).

Influence of cryopreservation medium

The influence of the medium used as CPA carrier during pre-incubation and cryopreservation on viability of slices is shown in Fig. 4. Neither UW nor FCS appeared to be a better cryopreservation medium than WME (Fig. 4, a and b).

The role of slice thickness

When slices of various thickness were prepared, the results after cryopreservation varied (Fig. 5). Because of this variation, data from each experiment are given separately. This shows that the trend in the experiments is consistent and statistically significant: both potassium content and the number of intact cells were considerably decreased in thicker slices. Potassium retention of the cryopreserved slices reached an optimum in slices with approximately 10 cell layers. Histomorphological examination of slices revealed that damaged cells in relatively thick cryopreserved slices are mainly located at the slice edges and in the cell layers central in the slice. In thin slices, only the outer cell layers are damaged. Potassium retention(Fig. 5a), but not the number of intact cells (Fig. 5b), in fresh slices was also related significantly to slice thickness, although less marked than in cryopreserved slices.

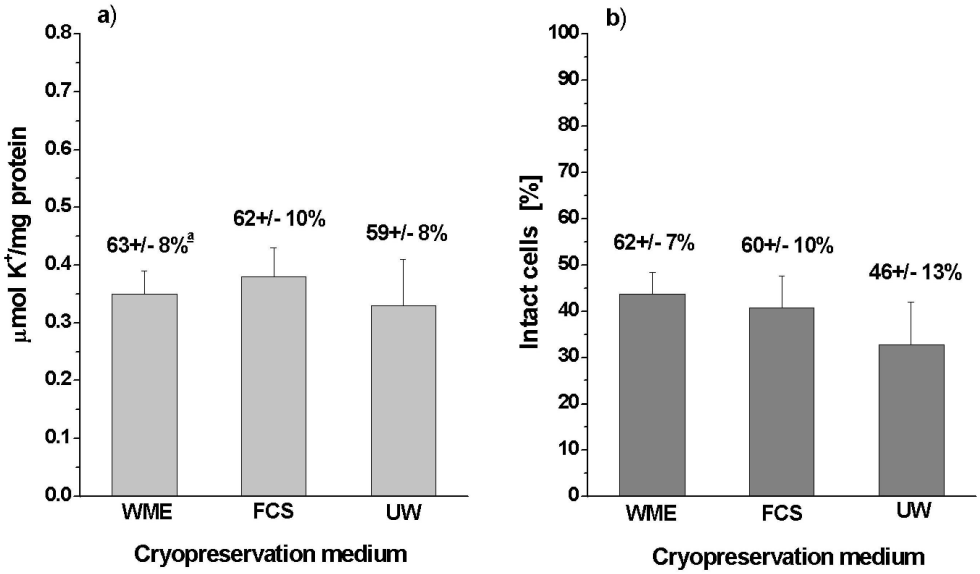


Fig. 4 Potassium content **[a]** and histomorphology **[b]** of cryopreserved slices pre-incubated with 18% DMSO in WME, FCS or UW as pre-incubation medium. Slices were cultured for 4 h after thawing. Values are shown as mean of individual experiments (5-7 experiments, 3 slices per experiment) +SEM. [a] % of value of fresh slices (i.e. slices that were not pre-incubated with DMSO or cryopreserved) + SEM.

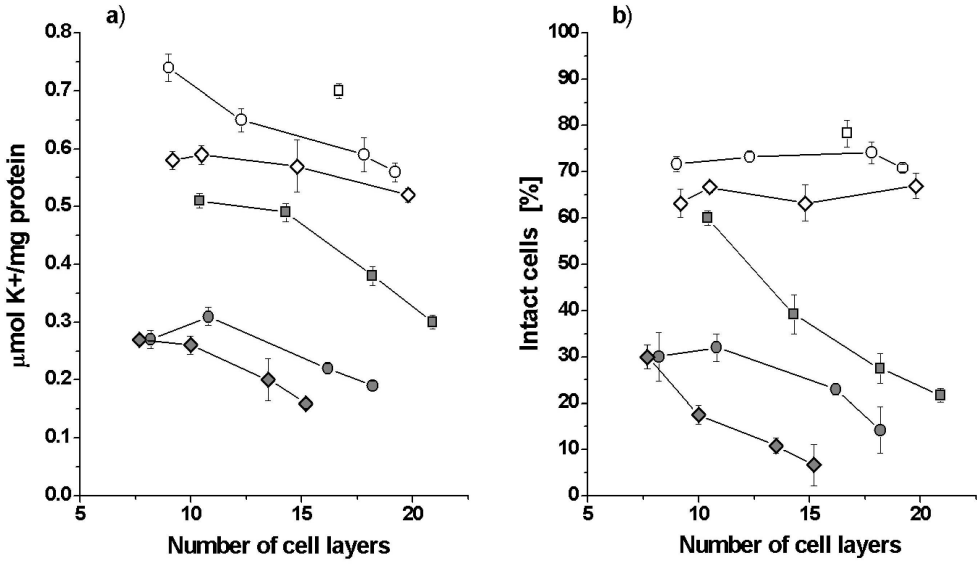


Fig. 5 Influence of slice thickness on potassium content **[a]** and histomorphology **[b]**) of fresh and cryopreserved slices. Open symbols: fresh slices not pre-incubated with DMSO, nor cryopreserved and closed symbols: cryopreserved slices. Analogous symbol forms represent mean of values of individual fresh and cryopreserved slices from the same experiment (6 slices per experiment) +SEM. Slices were cultured for 4 h. Potassium content of both fresh and cryopreserved slices is statistically significantly related to slice thickness ($p<0.05$). The % intact cells is only for cryopreserved slices statistically significantly related to slice thickness.

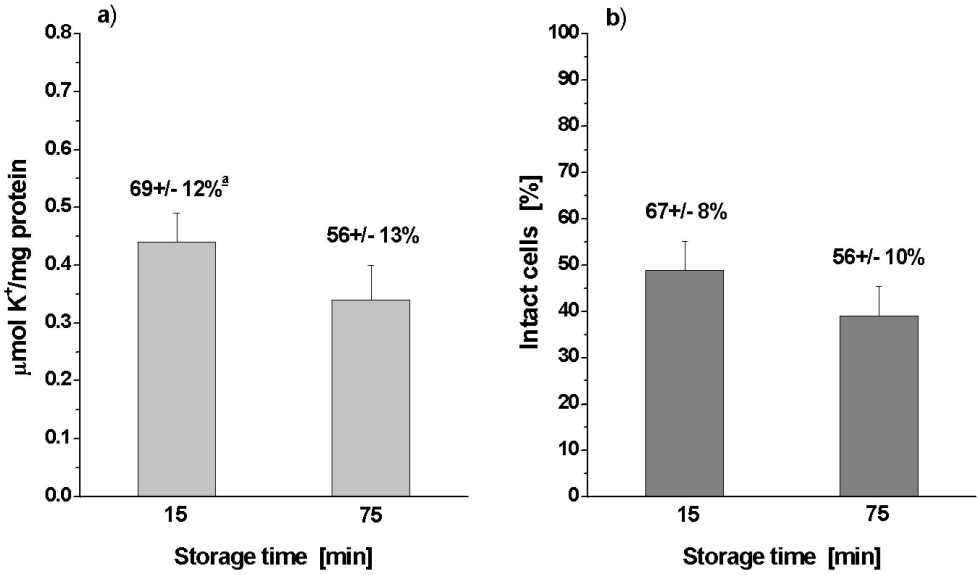


Fig. 6 Influence of storage time at 4°C after slice preparation on fresh and 18% DMSO treated cryopreserved slices, on potassium content **[a]** and histomorphology **[b]**. Slices were cultured for 4 h. Values are shown as mean values of individual experiments (5-6 experiments, 3 slices per experiment) +SEM. [a] % of value of fresh slices (i.e. slices that were stored at 4°C during the same amount of time, but were not pre-incubated with DMSO or cryopreserved) +SEM.

The role of cold storage prior to cryopreservation

Liver tissue and slices are generally kept on ice for approximately 60 min (counting from liver excision) during slice preparation before the cryopreservation pre-incubation is started. As shown in Fig. 6, varying this time from 15 to 75 min, decreased the number of intact cells and potassium content, although not statistically significant.

Biotransformation in liver slices with optimized viability

In thin slices, cryopreserved according to the improved method (using 18% DMSO in WME), biotransformation activity was determined. Results are shown in Table 1. Formation of 2α-hydroxytestosterone, an important hydroxymetabolite of testosterone in rat slices and a measure for P450-mediated phase I biotransformation, was maintained at 60-85% of levels of fresh slices. Peak areas of other metabolites (6β-OH, 19-OH, 14α-OH, 16α-OH, 16β-OH and androstenodione) were maintained at similar levels, as was the total area of all metabolite peaks. Also phase II biotransformation, that is the glucuronidation and sulfation of 7-HC, was maintained at 50-90% of fresh values, with a relative constant fraction formed of each of the two metabolites. The biotransformation capacity of cryopreserved slices correlates well with potassium content and slice histomorphology within the same experiment.

Table 1 Viability and biotransformation capacity of fresh and cryopreserved liver slices after 4 h in culture

	2α-OH testosterone[a]	7-HC-gluc[b]	7-HC-sulf[b]	K^+ content[c]	Histomorphology[d]
liver 1					
fresh	248 ± 37	10 ± 96	65 ± 8	0.74 ± 0.07	67 ± 2
cryopreserved	207 ± 39	816 ± 11	52 ± 0	0.49 ± 0.03	53 ± 8
% of fresh	84 ± 20	79 ± 7	80 ± 9	67 ± 7	79 ± 11
liver 2					
fresh	338 ± 28	1298 ± 66	63 ± 9	ND	75 ± 0
cryopreserved	214 ± 20	693 ± 110	41 ± 8	ND	32 ± 2
% of fresh	63 ± 8	53 ± 9	65 ± 16	ND	42 ± 2
liver 3					
fresh	156 ± 11	1301 ± 30	64 ± 5	0.71 ± 0.03	72 ± 2
cryopreserved	133 ± 16	904 ± 26	58 ± 3	0.46 ± 0.02	47 ± 2
% of fresh	85 ± 12	70 ± 3	90 ± 9	65 ± 4	65 ± 3
mean					
fresh	247 ± 52	1210 ± 90	64 ± 1	0.73 ± 0.01	71 ± 24
cryopreserved	184 ± 34	804 ± 52	50 ± 4	0.48 ± 0.01	44 ± 6
% of fresh	77 ± 7	67± 8	79 ± 7	66 ± 0.8	62 ± 11

Mean values are given ±SEM, ND=not determined

[a] metabolite production in pmol/min/slice. Peak areas of other metabolites (6β-OH, 19-OH, 14α-OH, 16α-OH, 16β-OH and androstenodione) were maintained at 62-114%, 54-71% and 73-117% of fresh slices for liver 1, 2 and 3 respectively. Total peak area of metabolites formed was 97%, 59% and 94% of fresh slices respectively

[b] metabolite production in pmol/min/slice

[c] µmol/mg protein

[d] % intact cells in slice cross section

Discussion

In an attempt to improve an existing cryopreservation method for liver slices, we found that pre-incubation with 18% DMSO in WME and direct immersion in liquid nitrogen maintained cellular integrity and functionality of thin slices (8-10 cell layers) at high levels. Since the potassium content, the number of intact cells and the functionality/drug metabolism parameters are all correlated, the intact cells can be tentatively indicated as viable.

In earlier studies in our laboratory (de Kanter and Koster, 1995) and by others (Glöckner *et al.* 1996; Glöckner *et al.* 1998), high viablity was found after fast freezing with lower concentrations of DMSO (10-12%). However, the viability parameters (i.e. testosterone oxidation, urea synthesis and retention of ALT) used by de Kanter and Koster (1995) to optimize this cryopreservation method, were found to be relatively insensitive to cryopreservation damage comparing to parameters that indicate cell

integrity like retention of small molecules (GSH, ATP) and potassium and preservation of intact histomorphology (Maas *et al.*, 2000b). To prolong incubation with thawed slices, it is important to maintain particularly these sensitive parameters at high levels. Since the biological machinery of the cell is required for the complete expression of cryopreservation damage, slices in the present study were cultured at 37°C for 4 h, prior to viability testing. Apparently, during a 4 h incubation, 18% DMSO maintains sensitive viability parameters better than 12%.

As shown by other authors, oxidative drug metabolism (i.e. testosterone oxidation) is well maintained by cryopreservation (Wishnies *et al.*, 1991; de Kanter and Koster, 1995; Ekins, 1996a; Ekins *et al.*, 1996a; Glöckner *et al.*, 1996; de Kanter *et al.*, 1998; Glöckner *et al.*, 1998; Maas *et al.*, 2000b). However, phase II metabolism is more sensitive to cryopreservation damage (Wishnies *et al.*, 1991; Ekins, 1996a; Ekins *et al.*, 1996a; de Kanter *et al.*, 1998; Maas *et al.*, 2000b). GSH and sulfate conjugation are more sensitive to cryopreservation damage than other metabolic pathways (Steinberg *et al.*, 1999). However, in the present study sulfation was maintained at a high level. There is evidence that conjugation is impaired by cofactor leakage rather than by enzyme inactivation (Maas *et al.*, 2000b). The preservation of sulfation in the present study presumably indicates therefore, that small molecules like the cofactor for sulfation, phosphoadenosine phosphosulfate, are adequately maintained.

In the present study we selected potassium and histomorphology as end-points to improve cryopreservation, since we found previously that these parameters not only are very sensitive to cryopreservation damage but also correlate well with cofactor (ATP/GSH) levels in the slice (Maas *et al.*, 2000b) and thus we expected these parameters to be reliable predictors of biotransformation activity. As expected, the maintenance of cellular integrity coincided well with the preservation of oxidation and conjugation capacity of cryopreserved slices: 2α-hydroxytestosterone and 7-HC-glucuronidates and 7-HC-sulfates were produced at levels close to that of fresh slices. Moreover, production of all metabolites was maintained at similar levels, indicating that all metabolic routes of testosterone and hydroxycoumarin were maintained.

The mechanism by which the present procedure protects cells from damage remains a subject of speculation: intracellular ice crystal formation (IIF) might be (largely) prevented by the higher DMSO concentration or the ice crystals may remain smaller. Moreover, DMSO may render the cellular membranes less susceptible for ice crystals formed. It is known that high

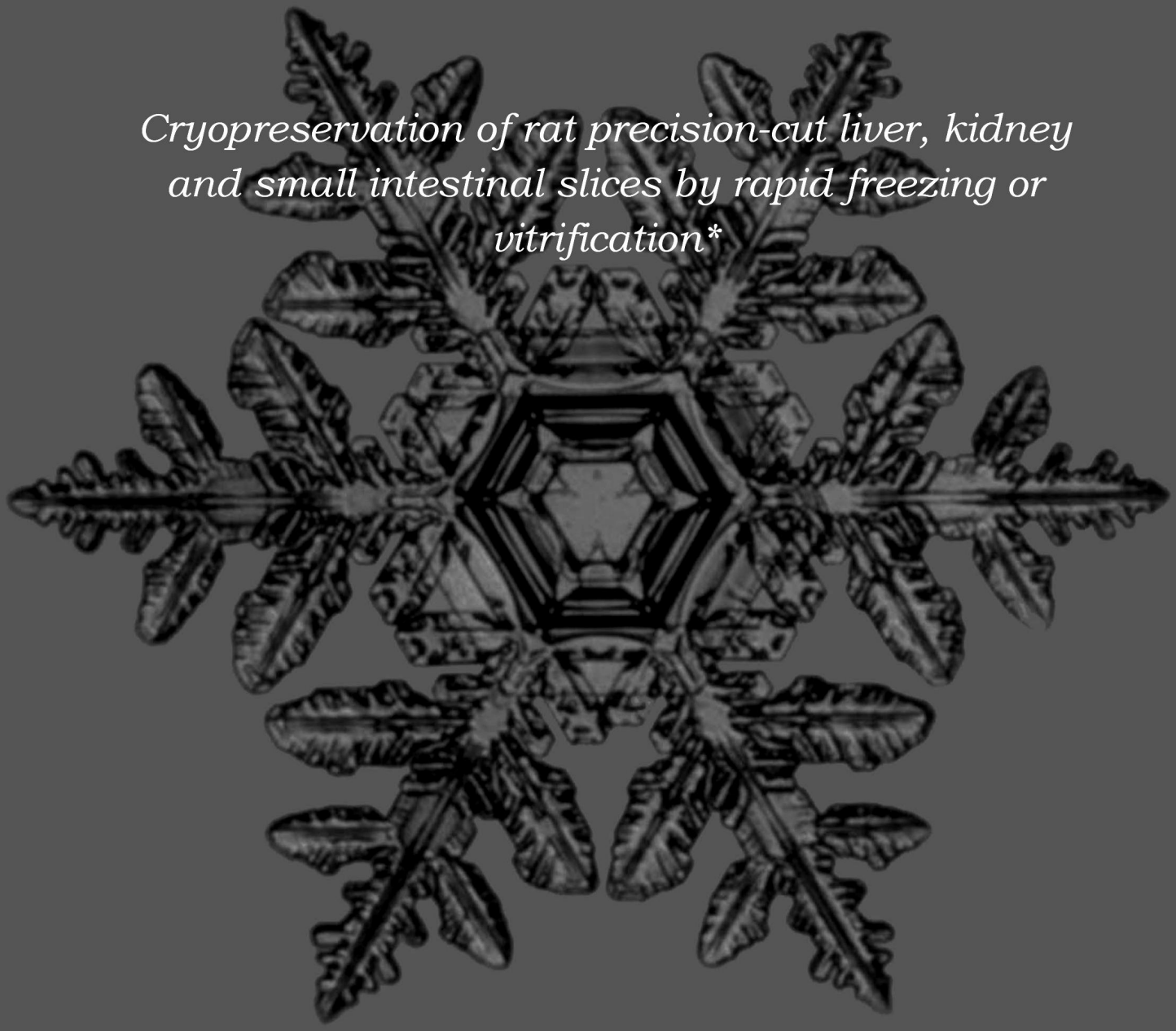
and toxic concentrations of DMSO (>40%) are required to completely prevent ice-formation in aqueous solutions (Boutron and Kaufmann, 1978; Baudot *et al.*, 1999). Therefore, with 18% DMSO, the cryopreservation medium *surrounding* the slices will not vitrify during freezing. An even higher concentration, 30% DMSO, did not further increase viability after cryopreservation, probably because of its toxicity.

Other attempts to stimulate vitrification, for example replacing WME by other cryopreservation media (FCS and UW) that contain substances that can behave as CPA (BSA and HES respectively) did not improve viability. Since these substances remain extracellular, they will only influence extracellular vitrification, which, with this method, does not seem to have an added value.

It should be noticed that viability varied within experiments and even more so between the experiments, in contrast to fresh slices. This could be caused by differences between slices derived from different parts of the liver and between livers from different animals. Moreover, the starting conditions of the slices may be critical. We found, for example, that storage time prior to cryopreservation influences slice viability after cryopreservation.

It can be concluded from the present study that the cryopreservation method improved here, using thin slices pre-incubated in WME with 18% DMSO as CPA, offers a simple way to store liver slices and can maintain their viability and biotransformation activity close to that of fresh slices. Variability between slices cryopreserved from different livers, may be of concern, but for *in vitro* metabolism studies, slices from successful cryopreservation experiments can be easily selected by measuring the potassium content of a few cryopreserved slices of that particular liver. Because both phase I and II metabolism are maintained quantitatively comparable to fresh slice levels, and more importantly, because even in less successful cryopreservation experiments, metabolite patterns are similar to that of fresh slices, cryopreserved slices seem to be a promising tool to study *in vitro* metabolism both qualitatively as (semi-) quantitatively.

Chapter 4



*Cryopreservation of rat precision-cut liver, kidney and small intestinal slices by rapid freezing or vitrification**

*Based on:
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Cryopreservation of rat precision-cut liver, kidney
and intestinal slices by rapid freezing or vitrification,
submitted

Abstract

Precision-cut tissue slices of both hepatic and extra-hepatic origin are extensively used as *in vitro* system to assess drug metabolism *in vivo*. Cryopreservation would greatly facilitate their use. In the present study, attempts are made to cryopreserve precision-cut rat liver, intestinal and renal cortical and medullary slices (1) by using 18% DMSO and a cooling and warming rate of approximately 200°C/min and (2) by vitrification with high molarity mixtures of cryoprotectants, VM3 and VS4.

Contrary to rat liver slices, intestinal and kidney slices were found not to survive cryopreservation by rapid freezing. Vitrification of liver slices with VS4 by ultra-rapid cooling and warming (800°C/min) was reasonably successful, but cryopreservation damage still occurred, despite the fact that cryoprotectant toxicity was low and no ice was formed during cooling or warming. Vitrification of kidney cortical slices with VS4 was not successful, partly because of cryoprotectant toxicity. Viability of kidney medullary slices was completely maintained after vitrification with VS4. Viability of intestinal slices was severely decreased by VS4 toxicity and no further attempts were made to actually vitrify them.

The protocol for pre-incubation with VM3 was optimized to decrease cryoprotectant toxicity and tissue slices were vitrified with varying cooling rates. It appeared that viability of both kidney cortical slices and liver slices increased gradually with decreasing cooling rate. A cooling rate of ca. 1.5°C/min resulted in viability close to that of fresh slices.

In conclusion, vitrification is a promising approach to cryopreserve tissue slices. However, contrary to prevailing thoughts, cooling rate determines cell survival, even when water crystallization is prevented.

Introduction

Precision-cut liver slices are increasingly used as *in vitro* tool to serve as a model for *in vivo* drug metabolism (for review see: Gandolfi *et al.*,1995; Parrish *et al.*, 1995; Ekins, 1996b; Bach *et al.*, 1996; Olinga *et al.*, 1997b; Lerche-Lagrand and Toutain, 2000; de Kanter *et al.*, 2002b). Cryopreservation would offer a possibility to store slices of human and animal origin, forming a tissue slice-bank from which slices can be derived and used for studies on drug metabolism and toxicity at any desired time-point. A number of cryopreservation methods for liver slices have been developed, amongst others by our laboratory (Fisher *et al.*, 1991; Wishnies *et al.*, 1991; Fisher *et al.*, 1993; de Kanter and Koster, 1995; Fisher *et al.*, 1996; Ekins, 1996a; Ekins *et al.*, 1996a; Glöckner *et al.*, 1998; Day *et al.*, 1998; Maas *et al.*, 2000a; de Graaf *et al.*, 2000b, Glöckner *et al.*, 2001). There is an increasing awareness that extra-hepatic organs contribute substantially to drug metabolism and studying metabolism with liver slices alone may give an incomplete view of *in vivo* metabolism (Sandker *et al.*, 1994; Pahernik *et al.*, 1995; Fisher *et al.*, 1994; Vickers *et al.*, 1995; de Kanter *et al.*, 1999; de Graaf *et al.*, submitted-see also chapter 7). However, the cryopreservation of precision-cut slices of extra-hepatic origin has not received much attention, with only a method for dog and human kidney slices reported (Fisher *et al.*, 1993; Fisher *et al.*, 1996).

In the present study, attempts were made to cryopreserve slices from rat liver, kidney medullary and cortical slices, and small intestinal slices. Two approaches were used for this purpose. The first approach was rapid freezing. For liver slices, we have developed a simple, rapid (200°C/min) freezing method (de Kanter and Koster, 1995; de Kanter *et al.*, 1998; de Graaf *et al.*, 2000b), using 18% DMSO and showed that post-thaw viability and phase I and II biotransformation activity of cryopreserved rat liver slices were maintained at least during 4 h after thawing (de Graaf *et al.*, 2000b). The second approach used was vitrification. Essentially, vitrification is the solidification of a liquid not induced by crystallization but by an extreme elevation in viscosity during cooling, so that the solution becomes a glass (Fahy *et al.*, 1984). Previously, with liver slices some success was reported when vitrification was used as starting point for cryopreservation (Wishnies *et al.*, 1991; Ekins, 1996a; Ekins *et al.*, 1996a; de Graaf and Koster, 2001). In the present study, highly concentrated cryoprotectant (CPA) mixtures, VS4 and VM3, were used for vitrification of tissue slices. VS4 consists of 1,2 propanediol, DMSO and formamide in buffered saline with glucose, with a

total molarity of 7.5. VM3 is a low toxic high molarity mixture of CPAs as well, however the composition can not be given because of proprietary reasons, but it is expected to be commercially available soon. The cooling rate required to provoke vitrification (critical cooling rate, V_{crc}) of VS4 in liver slices is ca. 10°C/min (de Graaf and Koster, 2001). The warming rate required to prevent devitrification of liver slices (critical warming rate, V_{crw}) varies between 40 and 1600°C/min, depending on the liver from which the slices were derived (de Graaf and Koster, 2001). These rates can be reached by freezing and thawing slices between aluminium foil sheets (de Graaf and Koster, 2001). The V_{crc} and V_{crw} of VM3 are lower than of VS4, respectively <1°C/min and <10°C/min (dr. Greg Fahy, personal communication).

The viability of slices was tested by measuring the ATP content of the kidney and liver slices, alkaline phosphatase (AP) retention in the intestinal slices and by evaluating the histomorphology of all. Both ATP content and slice histomorphology have been shown to be sensitive to cryopreservation damage (Maas *et al.*, 2000b) and to correlate with phase II biotransformation activity in cryopreserved slices (unpublished data). AP retention was chosen over ATP content as a parameter for the intestinal slices, because this protein is predominantly located in the mucosa, which is the metabolizing part of the intestine, thus preventing a possible influence of the muscle cells on the viability tests.

Materials and methods

Chemicals

Formamide, 1,2-propanediol, Krebs Henseleit (KH) buffer, HEPES buffer, insulin, gentamycine, low melting agarose, adenine, trehalose and glutathione were obtained from Sigma, Axel, The Netherlands; William's Medium E (+glutamax) (WME), phosphate buffered saline (PBS) and fetal calf serum (FCS) were from Gibco BRL, Breda, The Netherlands; University of Wisconsin medium (UW) was from Lamepro b.v., Raamsdonkveer, The Netherlands; VM3 was kindly provided by dr. Greg Fahy (21st Century Medicine, Charleston, USA), the exact composition cannot be given because of proprietary reasons. The Celsis Biomass assay kit was derived from Omnilabo, Breda, The Netherlands. The IL Test™ Alkaline Phosphatase test kit was derived from Instrumentation Laboratory (Lexington, USA). DMSO (>99.9% pure) and all other chemicals were from Baker, Deventer, The Netherlands.

Table 1 Stepwise CPA impregnation and removal

VS4 pre-incubation				VS4 out washing		
25%VS4 [a] 15 min	50% VS4 15 min	75% VS4 15 min	100% VS4 30 min	75% VS4 10 min	50% VS4 10 min	25% VS4 10 min
VM3 pre-incubation				VM3 out washing		
8% Pre-V[b] 15 min	15% Pre-V 15 min	26% Pre-V 15 min	VM3 30 min	26% Pre-V 10 min	15% Pre-V 10 min	8% Pre-V 10 min

Note. Values in the table represent the time span of each step, given in min with each row representing consecutive pre-incubation or washing steps
[a] % VS4 in VS4 buffer (v/v)
[b]% Pre-V in VS4 buffer (g/v)

Preparation of liver kidney and small intestinal slices

Male rats (Wistar, obtained from Harlan CPB, Horst, The Netherlands) had free access to food and water. They were anaesthetised with 65% CO_2, 35% O_2 before the small intestine and/or kidneys and liver were removed.

Precision-cut intestinal slices were made according to de Kanter *et al.* (submitted). From the small intestine, a part of ca. 10 to 20 cm, starting ca. 10 cm from the stomach was taken and flushed with ice-cold UW and subsequently filled with 37°C low-melting agarose. After the agarose had solidified, the filled intestine was carefully cut into pieces of ca. 1 cm. These pieces were then put into a cooled 8 mm diameter tissue core holder of the Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA), which was then filled with low-melting agarose. In later experiments a tissue-embedding tool (Alabama R&D) was used for this purpose. Slices were then made perpendicular to the intestinal wall.

For the liver and kidney, 8 mm cores were produced using a 9 mm tissue-coring tool (Alabama R&D) attached to a mechanical drill (Metabo, The Netherlands). Sometimes from the kidney 5 mm cores were produced using a 6 mm coring tool to better be able to make separate medullary and cortical slices. Subsequently, these cores were sliced using a Krumdieck tissue slicer (Krumdieck *et al.*, 1980) filled with ice-cold oxygenated KH buffer. After slicing, kidney medullary and cortical slices were separated. All slices were stored in ice-cold oxygenated WME with 10% FCS until further use.

Pre-incubation with the CPA

Slices were pre-incubated in 25 or 100-ml Erlenmeyer flasks (until 6 slices in a 25-ml flask, until 18 slices in a 100-ml flask) placed in a gently shaking

waterbath filled with ice. Each Erlenmeyer flask contained 5 (25-ml flask) or 15 ml CPA (100-ml flask) mixed in various proportions with oxygenated medium. As CPA DMSO, VS4 or VM3 were used. VS4 consists of DMSO, 1,2-propanediol and formamide (weight ratio 21.5: 15: 12.4) mixed with buffered saline, glucose, adenine and glutathione (for the exact composition see Fahy and Ali, 1997). The mixture has a total concentration of 7.5 M. VS4 buffer has the same composition as VS4, but without formamide, 1,2-propanediol and DMSO. VM3 is a CPA mixture under development for commercial sales and kindly provided by 21st Century Medicine. Because both VS4 and VM3 are highly concentrated CPA solutions, pre-incubation was executed stepwise with the intention to limit osmotic damage. For this purpose, VS4 was mixed to various concentrations with VS4 buffer. For VM3 impregnation, a special pre-incubation solution (called 'pre-V') was mixed in various proportions with VS4 buffer (the exact pre-incubation schedule is given in Table 1). In some experiments we deviated from this protocol, to further decrease CPA toxicity, by shortening the last pre-incubation step (with undiluted VS4 or VM3) from 30 to 15 min. 18% DMSO (v/v), used for rapid freezing was applied to the slices in one single step of 30 min on ice. In some experiments, the effect of this protocol on viability of the slices was compared to that of a two-step protocol, with the intention to limit osmotical damage. In that case, the slices were incubated for 15 min in 10% DMSO before the last step of 15 min in 18% DMSO.

Cryopreservation

For rapid freezing with 18% DMSO, 3-6 slices were placed in 2-ml cryovials (Greiner, Alphen a/d Rijn, The Netherlands) with 0.5 ml 18% DMSO. Hereafter, the cryovials were directly submersed in liquid nitrogen (de Kanter and Koster, 1995, de Graaf *et al.*, 2000b).

To prevent ice-crystal formation during cooling and warming with VS4, higher cooling and warming rates were required than were reached with the cryovials (de Graaf and Koster, 2001). Therefore, slices pre-incubated with VS4 were cryopreserved after placing them between pads of aluminium foil followed by direct immersion in liquid nitrogen. Cooling rates than were ca. 800°C/min (de Graaf and Koster, 2001). VM3 has a lower V_{crc} and V_{crw} than VS4, i.e. <1°C/min and <10°C/min respectively, so cooling rates of slices could be varied: depending on the experiment, some VM3 pre-incubated slices were cooled in cryovials with 0.5 ml VM3 by direct immersion in liquid nitrogen (-200°C/min), others were frozen in cryovials placed in a

polystyrene box in a –80°C freezer for 1 h (cooling rate approx. 1.5°C/min (de Kanter and Koster, 1995)), followed by freezing in liquid nitrogen.

Thawing and CPA removal

Slices in cryovials were thawed by placing the cryovials in a 37°C waterbath (18% DMSO) or on ice-water (VM3) until ice was no longer visible (or for VM3, the glass became fluid again). VS4 and VM3 pre-incubated slices that were vitrified after being sandwiched between aluminium foil were thawed by placing the foil in 100% VS4 or VM3 on melting ice. After thawing, VS4 and VM3 were washed away according to the stepwise protocol in Table 1. In some experiments we deviated from this protocol with the intention to further decrease osmotic damage by adding 300 mM trehalose, an impermeable sugar, to the washing medium. Slices that were rapidly frozen in 18% DMSO were normally directly incubated at 37°C after thawing and quick washing in WME, 10% FCS. In some experiments, a two-step washing protocol was used, with the intention to limit osmotic damage. In that case, the slices were incubated for 10 min in 10% DMSO before incubation at 37°C.

To determine possible adverse effects of pre-incubation with the CPAs on the slices, some slices underwent the procedure of pre-incubation with outwashing of the CPA and subsequent incubation, but were not cryopreserved.

Incubation and viability testing

Slices were incubated in a 25-ml Erlenmeyer flask (1 slice/flask for 8 mm liver and kidney slices or 2 slices per flask for 5 mm kidney slices or intestinal slices) in a shaking water bath (110 strikes/min), under humid carbogen (95% O_2, 5%CO_2) in 5 ml WME, supplemented with FCS (5%), 0.1 µM insulin, 50 mg/l gentamycine and *d*-glucose (to a medium concentration of 25 mM). After incubation for 4 h at 37°C viability was determined.

The ATP content of liver and kidney slices was determined as follows: Slices were cut in two equal parts. One part was used for histomorphological examination (see below). The other part was immersed in 70% ethanol in HPLC water, containing 2 mM EDTA (pH 10.9). In case 5 mm kidney slices were used instead of 8 mm ones, one of the slices from an Erlenmeyer flask was used for the determination of ATP content and the other for histomorphology examination. Then, slices were homogenized using a Branford sonifier (50% duty cycle, 5 s). An aliquot of the homogenate was taken and mixed 1/1 with Nucleotide Releasing Medium

from the assay kit. Thereafter, the solution was diluted 1/10 with 0.025M HEPES buffer to decrease the ethanol concentration. Subsequently, to 100 µl of the solution, 50 µl luciferin/luciferase solution from the assay kit was added and the amount of luminescence as a measure of ATP content was measured using a Lumac Biocounter M500 (Lumac, The Netherlands). ATP content was determined relative to the protein content of the slices.

Slice protein and potassium content were determined in the slice homogenate as described previously (de Graaf *et al.*, 2000b), except that now an I-lab 600 automatical spectrophotometer (Instrumentation Laboratory, Lexington, USA) was used for the measurements.

For histomorphological examination slices were fixed in 70% ethanol and further processed as described in de Graaf *et al.* (2000b). Slice integrity was determined in the haematoxylin and eosin stained cross sections by estimating the percentage of viable cells. It should be noted that also slice edges (1-2 cellayers at each edge in case of liver and kidney slices) that are damaged by cutting the slices during slice preparation were taken into account with viability scoring.

AP retention in the mucosa of intestinal slices was determined as follows. After 4 h of incubation, the agarose was stripped from intestinal slices. Subsequently the slices were blotted dry, put into 0.5 ml PBS, homogenized using a Branson sonifier (50% duty cycle, 5 s) and subsequently centrifuged at 600 rcf. The AP content was subsequently measured in the supernatant and the slice incubation medium with the ILab 600, using IL Test™ Alkaline Phosphatase test kit. Then the percentage retention per slice was determined by dividing the amount of AP in the slice by the amount of AP in the incubation medium that leaked out of the slices + the amount of AP in the slice.

Differential Scanning Calorimeter Measurements

A differential scanning calorimeter (DSC7, Perkin Elmer, Oosterhout, The Netherlands) was used for determination of V_{crw} of slices pre-incubated with the CPAs for 2 purposes a) to monitor whether or not optimal equilibration with a CPA had taken place (in case of optimal equilibration the V_{crw}s of the slices does not further decrease with increasing pre-incubation time) and b) to determine whether or not the feasible warming rate of 800°C/min was higher than the V_{crw} of the pre-incubated slices, so that devitrification was prevented. For detailed description of DSC measurements on slices, see de Graaf and Koster (2001).

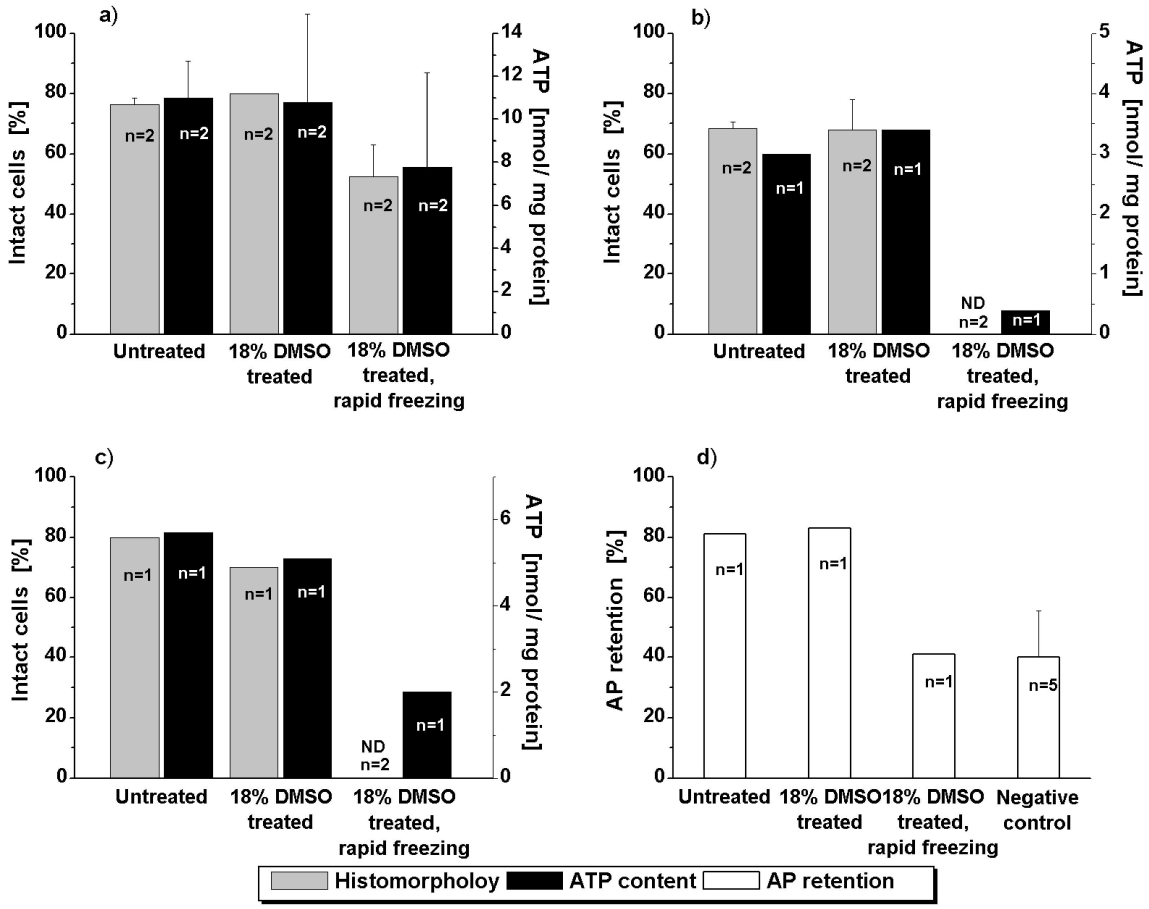


Fig. 1 Viability of tissue slices after pre-incubation with 18% DMSO (v/v) and rapid freezing. **(a)** liver slices, **(b)** kidney cortical slices, **(c)** kidney medullary slices **(d)** small intestinal slices. Untreated slices were not pre-incubated with 18% DMSO or cryopreserved, 18% DMSO treated slices were only pre-incubated with 18% DMSO, but not cryopreserved. 18% DMSO pre-incubation and out-washing was performed stepwise with kidney slices. All slices were incubated for 4 h prior to viability testing. Histomorphologic appearance of slices is quantified as the percentage of intact (not necrotic) cells in the slice cross section. AP retention is quantified as the percentage of the initial AP content of the slices. The number of experiments (n) is given in the bars, 2 or 3 slices were used per experiment. Values are given +SD where appropriate. ND=no (intact cells) detected.

Results

Tissue slice viability after pre-incubation with 18% DMSO and rapid freezing. Previously, we have reported that viability and functionality of liver slices were maintained close to fresh slices levels for at least 4 h after thawing. Results in the present study are consistent with this observation (Fig. 1a). Both the amount of viable cells in cryopreserved slices (see also Fig. 7b), 4 h after thawing, and the ATP content were approximately 70% of fresh slice levels. Also in accordance with previous studies, viability of fresh liver slices was not affected by pre-incubation with 18% DMSO.

Preliminary studies with kidney slices showed that pre-incubation with 18% DMSO significantly decreased viability (data not shown). For this reason, a protocol was developed to minimize DMSO toxicity by pre-incubating stepwise (15 min in 10% DMSO, 15 min in 18% DMSO). Moreover, while liver slices were washed in WME, 10% FCS directly after thawing, kidney slices were first placed in 10% DMSO for 15 min before washing. Treated in this manner, both fresh cortical and medullary slices were unaffected by 18% DMSO (Fig. 1b and c). Nevertheless, ATP levels of kidney slices that were treated in this way and then cryopreserved by rapid freezing were very low, <20% of fresh levels for cortical slices and <40% for medullary slices, while few unaffected cells were found upon histomorphological examination either cortical or medullary slices (see also Fig. 8b and 9b).

Viability of intestinal slices was deteriorated by rapid freezing as well: after thawing and subsequent culturing for 4 h, only 40% of alkalic phosphatase (AP) was retained in the slice, which is close to levels of slices that were cryopreserved without CPA, used as negative control (Fig. 1d). The decreased viability was not caused by 18% DMSO toxicity, since the amount of AP retained was equal in slices that were treated with DMSO and untreated slices.

Tissue slice viability after pre-incubation with VS4 and vitrification

Initially, to vitrify kidney and intestinal slices with VS4, a method was used that was found to maintain viability of rat liver slices at acceptable levels (de Graaf and Koster, 2001). However, Fig. 2 shows that viability of fresh kidney and intestinal slices already decreased markedly by treatment with VS4 without vitrification. Viability of liver slices after VS4 treatment was maintained at levels >75% of those of untreated slices (Fig. 2a), while that of kidney slices was reduced to approximately 50% (Fig. 2b). Data are given for kidney slices as a whole, but upon histomorphological examination it appeared that particularly the cortex was affected by VS4 treatment. Small intestinal slices retained only 45% of their AP after VS4 treatment, slightly more than slices used as negative control, that were cryopreserved without CPA and retained approximately 35% of their AP. Upon histomorphological examination of the intestinal slices it appeared that all mucosal cells became necrotic after VS4 treatment.

As shown in Fig. 3a, reducing the duration of the pre-incubation with 100% VS4 from 30 to 10 min increased viability of kidney cortical slices. Shortening pre-incubation did not improve the AP retention of intestinal slices.

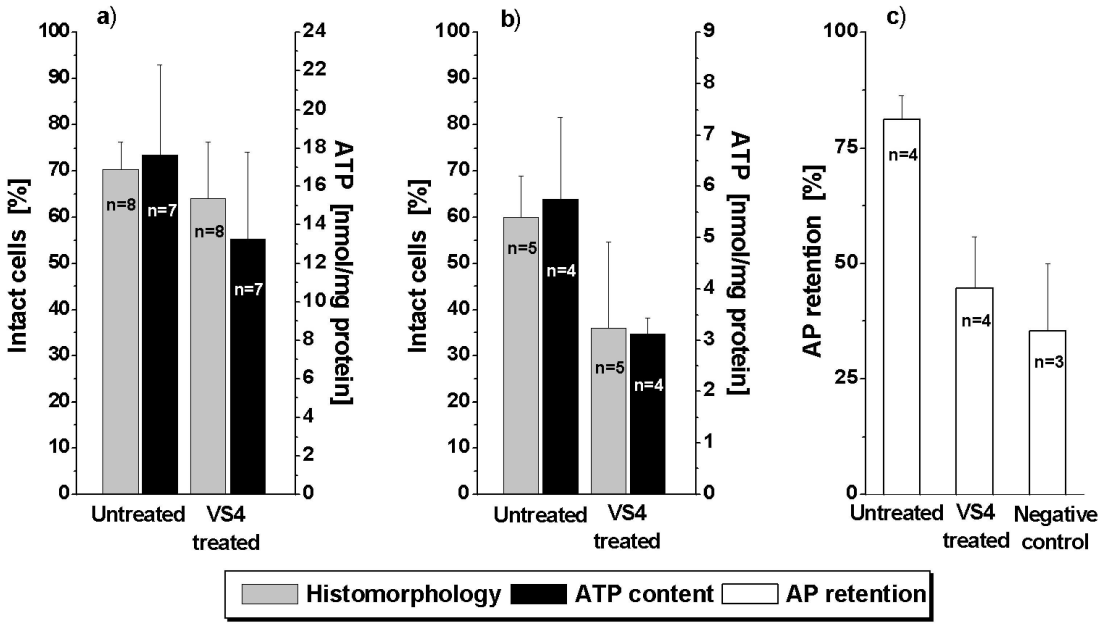


Fig. 2 Toxicity of VS4 to tissue slices. **(a)** liver slices, **(b)** kidney slices (not separated), **(c)** small intestinal slices. Untreated slices were not pre-incubated with VS4 or vitrified, VS4 treated slices were pre-incubated with VS4 but not vitrified. All slices were incubated for 4 h prior to viability testing. Histomorphologic appearance of slices is quantified as the percentage of intact (not necrotic) cells in the slices cross section. AP retention is quantified as the percentage of total AP content of the slices. The number of experiments (n) is given in the bars, 3 slices were used per experiment. Values are given +SD.

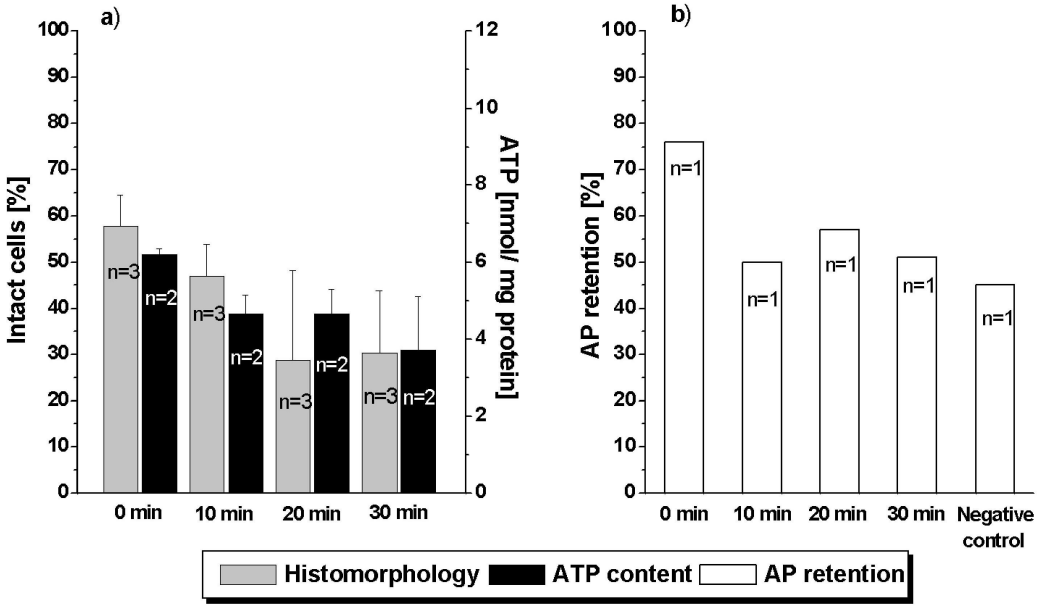


Fig. 3 The effect of shortening VS4 pre-incubation on viability of tissue slices. **(a)** kidney cortical slices and **(b)** small intestinal slices. All slices were incubated for 4 h prior to viability testing. Histomorphologic appearance of slices is quantified as the percentage of intact (not necrotic) cells in the slices cross section. AP retention is quantified as the percentage of total AP content of the slices. Data points are labeled with the number of experiments performed (n), 3 slices were used per experiment. Values are given + SD where appropriate.

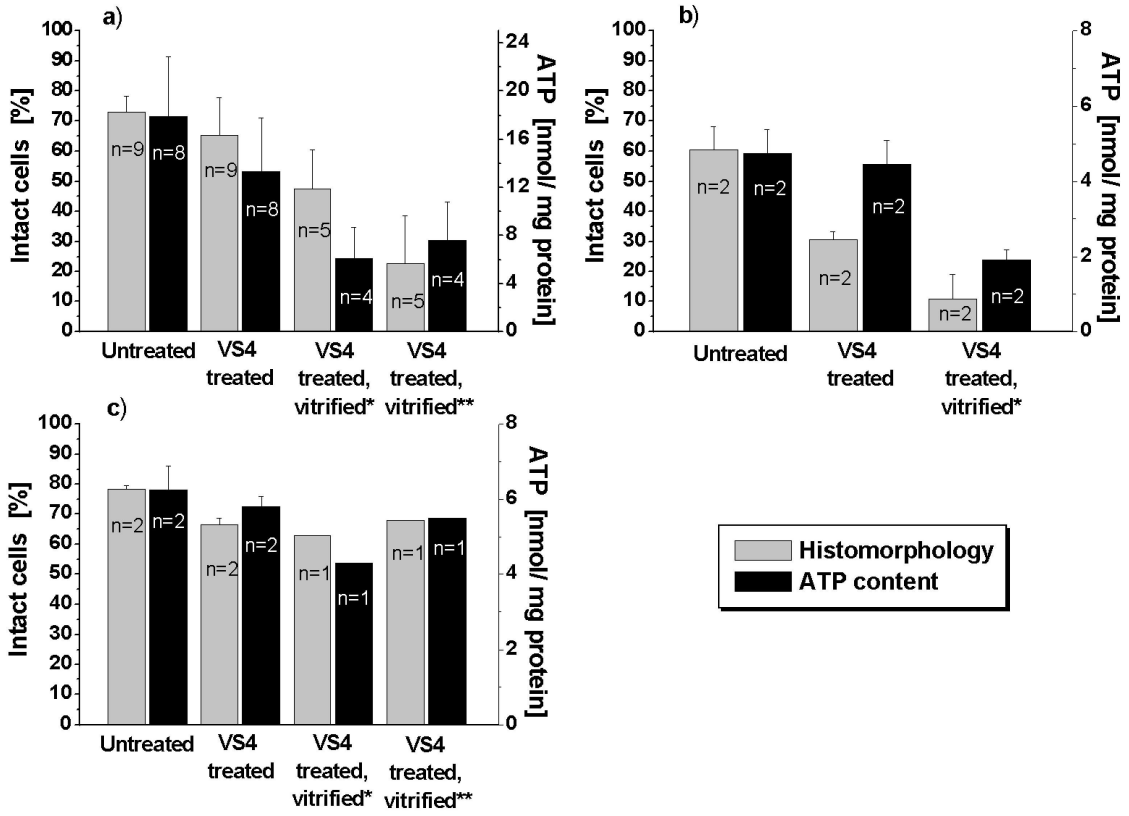


Fig. 4 Viability of tissue slices after treatment and vitrifcation with VS4. **(a)** liver slices, **(b)** kidney cortical slices and **(c)** kidney medullary slices. Untreated slices were not pre-incubated with VS4 or vitrified, VS4 treated slices were pre-incubated with VS4 but not vitrified. ***V_{crw}**s measured were below feasible warming rates, ****V_{crw}**s were higher than feasible warming rates. The final pre-incubation step with 100% VS4 was 30 min with liver slices and 10-15 min with kidney slices. All slices were incubated for 4 h prior to viability testing. Histomorphologic appearance of slices is quantified as the percentage of intact (not necrotic) cells in the slices cross section. The number of experiments (n) is given in the bars, 3 slices were used per experiment. Values are given +SD.

De Graaf and Koster (2001) have shown that the critical warming rate (i.e. the warming rate required to prevent devitrification, V_{crw}) of liver slices is in most cases near the warming rate that can be reached when slices are vitrified and warmed between aluminium foil sheets (800°C/min, from now on called feasible warming rate). When equilibration with VS4 is sub-optimal, the V_{crw} is expected to be above this level. To ensure that shortening of the pre-incubation period did not cause incomplete penetration of VS4, kidney and intestinal slices were pre-incubated for 10, 20 or 30 min with VS4 and V_{crw}s determined. With kidney slices, V_{crw}s were not changed by shorter pre-incubation, indirectly indicating that VS4 penetration in all cases was complete. With intestinal slices, V_{crw}s gradually increased from 216 to 595 and 1291°C/min for pre-incubation periods of 30, 20 and 10 min respectively. Nonetheless, even for 10 min pre-

incubation, V_{crw}s were still close to the feasible warming rate of 800°C/min. Nonetheless, because of the high sensitivity of intestinal slices for VS4 toxicity, no further attempts were made to actually vitrify them.

After optimization of the pre-incubation protocol for VS4, liver and kidney slices were vitrified. Results are shown in Fig. 4. Of each individual experiment (organ), one slice was used to check whether the V_{crw} was lower than the feasible warming rate of 800°C/min. Measured V_{crw}s varied between 350 and 3500°C/min for liver slices and were 298 and 194°C/min for kidney cortical and 2578 and 498°C/min for medullary slices. So, for part of the liver and kidney medullary slices the V_{crw}s were higher that the feasible warming rates and some crystallization may have occurred upon warming. In Fig. 4, the results of experiments with V_{crw}s lower than the feasible warming rates and of experiments with higher V_{crw}s than the feasible warming rates are shown separately. It appears that VS4 vitrified liver slices had a viability of (only) 35-60% of that of untreated slices, despite of the fact that VS4 toxicity was low and no ice was formed either during cooling or warming (see Fig. 7c for histomorphology of liver slices that were treated and vitrified with VS4). The same holds true for kidney cortical slices: although V_{crw}s were well below the feasible warming rates, viability after vitrification decreased to approximately 10% (histomorphology, see also Fig. 8c) or <40% (ATP content) of untreated slices. Only a part of this decrease can be attributed to VS4 toxicity. Kidney medullary slices were well preserved by vitrification with VS4 (see also Fig. 9c). Remarkably, viability of liver and kidney medullary slices that had V_{crw}s higher than the feasible warming rates did not differ much of those with V_{crw}s below the feasible warming rates.

Vitrification of liver and kidney slices with VM3

Initially, to pre-incubate slices with VM3, a stepwise protocol was used with a 30 min pre-incubation period with VM3 as final step. As with VS4 however, the viability of kidney cortical slices was decreased by VM3 treatment, while liver slices and kidney medullary slices were barely affected. To increase viability of VM3 treated cortical slices, several variations were made in the pre-incubation protocol. As shown in Fig. 5, slices incubated with VM3 for 15 min instead of 30 min and washed with 300 mM trehalose added to the washing medium with the intention to decrease osmotic effects, had the highest viability.

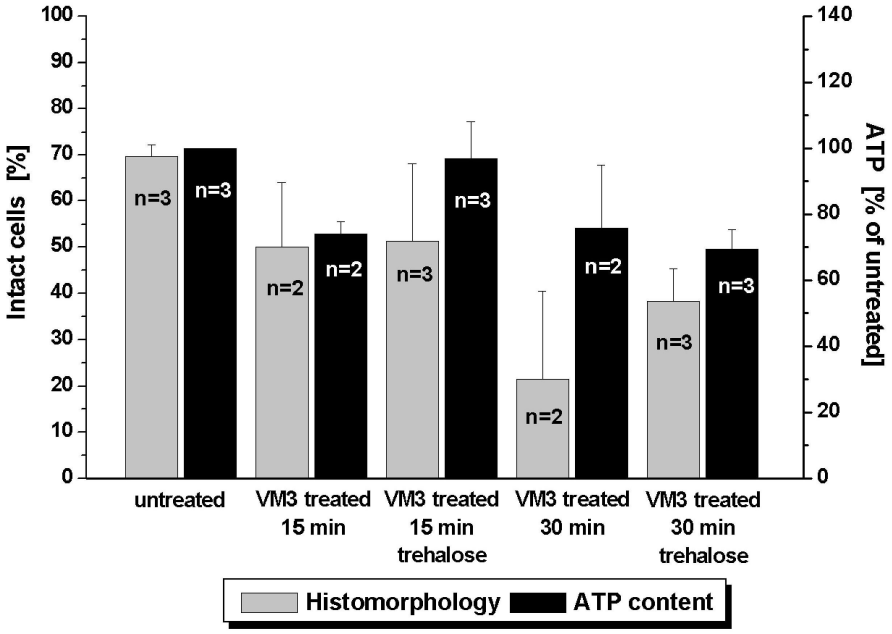


Fig. 5 The effect of shortening VM3 pre-incubation and adding 300 mM trehalose to the washing medium on viability of kidney cortical slices after VM3 treatment. Untreated slices were not pre-incubated with VM3 or vitrified, VM3 treated slices were pre-incubated with VM3 for 15 or 30 min, not vitrified and washed with or without 300 mM trehalose in the washing medium. Slices were incubated for 4 h prior to viability testing. Histomorphologic appearance of slices is quantified as the percentage of intact (not necrotic) cells in the slice cross section. ATP content is given relative to untreated slices of the same experiment because of the great variation in absolute values between experiments (absolute values for untreated slices were 10.3, 5.6 and 3.4 nmol ATP/mg protein). The number of experiments (n) is given in the bars, 3 slices were used per experiment. Values are given +SD.

Because the critical cooling rate (V_{crc}) and the V_{crw} (respectively <1 and <10°C/min) of VM3 are much lower than of VS4, it was possible to vary cooling rates of VM3 pre-incubated slices. Cooling rates of 800, 200 and 1.5°C/min were used. In preliminary experiments it was noticed that the glass that was formed from VM3 in the cryovials was susceptible to cracking, leading to fragmentation. This pheno-menon is probably caused by temperature stress within the sample. It is known that cracking in a glass at low temperatures can be avoided by lowering the cooling rate around the glass transition point (Baudot *et al.*, 2001). Optimization of the freezing technique for VM3 has led to the following protocol: Cryovials containing VM3 and slices were initially cooled to –80°C with 200 or 1.5°C/min and then placed in a piece of polystyrene floating on liquid nitrogen in a small tank. The vials were not in direct contact with the liquid nitrogen or the nitrogen gas. Then, the tank was closed and after 40 min (the temperature of the samples was than around –140°C), the vials were submerged directly in liquid nitrogen. In this manner, the obtained glass was virtually unfractured and the slices were not fragmented after thawing.

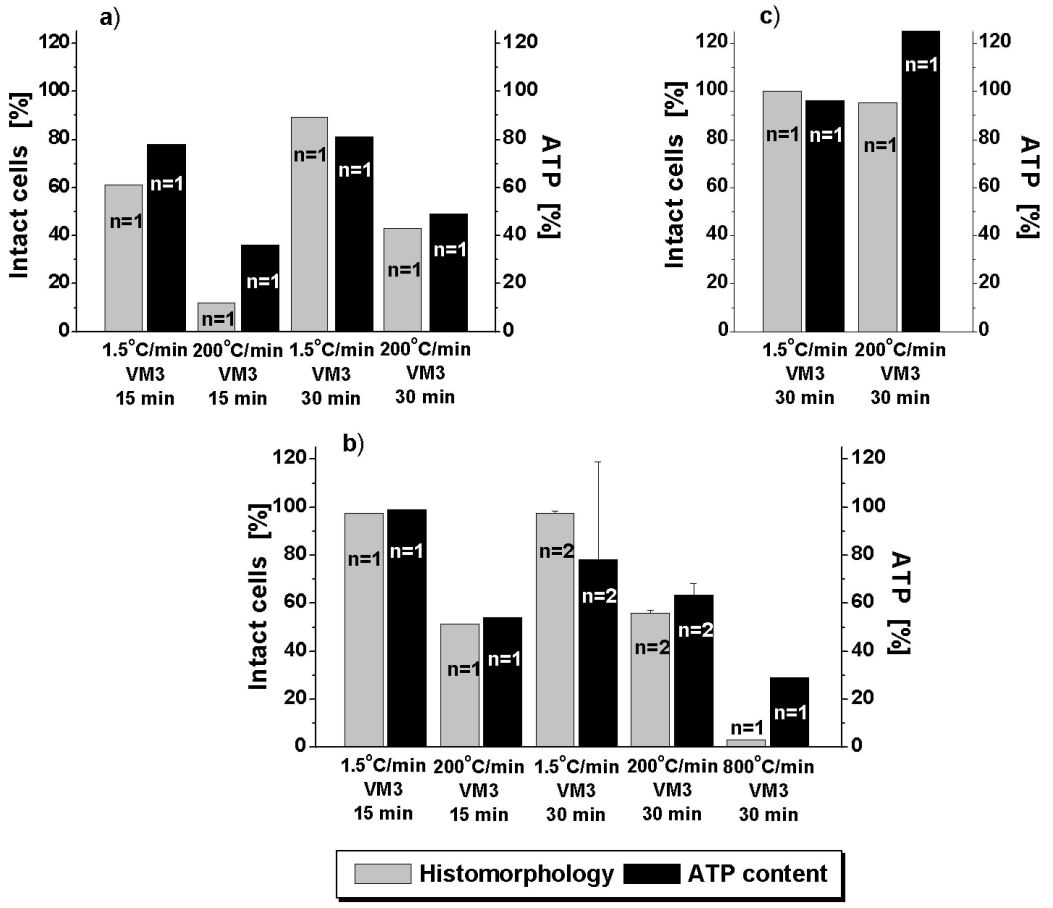


Fig. 6 The effect of cooling rate on normalized viability of vitrified tissue slices after VM3 pre-incubation. **(a)** liver, **(b)** kidney cortical slices and **(c)** kidney medullary slices. Slices were pre-incubated with VM3 for 15 or 30 min, vitrified with various cooling rates, thawed rapidly and washed with 300 mM trehalose in the washing medium. All slices were incubated for 4 h prior to viability testing. Viability is given relative to VM3 treated slices, to normalize for CPA toxicity. The number of experiments (n) is given in the bars, 3 slices were used per experiment. Values are given +SD, where appropriate.

Fig. 6 shows that viability, normalized for CPA toxicity, of both liver and kidney cortical slices decreased with increasing cooling rate. With a cooling rate of approximately 1.5°C/min, normalized viability of kidney cortical slices (Fig. 6b) was nearly completely maintained by vitrification. Also liver slices (Fig. 6a) were virtually unaffected by vitrification at 1.5°C/min, but only when they were incubated for 30 min with VM3, indicating that pre-incubation for 15 min possibly caused incomplete penetration with the CPA. Viability of renal medullary slices (Fig. 6c), pre-incubated for 30 min with VM3, was not clearly affected by the cooling rate.

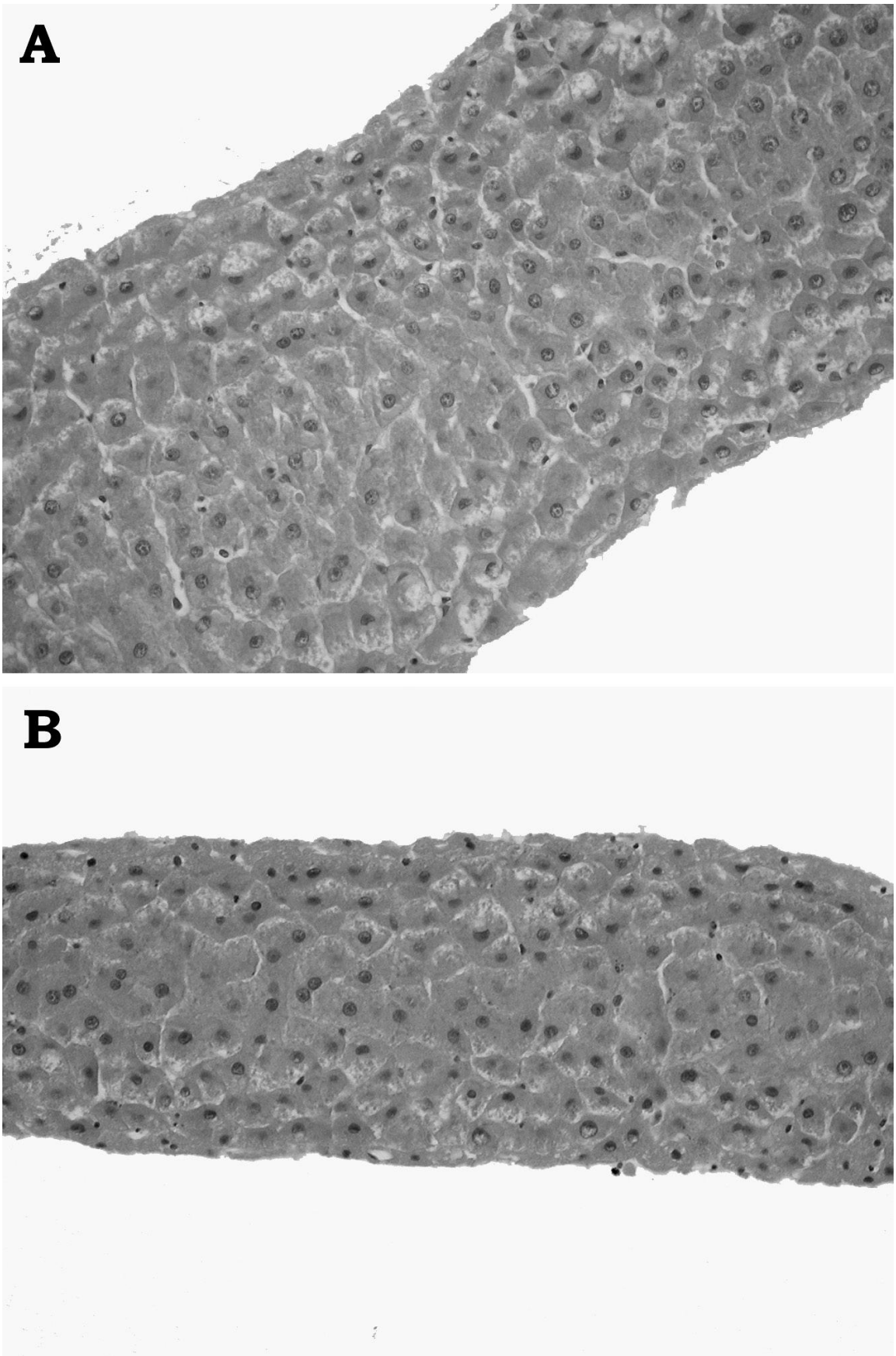


Fig. 7 Histomorphology of liver slices **(A)** untreated slice **(B)** slice cryopreserved by rapid freezing after pre-incubation with 18% DMSO.

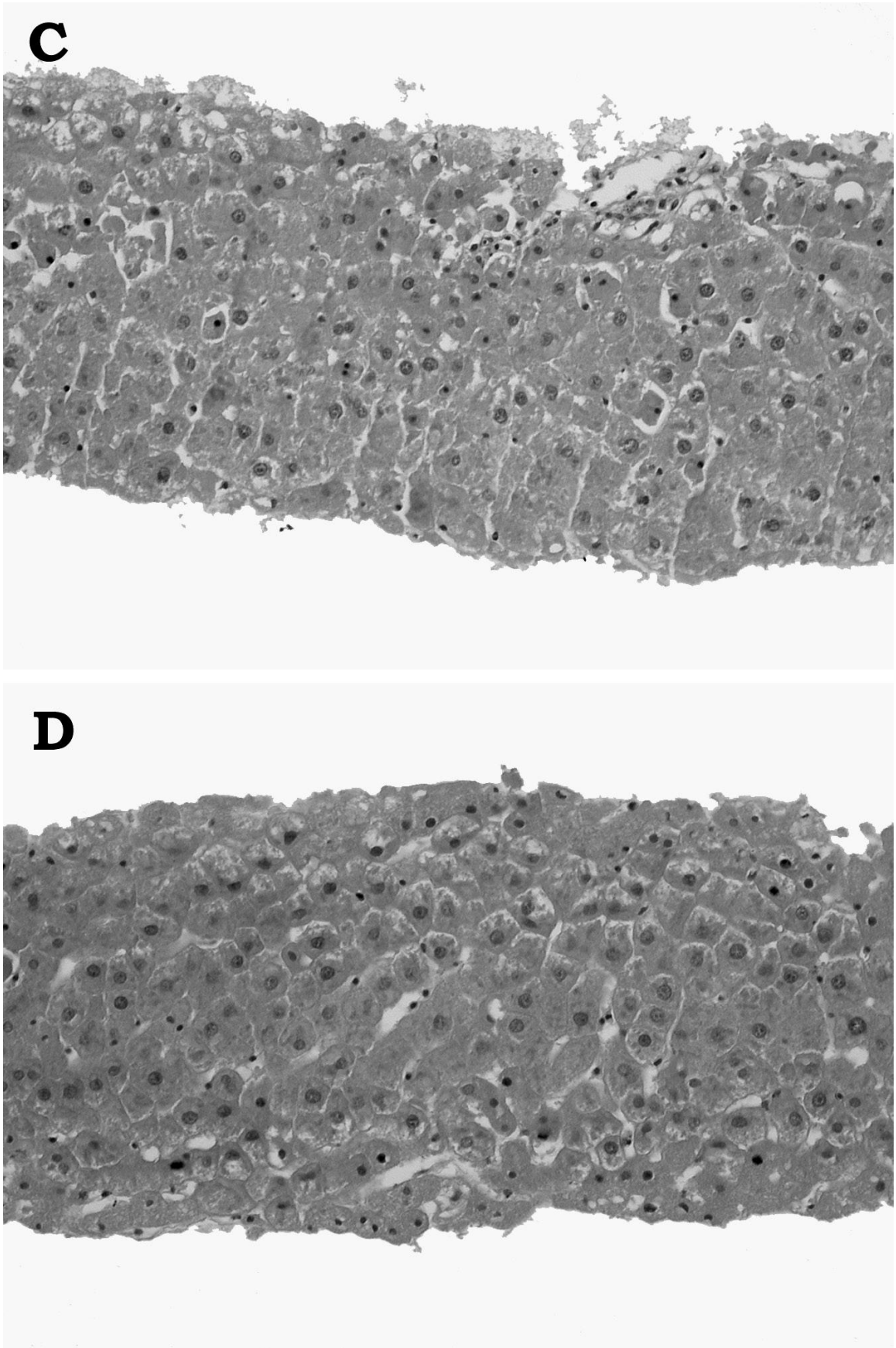


Fig. 7, continued **(C)** slice vitrified with VS4 **(D)** slice vitrified with VM3. All slices were incubated for 4 h.

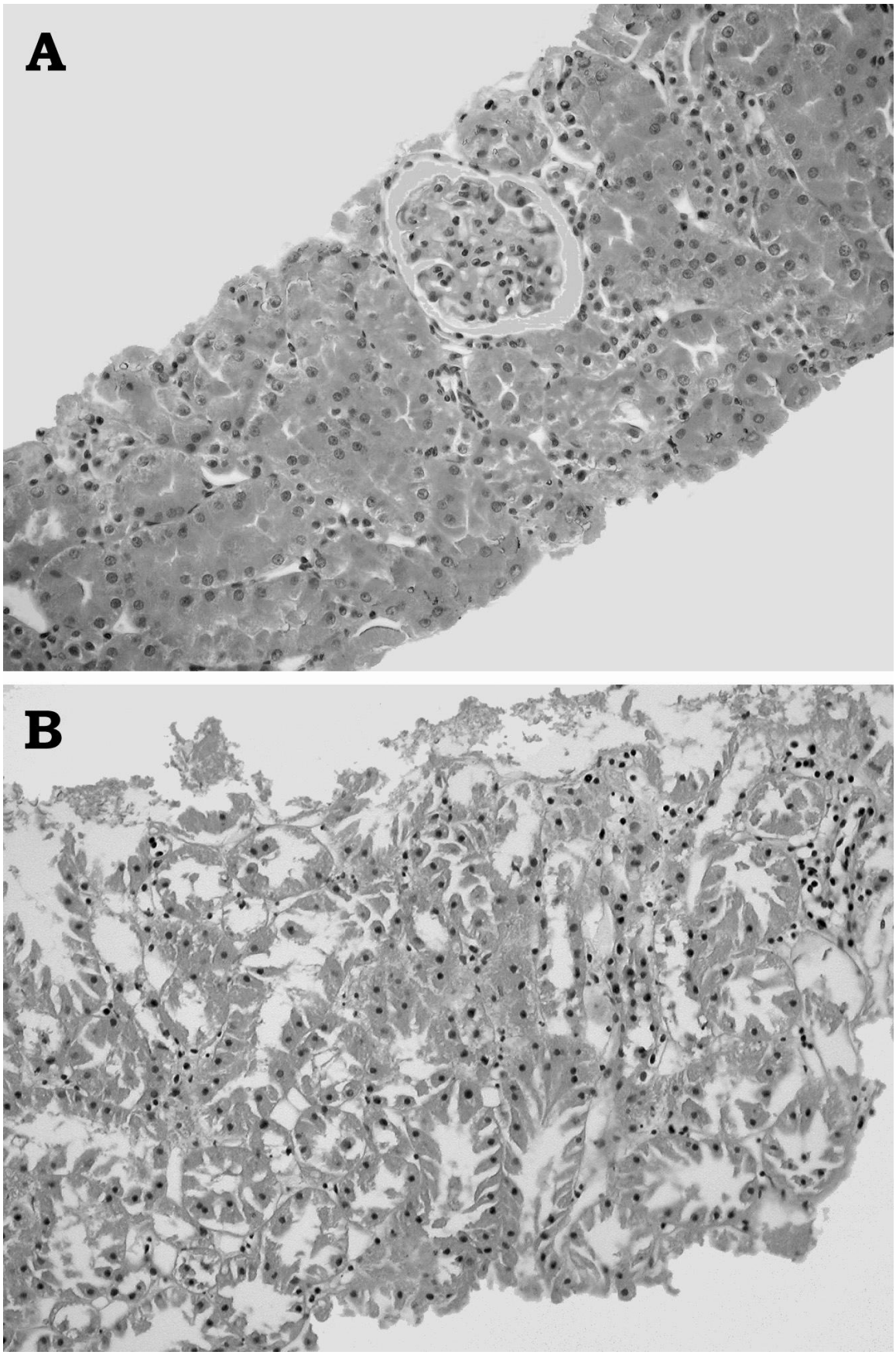


Fig. 8 Histomorphology of kidney cortical slices **(A)** untreated slice **(B)** slice cryopreserved by rapid freezing after pre-incubation with 18% DMSO.

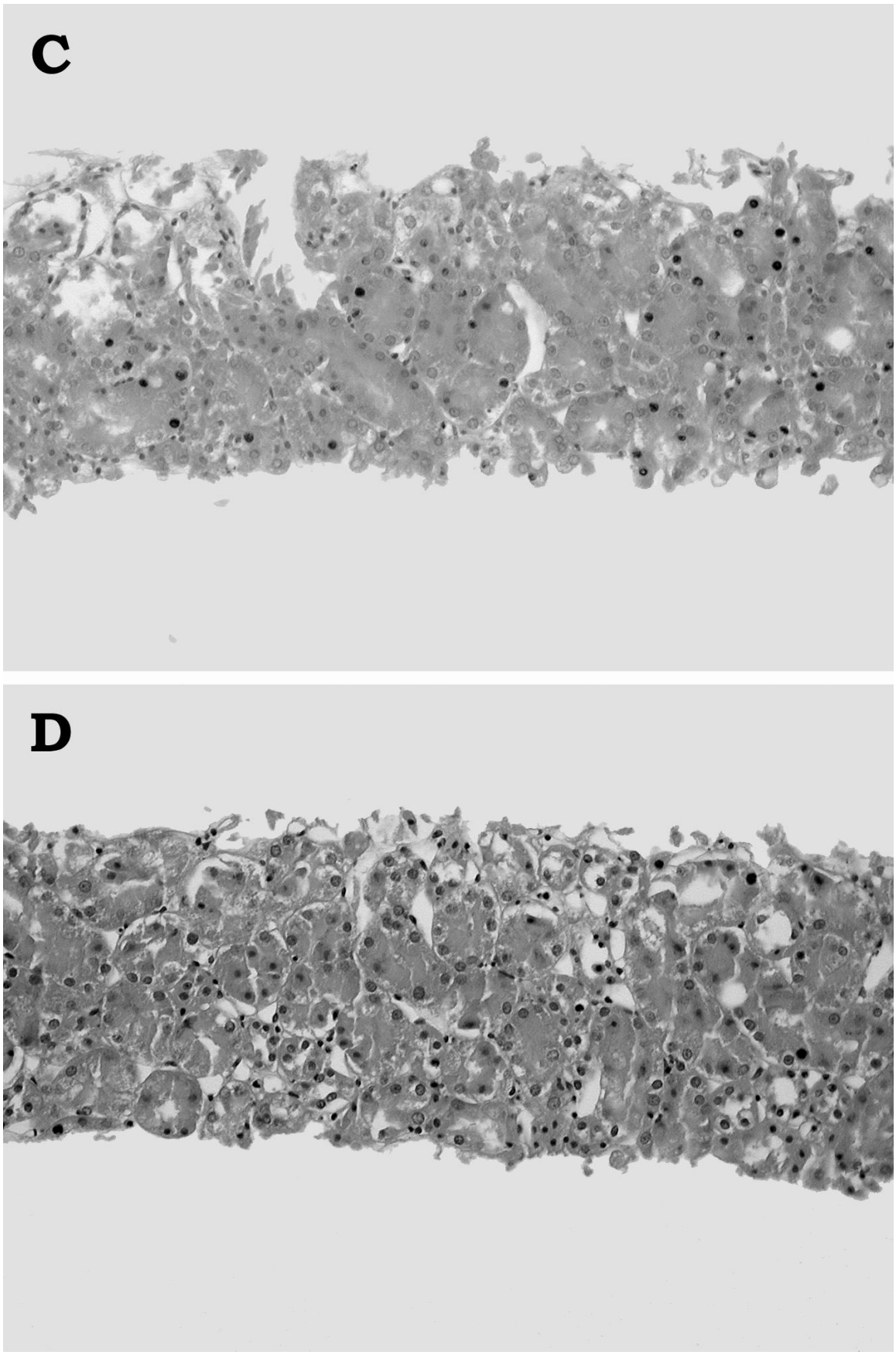


Fig. 8, continued **(C)** slice vitrified with VS4 **(D)** slice vitrified with VM3. All slices were incubated for 4 h.

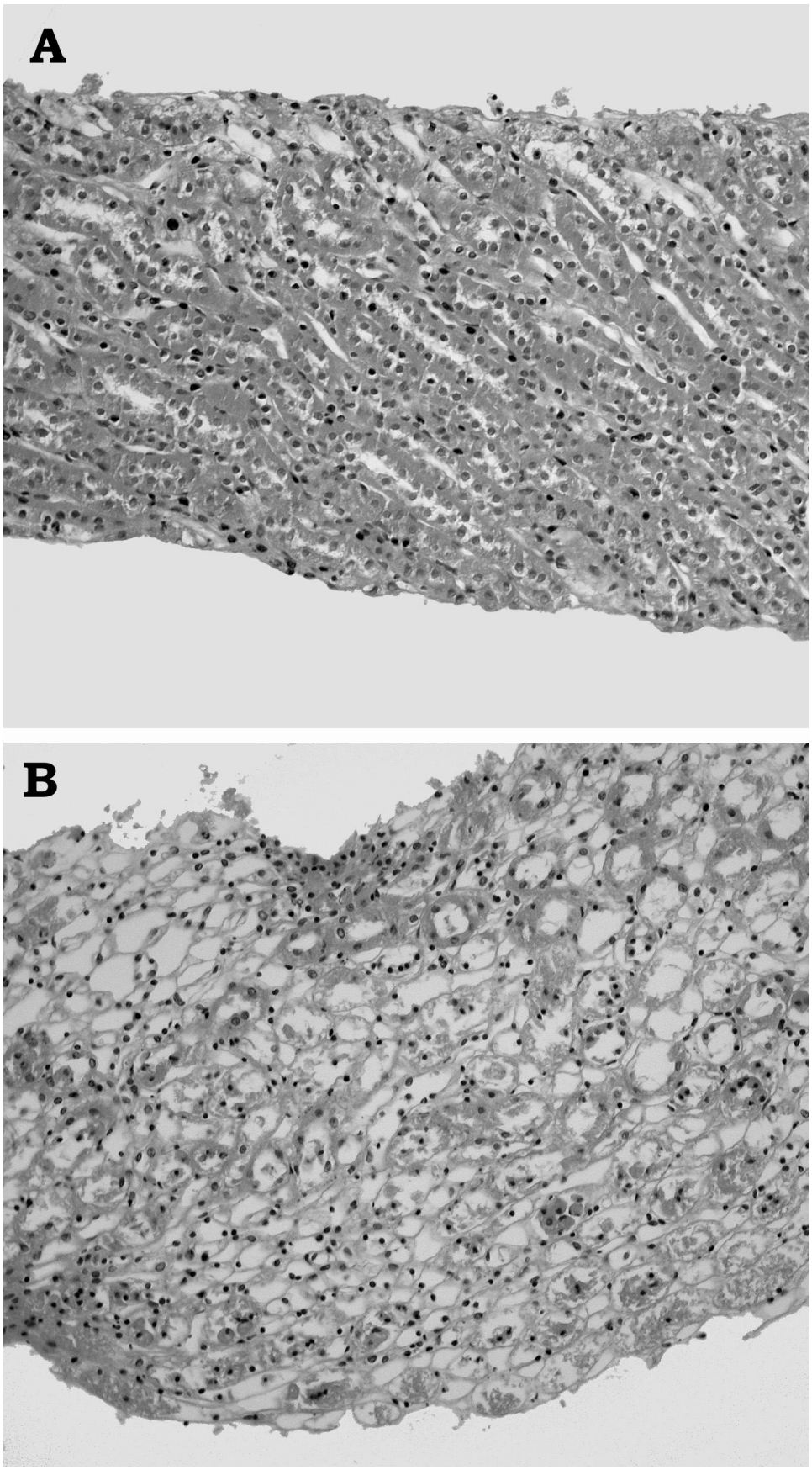


Fig. 9 Histomorphology of kidney medullary slices **(A)** untreated slice **(B)** slice cryopreserved by rapid freezing after pre-incubation with 18% DMSO.

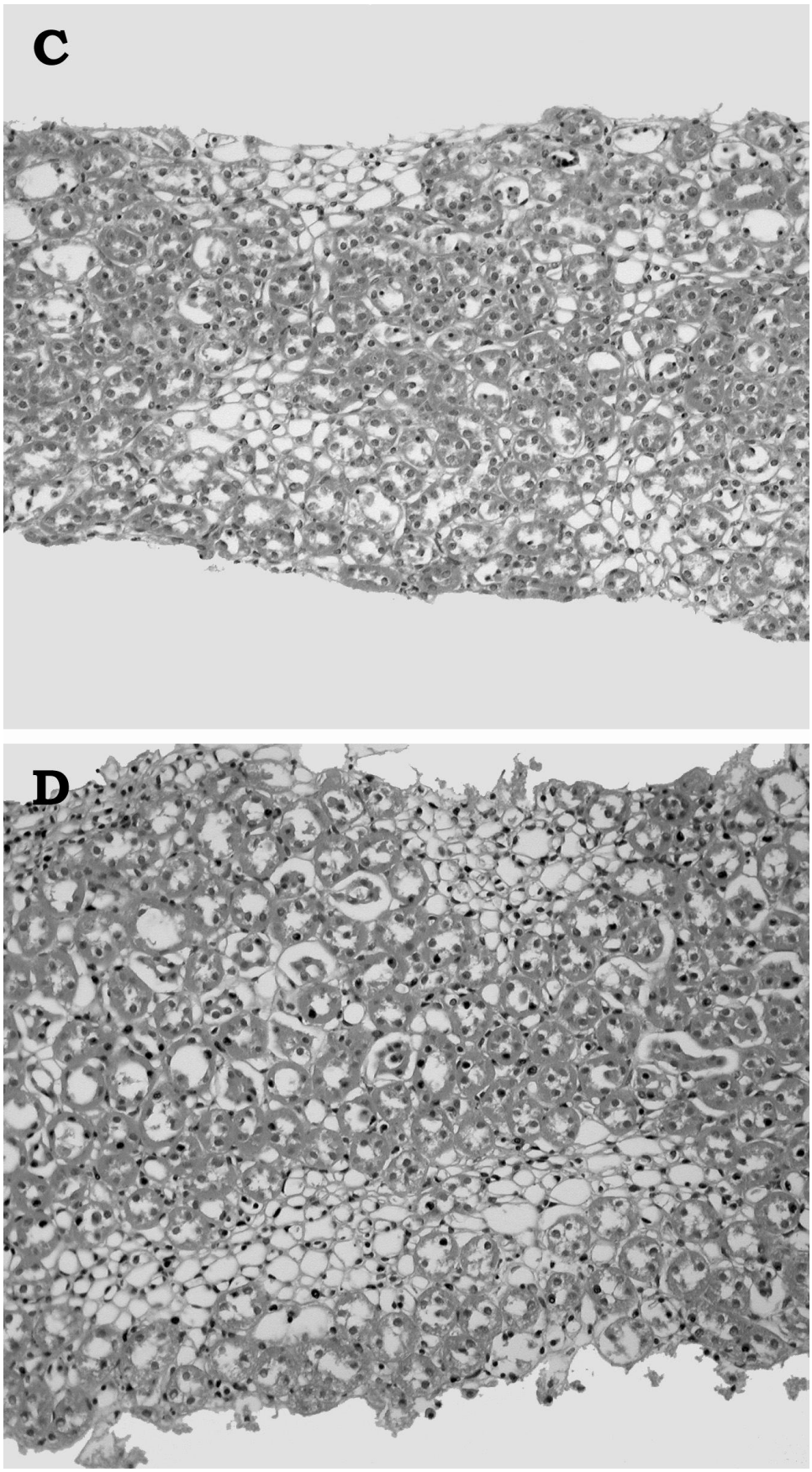


Fig. 9, continued **(C)** slice vitrified with VS4 **(D)** slice vitrified with VM3. All slices were incubated for 4 h.

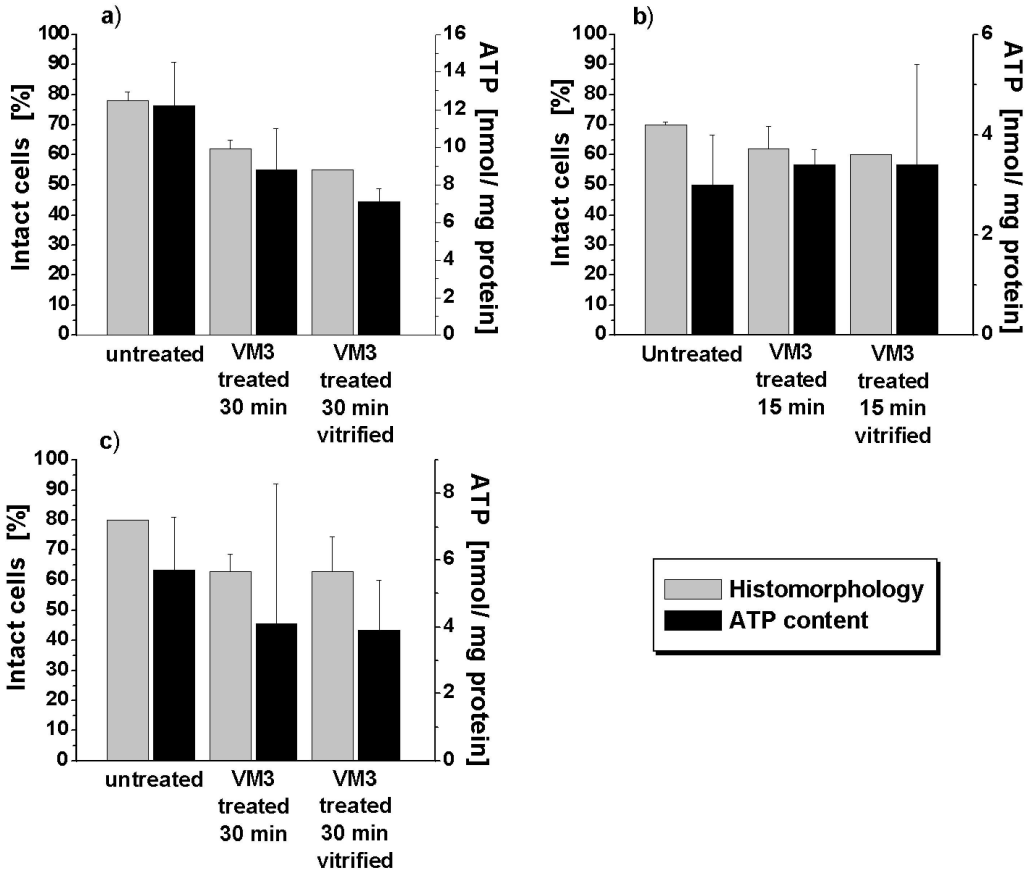


Fig. 10 Viability of tissue slices after vitrification with VM3 **(a)** liver slices, **(b)** kidney cortical slices and **(c)** kidney medullary slices. Slices were pre-incubated with VM3 for 15 or 30 min, vitrified with 1.5°C/min, thawed rapidly and washed with 300mM trehalose in the washing medium. All slices were incubated for 4 h prior to viability testing. Histomorphologic appearance of slices is quantified as the percentage of intact (not necrotic) cells in the slices cross section. Data are the average of 3 slices from 1 experiment, +SD.

In Fig. 10, results are shown of the 'best' vitrification method we obtained with VM3 for liver (Fig. 10a) and renal cortical (Fig. 10b) and medullary slices (Fig. 10c), i.e. the method resulting in the highest viability of vitrified slices regarding the effect of both CPA toxicity and actual vitrification. Kidney cortical slices were incubated for 15 min with VM3 and liver and kidney medullary slices for 30 min. All slices were slowly cooled with approximately 1.5°C/min while avoiding fracturing as described above. After fast thawing, slices were washed with 300 mM trehalose in the washing medium. With this protocol, both kidney cortical and medullary slices and liver slices maintained viability 80-100% of that of untreated slices. Fig. 7d, 8d and 9d show histomorphology of respectively liver, kidney cortical and kidney medullary slices that were treated and vitrified with VM3.

Discussion

Rapid freezing with moderately concentrated DMSO solutions has been proposed by our laboratory as a simple and adequate way to cryopreserve rat liver slices, maintaining viability and phase I and II biotransformation activity of cryopreserved rat liver slices for at least 4 h after thawing. Others who have used rapid freezing for the cryopreservation of liver slices (using 18% DMSO or more) have found similar results (Sohlenius-Sternbeck et al., 2000; Glöckner et al., 2001). With this method, we also obtained a satisfying cryopreservation outcome with cynomolgus monkey liver slices, maintaining ATP content, slice histomorphology and drug metabolism on levels comparable to fresh slices (unpublished data). Others have reported short-term maintenance of phase I and II biotransformation in cryopreserved liver slices of beagle dogs, mice, monkeys and humans (Monshouwer et al., 2000). In the present study, liver slices again remained viable after cryopreservation by rapid freezing, but kidney and intestinal slices were severely damaged.

The mechanism by which rapid freezing maintains viability of liver slices is not completely clear. De Kanter and Koster (1995) have proposed that vitrification within the slice promoted by partitioning of water within the tissue could possibly explain the success of rapid freezing. Recent studies by DSC measurements, however, have pointed out that with 18% DMSO and rapid freezing ice was formed in liver slices in the same amount as in bulk cryopreservation solution, with ice crystals probably formed both within the liver cells and in the intercellular space (de Graaf and Koster, 2001). A number of authors have reported examples of cell types that survive intracellular ice formation caused by freezing rapidly (MacKenzie, 1970; Mazur et al., 1972; Zieger et al., 1996; Acker and McGann, 2000), provided that these cells were rapidly thawed as well. It was proposed that small intracellular ice crystals would re-arrange during slow warming, growing to a size deleterious to the cells (Mazur et al., 1972). With liver slices, slow warming after rapid freezing also results in loss of viability (unpublished data) indicating that the same mechanism plays a role. The question arises why kidney and intestinal slices do not survive rapid freezing. Possibly, they are more susceptible for ice crystals formed intracellularly. Studies to compare the exact location and size of ice-crystals in rapidly frozen liver, kidney and intestinal slices may illuminate our observations.

Vitrification overcomes the problems of intra- (or inter-)cellular ice formation since it allows ice-free cooling to liquid nitrogen temperatures. When vitrification is chosen as an approach for cryopreservation, the main problem to conquer is the toxicity of the CPAs that are used in high concentrations (Fahy and Hirsh, 1982). In the present study we noticed that the various tissue types are unequally affected by CPA toxicity. Particularly kidney cortical slices and small intestinal slices were highly sensitive to VS4 exposure. CPA toxicity can be both chemical and osmotically of origin. One approach to reduce chemical toxicity of a CPA solution, without diminishing its glass-forming tendency, is to combine different CPAs with different mechanisms of toxicity and that may even antagonize each others toxicity (Baxter and Lath, 1971; Fahy, 1984, Fahy et al., 1987). This is the scientific base of the development of high molarity mixtures of CPAs like VS4 and VM3. Another manner to reduce chemical toxicity is to reduce the exposure time to the CPA. When decreasing the exposure time, one should affirm that the tissue is still fully equilibrated with the CPA. The use of a DSC can be of help for this purpose, monitoring possible elevation of critical cooling and warming rates when the exposure time is reduced. In the present study, we indeed found that reducing the exposure time to VS4 and VM3 reduces toxicity, but may also elevate critical warming rates of the tissue, as measured by DSC. Reducing exposure time of liver slices to VM3 from 30 to 15 min resulted in a higher viability of VM3 treated slices, yet viability (normalized for toxicity) of vitrified slices was lower, which can be explained by incomplete penetration of VM3. Similar observation were reported with human corneas treated with VS41a (Bourne and Nelson, 1994), confirming the necessity to optimize loading protocols for different tissues regarding both toxicity and tissue penetration of the CPA.

Osmotic effects of CPA exposure occur, because the cell membrane is less permeable for most CPAs than for water. Consequently, when a cell is exposed to a certain CPA solution, it will first shrink, because of the higher osmolarity of the CPA solution, and eventually return to its normal volume when the CPA enters the cell. Reversibly, if cells permeated with CPAs are brought into CPA free buffer solution for washing, water first enters the cell before the CPA gets out, resulting in (extensive) cell swelling and eventually lysis (Levin and Miller, 1984; Pegg and Diaper, 1990). In the present study we tended to diminish osmotic damage by introducing and removing the CPA stepwise, with the intention to allow the cells to recover their volume before each additional step. The use of impermeable sugars is known to prevent cell swelling during CPA removal, because it elevates osmolarity of

the washing medium (Pegg and Diaper, 1990). In agreement with this, we found that the use of 300 mM trehalose in the washing medium increased viability of VM3 treated kidney cortical slices.

Vitrification has been put forward by Fahy and Hirsh (1982) as a mechanism to avoid all damaging events associated with freezing, as long as CPA toxicity would be prevented. Furthermore they state that with vitrification, there is no need to worry about finding optimal cooling and warming rates for different cell types in an organ. Of course, this statement only holds true if cooling and warming rates are rapid enough to avoid ice crystal formation during freezing and thawing. The cooling rate to provoke vitrification of 10 mg medium samples of VS4 (approximately 10°C/min) is easily achievable with slices. The warming rate to prevent devitrification of VS4-medium samples has been found to be much higher (1500-6990°C/min) (de Graaf and Koster, 2001). The feasible warming rate of slices is approximately 800°C/min, hence too low to prevent ice-formation in medium samples. However, we have found that liver slices in general have less tendency to devitrify than bulk medium (de Graaf and Koster, 2001). In the present study we also found that tissue slices in general had lower V_{crw}s than VS4-medium samples. In most tissue samples devitrification is prevented using warming rates of 800°C/min, but not in all. Remarkably, viability of liver and kidney medullary slices that had V_{crw}s higher than the feasible warming rates did not differ much of those with V_{crw}s below the feasible warming rates.

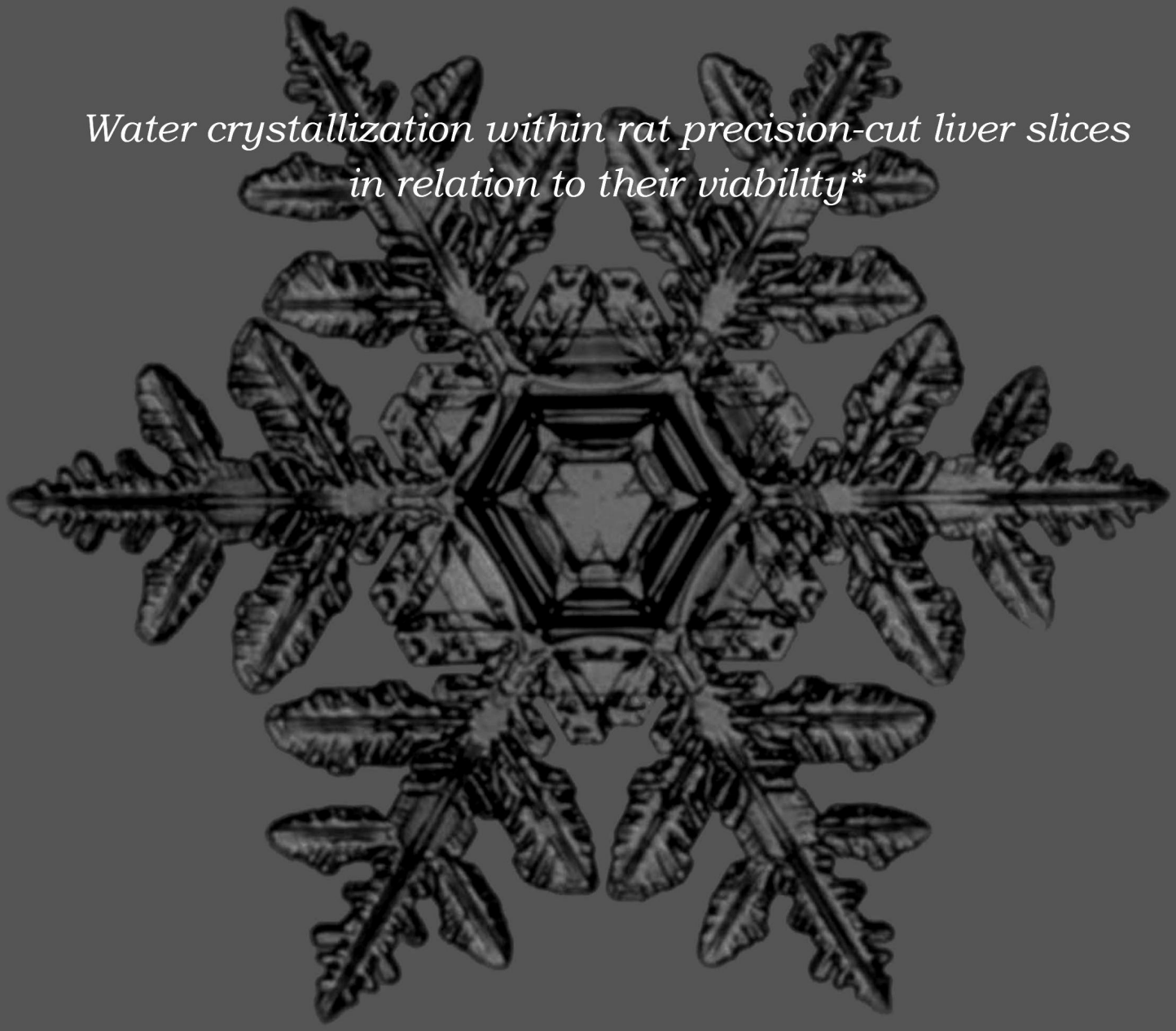
Nevertheless, according to the proposition of Fahy and Hirsh (1982), in slices with a V_{crw} lower than the feasible warming rate, vitrification should not further decrease viability. In the present study, with VS4 pre-incubated kidney medullary slices, this was indeed the case. However, both liver and kidney cortical slices had a clearly lower viability after vitrification at 800°C/min than slices that were treated with VS4 but not vitrified. In slices vitrified with high cooling rates after pre-incubation with VM3, we noticed the same. With VM3 however, it was possible to vary the cooling and warming rates because it has much lower critical cooling and warming rates than VS4. In slices slowly cooled after pre-incubation with VM3, viability was maintained at high levels. So, despite of the fact that the cooling rate does not determine whether ice formation is prevented or not in VM3 solutions (as long as those rates are >1°C/min), it yet seems to determine slice viability, at least in the case of liver and kidney cortical slices. Similar observations have been done by Greg Fahy's group (Greg Fahy, personal

communication). As yet, we have no explanation for this observation, but possibly thermal stresses induced by high cooling rates play a role.

In conclusion, rapid freezing after pre-incubation with 18% DMSO maintains viability of liver slices as determined by measuring ATP content and examining slice histomorphology. Yet for unclearified reasons, rapid freezing is deleterious for rat kidney and small intestinal slices. Vitrification with relatively non-toxic, high molarity mixtures as VS4 and particularly VM3 is a promising approach for cryopreservation of tissue slices, maintaining viability of these slices on levels comparable to those of fresh slices. However, preventing toxicity and ice formation is not sufficient to maintain viability of particularly liver and kidney cortical slices by vitrification: the use of slow cooling rates, preventing thermal stress in the sample, seems to be a prerequisite.

Chapter 5

*Water crystallization within rat precision-cut liver slices in relation to their viability**



*Based on:
I.A.M. de Graaf and H.J. Koster. Water crystallization within rat precision-cut liver slices in relation to their viability, *Cryobiology* **43**, 224-237 (2001)

Abstract

The present study examines whether tissue vitrification, promoted by partitioning in the tissue, could be the mechanism explaining the high viability of rat liver slices, rapidly frozen after pre-incubation with 18% DMSO or VS4 (a 7.5M mixture of DMSO, 1,2-propanediol and formamide with weight ratio 21.5: 15: 12.4).

To achieve this, we first determined the extent to which crystallization or vitrification occurred in CPA solutions (DMSO and VS4) and within liver slices impregnated with these solutions. Secondly, we determined how these events were related to survival of slices after thawing. Water crystallization was evaluated by Differential Scanning Calorimetry and viability was determined by histomorphological examination of the slices after culturing at 37°C for 4 h.

VS4 pre-incubated liver slices indeed behaved differently from bulk VS4 solution, because, when vitrified, they had a lower tendency to devitrify. Vitrified VS4 pre-incubated slices that were warmed sufficiently rapid to prevent devitrification had a high viability. When VS4 was diluted (to 75%) or if warming was not fast enough to prevent ice formation, slices had a low viability. With 45% DMSO, low viability of cryopreserved slices was caused by CPA toxicity. Surprisingly, liver slices pre-incubated with 18% DMSO or 50% VS4 had a high viability despite the formation of ice within the slice.

In conclusion, tissue vitrification provides a mechanism that explains the high viability of VS4 pre-incubated slices after ultra-rapid freezing and thawing (>800°C/min). Slices that are pre-incubated with moderately concentrated CPA solutions (18% DMSO, 50% VS4) and cooled rapidly (100-200°C/min) survive cryopreservation despite the formation of ice crystals within the slice.

Introduction

A major goal for cryobiologists is the prevention of ice crystal formation when cryopreserving biological samples. With single cells, this goal may be achieved by freezing sufficiently slowly to allow equilibrium freezing (Mazur, 1984). For whole organs, equilibrium freezing is not an option, since ice that is formed in the intercellular space may harm the cells from the outside. Therefore, cryopreservation of organs is focussed on the promotion of vitrification, in which an amorphous glassy state is formed (Fahy *et al.*, 1984; Armitage and Rich, 1990).

Precision-cut liver slices are becoming increasingly popular as an *in vitro* tool for metabolic and toxicity studies in drug research, because their micro-architecture resembles the liver architecture *in vivo*, (Bach *et al.*, 1996; Olinga *et al.*, 1997). Liver slices have physical dimensions (8 mm x 200 μm) that are intermediate between those of isolated cells and whole organs. A number of cryopreservation methods for liver slices have been published in the past decade. These methods are based either on equilibrium freezing using low cooling rates and cryoprotectant (CPA) concentrations (Fisher *et al.*, 1991; Fisher *et al.*, 1993; Fisher *et al.*, 1996, Maas *et al.*, 2000a) or on vitrification protocols with highly concentrated CPA solutions and (ultra-) rapid freezing (Wishnies *et al.*, 1991; Ekins, 1996a; Ekins *et al.*, 1996a).

A third group of methods has been published more recently, including some from our laboratory. These methods are successfully combining rapid freezing (100-1000°C/min) with moderately concentrated CPA solutions (12-30% dimethylsulfoxide (DMSO)) (de Kanter and Koster, 1995; Glöckner *et al.*, 1996; Glöckner *et al.*, 1998; de Kanter *et al.*, 1998, Day *et al.*, 1999; de Graaf *et al.*, 2000b; Sohlenius-Sternbeck *et al.*, 2000, Glöckner *et al.*, 2001), and maintain the viability of slices at relatively high levels, even 24 h after thawing (Glöckner *et al.*, 2001 and own unpublished research). The mechanism that explains the success of this approach is not understood: the high cooling rates do not allow prevention of intracellular ice formation (IIF) by equilibrium freezing (Karlsson, 1993). With the applied cooling rates the CPA concentration is too low to achieve vitrification (Boutron and Kaufmann, 1978; Baudot *et al.*, 2000). Evidence exists however, that cells or tissues that are impregnated with a CPA vitrify more readily than bulk solutions of the same CPAs and that the amorphous state also has a higher stability (Takahashi and Hirsch, 1985; Peridieu *et al.*, 1995). It has been suggested previously that this may be caused by the fact that liquid within

a tissue behaves as if it were compartmentalized like in hydrogels (Peridieu *et al.*, 1995). Partitioning of liquids increases the tendency to supercool and therefore facilitates vitrification (Yamane *et al.*, 1992). Therefore, the liquid within the slice may vitrify while the CPA medium surrounding the slice crystallizes. This would explain why liver slices survive rapid freezing when relatively low CPA concentrations are used.

Preliminary studies within our laboratory showed that (ultra) rapid freezing of slices impregnated with a relatively non-toxic CPA called Vitrification Solution 4 (VS4) gave similar survival rates as 18% DMSO. VS4 is composed of DMSO, 1,2-propanediol and formamide (weight ratio 21.5: 15: 12.4) with a total concentration of 7.5 M (Fahy and Ali, 1997; Kheirabadi and Fahy, 2000) and vitrifies as a bulk solution under pressure. The experiments performed in the present study try to clarify whether or not tissue vitrification is the mechanism responsible for the maintenance of viability by either method. To achieve this, we first used Differential Scanning Calorimetry (DSC) to determine the extent to which crystallization and vitrification occurred in CPA solutions (DMSO and VS4) and within liver slices that were impregnated with these solutions. Secondly we determined how these events were related to the viability of slices after thawing.

Slice viability was determined by histomorphological examination. This parameter was selected because previous studies have shown that this correlated with results obtained with other viability parameters (e.g. slice ATP- and potassium content) and with slice functionality (e.g. metabolism of testosterone and 7-hydroxycoumarin). The latter parameters even appeared to be less readily affected by cryopreservation than slice histomorphology (de Graaf *et al.*, 2000b, Maas *et al.*, 2000b). The relative insensitivity of functionality parameters to tissue damage in comparison with histomorphology is also reported by Gokhale *et al.* (1995). The thawed slices were incubated for 4 h at 37°C prior to microscopic examination of morphology, to allow sufficient time for damage caused by CPA toxicity or cryopreservation to be expressed.

Materials and Methods

Chemicals

Formamide, cyclohexane, insulin, gentamycine, adenine and glutathione were obtained from Sigma, Axel, The Netherlands; William's Medium E + glutamax (WME) and Fetal calf serum (FCS) were from Gibco BRL, Breda, The Netherlands; DMSO (>99.9% pure) and all other chemicals were from Baker, Deventer, The Netherlands.

Preparation and incubation of liver slices and viability testing

Male rats (Wistar, obtained from Harlan CPB, Horst, The Netherlands) had free access to food and water. They were anaesthetised with 65% CO_2, 35% O_2 before the livers were removed. Subsequently tissue slices (thickness 200-250 μm) were prepared from 8 mm tissue cores with a Krumdieck tissue slicer (Krumdieck *et al.*, 1980). Slices were incubated at 37°C in WME-medium, supplemented with FCS (5%), 0.1 μM insulin, 50 mg/l gentamycine and D-glucose (to a medium concentration of 25 mM) using a shaking flask incubation system (de Kanter and Koster, 1995; de Graaf *et al.*, 2000b; Olinga *et al.*, 1997b). Slice viability was evaluated by histomorphological examination after 4 h of incubation at 37°C. To examine histomorphology, a 5 μm cross-section of the slice was made and stained with haematoxylin and eosin. Cells were considered to be viable if they did not show signs of degeneration such as hydropic cell swelling, pyknotic nuclei, karyolysis or karyorhexis. The percentage of intact cells was the number of viable cells divided by the number of viable plus non-viable cells x 100.

Pre-incubation with the CPA and cryopreservation

Slices were pre-incubated in 25-ml Erlenmeyer flasks (6 slices/Erlenmeyer) placed in a shaking water bath (110 strikes/min) on ice. Each Erlenmeyer flask contained 5 ml CPA (DMSO or VS4) mixed in various proportions with oxygenated medium (WME or VS4 buffer). VS4 buffer has the same composition as VS4, but without formamide, 1,2-propanediol and DMSO. Pre-incubation with 10 or 18% v/v DMSO and 25% v/v VS4 was executed in one single step, during 30 min. When higher concentrations of DMSO or VS4 were used, the CPA was added stepwise with the intention of limiting possible osmotic damage, according to the empirically derived protocol shown in Table 1.

Table 1 Stepwise cryoprotectant impregnation and removal

	Pre-incubation medium concentration[a]					Washing medium concentration[a]				
End-concentration VS4	0%	25%	50%	75%	100%	100%	75%	50%	25%	0%
0%	10									
25%		30								10
50%		15	30						10	10
75%		15	15	30				10	10	10
100%		15	15	15	30		10	10	10	10
	Pre-incubation medium concentration[b]					Washing medium concentration[b]				
End-concentration DMSO	0%	10%	18%	30%	45%	45%	30%	18%	10%	0%
0%	10									
10%		30								10
18%			30							10
30%			15	30				10		10
45%			15	15	30		10	10		10

Values in the table represents the time span of each step, given in min with each row represen-ting consecutive pre-incubation or washing steps
[a] % VS4 in VS4 buffer (v/v)
[b] % DMSO in WME (v/v)

Immediately after pre-incubation, slices were cryo-preserved by placing 3-6 slices in 2 ml cryovials (Greiner, Alphen a/d Rijn, The Netherlands) with 0.5 ml of the CPA solution. The cryovials were then submerged directly in liquid nitrogen. Some slices that were pre-incubated in 100% VS4 or 45% DMSO were cryopreserved by placing them between aluminium foil followed by direct immersion in liquid nitrogen. The aim of this procedure was to increase cooling and warming rates as described by Day *et al.* (1999).

After storing the cryopreserved slices in liquid nitrogen for 30 min or more, slices were thawed by placing the cryovials in a 37°C waterbath until ice was no longer visible. The slices that were sandwiched between aluminium foil were thawed by placing the foil in VS4 or 45% DMSO on melting ice. After thawing, the cryopreservation medium was washed away, depending on the cryopreservation medium used, according to Table 1, immediately followed by 4 h incubation at 37°C prior to viability determination.

To determine possible adverse effects of pre-incubation with the CPAs on the slices, some slices underwent the same procedure of pre-incubation and washing, but were not cryopreserved. Other liver slices were only pre-incubated with CPA and subsequently used to determine the 'liquid fraction' (see below) of the slice or for DSC measurements.

Determination of slice 'liquid fraction'

The slice liquid fraction is the relative liquid content (cellular water and CPA) of a slice by weight, and is determined as follows: The slice wet weight ($Mass_w$) was measured after carefully removing CPA solution attached to the slice with paper tissue. The slice was then dried overnight in a 60°C oven. After measuring the dry weight ($Mass_d$) the liquid fraction (g/g) was determined by Eq. [1].

$$\text{liquid fraction (g/g)} = (Mass_w - Mass_d)/ Mass_w \qquad [1]$$

Determination of cooling and warming rate in cryovials

The cooling rate of cryovials filled with 0.6 ml medium was determined by placing the temperature sensor of an Icecube 1610 computer freezer (Sylab) in the vial, followed by direct submersion of the vial in liquid nitrogen. The sensor was calibrated with ice-water (0°C) and liquid nitrogen (-196°C). Warming rates were measured during the warming of the vials (with the sensor) in a 37°C water bath. Cooling rates of slices that were sandwiched between aluminium foil after pre-incubation with 45% DMSO or VS4 were determined by including the sensor between two slices in the aluminium foil sandwich. Warming rates were determined in VS4 or 45% DMSO on melting ice, during warming with the sensor in between the aluminium foil pads.

Determination of sample cooling rate in the Differential Scanning Calorimeter

A small adaptation in the Differential Scanning Calorimeter (DSC-7) (Perkin Elmer, Oosterhout, The Netherlands) made it possible to determine both the programmed and the actual sample warming and cooling rates from the diagrams. For this purpose, in the Thermal Analyser Controller (TAC-7) of the DSC-7, the PIN 6 from IC U8 (which is located on the motherboard of the TAC-7) was disconnected from its socket. For further information consult Perkin Elmer (Oosterhout, The Netherlands).

DSC measurements

DSC measurements were performed using a DSC-7. The apparatus was calibrated with cyclohexane and pure water.

Sample preparation

At the end of pre-incubation with the CPA, a part of a slice (weighing 5-9 mg) was transferred into a 50 µl DSC sample pan (Perkin Elmer, Oosterhout, The Netherlands). Cryopreservation medium attached to the slice was carefully removed with a paper tissue before the pan was closed hermetically. From the pre-incubation medium used, approximately 9 mg was pipetted into another sample pan. To prevent evaporation, the pan was directly closed tightly and kept at 4°C until use.

Determination of the percentage of a sample water that crystallised

From DSC thermograms, the heat of crystallization and the heat of melting were determined from the area of the crystallization and melting peaks. From these values q (in %), which can be defined as the percentage of a sample that crystallized as pure ice (Boutron, 1986), was calculated by dividing the liberated heat of crystallization per gram sample by the specific crystallization heat of pure water that freezes at 0°C (*100%). For liver slices, q was corrected for the solid matter of the slice by dividing q by the liquid fraction (see above) of the slice. When water only crystallized on cooling (i.e. no devitrification occurred), the melting peak was used to calculate q, since this peak was better shaped and therefore more reliably quantified than the crystallization peak.

Determination of critical cooling rates in slices and CPA solutions

For determination of the critical cooling rate (V_{crc}) of a sample, i.e. that cooling rate just high enough to almost prevent crystallization in the sample, q (in %) was determined on cooling with the DSC cooling rate (υ) set at 2.5, 5, 10, 20, 40, 80 or 160°C/min. Subsequently, q was plotted against the logarithm of υ. The theoretical relation between q and υ was described by Boutron (1986) in the fourth semi-empirical model of Boutron. We used this model to calculate the theoretical curves, fitting the experimental values in our experiments. V_{crc} was then determined from the theoretical curves by calculating υ when q = 0.2%. The value of 0.2% for q was chosen by Boutron for practical reasons. It was assumed that this minimal amount of ice would not damage cells (Boutron, 1986).

Determination of critical warming rates in slices and CPA solutions

The stability of the vitrified state was assessed in samples that vitrified during cooling, by determining the critical warming rates V_{crw}. For this purpose, the samples were warmed with the DSC warming rate (υ) set at 5,

10, 20, 40 or 80°C/min after cooling with the DSC set at 300°C/min. From the thermograms the temperature at the end of melting (T_m) and the temperature of the devitrification peak (T_d) were determined. A linear relationship exists between T_m/T_d and the logarithm of the warming rate (Boutron and Mehl, 1990). The critical warming rate (V_{crw}), i.e. the warming rate just high enough to prevent devitrification, can be determined by extrapolating the curve, determining v when T_d = 0.95 T_m (Boutron and Mehl, 1990; Boutron, 1993, Boutron and Peridieu, 1994)

To assess V_{crc} and V_{crw} in an individual sample, various cooling and warming scans were required. The impact of these multiple freeze and thaw cycles on the sample was minimized by first scanning with the highest cooling and warming rates (at which the lowest amount of ice was expected). The effect of the repetitive freeze-thaw cycles on the devitrification temperature (T_d) of a VS4 pre-incubated slice was tested by repeating a cycle of cooling with 300°C/min and warming with 20°C/min 3 times. No difference was found between the T_d of the first and the subsequent cycles. The same was done for the crystallization peak while repeatedly cooling the slice with 5°C/min and warming with 80°C/min. In this case also, no significant change in the size of the crystallization peak was seen (data not shown).

Determination of q in slices that were pre-incubated with various CPA solutions and subsequently cryopreserved

In slices pre-incubated with aqueous solutions of DMSO (0, 10, 18, 30 and 45% v/v in WME) or VS4 (0, 25, 50, 75 and 100% v/v in VS4 buffer) the amount of ice (*q,* in %*)* formed during cryopreservation (freezing and thawing) was determined. For this purpose, the cooling rates in the cryovials during cryopreservation had to be determined first (see above). Subsequently for each sample a cooling program was written in the DSC, mimicking the cooling rates in the cryovials. For samples that did not devitrify on thawing (because all crystallizable water had already crystallized on cooling), the warming rate in the DSC was set to 80°C/min. For vitrified samples that devitrified during thawing, *q* was calculated from the devitrification peak. In this case, *q* was dependent on the warming rate and therefore the DSC was also programmed to mimic the warming rates as measured in the cryovials.

Table 2 Critical cooling and warming rates in liver slices impregnated with VS4 and in a sample of VS4 solution taken at the end of pre-incubation in the same experiment

	VS4 impregnated liver slices		VS4 solution	
	V_{crc} (°C/min)	V_{crw} (°C/min)	V_{crc} (°C/min)	V_{crw} (°C/min)
Liver 1 (slice 1)		26	9	6856
(slice 2)	<2.5	29.5		
(slice 3)	23			
Liver 2 (slice 1)	9.5	238	9	2400
Liver 3 (slice 1)	11	354	8	2412
Liver 4 (slice 1)	22	1589	4	1570
Liver 5 (slice 1)	12	351	12	6991
Liver 6 (slice 1)		634		3472

Results

Determination of critical cooling and warming rates in slices and bulk CPA solutions

To determine the critical cooling rates, the quantity of ice formed within a sample (q) was measured at various cooling rates (v). When slices were impregnated with 18% v/v DMSO, q did not vary with the cooling rate (Fig. 1). Moreover, there was no difference between slices and the CPA solution. However, in VS4 bulk solution and in slices that were pre-incubated with VS4, q did depend on the cooling rate (a typical example is shown in Fig. 2). For both slices and solutions, there was a good correlation between the experimental data points and the theoretical curves, calculated with the fourth semi-empirical model of Boutron. In Table 2, for liver slices from 6 different livers, the critical cooling rates required to achieve (almost) complete vitrification of the sample (V_{crc}), are shown. The V_{crc}s of slices and the CPA solutions are in the same range (2.5-23°C/min and 4-12°C/min respectively).

Critical warming rates (V_{crw}s), however, *did* differ between slices, pre-incubated with VS4, and VS4 bulk solution (Fig. 3). Although V_{crw}s varied considerably, they were lower for slices than for medium in 5 out of 6 experiments. In one experiment there was no difference between the V_{crw}s. To prevent devitrification, slices that were vitrified after pre-incubation with VS4 had to be warmed at 26 to 1589°C/min, while the prevention of devitrification of vitrified CPA solutions required warming rates between 1570 and 6991°C/min.

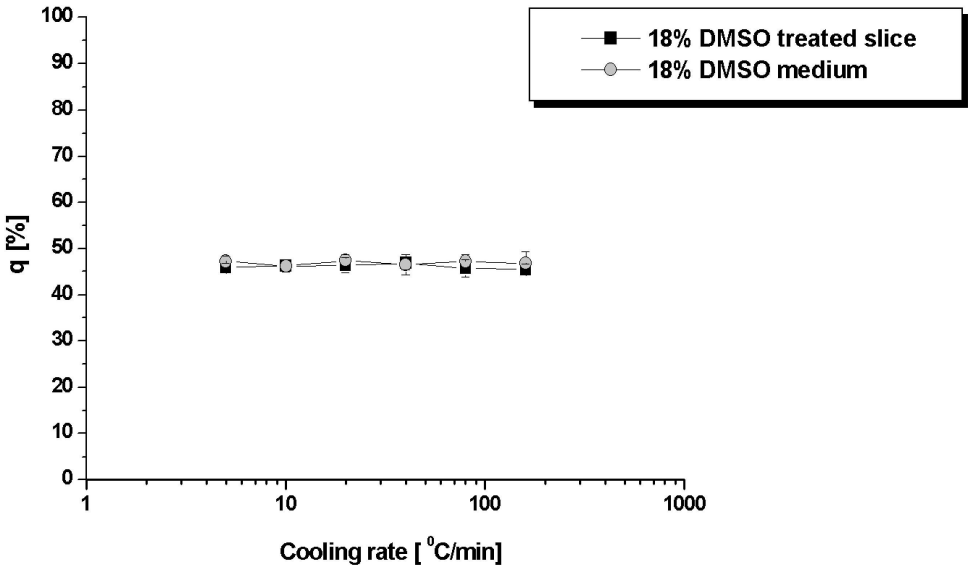


Fig.1 Relation between the cooling rate and *q* (the percentage of a sample that crystallized as pure ice) in an 18% (v/v) DMSO solution and in a slice impregnated with 18% DMSO. Data-points represent the mean of 3 experiments (livers), 1 sample (medium or slice) per experiment.

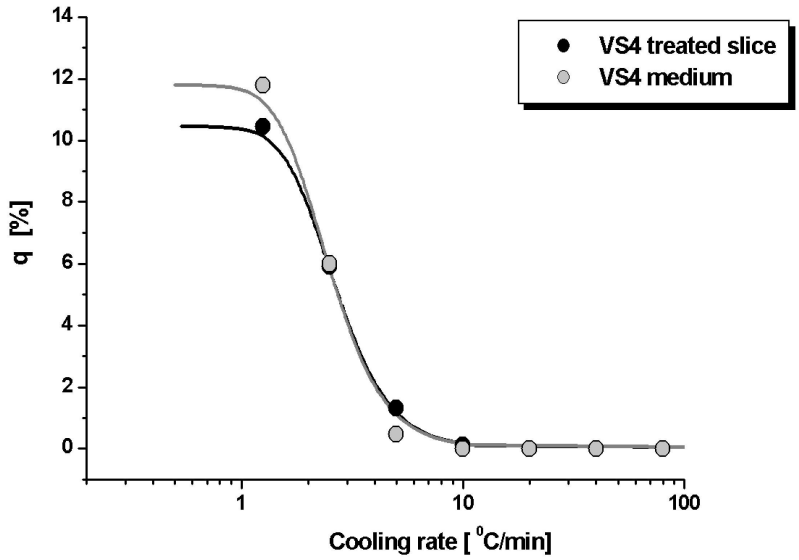


Fig. 2 Relation between the cooling rate and *q* (the percentage of a sample that crystallized as pure ice) in a VS4 solution and in a slice impregnated with VS4. Data-points represent one sample (medium or slice), from one representative experiment (liver). Solid lines represent semi-empirical curves calculated according to the fourth model of Boutron. Critical cooling rates were calculated by extrapolating the theoretical curves to *q*=0.2% (Boutron, 1986).

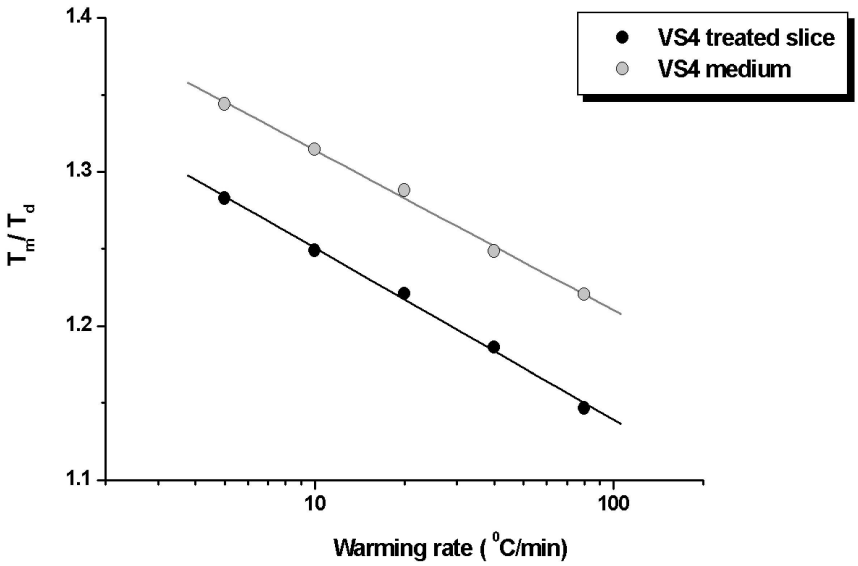


Fig. 3 Relation between the warming rate and T_m/T_d in a VS4 solution and in a slice impregnated with VS4. Data-points represent one sample (medium or slice), from one representative experiment (liver). Solid lines are theoretical regressions used to calculate the critical warming rates. To calculate critical warming rates, theoretical regression curves were extrapolated to T_d =0.95 T_m (Boutron and Mehl, 1990; Boutron, 1993; Boutron and Peridieu, 1994).

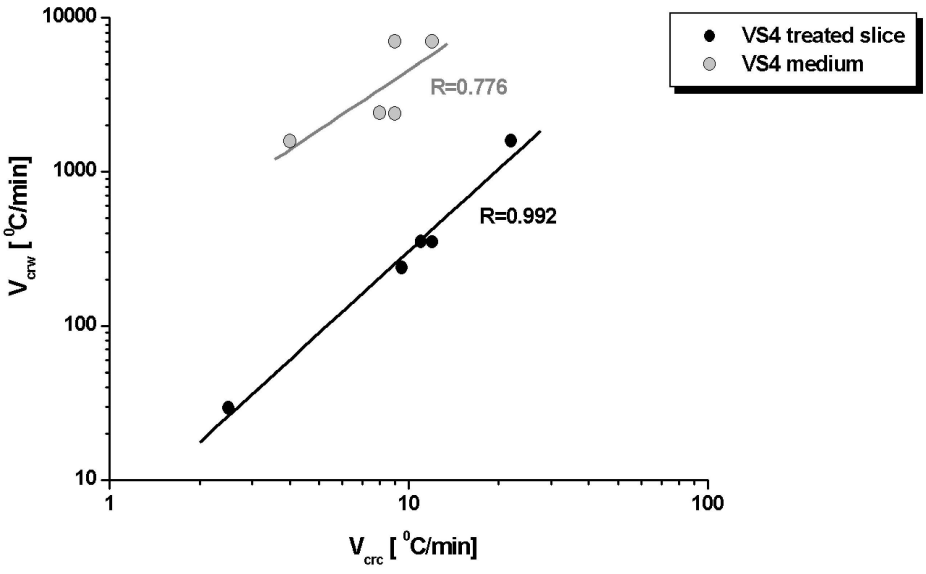


Fig. 4 Relation between critical cooling and warming rates in VS4 solutions and in slice impregnated with VS4. Data are derived from 5 different experiments (livers), each data-point represents one sample (medium or slice).

For both slices and CPA solution, there is a correlation between the critical cooling and warming rates of the same sample (Fig. 4). For slices, warming rates required to prevent devitrification of a vitrified sample were on the average 50 times higher than the cooling rates required to achieve vitrification. For the CPA solutions, critical warming rates were approximately 500 times higher than the critical cooling rates.

Determination of cooling and warming rates during cryopreservation

To make an accurate comparison between the crystallization of ice in a liver slice during cryopreservation and liver slice viability, it is essential to use approximately the same cooling rates and warming rates with the DSC as those that are normally reached during cryopreservation and warming. For this reason, we first measured cooling and warming rates in cryovials with CPA solution and of slices sandwiched between aluminium foil pads, following the normal cryopreservation procedure. As shown in Fig. 5a (VS4) and 5b (DMSO), cooling rates in cryovials are dependent on the composition of the cryoprotective solution and on the state of the sample (fluid or frozen): the highest maximum cooling rates were 200-300°C/min and occurred in samples that were frozen with low CPA concentrations. Freezing of VS4 and 45% DMSO pre-incubated slices sandwiched between aluminium foil increased the cooling rate from 100 to at least 800°C/min. The cooling rate of all samples decreased as the liquid nitrogen temperature was approached. It should be noted that the cooling rates in the cryovials were measured in the CPA solution without slices. However, the addition of slices did not alter the cooling rates (data not shown).

The warming rate was also determined in order to quantify crystallization accurately. However, since q in non-vitrified samples is dependent only on the cooling rate, Fig. 5c shows just the warming rates for slices that were *vitrified* in cryovials or clamped between aluminium foil pads after pre-incubation with 45% DMSO or 100% VS4. Warming of sandwiched slices was at least 8 times more rapid than of slices in cryovials.

The DSC was set to match the cooling rates of the slices under normal cryopreservation circumstances. For vitrified slices the warming rate was also mimicked since ice formation in these slices occurred particularly during warming (devitrification) and was dependent on the warming rate. For some samples the cooling rates of the samples after crystallization under normal cryopreservation conditions, was somewhat faster than the maximum sample cooling rates in the DSC.

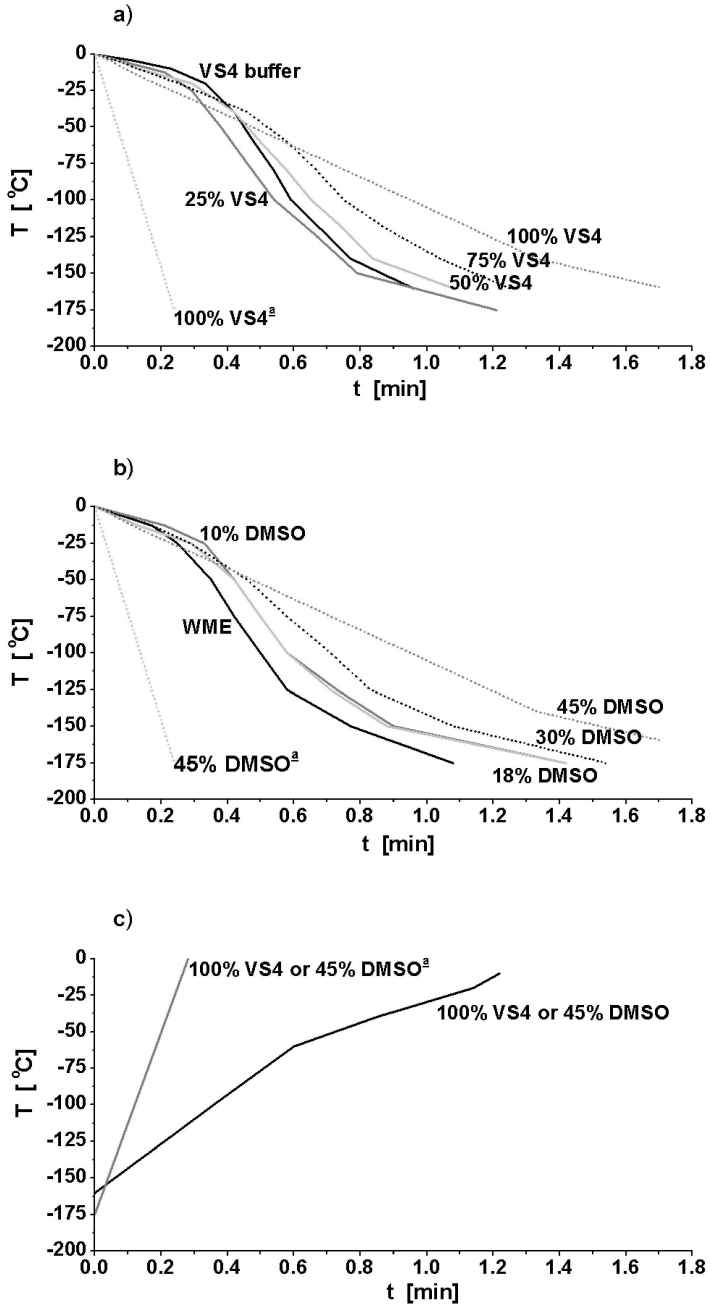


Fig. 5 (**a**) Sample temperature at various time points after direct immersion in liquid nitrogen of various VS4 solutions in cryovials or of [a]slices sandwiched between aluminium foil after pre-incubation with 100% VS4 **(b)** Sample temperature at various time points after direct immersion in liquid nitrogen of various DMSO solutions in cryovials or of [a]slices sandwiched between aluminium foil after pre-incubation with 45% DMSO. **(c)** Sample temperature at various time points after placing cryovials with 45% DMSO or 100% VS4 in a 37°C waterbath or after [a]placing the alumnium foil sheets containing slices pre-incubated with 45% DMSO or 100% VS4 in 4°C 45% DMSO or VS4.

Relation between the amount of ice formed in a slice and viability

The relation between the amount of ice (*q*) formed within a slice and slice viability is presented in fig. 6a for VS4 as the CPA and in Fig. 6b for DMSO. These figures also show the influence of pre-incubation with the CPA, without cryopreservation, on the viability of fresh slices (closed symbols). To determine viability, the slices were histomorphologically examined. Our experience is that within 4 h of culturing eventual damage by cryopreservation or by CPA toxicity becomes evident using this parameter.

For VS4, a slight decrease in viability can be seen at the highest concentrations, compared to the viability of slices that were not exposed to VS4 (Fig. 6a). According to Fig. 6b, DMSO was not toxic to rat liver slices at concentrations up to 30% v/v but no cells survived exposure to 45% DMSO. Even in slices that were not exposed to CPAs, no more than 75% of the cells in a cross section were viable. It should be noted, however, that because of the procedure of slice preparation, 1 or 2 outer cell layers are always damaged, leading to a maximal 'viability score' of approximately 80% for very good slices.

For slices cryopreserved after pretreatment with VS4 (Fig. 6a), two optima in the curve relating *q* to viability occurred. Because of the low toxicity, 58% of the cells in a slice were still intact after pre-treatment with 100% VS4 and subsequent culturing for 4 h. After cryopreservation and thawing of these slices between aluminium foil sheets, and 4 h of culturing, 45% of the cells on average were still intact. It should be noted that the amount of ice formed in the slices that were cryopreserved and thawed between aluminium foil was not actually measured, because the cooling and warming rates (ca. 800°C/min) required were too high to simulate with the DSC. We may assume, however, that with these rates all slices vitrified on cooling, but some devitrified during warming and (a small amount of) ice was formed after all (see Fig. 3 and Table 2). Just a few cells in a slice pre-incubated with 100% VS4 survived cryopreservation in cryovials (cooling and warming rate approximately 100°C/min, *q*= ca. 8%). Surprisingly, despite the fact that ice does form during cryopreservation, viability of slices pre-treated with 50% VS4 (*q*= ca. 40%) was relatively high after thawing. Slices permeated with VS4 buffer, 25% VS4 or 75% VS4 and then cryopreserved had a very low viability.

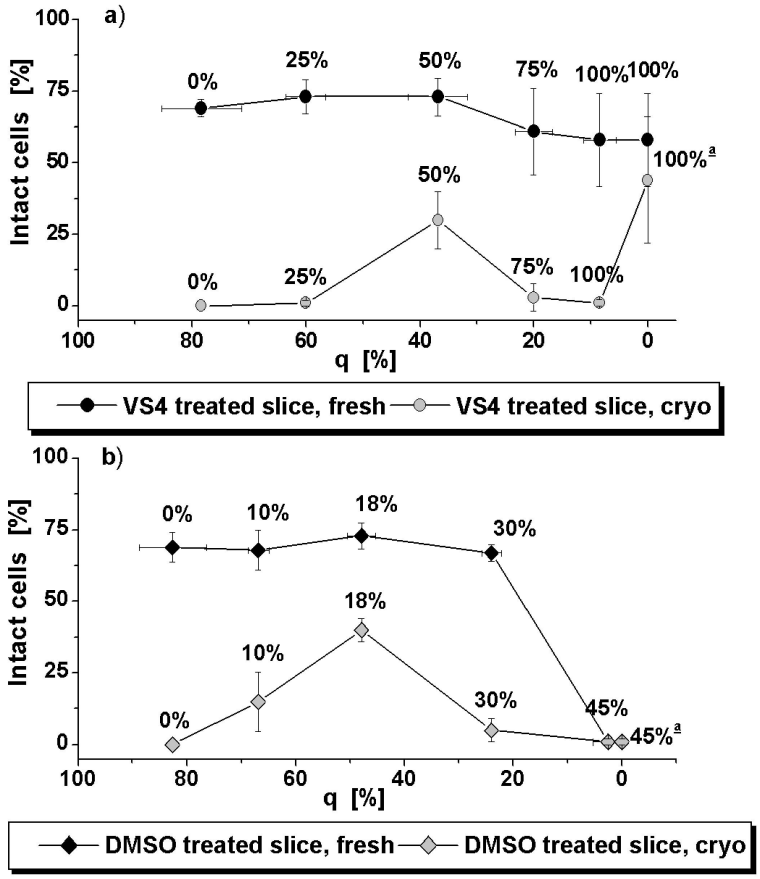


Fig. 6 Relation between *q* (the percentage of a sample that crystallised as pure ice) and viability as determined by histomorphological examination. **(a)** Slices were pre-incubated with VS4 and cryopreserved in cryovials except for [a], these slices were cryopreserved between aluminium foil. Data-points are labeled with the used VS4 concentrations and represent 3-4 experiments, 2 or 3 slices per experiment.. Fresh slices were only pre-incubated with VS4, but not cryopreserved. **(b)** Slices were pre-incubated with DMSO and cryopreserved in cryovials except for [a], these slices were cryopreserved between aluminium foil. Fresh slices were only pre-incubated with DMSO, but not cryopreserved. Data-points are labeled with the used DMSO concentrations and represent 3 experiments, 2 or 3 slices per experiment. Data are given $^{+}$/- SD.

Despite the fact that no ice was formed during the cooling or warming of 45% DMSO pre-incubated slices, no cells survived cryopreservation (Fig. 6b). In this case however, the low viability was caused by CPA toxicity. Therefore, only one viability optimum was observed, namely when 18% DMSO (*q*= ca. 50%) was used as CPA. With a higher *q*, obtained with 0 or 10% DMSO, more ice was formed and few cells survived. Surprisingly, with 30% DMSO, despite the facts that less ice was formed than with 18% DMSO and that toxicity appeared to be low, no cells survived.

Discussion

Calorimetric studies performed in the present study showed that ice was formed within slices that were rapidly frozen after pre-incubation with 18% DMSO and therefore, the relatively high viability of these slices cannot be explained by the occurrence of tissue vitrification. The total amount of ice formed within these slices was equal to that formed in the CPA solution, indicating that the crystallization of water was maximal for the given DMSO concentration. Also, the cooling rates needed to achieve vitrification in VS4 treated slices were the same as in VS4 bulk solution and, hence, no effect of water partitioning in the tissue was evident.

The critical cooling rate for VS4 bulk solution found in the present study was approximately 10ºC/min, however, to achieve vitrification of VS4 when cooling at 10ºC/min, Kheirabady and Fahy (2000) had to apply a high hydrostatic pressure to the sample. This discrepancy can be explained by the fact that different methods were used to assess V_{crc}. Fahy and coworkers used a macroscopic method and defined V_{crc} as that rate at which no visible ice crystals or nuclei were formed during cooling (Fahy and Hirsh, 1982). The method of Boutron, which is used in the present study, allows 0.2% of the sample volume to crystallise at the V_{crc}. Moreover, Fahy and coworkers used a sample of approximately 8 ml to assess V_{crc} (Fahy and Hirsh, 1982), so the chance of nucleation was much higher than in our sample, which was approximately 10 µl. The effect of a smaller sample volume was also shown by Mehl (1993): With a 15-30 mg sample of VS41A, which should vitrify at 10ºC/min under atmospheric pressure according to Fahy, he found a V_{crc} of less than 1ºC/min.

In agreement with data of Peridieu *et al.* (1995) and Takahashi and Hirsh (1985), the vitrified state in slices appeared to be more stable than in the cryoprotective solutions, with critical warming rates up till 200 times lower in slices than in CPA solutions. The V_{crw}s varied considerably between slices incubated with VS4, by a factor 50. We do not believe that the measurement itself and the subsequent calculation introduced this variation, because the V_{crw}s varied only by a factor of 4 in the cryopreservation solutions from the same experiments. Furthermore, slices with high V_{crw}s also had high critical cooling rates. It is not likely that insufficient equilibration of slices with the CPA caused the variation, since the maximal amount of water that crystallized, which is dependent on the CPA concentration, did not vary substantially between the different slices. So, the variation found may be caused by differences in the intrinsic

properties of the slices such as vascularization, collagen content and glycogen content, initiating differences in the extent of partitioning. Variation of critical warming rates between kidney samples was also reported by Peridieu *et al.* (1995). They found a correlation between V_{crw} and the degree of vascularization in samples from different parts of the kidney.

The critical warming rates of slices (26-1600°C/min) are sufficiently low to prevent devitrification in the majority of VS4 treated slices that were warmed between aluminium foil sheets (warming rate at least 800°C/min). In some slices, however, the V_{crw}s were so high that devitrification occurred. Indeed, slices from liver 4 (see Table 2), which had the highest V_{crw} (>1500°C/min), appeared to be less viable than slices in all the other experiments. However, DSC measurements and viability testing could not be done with the same slice and so it was not possible to directly correlate the amount of ice generated by devitrification to the slice survival. In slices that were cooled and warmed in cryovials at only 100°C/min after pre-incubation with VS4, devitrification on warming lead to the death of even more cells.

The question arises why slices remain relatively viable when they are treated with 18% DMSO (or to less extent, 50% VS4) and cryopreserved by rapid freezing (100-200°C/min), since a considerable amount of ice was formed in these slices. One explanation could be that the liver cells (partially) dehydrated and vitrified internally after reaching intracellular DMSO levels high enough to achieve this. Ice would then be formed mainly extracellularly. Karlsson (1993), however, has shown that collagen-sandwiched hepatocytes in culture should not be cooled faster than about 10°C/min in order to allow enough time for the extrusion of sufficient water by the cells to enable them to reach vitrifiable concentrations of CPA intracellularly. Equilibrium freezing rates required for optimal survival of hepatocytes are often even lower than this (Powis *et al.*, 1987; Condouris *et al.*, 1990; Diener *et al.*, 1993; Maas *et al.*, 2000a). Cooling rates applied to slices treated with 18% DMSO in the present studies (100-200°C/min) are well above these levels and we know that the application of cooling rates of at least 800°C/min (by placing the slice between aluminium foil sheets) does not decrease the viability of slices cryopreserved after pre-incubation with DMSO (Wilfred Maas, personal communication and own unpublished research). Moreover, when higher, non-toxic CPA concentrations are used (30% DMSO or 75% VS4) and therefore less water has to leave the cell to obtain vitrifiable concentrations, slices do not survive cryopreservation. For

these reasons, we do not expect cells within the slices to dehydrate sufficiently to avoid IIF.

Hence, when tissue vitrification and cellular dehydration are eliminated as possible mechanisms to explain the viability of slices permeated with 18% DMSO and cryopreserved, it seems that the cells within the slice are to some extent resistant to IIF. Indeed, some evidence exists that cells can survive when a fraction of their volume exists of ice (Mazur *et al.*, 1972; Karlsson, 1993). Cellular resistance to ice seems to be dependent on the warming rate, which must be high enough to avoid the re-arrangement of small ice crystals to form larger crystals (recrystallization) (Mazur *et al.*, 1972), and on whether the cells are in a suspension or connected to other cells (Acker and McGann, 2000). Nevertheless, the low survival of slices permeated with 30% DMSO or 75% VS4 seems to be contradictory to this proposal, since q is lower for these concentrations and thus even less ice, or smaller ice-crystals will have to be resisted. One might speculate that the characteristics of the CPAs underlie the existence of two optima in the curve relating q to viability. It may be possible, that DMSO (whether or not in combination with 1,2-propanediol and formamide in VS4) in an optimal concentration (18% DMSO/50% VS4) protects cellular membranes and proteins by stabilizing them, while in higher concentrations these cellular components are slightly damaged by exposure to these compounds. This damage may be sublethal and therefore no signs of toxicity may be seen in fresh slices, but it may make slices more susceptible to subsequent damage by ice crystals. With the second optimum the high concentration of cryoprotective agent may allow vitrification so that the eventual decrease in viability is the result of CPA toxicity and no additional damage by ice crystals occurs.

In conclusion, liver slices pre-incubated with VS4 behave differently from the bulk CPA solution because, when vitrified, they have a lower tendency to devitrify. Slices that are vitrified and warmed sufficiently rapidly to prevent devitrification have a high viability. However, liver slices permeated with 18% DMSO also have a high viability after cryopreservation, despite of the fact that ice is formed within the slice. The exact mechanism that might explain the survival of these slices remains a subject of speculation and requires further research.

Acknowledgements

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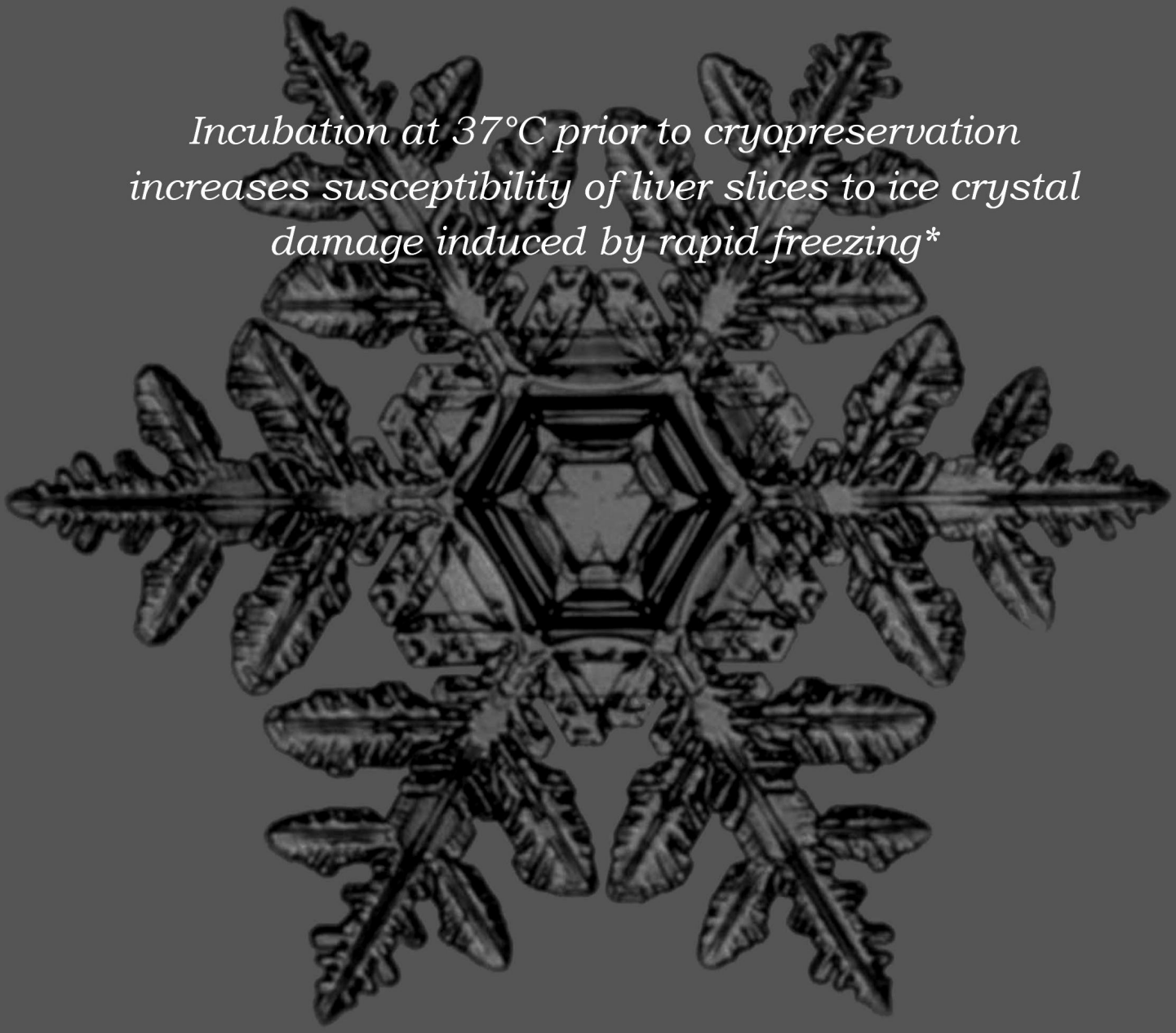
Chapter 6

*Incubation at 37°C prior to cryopreservation increases susceptibility of liver slices to ice crystal damage induced by rapid freezing**



*Based on:
I.A.M. de Graaf, A. Geerlinks and H.J. Koster.
Incubation at 37°C prior to cryopreservation increases susceptibility of liver slices to ice crystal damage induced by rapid freezing, *submitted*

Abstract

Precision-cut liver slices are to some extent resistant to ice formation induced by rapid freezing. Susceptibility to rapid freezing damage has been shown to be (partly) dependent on intrinsic properties of cells. In the present study an attempt was made to decrease the susceptibility of rat liver slices to rapid freezing damage: to recover from low ATP levels, impaired ion regulation and cell swelling induced by their preparation, the slices were pre-incubated at 37°C under oxygen, prior to cryopreservation.

It was shown that unexpectedly recovery of cellular homeostasis prior to the cryopreservation procedure by the 37°C pre-incubation markedly decreased viability of rapidly frozen slices (in which ice was formed), but not of vitrified slices (in which no ice was formed), in a time- and temperature dependent manner. UW was found to protect slices from this 'warm pre-incubation phenomenon'. Apparently, pre-incubation prior to freezing causes certain cellular alterations, that render slices more susceptible to ice crystals.

Introduction

A considerable number of relatively successful methods for the cryopreservation of precision-cut liver slices have been developed in the past decade, with the goal to facilitate the use of these slices in *in vitro* drug metabolism and pharmaco-toxicological research (Fisher *et al.*, 1991; Wishnies *et al.*, 1991; Fisher *et al.*, 1993; de Kanter and Koster, 1995; Fisher *et al.*, 1996; Ekins, 1996a; Ekins *et al.*, 1996a; Glöckner *et al.*,1998; Day *et al.*, 1998; Maas *et al.*, 2000a; de Graaf *et al.*, 2000b, Glöckner *et al.*, 2001). Many of these cryopreservation methods are based on 'rapid' (200-1000°C/min) freezing and thawing of the slices after pre-incubation with moderately concentrated (12-30% v/v) DMSO solutions (de Kanter and Koster, 1995; Glöckner *et al.*,1998; Day *et al.*, 1998; de Graaf *et al.*, 2000b, Glöckner *et al.*, 2001). An interesting feature of these methods is that reasonably high survival of the liver slices is obtained, even though the formation of inter- or even intracellular ice is most probably not prevented (de Graaf and Koster, 2001).

The resilience of cells to ice has been discussed by other authors. Survival of cells despite of exposure to ice crystals has been shown to be dependent on extrinsic (physical) factors, like the size and location of ice crystals (MacKenzie, 1970; Mazur, 1977; Hunt, 1984; Zieger *et al.*, 1996), but also on intrinsic (biological) factors. The presence of intercellular connections is one of the intrinsic factors of cells reported to determine their survival after rapid freezing: isolated cells seem more prone to rapid freezing damage (and intracellular ice formation associated with it) than cells of the same type that are integrated within a tissue or monolayer (Zieger *et al.*, 1996; Acker and McGann, 2000). Other intrinsic factors that determine the outcome of cryopreservation of liver slices involve the condition of the slices prior to cryopreservation, influenced by for example cold storage (Glöckner *et al.*, 1996; de Graaf *et al.*, 2000b) or the quality of liver donor material (Fisher *et al.*, 2001)

In cryobiology research, focus is normally put on influencing *extrinsic* factors that determine cryopreservation outcome, like prevention of intracellular ice formation or of cryoprotectant (CPA) toxicity. With the knowledge in mind that cells seem to be able to resist ice crystals under certain conditions, we decided to examine to which extent modification of *intrinsic* factors, like tissue condition, could influence cryopreservation outcome. Our hypothesis was that improving the condition of slices prior to cryopreservation would positively influence survival of those slices after

thawing. In liver slice preparation, a period of warm ischemia during liver excision and low temperatures during preparation and storage of the slices is known to lead to an initially low cellular ATP content, disturbed cellular ion levels and cell swelling in freshly cut slices (Smith *et al.*, 1986; Smith *et al.*, 1987; Dogterom *et al.*, 1993; Toutain *et al.*, 1998; De Kanter *et al.*, 1999; Maas *et al.*, 2000a; Maas *et al.*, 2000b and own unpublished observations). Upon subsequent incubation at physiological temperatures, cellular homeostasis is normally regained within 1-4 h. In the present study we attempted to improve slice condition by pre-incubating them at 37°C prior to cryopreservation. In this manner we allowed the slices to regain cellular homeostasis. Subsequently, slices were rapidly frozen after impregnation with 18% DMSO.

On the other hand, if improvement of slice condition indeed reduces its susceptibility to ice crystal damage, one would expect that this effect would be absent when slices are cryopreserved by vitrification (thus no ice crystals are formed within the slice). To confirm this, in the present study, some 37°C pre-incubated slices were vitrified after treatment with VS4 (a high molarity mixture of CPAs; (Fahy and Ali, 1997)) instead of rapidly frozen.

Viability of slices was assessed after 4 h of culturing after treatment by measuring intracellular potassium and/or ATP levels and by examining slice histomorphology, parameters that are considered to be very sensitive to cell damage (Maas *et al.*, 2000b). We will show that, unexpectedly, pre-incubation prior to the cryopreservation procedure, markedly *decreased* viability of *rapidly frozen* slices, but not of *vitrified* slices, in a time- and temperature dependent manner. We will discuss what possible alterations within the liver slices could be inflicted during this warm pre-incubation period and how these alterations could render the slices more susceptible to ice crystal damage.

Materials and methods

Chemicals

Formamide, Krebs Henseleit (KH) buffer, HEPES buffer, insulin, gentamycine, adenine and glutathione were obtained from Sigma, Axel, The Netherlands; William's Medium E/glutamax (WME), Phosphate buffered saline (PBS) and Fetal calf serum (FCS) were from Gibco BRL, Breda, The Netherlands; University of Wisconsin medium (UW) was from Lamepro b.v., Raamsdonkveer, The Netherlands; The Celsis Biomass assay kit was derived from Omnilabo, Breda, The Netherlands. The Coomassie Protein kit No. 23200, purchased from Pierce (Oud-Beyerland, The Netherlands). DMSO (>99.9% pure) and all other chemicals were from Baker, Deventer, The Netherlands.

Preparation of slices

For a more detailed description of the preparation and incubation of the slices, we refer to previous publications (de Graaf *et al.*, 2000b; de Graaf and Koster, 2001). Shortly, the liver was excised from male rats (Wistar, obtained from Harlan CPB, Horst, The Netherlands) that had free access to food and water, after anaesthesia with 65% CO_2, 35% O_2. Depending on the experiment, sometimes the liver was perfused with UW prior to further treatment. Precision-cut liver slices were made from 8 mm tissue cores using a Krumdieck tissue slicer (Krumdieck *et al.*, 1980) filled with ice-cold oxygenated KH buffer. In some experiments, other media were used for slicing. After preparation, slices were stored in ice-cold oxygenated WME with 10% FCS (or other media, dependent on the experiment) for approximately 45 min until further use.

To study the influence of regaining cellular homeostasis on cryopreservation outcome, slices were pre-incubated at 15, 25 or 37°C in oxygenated WME+supplements (for the description of the supplements, see the slice culturing section), for various time periods prior to the cryopreservation procedure. To study the influence of the pre-incubation medium, in some experiments UW was used instead of WME. In other experiments calcium chelators, allopurinol or oxygen radical scavengers were added to WME.

Cryopreservation and thawing

Slices were cryopreserved and thawed as previously described (de Graaf *et al.*, 2000b). First, slices were impregnated with 18% DMSO in oxygenated WME on ice for 30 min under gentle shaking. Subsequently, they were brought over to cryovials together with 0.5 ml of the pre-incubation medium, and directly submerged into liquid nitrogen (cooling rate approximately 200°C/min). After storing the cryopreserved slices in liquid nitrogen, they were thawed by placing the cryovials in a 37°C waterbath until ice was no longer visible (warming rate approximately 200°C/min).

In some experiments, slices were vitrified instead of rapidly frozen. For this purpose, slices were impregnated stepwise with increasing concentrations of oxygenated VS4 solutions, like previously described (de Graaf and Koster, 2001). VS4 consists of DMSO, 1,2-propanediol and formamide (weight ratio 21.5: 15: 12.4 with a total concentration of 7.5M) mixed with saline, glucose, adenine and glutathione (for the exact composition see Fahy and Ali (1997)). Slices were vitrified by placing them between pads of aluminium foil followed by direct immersion in liquid nitrogen (cooling rate approximately 800°C/min (de Graaf and Koster, 2001)). Vitrified slices were thawed by placing the foil with the slices in ice-cold VS4 (warming rate approximately 800°C/min (de Graaf and Koster, 2001)). Subsequently, slices were washed stepwise in solutions with decreasing VS4 concentrations. To detect any adverse effects of VS4 on the slices, some slices were treated with VS4, not vitrified but directly incubated at 37°C for 4 h to test viability. In each experiment, one VS4 impregnated slice was taken and measured by Differential Scanning Calorimetry (DSC) as previously described (de Graaf and Koster, 2001) in order to determine whether the slices actually vitrified upon cooling and devitrification was prevented upon warming (thus the critical cooling and warming rates (V_{crc} and V_{crw}) were lower than the attained cooling and warming rates of 800°C/min).

Slice incubation and viability testing

Fresh and thawed slices were incubated in a 25-ml Erlenmeyer flask (1 slice/flask) in a shaking waterbath (110 times/min), under humid carbogen (95% O_2, 5%CO_2) in WME+glutamax, supplemented with FCS (5%), 0.1 µM insulin, 50 mg/l gentamycine and D-glucose (to a medium concentration of 25 mM). After culturing for 4 h at 37°C viability was determined.

The ATP content of liver and kidney slices was determined using a Celsis Biomass Assay kit, as follows: Slices were cut in two equal parts. One part was used for histomorphological examination (see below). The other part was immersed in 70% ethanol in HPLC water, containing 2 mM EDTA (pH 10.9). Then, slices were homogenized using a Branford sonifier (50% duty cycle, 5 s) for determination of ATP and potassium content. For determination of the ATP content, an aliquot of the homogenate was taken and mixed 1/1 with Nucleotide Releasing Medium from the assay kit. If necessary, the homogenate was subsequently diluted in 70% ethanol/2 mM EDTA to ensure the values were in the range of the counter. Thereafter, the solution was diluted 1/10 with 0.025M HEPES buffer to decrease the ethanol concentration. Subsequently, to 100 µl of the solution, 50 µl luciferin/luciferase solution from the assay kit was added and the amount of luminescence as a measure of ATP content was determined using a Lumac Biocounter M500 (Lumac, The Netherlands). ATP content was determined relative to the protein content of the slices. Slice protein and potassium were determined in the slice homogenate as described previously (de Graaf *et al.*, 2000b), except that now an I-lab 600 automatical spectrophotometer (Instrumentation Laboratory, Lexington, USA) was used for the measurements.

For histomorphological examination, liver slices were fixed in 70% ethanol and further processed as described by de Graaf *et al.* (2000b). Slice viability was determined in the haematoxylin and eosin stained cross sections by estimating the percentage of viable cells. For determination of viability upon microscopical examination, nuclear shape and staining and cytoplasmatic staining were taken into account. It should be noted that also slice edges (1-2 cellayers at each edge) that are damaged by cutting the slices during slice preparation were included in viability scoring.

Hypothermic cell swelling can be assessed in slices by determination of the total tissue water (TTW) of the slice (Sundberg *et al.*, 1991). TTW is defined as (the wet weight-slice dry weight)/dry weight (Little, 1964). The slice wet weight was measured after carefully removing medium attached to the slice with paper tissue. The slice was then dried overnight in a 60°C oven and hereafter the dry weight was measured.

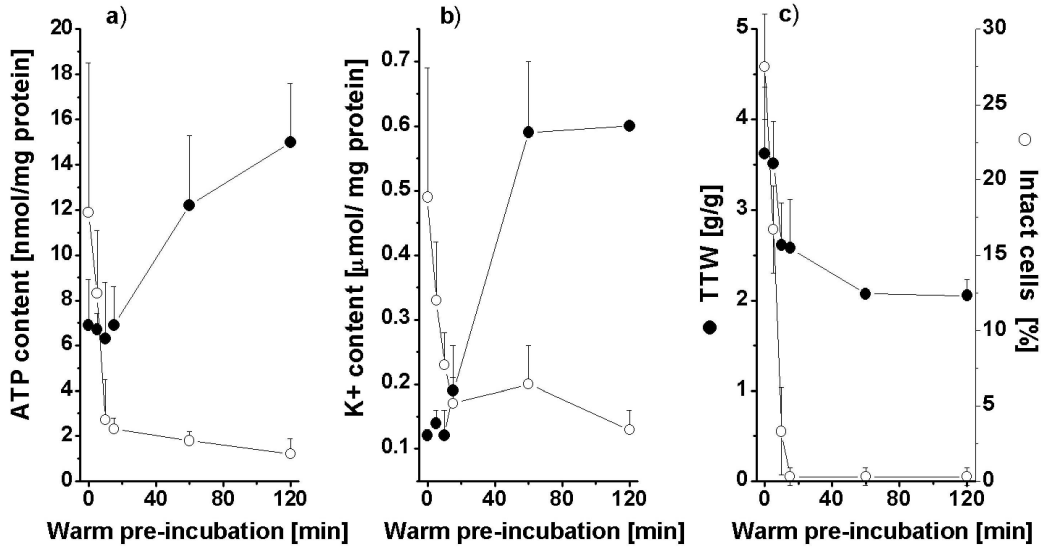


Fig. 1 The influence of incubation at 37°C on cellular parameters prior to cryopreservation (closed symbols) and after cryopreservation (open symbols). **(a)** ATP content, **(b)** K^+ content, **(c)** TTW and histomorphology. Parameters of slices were monitored after 37°C pre-incubation *and* CPA impregnation, so directly prior to freezing (closed symbols). Cellular parameters of cryopreserved slices were determined after 37°C pre-incubation, CPA impregnation, rapid freezing, thawing and 4 h of culturing (open symbols). Data-points represent the mean of 3 slices from one liver (+SD). For comparison: control slices (which were directly incubated for 4 h and not pre-incubated, treated with CPAs or cryopreserved) had a mean ATP content of 24.6 ± 1.3 nmol/mg protein, a K^+ content of 0.68 ± 0.05 µmol/mg protein and 72.5 ± 3.5 % intact cells in the slice cross section.

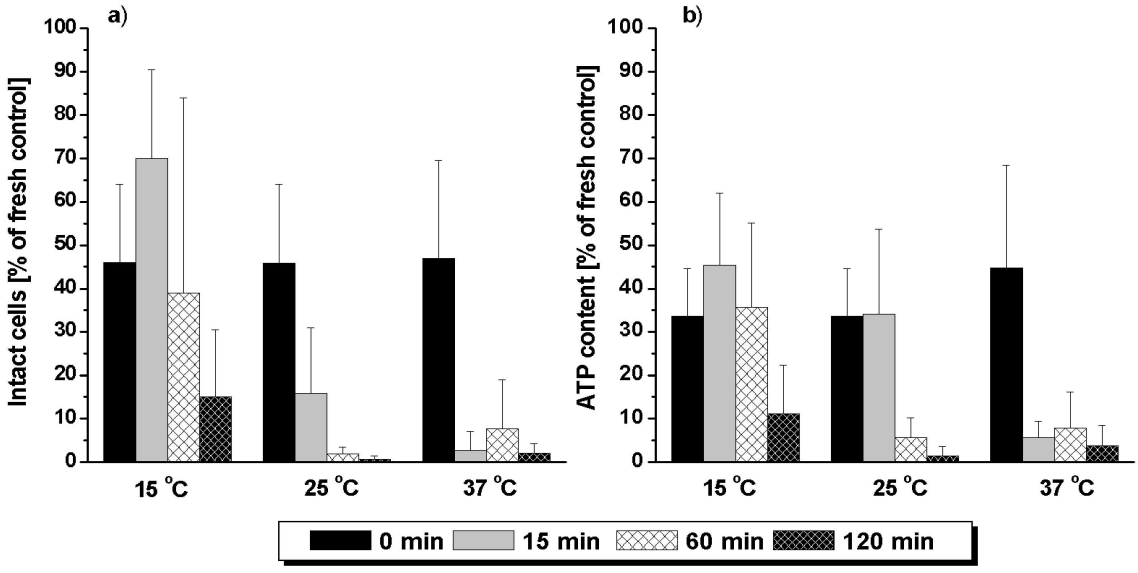


Fig. 2 The influence of duration and temperature of pre-incubation prior to cryopreservation on viability of rapidly frozen slices after 4 h of culturing after thawing. **(a)** histomorphology (% of intact cells) and **(b)** ATP content. Bars represent the mean of 3-7 livers + SD, 3 slices were used per liver. Values are given as relative to those of control slices of the same experiment. Control slices were cultured for 4 h, but not impregnated with CPA or cryopreserved. Mean ATP content of fresh controls after 4 h of culturing: 19.05 ± 6.1 nmol/mg protein (7 livers), mean % of intact cells in slice cross section: 70.1 ± 6.4 (7 livers).

Results and Discussion

We hypothesized that allowing the slices to regain normal cellular homeostasis prior to freezing would improve cryopreservation results. Surprisingly, the opposite is true: 37°C incubation prior to cryopreservation actually allowed slices to regain cellular homeostasis, but at the same time deteriorated the viability of slices after cryopreservation by rapid freezing. Indeed the restoration of cellular homeostasis during pre-incubation at 37°C (Fig. 1, closed symbols) is shown by the cellular ATP and potassium levels gradually increasing and reaching levels after 2 h of incubation that are close to the maximum values reported by other authors to be reached after re-equilibration of slices at physiological temperatures (Miller *et al.*, 1993; Maas *et al.*, 2000b). As shown in Fig. 1c, in the wake of ATP and potassium levels, cellular water content is recovered to near physiological levels (2.08 gram water/gram dry weight as reported by Sundberg *et al.* (1991)). Slices that were cryopreserved after regaining homeostasis during the 37°C incubation, had a very low viability compared to slices that were not pre-incubated prior to freezing (compare open symbols after 120 min of pre-incubation with those at 0 min of pre-incubation). While viability of non-pre-incubated cryopreserved slices was reasonably high (approximately 40% (histomorphology) to 70% (potassium content), in slices that were pre-incubated for only 15 min at 37°C prior to freezing, no viable cells were detected after thawing and subsequent culturing and ATP and potassium levels were only 10-25% of fresh slice levels (compare Fig. 1, open symbols at t=0 with those at t=15 min). Remarkably, slices that were not pre-incubated prior to freezing, managed to increase their initially low ATP content from 7 nmol/mg protein at the moment of freezing (Fig 1a, closed symbols, t=0) to 12 nmol/mg protein after thawing and culturing (Fig. 1a, open symbols, t=0). Correspondingly, slice potassium content was increased from 0.12 µmol/mg protein directly prior to freezing (Fig 1b, closed symbols, t=0) to 0.5 µmol/mg protein after thawing and culturing (Fig. 1b, open symbols, t=0). On the opposite, slices that were pre-incubated at 37°C prior to freezing, lost most of the regained ATP and potassium after freezing, thawing and culturing.

Fig. 2 shows that the impact of the pre-incubation prior to freezing is not only time-, but also temperature-dependent. If a 15 min pre-incubation took place at 15°C, it even seemed to be beneficial to the slices. If the 15°C pre-incubation was persisted for more than 60 min, viability was decreased. A 25°C pre-incubation during 15 min decreased the percentage of intact

cells after cryopreservation, but not the ATP content. If pre-incubation was carried out at 37°C, both parameters showed a dramatic loss of viability of thawed slices.

Based on the foregoing, we may conclude that pre-incubation prior to freezing in a time- and temperature-dependent manner causes certain cellular alterations, that render slices more susceptible to cryopreservation by rapid freezing. The question rises whether the pre-incubation sets the slices to be more susceptible to ice formed within the slice or, alternatively, to other factors that are related to cryopreservation (such as CPA toxicity or chilling injury). To answer this question, we set up an experiment in which 37°C pre-incubated slices were either impregnated with 18% DMSO and rapidly frozen or impregnated with VS4 and vitrified by ultra-rapid freezing (cooling rate at least 800°C/min (de Graaf and Koster, 2001); DSC measurements confirmed that the V_{crc} and V_{crw}s of the VS4 treated slices were near or below the feasible cooling and warming rate of 800°C/min and negligible ice crystal formation occurred, data not shown). If the deleterious effect of warm pre-incubation prior to freezing indeed rendered slices more susceptible to ice crystal damage, this effect should only occur in rapidly frozen slices but not in vitrified slices. Fig. 3 shows the result of this experiment: Viability of slices that were cryopreserved directly after preparation and CPA impregnation by either rapid freezing or vitrification had a reasonably high viability after thawing. Viability of vitrified slices was not affected by 37°C pre-incubation, but viability of rapidly frozen slices was again markedly decreased. Hence, it can be concluded that warm pre-incubation renders slices more susceptible to ice crystal damage.

Possible explanations for the 'warm pre-incubation phenomenon'

The question remains what possible alteration is provoked during 37°C pre-incubation that renders slices more susceptible to ice crystal damage. Remarkably, alterations caused by warm pre-incubation are lethal to slices after cryopreservation, but not to fresh slices. Since the effect of the pre-incubation is depending on temperature and is most pronounced if pre-incubation takes place at 37°C, obviously, activation of the cellular machinery is required for this phenomenon to occur.

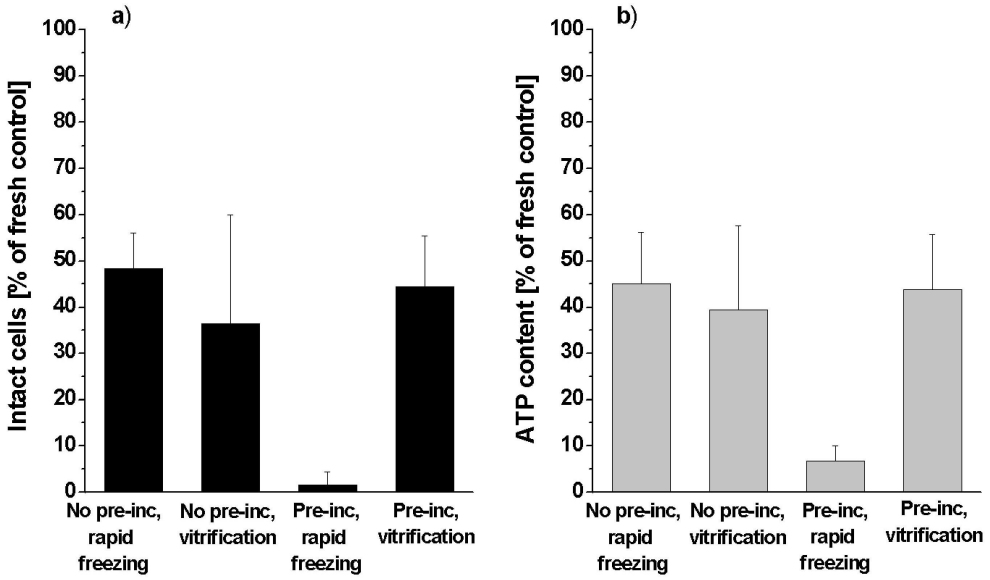


Fig. 3 The effect of 37°C pre-incubation prior to cryopreservation on viability of rapidly frozen or vitrified liver slices after thawing and 4 h of culturing. **(a)** histomorphology (% of intact cells) and **(b)** ATP content. Bars represent the mean values + SD of 4 (ATP) or 6 (histomorphology) livers, 3 slices were used per liver. Values are given as relative to those of control slices of the same experiment. Control slices were cultured for 4 h but not impregnated with CPA or cryopreserved. Mean ATP content of fresh controls: 20.1 ± 6.4 nmol/mg protein, mean % of intact cells was 71.9 ± 6.4. Pre-incubation with VS4 decreased ATP content of (37°C pre-incubated) fresh slices by approximately 25% and the % of intact cells by 5%.

From transplantation research it is known that if organs are re-oxygenized at physiological temperatures after a period of (cold) ischemia, 'reperfusion'/reoxygenation injury may occur. Pre-incubation at 37°C (under 95% O_2) could have caused a similar effect in the slices in the present study. Reperfusion injury is often associated with oxygen radical production leading to lipid peroxidation. Production of free radicals is associated with ATP catabolism and calcium influx leading to activation of xanthine oxidase. Since ATP levels are low after slice preparation and the low potassium content indicates that regulation of ion concentration is impaired, free radicals are likely to be produced leading to lipid peroxidation during the 37°C pre-incubation. Peroxidation of lipids in cellular membranes decreases their fluidity (Garcia *et al.*, 1997) and a decrease in membrane fluidity is associated with increased mortality of spermatozoa after cryopreservation (Giraud *et al.*, 2000). So we may hypothize that lipid peroxidation during warm pre-incubation may predispose the slices for additional cryopreservation damage.

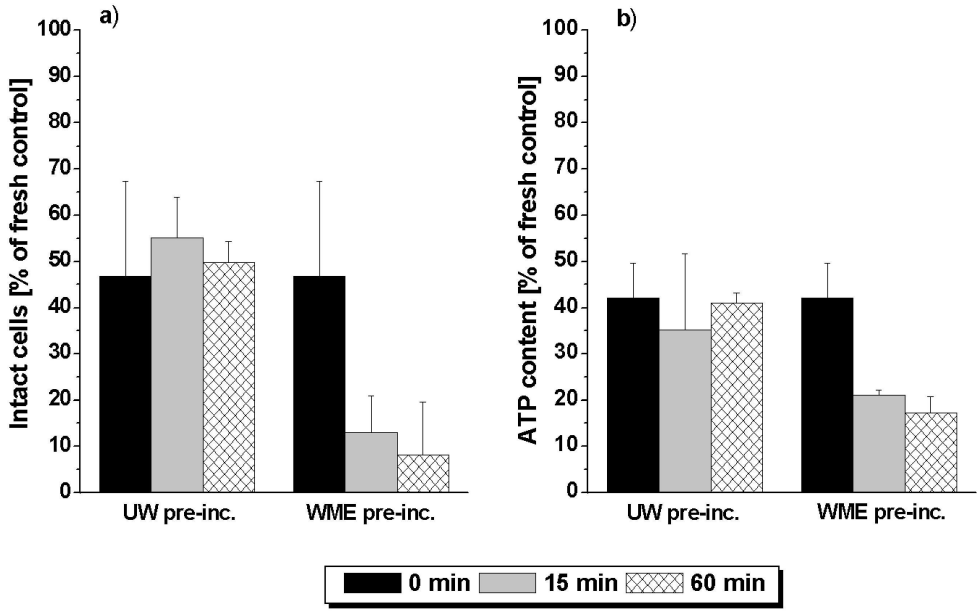


Fig. 4 The effect of 37°C pre-incubation in either UW or WME prior to cryopreservation on viability of rapidly frozen liver slices after thawing and 4 h of culturing. (**a**) slice histomorphology (% of intact cells) and (**b**) ATP content. For this experiment, livers were perfused in situ with UW and slices were cut and stored in UW as well. Bars represent the mean values + SD of 2 livers, 3 slices were used per liver. Values are given as relative to those of control slices of the same experiment. Control slices were cultured for 4 h but not impregnated with CPA or cryopreserved. Mean ATP content of fresh controls: 22 ± 4.5 nmol/mg protein, mean % of intact cells was 69.9 ± 2.6. There was no effect of 37°C pre-incubation in UW on fresh slices.

UW, an organ preservation solution, is known to prevent reperfusion injury. Critical components of this solution are allopurinol, that inhibits xanthine oxidase, and GSH, an oxygen radical scavenger. Furthermore, UW has an 'intracellular' ion composition thus preventing calcium influx during ischemia (Southard and Belzer, 1993). With this in mind we hypothized that perfusion of the liver with UW, followed by slice preparation and storage in UW might prevent the warm pre-incubation phenomenon. Fig. 4 shows viability of rapidly frozen slices of livers that were perfused in situ with UW. Slices were cut and stored in UW and subsequently either impregnated with 18% DMSO and cryopreserved (0 min pre-incubation), or pre-incubated at 37°C for 15 or 60 min in either UW or WME and than impregnated and cryopreserved. Apparently, UW completely protects slices from the warm pre-incubation effect, but only if it is used for all phases of slice preparation and the warm pre-incubation itself. However, the use of WME without calcium or WME with added calcium chelators did not prevent the pre-incubation effect, neither did the addition of allopurinol to WME, or the oxygen radical scavengers GSH, vitamin E and C (data not shown). Since these modifications of WME should prevent lipid peroxidation during 37°C

pre-incubation but did not prevent the deleterious effect of warm pre-incubation on cryopreservation outcome, the proposed oxygen free radical theory to explain the pre-incubation effect seems not to hold true.

Concluding remarks

In agreement with other reports we found that influencing the intrinsic properties of a tissue prior to cryopreservation can determine cryopreservation outcome. In the present study it was shown that pre-incubation under oxygenated conditions at physiological temperatures prior to cryopreservation renders slices more susceptible to ice crystal damage. UW apparently protects slices from the deleterious effects of warm pre-incubation, but probably not by prevention of oxygen free radical formation. Which cellular alteration is provoked by 37°C pre-incubation is not yet clear. Further research to this phenomenon seems worth the effort, since it may provide further insight into the mechanism of survival of cells that are cryopreserved at so called 'supra-optimal' cooling rates and may support the optimization of rapid freezing protocols for slices and other tissues.

Acknowledgements

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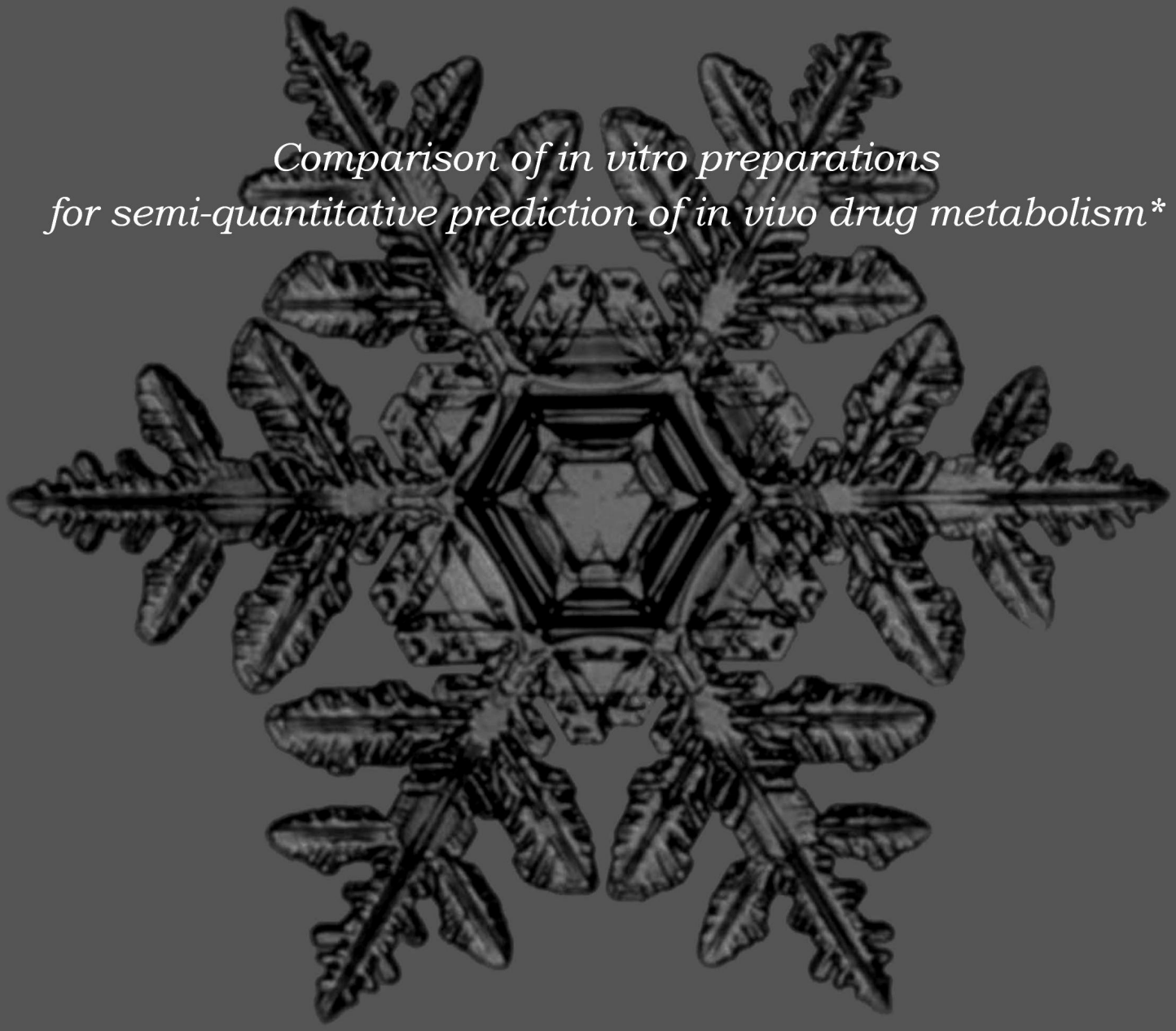
Chapter 7

*Comparison of in vitro preparations for semi-quantitative prediction of in vivo drug metabolism**



*Based on:
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Comparison of in vitro preparations for semi-quantitative prediction of in vivo drug metabolism, *submitted*

Abstract

Various *in vitro* preparations were compared with respect to their ability to mimic *in vivo* metabolism. For this purpose, S9-liver homogenate, microsomes, cryopreserved hepatocytes, cryopreserved liver slices and fresh liver, lung, kidney and intestinal slices were incubated with three drugs in development, which are metabolized *in vivo* by a wide range of biotransformation pathways. Metabolites were identified and quantified with LC-MS/UV from the *in vitro* incubations and compared with metabolite patterns in feces, urine and bile of dosed rats.

In vitro systems with intact liver cells produced the same metabolites as the rat *in vivo* and are a valuable tool to study drug metabolism. Phase I metabolites were almost all conjugated in intact cells, whereas S9-homogenate only conjugated by sulfation and *N*-acetylation. Microsomes and S9-homogenate are useful to study phase I metabolism, but not for the prediction of *in vivo* metabolism. Extra-hepatic organ slices did not form any metabolites that were not produced by liver cells, but the relative amounts of the various metabolites differed considerably. Small intestinal slices were more active than liver slices in the formation of the *N*-glucuronide of compound C, which is the major metabolite *in vivo*. When the relative contribution of liver and small intestinal slices to the metabolism of this compound was taken into account, it appeared that the *in vivo* metabolite pattern could be well predicted. Results indicate that for adequate prediction of *in vivo* metabolism, fresh or cryopreserved liver slices or hepatocytes in combination with slices of the small intestines should be used.

Introduction

In vitro research has been under growing interest for many disciplines within drug safety research, but pre-eminently for the selection of animal species as models for human drug toxicity in late drug discovery and early development. In this phase, this is the only way to compare metabolism of a particular drug between animals and humans. From such a species comparison study, the animal species could be selected of which the metabolism of the tested compound resembles metabolism by humans. A prerequisite for making an adequate selection is that the used *in vitro* system reliably predicts *in vivo* biotransformation of the drug, i.e. *in vivo* and i*n vitro* patterns of the major metabolites should be comparable.

The *in vitro* approach in early drug development is normally very straightforward to serve speed. In our lab, normally a low and a high drug concentration are used, that are hopefully not too high such that toxicity occurs but high enough to produce detectable amounts of metabolite(s). In this phase, linearity in relation to drug concentration of the metabolite production is not studied and other possible incubation variables are not optimized, like cell number used and incubation times. Only the major human metabolites are studied for their production by animal preparations.

As *in vitro* tool, both subcellular fractions and intact cells are intensively used. Subcellular fractions (i.e. S9-homogenates and microsomes) from various animal species and humans are commercially available and can be easily preserved for long periods of time. Disadvantages are the necessity of cofactor addition to facilitate metabolism and the lability (e.g. flavine mono-oxygenases) (Ekins, 1999), absence (e.g. cytosolar enzymes with microsomes) or inaccessibility (e.g. glucuronyl-transferases) of some metabolic enzymes.

In contrast, *in vitro* preparations with intact cells (hepatocytes, tissue slices) possess the 'complete' cellular machinery and have the ability for an integrated phase I and phase II metabolism of xenobiotics. A disadvantage of the use of hepatocytes is that isolation needs to be optimized for livers of every different animal species and involves collagenase digestion for disrupting cell-cell contacts. These problems are overcome when precision-cut tissue slices are used, which can be easily prepared from organs from various animal species, while the tissue architecture remains intact (Krumdieck *et al.*, 1980). Several informative reviews discuss the applicability of liver slices in pharmaco-toxicological settings (Bach *et al.*, 1996; Ekins, 1996b, Olinga *et al.*, 1997b; Lerche-Langrand and Toutain,

2000). Recent studies have shown that extra-hepatic organ slices (lung, kidney and intestinal slices) are almost as actively as liver slices metabolizing some drugs (Vickers, 1994; Vickers *et al.*, 1995; de Kanter *et al.*, 1999; Vickers *et al.*, 2001, de Kanter *et al.*, in press)

Long-term storage of slices and hepatocytes is more complicated than storage of subcellular fractions. Recently, we have developed a simple rapid freezing method for liver slices (de Kanter and Koster, 1995; de Kanter *et al.*, 1998; de Graaf *et al.*, 2000b) and showed that post-thaw viability and phase I and II biotransformation activity of cryopreserved rat liver slices were maintained at least during 4 h after thawing. More complicated cryopreservation protocols exist for hepatocytes (Powis *et al.*, 1987; Condouris *et al.*, 1990; Diener *et al.*, 1993). Cryopreserved hepatocytes are now commercially available.

In the present study rat liver microsomes, S9-homogenate, cryopreserved hepatocytes, cryopreserved liver slices and fresh liver, lung, kidney and intestinal slices are compared in their ability to predict *in vivo* metabolism. Furthermore, the metabolite patterns of precision-cut slices of liver, kidney, lung and small intestine are combined on the base of the relative contribution of these slices to the metabolism of the compound. Metabolites formed by the *in vitro* preparations are compared with those found in feces and urine of dosed rats. In addition, comparisons are made with the metabolite profile in bile, because some of the conjugates formed are deconjugated by microflora in the gut *in vivo*. The *in vitro* preparations are considered to be adequate predictors of *in vivo* metabolism if they meet two criteria: 1) they should produce the same metabolites as the intact animal 2) they should produce these metabolites in approximately the same relative amounts as *in vivo*.

Three compounds from Solvay Pharmaceuticals Research, from here called Compound A, B and C were selected as model compounds, since these are metabolized *in vivo* via a wide range of metabolic routes. Metabolites were analyzed by LC-MS/UV.

Materials and methods

Materials

Compound A, B and C are products of Solvay Pharmaceuticals Research, UDPGA, PAPS, $NADP^+$, NADPH, isocitrate, isocitrate dehydrogenase, Phenylmethylsulfonyl fluoride (PMSF), glycerol, glutathione, Acetyl CoA, Triton X-100, Krebs Henseleit buffer, insulin, gentamycine and low melting agarose were obtained from Sigma, Axel, The Netherlands; William's Medium E (WME), Fetal calf serum (FCS) and phosphate buffered saline (PBS) were from Gibco BRL, Breda, The Netherlands; DMSO (>99.9% pure), The Netherlands; Haematoxylin and eosin were from Sigma, St. Louis, MO, USA. *D*-glucose, HPLC-water and all other chemicals were from Baker, Deventer, The Netherlands.

Preparation of S9-homogenates and microsomes of rat liver and slices of rat liver, lung, kidney and small intestine

For preparation of S9-homogenates, microsomes and slices, male rats (Wistar, obtained from Harlan CPB, Horst, The Netherlands) were used. Rats had free access to food and water. They were anesthetized with 65% CO_2, 35% O_2 before the organs were removed.

For preparation of liver S9-homogenates, freshly excised liver was homogenized on ice using a Potter-Elvehjem homogenator. Subsequently, homogenates were ultracentrifuged at 9000g for 20 min at 2-4 ^{0}C. To the supernatant PMSF (final concentration 100 μM) was added. The S9-homogenate was stored at -80°C until use.

For preparation of microsomes, the S9-homogenate was subsequently centrifuged at 100.000g for 30 min (Williams and Wilson, 1978). The pellet was resuspended in KCl-Tris-EDTA buffer with a volume corresponding to that of 80% of the original volume of the liver. Then, the pellet was homogenized again using a Potter-Elvehem homogenator (400 rpm). Finally, a volume glycerol corresponding to 20% of the original volume of the liver was added to the microsomal suspension. The microsomes were frozen in liquid nitrogen and stored at -80°C until use.

Tissue cores (8 mm) from freshly excised kidneys and livers were prepared using an electrical drill (Metabo BSE 5010) with a tissue-coring tool (Alabama R&D, Munford, AL, USA). For preparation of lung tissue cores, the lung was filled with 37°C 1.5% (w/v) low melting agarose with 0.9% (w/v) NaCl in distilled water and kept on ice for up to 1 h prior to coring. Cores from small intestines were prepared as follows: a 10 to 20 cm

piece of the small intestine, starting 10 cm from the stomach was dissected, directly flushed with ice-cold UW-medium and subsequently filled with 37°C 3% (w/v) low melting agarose with 0.9% (w/v) NaCl in distilled water. Both ends were tied and the filled intestine was submerged in ice-cold UW medium for 5 to 10 min. After the agarose had solidified, the intestine was carefully cut into pieces of approximately 2 cm. Subsequently, the bottom of the sample holder of the tissue-slicer was sealed with a piece of parafilm and filled with 37°C agarose. A piece of the intestine was quickly submerged into the liquid agarose and the sample holder was then stored at 4°C for about 15 min until the agarose had solidified. The technique of intestinal slice preparation was obtained from Ruben de Kanter of the Groningen University Institute for Drug Exploration (publication reporting evaluation of viability of these slices is submitted).

Slices (10-14 mg for kidney and liver slices, 25-30 mg for lung slices and 10-12 mg (1-2 mg without agarose) for small intestinal slices were prepared subsequently using a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA), filled with oxygenated (95% O_2, 5% CO_2) ice-cold KH buffer, supplemented with $NaHCO_3$ (25 mM) and $CaCl_2$ (2.5 mM). After preparation, slices were washed and kept on ice in WME with 10% FCS until use.

Cryopreservation and thawing of slices and hepatocytes

Slices were cryopreserved according to the method developed by de Kanter and Koster (1995) and optimized by de Graaf *et al.* (2000b). After storage in liquid nitrogen for 1-12 weeks, slices were thawed by placing the cryovials in a 37°C waterbath. After visible ice had vanished, slices were washed shortly with WME, 10% FCS. Since in earlier studies (de Graaf *et al.*, 2000b) cryopreservation success has appeared to be variable to some extent, cryopreserved slices were selected based on viability. Only slices from livers with successful cryopreservation results (viability of at least 70% of fresh slices of the same liver after 4 h incubation or/and 50% after 24 h incubation) were selected. Viability was measured by estimating the percentage viable cells in a slice cross section upon histomorphological examination as described previously (de Graaf *et al.*, 2000b).

Cryopreserved male Sprague-Dawly rat hepatocytes were obtained from Sanvertech (Heerhugowaard, The Netherlands). Hepatocytes were thawed according to recommendations of the supplier. Shortly, hepatocytes were quickly thawed by placing the cryovial in a 37°C water-bath until visible ice vanished. Subsequently, the cryoprotectant concentration was lowered by

dropwise addition of WME. Finally, the diluted cryoprotectant solution was removed from the hepatocytes after centrifugation and the pellet was resuspended in WME supplemented with FCS (5%), 0.1 µM insulin, 50 mg/l gentamycine and *d*-glucose (to a medium concentration of 25 mM).

Incubation of S9-homogenate, microsomes, hepatocytes and tissue slices
A volume S9-homogenate or microsomes containing 1 mg protein was incubated with 175, 110 and 104 µM compound A, B or C respectively. To the S9-homogenate UDPGA (final concentration 200 µM), PAPS (final concentration 100 µM), Acetyl Co-A (final concentration 0.25 mM) were added to a total volume of 900 µl. Subsequently the incubation mixtures were placed in a 37°C water bath for 10 min under air. The reaction was started subsequently by adding 100 µl NADPH-regenerating system (containing $NADP^+$, NADH, isocitrate, isocitrate dehydrogenase and $MgCl_2$ to a final concentration of 0.57, 0.57, 6.4 and 23.4 mM respectively). After 2 h the incubation was terminated by freezing the samples in acetone-CO_2 ice.

Fresh and cryopreserved slices were incubated with 175 or 110 µM compound A and B respectively or 21 µM compound C during 24 h at 37°C, while floating in 5 ml medium in a 25-ml Erlenmeyer flask (1 slice/Erlenmeyer), placed in a shaking waterbath (110 strikes/min), under humid carbogen (95% O_2, 5% CO_2). This incubation system has proven to maintain viability of fresh liver slices for at least 24 h (Olinga *et al.*, 1997b). As incubation medium WME was used, supplemented with FCS (5%), 0.1µM insulin, 50 mg/l gentamycine and *D*-glucose (to a medium concentration of 25 mM).

Thawed hepatocytes were diluted with WME supplemented with FCS (5%), 0.1 µM insulin, 50 mg/l gentamycine and *d*-glucose (to a medium concentration of 25 mM) to a concentration of approximately $1*10^6$ living cells per ml. Subsequently, a 12-wells plate was filled with 1 ml of this solution per well. Hepatocytes were incubated for 6 h with 175 or 110 µM compound A or B or 21 µM compound C by placing the 12-wells plate in a shaking water-bath (37°C) under humid carbogen atmosphere.

In vivo studies with compound A, B and C
In vitro data were compared with data from various *in vivo* studies that were performed in the past. *In vivo* metabolism data of Compound A were from one male Wistar rat, orally dosed with 273 µmol/kg ^{14}C-compound A. *In vivo* data about the metabolism of compound B were from one male

Sprague Dawley (SD) rat that was dosed orally with 11 µmol/kg ^{14}C-compound B. In addition, one liver of a male Wistar rat was isolated and perfused with 172 µmol/kg ^{14}C-compound B and bile was collected. *In vivo* data of Compound C were derived from 2 SD rats dosed with 1.6 µmol/kg of ^{14}C-Compound C. In addition, for compound C, an *in vivo* bile excretion study was performed. For this purpose, a male SD rat was dosed with 4 times 31 µmol/kg (1 time per 12 h) ^{14}C-compound C.

Because both SD and Wistar rats were used for the various *in vivo* and *in vitro* studies, a study was done to assess possible differences in metabolism between the two strains. For this purpose, two Wistar rats were dosed with 110 µmol/kg Compound B or 12 µmol/kg ^{14}C-Compound C. Metabolite patterns were compared with those of SD rats.

Analysis by HPLC and LC-MS

Metabolites of compound A, B and C were determined (after sonication of the slices in their incubation medium) in the homogenate, after de-proteinization with methanol. For analysis by HPLC and LC-MS (both Hewlett Packard) an Inertsil ODS-3 column (150 x 2.1, 5µm) was used with an OPTI-GUARD 1mm C18 as pre-column. As eluens a gradient of 100% 3 g/l aqueous ammonium acetate to 100% methanol with a flow of 0.2 ml/min was used. UV-detection occurred at 320 nm for compound A and at 236 and 254 nm for compound B and C respectively. The mass spectrum was taken at 100-800 m/z by Electron Spray Ionisation using ICL/ICIS software for data acquisition and processing.

Quantification of metabolites

Peaks occurring in the UV signal were identified by MS, by searching for expected MH^+ signals of possible metabolites. UV peak areas (% of total of metabolite peak areas) were used to evaluate the relative abundance of the metabolites in the chromatograms.In some cases, 2 different metabolites were identified by MS representing one single UV-peak. In this case the relative amounts of the metabolites involved are estimated using their MS peak area, taking the difference in sensitivity of the MS for the different compounds into account.

Criteria for judgment

We considered the i*n vitro* systems to be adequate for the prediction of *in vivo* metabolism if they met two criteria: 1) they should produce the same metabolites as the intact animal 2) they should produce these metabolites

in approximately the same relative amount as *in vivo*. To evaluate the latter, only metabolites that were formed in considerable amounts *in vivo* (i.e. with a peak area of at least 10% of the major metabolite) were taken into account. Of these metabolites, the percentage that they made up of the total amount of metabolites was calculated. The *in vitro / in vivo* ratio of this percentage should lie between 0.5 and 2. For example, if a metabolite made up 15% of the total amount of metabolites *in vivo* and 10% *in vitro*, the *in vitro/ in vivo* ratio was 0.66, so in this case the *in vitro* system was considered to adequately predict the relative *in vivo* amount of the metabolite. The marginsof 0.5 and 2 were chosen arbitrarily but reflect the intuitive way *in vitro* data are perceived as reasonably predictive for the *in vivo* situation.

Up-scaling

The combined *in vitro* metabolite profile of the different organ slices and liver slices was calculated as follows: First, the metabolism of the different slices was scaled up to the whole organ. For this purpose, the ratio of the organ weight and the slice weight was multiplied with the percentage of parent compound that was metabolized by the slices of that particular organ. Subsequently, these organ contributions were summed and normalized to 1. Thus, the relative contribution of each organ to the metabolism of the drug was obtained and used as a scaling factor. Then, for each organ, the relative abundance of the metabolites formed were multiplied with the scaling factor of that organ (giving the predicted relative contribution of that organ in the formation of the metabolites). Finally, for each of the metabolites, the relative contribution of each organ was summed to give the *in vivo* metabolite pattern.

For example: both liver and kidney slices metabolize compound X. For liver slices, a scaling factor of 0.4 is calculated (so, 40% of the *in vivo* metabolites is predicted to be made by the liver) and for kidney slices this factor is 0.6. Liver slices make metabolite 1 (10%), metabolite 2 (30%) and metabolite 3 (60%). Kidney slices make respectively 40, 20 and 40% of metabolite 1, 2 and 3. The combined metabolite pattern is calculated as follows: metabolite 1: 0.4*10 + 0.6*40= 28%, metabolite 2: 0.4*30 + 0.6*20 = 24% and metabolite 3: 0.4*60 + 0.6*40= 48%.

Table 1 Viability as determined by studying histomorphology of fresh and cryopreserved liver slices after 4 or 24 h in culture

	4 h	24 h		4 h	24 h
Liver 1			Liver 4		
fresh	68[a] ± 3	68 ± 3	fresh	77 ± 2	72 ± 2
cryopreserved	57 ± 2	40 ± 2	cryopreserved	65 ± 2	62 ± 2
% of fresh	84 ± 4	60 ± 5	% of fresh	85 ± 4	86 ± 3
Liver 2			Liver 5		
fresh	70 ± 0	60 ± 3	fresh	67 ± 2	55 ± 3
cryopreserved	38 ± 3	20 ± 5	cryopreserved	27 ± 3	8 ± 3
% of fresh	54 ± 5	33 ± 9	% of fresh	40 ± 5	15 ± 6
Liver 3			Liver 6		
fresh	78 ± 2	65 ± 3	fresh	ND	68 ± 2
cryopreserved	58 ± 3	43 ± 4	cryopreserved	ND	48 ± 2
% of fresh	73 ± 4	67 ± 7	% of fresh	ND	70 ± 4

[a] % intact cells in slice cross section
Mean values are given +/- SEM from 3 slices per liver
ND=not determined

Results

Viability of cryopreserved liver slices

In previous studies we have noticed that viability of slices of different livers after cryopreservation varies considerably (de Graaf *et al.*, 2000b). For this reason, in the present study we decided to select slices from experiments with the best cryopreservation results for further use in the drug metabolism studies. The results are shown in Table 1. The viability of fresh liver slices did not vary much between the different livers with 67-78% of the cells in a slice histomorphologically intact after 4 h of culturing and 55-72% after 24 h. (Note that in all slices 1 or 2 outer cell layers are always damaged because of the slicing procedure, so that, even in very good slices, normally no more then approximately 80% of the cells are intact). In contrast with fresh slices, the quality of cryopreserved slices varied considerably, with 27-65% of the cells intact after 4 h of culturing and 8-62% after 24 h. For this reason, slices of liver 1, 3, 4 and 6 were thawed to be used in the drug metabolism studies.

Table 2 Relative activities of the *in vitro* systems in metabolism of compound A, B and C

	% Parent compound metabolized		
	Compound A	Compound B	Compound C
Microsomes	0	10 (2)	11 (5)
S9-homogenate	13 (3)	8 (2)	5 (1)
Cryopreserved hepatocytes	24 (16)	23 (2)	16 (5)
Cryopreserved liver slices	21 (3)	20 (5)	36 (1)
Fresh liver slices	17 (6)	26 (10)	37 (4)
Fresh lung slices	<1	<1	<1
Fresh kidney slices	7 (6)	3 (2)	<1
Fresh intestinal slices	10 (6)	<1	16 (8)

Values are given with SD in parentheses
Note that with compound C the initial concentration was 104 μM for microsomes and S9-homogenate, but 21 μM for other *in vitro* systems
For all systems, >75% of the HPLC metabolites-peaks were identified

Comparison of metabolism between Sprague Dawley and Wistar rats

For logistic reasons, in the present study different rat strains (Sprague Dawley (SD) and Wistar) were used for the various *in vitro* and *in vivo* studies. A study was undertaken to study possible differences in metabolism between the strains regarding compound B and C. The relative abundance of the metabolites of these compounds appeared to be well comparable for SD and Wistar rats (data not shown).

In vivo metabolism of Compound A, B and C

Of compound A, 75% of the radioactivity was excreted by the Wistar rat within 24 h: 12% in feces and 63% in urine. Ca. 69% of the radioactivity in urine originated from the parent compound as well as ca. 50% in the feces. Of compound B, 74% of the radioactivity was excreted by the SD rats within 48 h: 63% in feces and 11% in urine. In feces, 5% of the radioactivity was found to be the unchanged parent compound, in urine 25%. In bile, 3% of the radioactivity was represented by the parent compound B. The SD rats dosed with compound C excreted 95 % of the dose within 72 h, mainly in feces. Of the total dose, 50% was found to be metabolized. In bile, all administered Compound C was found to be metabolized.

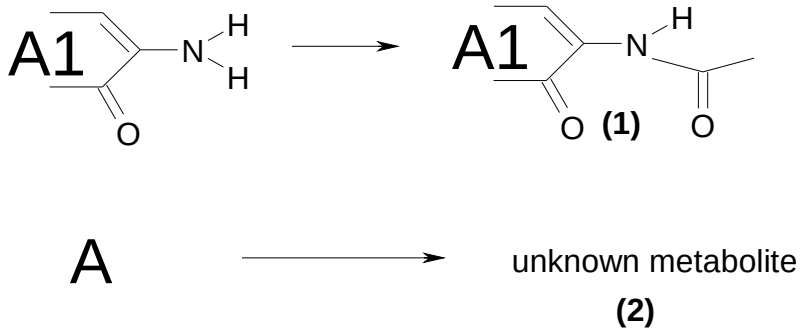

Fig. 1 Identified metabolic pathways of compound A. **A1**: molecular fragment attached to the part that is acetylated. **(1)-(2)**: metabolites referred to in the text

In vitro metabolism of compounds A, B and C.

The various preparations used varied in their activity of metabolism of the three compounds (Table 2). All three compounds were most actively metabolized by intact liver cell preparations, whereas intestinal slices also metabolized compounds A and C. Kidney slices had some activity but lung slices hardly produced metabolites.

In vivo and in vitro metabolite patterns of compound A

The major routes of biotransformation of compound A are given in Fig. 1. As shown in Fig. 2, by far the most important metabolite in the rat *in vivo* is the product of *N*-acetylation (metabolite no. 1). Another, still unknown metabolite was found *in vivo* (no. 2). All *in vitro* preparations with intact cells (organ slices and hepatocytes) produced the major N-acetyl metabolite, but lung slices only in negligible amounts. Metabolite no. 2 was only formed *in vitro* by the intact cell systems derived from the liver. Of the subcellular systems, S9 homogenate did acetylate compound A, while microsomes did not, because they lack the enzyme required for this reaction. Of the slices from the various organs, liver slices were the most active in the metabolism of compound A (Table 2). There is not much difference between the amounts of metabolites formed by fresh or cryopreserved slices. Cryopreserved hepatocytes were only incubated for 6 h with compound A, but metabolized the same amount of this compound as liver slices did in 24 h.

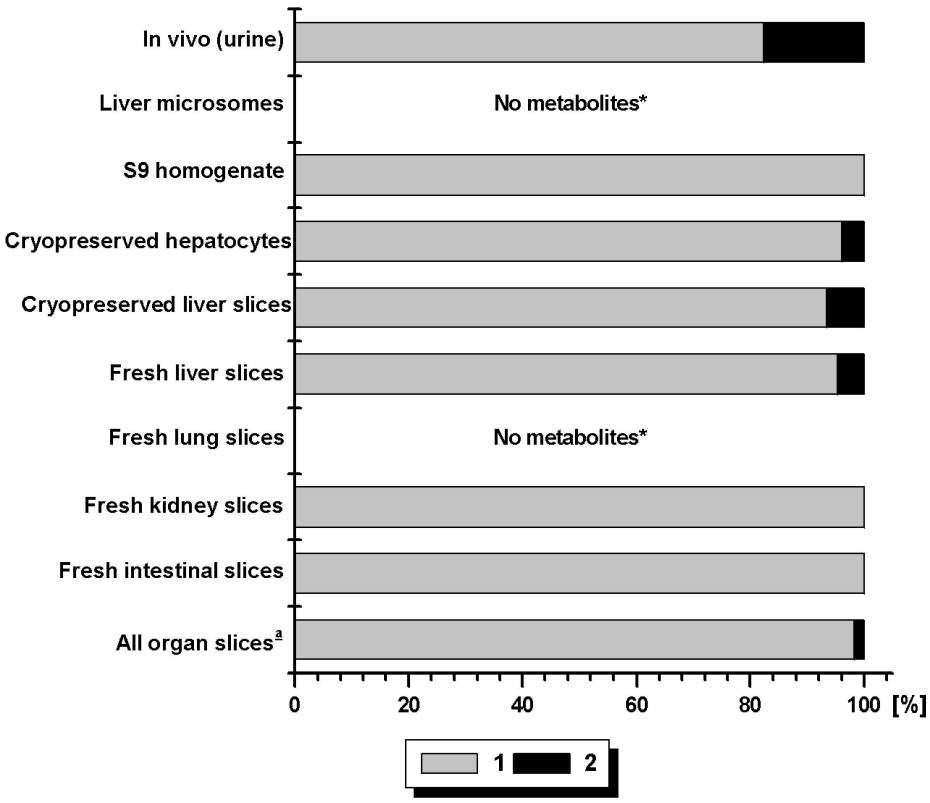

Fig. 2 Relative amounts of identified metabolites of Compound A formed by various test systems.
1: acetylated A
2: unknown metabolite
For all test systems >75% of the metabolites were identified. Slices were from 3 livers; Microsomes from 2 livers; S9-homogenate from 2 livers; hepatocytes from 1 batch. *In vivo* data were from 1 rat.
* <1% of the mother compound was metabolized
[a] relative amounts of metabolites are calculated as described in materials and methods section, with data in Table 3

Clearly, only intact cellular systems derived from liver made both important *in vivo* metabolites of Compound A. Therefore, to calculate a combined metabolite pattern taking extra-hepatic metabolism into account to predict the *in vivo* metabolite profile of compound A, data of liver slices and kidney and small intestinal slices were used. The scaling factors for the organ slices are shown in Table 3. Both metabolites are predicted but the amount of metabolite no. 2 is underestimated (Fig. 2). This is also seen when the ratios of the relative amounts of metabolites formed *in vitro* and *in vivo* are compared (Table 4); for all intact liver cell preparations, the ratio for this metabolite is below 0.5 and, when all organ slices are combined, this is even lower. However, the major metabolite, *N*-acetyl A, was predicted correctly; all ratio's were close to 1.

Table 3 Calculation of scaling factors for compound A

	Organ weight (A)[a]	Slice weight (B)	Fraction metabolized (C)[b]	(A / B) *C	Scaling factor
Liver	15.3 g	14 mg	0.21	230	0.38
Lung	6.9 g	30 mg	<0.01	0	0
Kidney	2.9 g	14 mg	0.07	14.5	0.02
Small intestine	7.2 g	2 mg	0.10	360	0.60

[a] derived from the Kanter *et al.*, 2002 (in press)
[b] data from Table 2

Table 4 Semi-quantitative prediction of *in vivo* metabolism of compound A, B and C by various *in vitro* systems

	In vivo[d]	*In vitro/in vivo* [e]			
Metabolite		Hepatocytes	Liver slices (fresh)	Liver slice (cryo)	All organ slices
Compound A[a]	urine				
1	**82.4**	**1.2**	**1.1**	**1.2**	**1.2**
2	**17.6**	**0.23**	**0.4**	**0.3**	**0.10**
Compound B[b]	bile+urine				
1	3.2	2.6	2.6	0.8	[f]
2	**8**	**0.9**	**0.7**	**0.6**	
3	**15**	**1.2**	**0.6**	**0.42**	
4	3	3.1	3.6	3.0	
5	1.5	7	2.0	2.5	
6	**19.5**	**0.8**	**0.7**	**0.9**	
7	2.5	2.1	0.6	0	
8	**47**	**0.5**	**1.0**	**1.2**	
Compound C[c]	bile				
1	**87.1**	**0.06**	**0.18**	**0.15**	**0.75**
2	0.7	0	0	0	0
4	**12.1**	**4.75**	**4.17**	**3.29**	**1.72**

bold: 'important' metabolites
[a] for the metabolic route see Fig. 1
[b] for the metabolic route see Fig. 3
[c] for the metabolic route see Fig. 5
[d] % of the total amount of metabolites *in vivo*
[e] ratio of the % of the total amounts of metabolites predicted *in vitro* and the % of the total amounts of metabolites actually formed *in vivo*
[f] extra-hepatic metabolism was insignificant

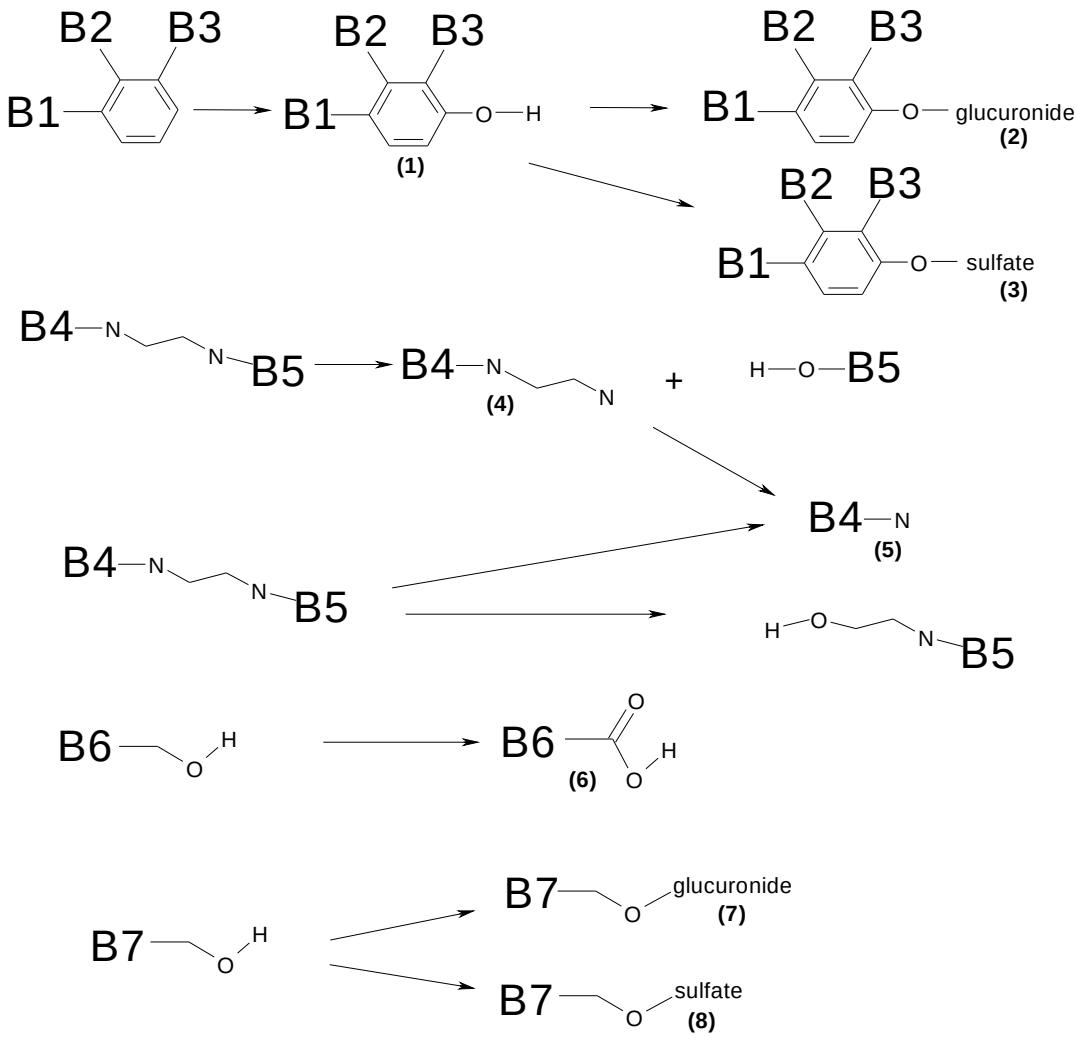

Fig. 3 Identified metabolic pathways of compound B. The molecular fragments attached to the part that is metabolized are indicated as **B1** through **B7**. **(1)-(8)**: metabolites referred to in the text.

In vivo and in vitro metabolite patterns of compound B

Compound B is metabolized *in vivo* via a wide range of metabolic pathways (Fig. 3), the major metabolite being the sulfate conjugate, no. 8. This metabolites was produced in considerable amounts by fresh and cryopreserved liver slices, cryopreserved hepatocytes, S9 homogenate and in small amount by kidney slices (Fig. 4). Only intact cell systems derived from liver formed all metabolites of compound B that are produced by the rat *in vivo*. Some conjugates (i.e. no. 3 and 7) produced *in vitro* were only found in bile and not in other excreta. Microsomes only formed phase I metabolites of compound B (i.e. no. 1, 4, 5 and 6). S9 homogenate produced all phase I metabolites except for no. 4, and it produced both sulfate conjugates (no. 3 and 8), but not the glucuronide conjugates (no. 2 and 7). These results show that only the intact-cell systems can be used for up-scaling to predict *in vivo* metabolite pattern.

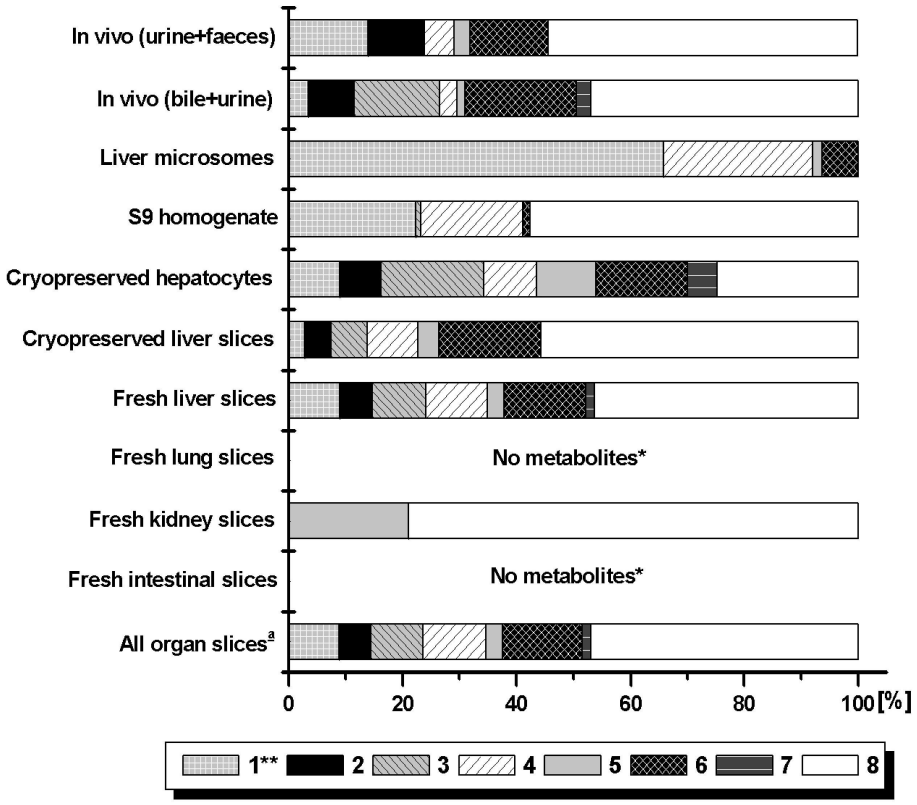

Fig. 4 Relative amounts of identified metabolites of compound B
1: hydroxy-B
2: [hydroxy-B]-*O*-glucuronide
3: [hydroxy-B]-*O*-sulfate
4, 5: fragments after *N*-dealkylation
6: B-carboxylic acid
7: B-glucuronide
8: B-sulfate
For all test systems >75% of the metabolites were identified.
Slices were from 3 livers; Microsomes from 2 livers; S9-homogenate from 2 livers; hepatocytes from 1 batch. *In vivo* data were from 1 rat, bile from 1 rat.
* <1% of the mother compound was metabolized
** in some *in vitro* incubations a second hydroxyl metabolite was found in small amounts
[a] metabolite pattern is calculated as described in materials and methods section, with data in Table 5

In vitro metabolite patterns are compared with those in bile+ urine. This is a more adequate comparison than that with feces+urine since in feces, some metabolites are found to be deconjugated (Fig. 4). Slices from kidney, lung and the small intestines metabolized small amounts of compound B and, therefore, the combined metabolite pattern based on metabolism by all organ slices (scaling factors given in Table 5) resembles that of liver slices (Fig. 4). Since cryopreserved hepatocytes and fresh and cryopreserved liver slices all give a similar metabolite profile, these preparations all predict *in vivo* metabolite patterns of Compound B well, with *in vitro* / *in vivo* ratios all being between 0.5 and 2 (Table 4).

Table 5 Calculation of scaling factors for compound B.

	Organ weight (A)[a]	Slice weight (B)	Fraction metabolized (C)[b]	(A / B)*C	Scaling factor
Liver	15.3 g	14 mg	0.26	284	0.98
Lung	6.9 g	30 mg	<0.01	0	0
Kidney	2.9 g	14 mg	0.03	6	0.02
Small intestine	7.2 g	2 mg	0	0	0

[a] derived from the Kanter *et al.*, 2002 (in press)
[b] data from Table 2

In vivo and in vitro metabolism of compound C

Compound C was metabolized *in vivo* to four major metabolites, notably an N-glucuronide (metabolite no. 1 in Fig. 5) and three hydroxylated metabolites excreted in feces in unconjugated form (no. 2, 5 and 7). In bile, the *N*-glucuronide was prominent, but also the glucuronidated hydroxy metabolite (no. 4) was seen. The sulfate conjugate (no. 3) was detected with the MS in bile, but the amount was too small to be quantified by HPLC/UV.

The *N*-glucuronide was only one of the minor metabolites in *in vitro* systems with intact cells derived from liver (Fig. 6). Subcellular fractions did not make this glucuronide conjugate at all. However, the *N*-glucuronide was formed in considerable amounts by slices of the small intestine. In fact, intestinal slices (1-2 mg tissue) produced approximately three times more of this metabolite than liver slices (10-15 mg tissue). A number of metabolites was formed by all systems with intact cells from liver, but not found in *in vivo* (no. 3, 6 and 8, Fig. 6). A considerable amount of a metabolite with the same molecular weight as the parent drug was found *in vitro* but not *in vivo*. Presumably, this metabolite was a C-*N*-oxide that was converted to the original drug by the mass spectrometer. Unconjugated hydroxy metabolites were not detected in (liver) slices or hepatocyte incubations. With microsomes and S9 homogenate, however, substantial amounts of these metabolites were found. Fresh liver slices and hepatocytes converted the same amount of compound C during incubation (Table 3). Cryopreserved liver slices and intestinal slices were a little less active. Negligible amounts of metabolites were found after incubation of compound C with lung and kidney slices.

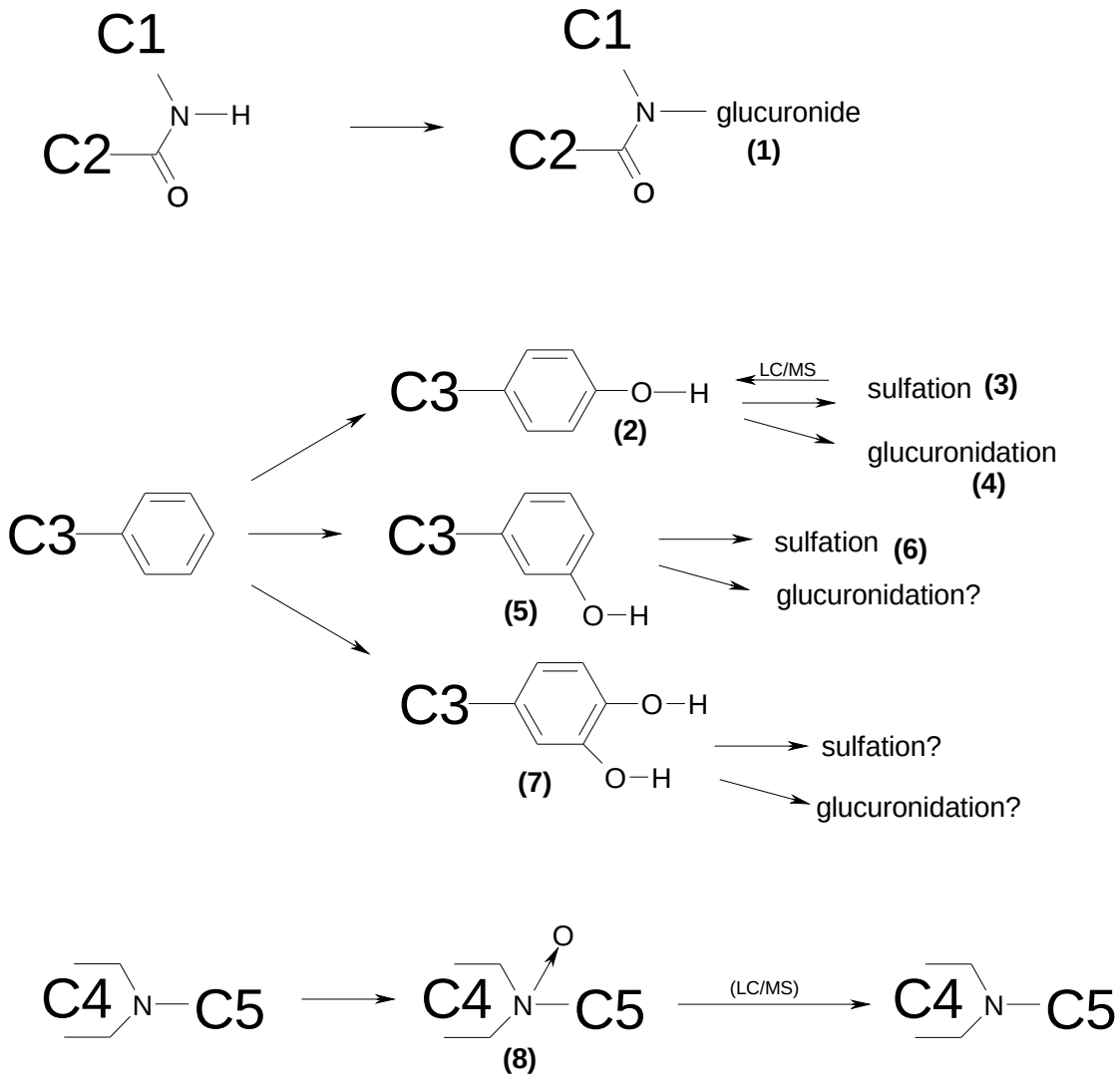

Fig. 5 Identified metabolic pathways of compound C. The molecular fragments attached to the part that are metabolized are indicated as **C1** through **C5**. **(1)-(8)**: metabolites referred to in the text.

Since only the intact cell preparations predicted all major *in vivo* metabolites, the data from these preparations were used for up-scaling. These preparations produced only conjugated hydroxyl metabolites (that were found to be deconjugated in feces) and, therefore, comparison was made with the combined bile and urine data. The results in Table 4 show that the liver preparations underestimate the *N*-glucuronide and overestimate the glucuronidated hydroxyl metabolite. When the relatively large contribution of intestinal slices to the *N*-glucuronidation of compound C is taken into account in the calculation of the combined metabolite pattern of the various organ slices, the obtained pattern closely resembles *in vivo* metabolism. (Table 4, last column; scaling factors are given in Table 7).

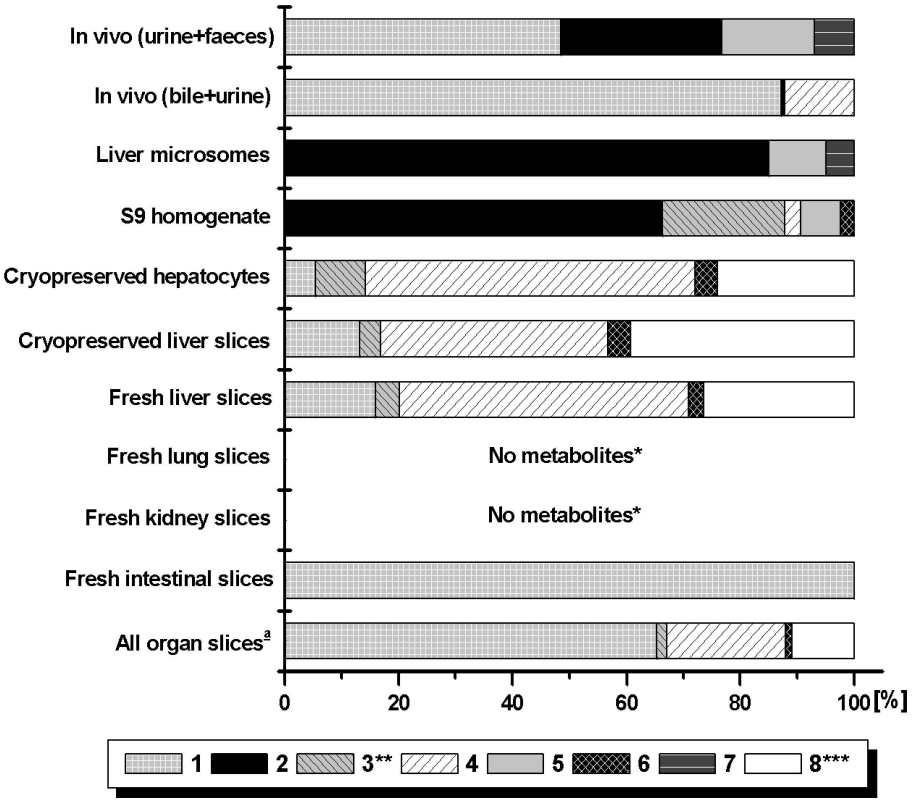

Fig. 6 Relative amount of identified metabolites of compound C formed by various test systems.
1: [C]-*N*-glucuronide
2: 3-hydroxy-C
3: [4-hydroxy-C]-*O*-sulfate
4: [4-hydroxy-C]-*O*-glucuronide
5: 3-hydroxy-C
6: [3-hydroxy-C]-*O*-sulfate
7: dihydroxy-C
8: C-*N*-oxide
For all test systems >75% of the metabolites were identified. Slices were from 3 livers; Microsomes from 2 livers; S9-homogenate from 2 livers; hepatocytes from 1 batch. *In vivo* data were from 2 rats, bile from one rat.
* <1% of the parent compound was metabolized.
** An hydroxy-C and [hydroxy-C]-*O*-sulfate were found at the same retention time because the sulfate was split off by the mass spectrometer.
*** A metabolite with the same mass as the parent was found, presumably because C-*N*-oxide was converted to the original drug by the mass spectrometer.
[a] Metabolite pattern is calculated as described in materials and methods section, with data in Table 6.

Table 6 Calculation of scaling factors for compound C

	Organ weight (A)[a]	Slice weight (B)	Fraction metabolized (C)[b]	(A / B)*C	Scaling factor
Liver	15.3 g	14 mg	0.37	404	0.41
Lung	6.9 g	30 mg	<0.01	0	0
Kidney	2.9 g	14 mg	<0.01	0	0
Small intestine	7.2 g	2 mg	0.16	576	0.59

[a] derived from the Kanter *et al.*, 2002 (in press)
[b] data from Table 2

Discussion

In the present study we compared *in vitro* metabolism by preparations from rat liver with increasing structural organization and by slices from other metabolizing organs (rat lung, kidney, intestines) with *in vivo* biotransformation. It appeared that there are substantial differences in metabolism, both qualitatively and quantitatively, between the different systems.

Microsomes and S9-homogenate produced considerable amounts of phase I metabolites of the drugs tested. However, as microsomes possess only inaccessible UDP-Glucuronyltransferases and no other phase II enzymes, this preparation did not produce the phase II metabolites formed *in vivo* from compounds A, B and C. S9-homogenate formed only the sulfate and acetyl conjugates since cytosolic enzymes phase II enzymes as acetyltransferases and sulfo-transferases are present, but glucuronyltransferases are inaccessible, like in microsomes. With compound C, small amounts of an *O*-glucuronide metabolite were found with S9-homogenate nevertheless. In contrast with the other compounds, compound C was dissolved in DMSO (medium concentration 1%). Possibly, DMSO disturbed the ER membrane, making it more permeable and activating the glucuronyl transferases. These results clearly show that intact cell preparations are required to reliably predict the metabolites formed *in vivo*.

In general, fresh and cryopreserved slices and cryopreserved hepatocytes produced the various metabolites in approximately the same relative amounts. Liver slices, containing approximately the same amount of hepatocytes per slice as were used for the hepatocyte incubations, however, needed a 24-h incubation to produce the same absolute amount of metabolites as hepatocytes in 6 h. The lower drug metabolizing activity of liver slices, which is also shown by Ekins *et al.* (1995) may be caused by slow drug transport into the center of the slice, limiting its metabolism.

Our results indicate that cryopreserved liver slices are as good to be used as *in vitro* tool to study drug metabolism as fresh liver slices are. However, selection of slices by viability is in our opinion necessary, because the success of cryopreservation differs between livers (de Graaf *et al.*, 2000b). In previous studies, we noticed that cryopreserved liver slices with a low viability did not only produce smaller amounts of metabolites, but also the relative amounts of the metabolites changed (unpublished observations). Particularly conjugation activity decreases in unsuccessfully cryopreserved slices, possibly by cofactor loss, while phase I biotransformation is preserved longer (Maas *et al.*, 2000b; Vanhulle *et al.*, 2001). The selection of slices on the base of their histomorphological appearance after thawing and culturing, as done in the present study, appeared to be useful.

Slices from extra-hepatic origin produced metabolites that were also formed by liver slices or hepatocytes, but the relative amount in which the various metabolites were formed differed considerably. With compound C the major metabolite *in vivo* was the *N*-glucuronide, while in liver slices and hepatocytes this was the *O*-glucuronide. Similar results were reported by Sandker *et al.* (1994) with Org 3770 and by Pahernik *et al.* (1995) with pimobendan. It was suggested that the *N*-glucuronide was mainly formed extra-hepatically (Sandker *et al.* 1994). In the present study, small intestinal slices appeared to be very active producers of the *N*-glucuronide conjugate of compound C in comparison to liver slices, indicating that the intestines indeed are important in the *in vivo* metabolism of compound C. Differences in relative activities of various biotransformation pathways between liver, lung and kidney slices were also reported with ethoxycoumarin and testosterone by de Kanter *et al.* (1999 and 2002). These results indicate that for a reliable prediction of *in vivo* metabolism of some compounds, the use of both hepatical and extra-hepatical slices is required.

The up-scaling procedure we applied combines the contribution of each of the organs by a scaling factor based on the amount of metabolism per slice of the various organs, the slice weights and the organ weights. This procedure assumes that the relative rates of metabolism of the various organs is the same *in vitro* and *in vivo*, as well as the relative amounts of metabolites formed by an organ. These assumptions are only valid when the degree of saturation of metabolism is similar *in vitro* and *in vivo*. This factor was not examined however in the present study. Nevertheless, *in vivo* metabolism was found to be well predicted by either intact liver cells and/or the combined organ slices. The predicted relative *in vivo* amounts of

metabolites were in most cases within the set limits of 0.5 and 2 of the amounts that were actually formed *in vivo*. So, this approach could be useful in a prospective study, predicting metabolite patterns of various animal species and humans for proper selection of an animal model for human drug toxicity studies.

In conclusion, *in vitro* systems with intact cells (slices and hepatocytes) from liver produced the same metabolites of the three compounds studied as the rat *in vivo* and for that reason seem to be a valuable tool to predict drug metabolism *in vivo*. Deconjugation of biliairy conjugates in feces should be taken into account when comparing *in vitro* metabolite patterns with that in feces and urine. Since phase I metabolites were found in only small amounts with intact cells, microsomes and S9-homogenate can be adequately used to study phase I metabolism, but are unsuitable to predict *in vivo* metabolite patterns. Extra-hepatic organ slices produced metabolites that were also formed by liver cells, but the relative amount of the various metabolites differed considerably. For compounds that are metabolized by N-glucuronidation, the small intestine may be an important metabolizing organ and the amount of N-glucuronides formed may be underestimated when only liver cell preparations are used. Therefore, for the prediction of the relative amounts of metabolites formed *in vivo*, the use of intestinal slices in combination with liver slices can be required.

Acknowledgements

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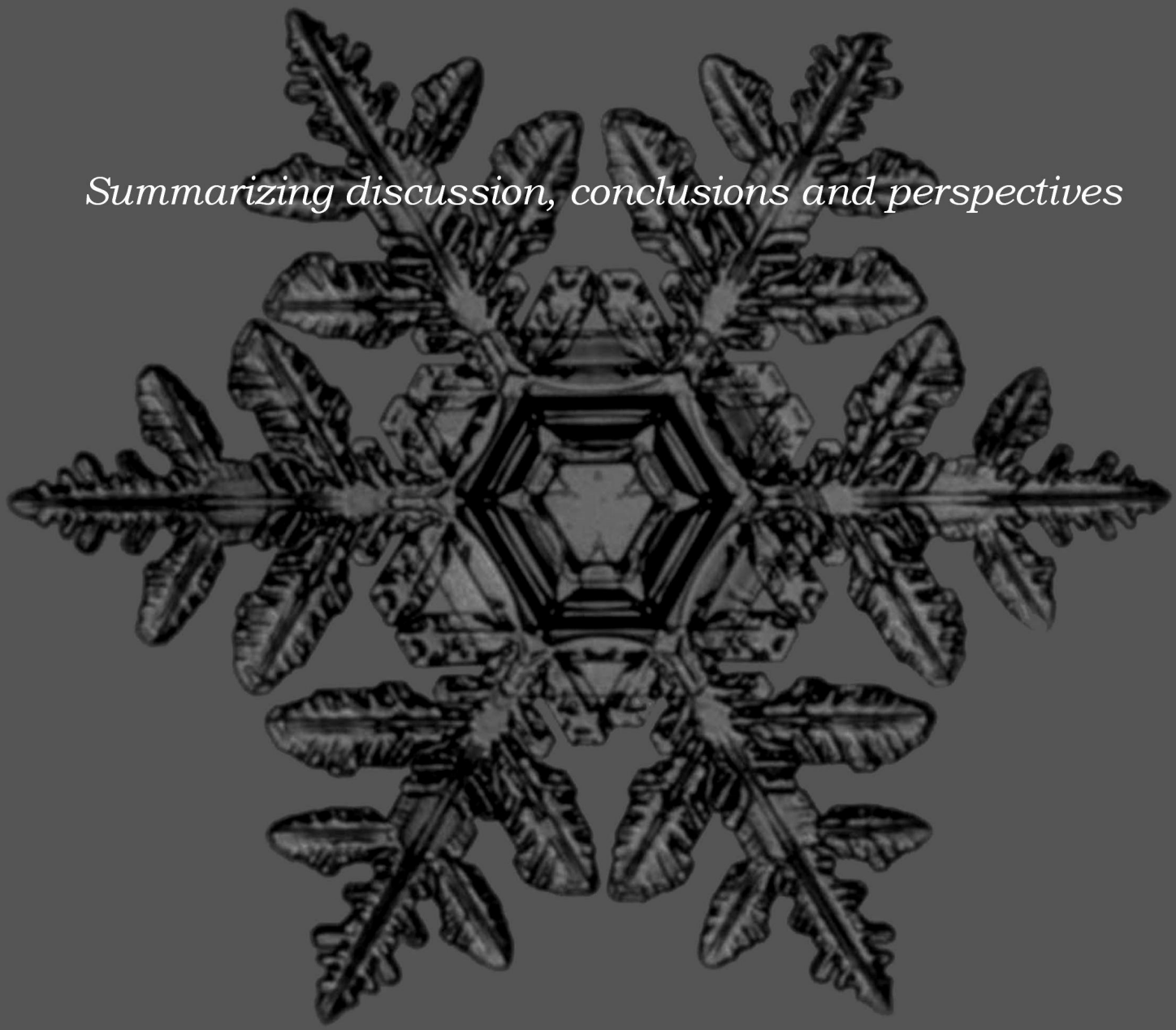
Chapter 8

Summarizing discussion, conclusions and perspectives



Introduction

In this chapter, the most important results and conclusions of this thesis as well as future perspectives of tissue slice cryopreservation for application in drug metabolism research are discussed.

Slice histomorphology, ATP, GSH and K^+ content and phase II biotransformation capacity are very sensitive parameters to determine cryopreservation outcome

Various end-points are used to determine whether cryopreservation of slices has been successful or not (see chapter 1). In chapter 2, it appeared that in practice, the judgement 'viable' or 'not viable' depends on the parameter used for evaluation. Of course, if damage by cryopreservation is severe enough, most (if not all) parameters will point this out ultimately, but a sensitive viability parameter will indicate loss of cell integrity within a short period after thawing. It was shown that slice histomorphology, potassium, ATP and GSH content and phase II biotransformation met this criterium. Other parameters tested (LDH retention, testosterone metabolism, enzyme activity, and urea synthesis) were less affected by cryopreservation. It should be noted that time is required for cryopreservation damage to become expressed: directly after thawing, viability of cryopreserved slices did not differ much of that of fresh slices.

These findings are in agreement with those of other authors who studied the viability of thawed slices (see chapter 1). Particularly phase II biotransformation has been found repeatedly to be severely affected by cryopreservation (Wishnies *et al.*, 1991; Ekins, 1996a; Ekins *et al.*, 1996a; Ekins *et al.*, 1996b; de Kanter *et al.*, 1998; Day *et al.*, 1998; Maas *et al.*, 2000a; Maas *et al.*, 2000b; Sohlenius-Sternbeck *et al.*, 2000; Vanhulle *et al.*, 2001). Several reasons can account for this observation. Enzymes may have been lost due to induced membrane leakage, cofactor availability may be limited or enzyme activity may be lost by denaturation. Enzyme activity is not prone to be affected by cryopreservation (Glöckner *et al.*, 1998 and chapter 2). Vanhulle *et al.* (2000) have shown that the content of enzymes responsible for phase II biotransformation (particularly those enzymes that are associated to cellular membranes) was not affected by cryopreservation, even 24 h post-thawing. However, the sulfation and glucuronidation of a model compound were decreased markedly. In chapter 2 we have shown

that while conjugation of CDNB with GSH decreased in cryopreserved slices after 4 h of culturing after thawing, conjugation in the slice homogenate after addition of GSH was barely affected. All these results indicate that not the enzyme content, nor the enzyme activity but the cofactor content limits conjugation in thawed slices. The low GSH content we have found in the thawed slices supports this. Contrary to the direct conjugation of model compounds, the conjugation of 7-HC to 7-HC-glucuronide and 7-HC-sulfate after *O*-deethylation of 7-EC has been shown to be much less affected by cryopreservation (Ekins, 1996a; Ekins *et al.*, 1996a). However, this might be explained by the fact that the *O*-deethylation step rather than the cofactor content is rate-limiting in this reaction. The fact that this assay is actually overestimating the ability of thawed slices to conjugate can be illustrated by the observation that thawed slices from the same experiments, directly incubated with 7-HC, produced its conjugates in small amounts compared to fresh slices (Ekins, 1996a; Ekins *et al.*, 1996a).

Remarkably, we have found that testosterone metabolism (as indicator for the capability of slices to perform phase I biotransformation) was well preserved in thawed slices, even when almost all other parameters used indicated severe loss of slice integrity. Several other authors have reported similar results (Wishnies *et al.*, 1991; Ekins, 1996a; Ekins *et al.*, 1996a; Ekins *et al.*, 1996b; de Kanter *et al.*, 1998; Glöckner *et al.*,1998; Glöckner *et al.*,1999; Maas *et al.*, 2000a; Sohlenius-Sternbeck *et al.*, 2000; Vanhulle *et al.*, 2001). These observations are surprising because also phase I biotransformation is dependent on cofactors and we have shown that particularly these molecules were lost by unsuccessful cryopreservation. We can speculate that for the phase I biotransformation not the cofactor content, but the transport of the compounds into the slice is rate-limiting and that damage of slices by cryopreservation increases substrate availability. Indeed, Ekins *et al.* (1995) have shown that substrate transport into slices inhibited metabolism compared to isolated hepatocytes. However, not only phase I, but also phase II biotransformation was found to be inhibited in slices, so enhanced transport cannot explain the different sensitivity of these parameters to cryopreservation damage.

Another observation in chapter 2, difficult to explain, is the relative insensitivity of urea synthesis to cryopreservation. Again, this reaction is directly dependent on cofactor availability (ATP). Potassium content, which is also directly related to ATP content, was found to correlate very well with ATP content and was low in unsuccesfully cryopreserved slices. However, regulation of intracellular ion concentrations requires by far the greatest

part of the cellular energy. It could well be, that the small amount of ATP left in a unsuccessfully cryopreserved slice is just enough to keep urea synthesis going on. This assumption is supported by the fact that in fresh slices, the ATP content directly after slice preparation was low and increased during culturing, while urea synthesis was linear from t=0 and did not increase at later time-points.

The relative insensitivity of LDH content to cryopreservation damage can be explained in correspondence to the maintenance of biotransformation enzymes found by Vanhulle *et al.* (2001): after cryopreservation the membranes apparently did not become leaky enough to become easily permeable for relatively large molecules. Also other authors have concluded that large molecules like LDH are only lost when a cell has reached 'a point of no return' (Danpure, 1984). With milder damage, it has been found that the leakage depends on the size of the molecule and small molecules are much easier lost than the large ones (Schmidt and Schmidt, 1967).

In chapter 2, histomorphology was found to be a sensitive and important parameter to determine slice viability, similarly to ATP and potassium content. Essentially, this is not surprising because depletion of energy and subsequent deregulation of ion concentration is known to directly lead to necrosis. But, more importantly, we have found that the percentage of intact cells in a slice after cryopreservation strongly correlated with phase II biotransformation (unpublished results). Furthermore, in chapter 7 we have shown that rapidly frozen slices, selected on the basis of their intact slice histomorphology, were capable to biotransform drugs to the same extent as fresh slices.

Summarizing, appropriate parameters should be used to evaluate cryopreservation outcome to be able to assess whether thawed slices can be used for drug metabolism studies. Amongst the parameters used for this purpose, we have found slice histomorphology, potassium, ATP and GSH content and phase II biotransformation of a model compound to be the most adequate.

Rapid freezing preserves liver slices viability better than slow freezing

In chapter 2 we have found that slices were better preserved by the rapid freezing method of de Kanter and Koster (1995) than by the slow freezingprotocol published by Maas *et al.* (2000a). Freezing rate was found to explain a great part of the differences in viability between the methods.

Comparable results have been reported by de Kanter *et al.* (1995) and Glöckner *et al.* (1996) with rat liver slices. Rapid freezing has also been shown to be successful with other tissue; For example Zieger *et al.* (1996) have found that skin tissue, rapidly frozen in the presence of 2M DMSO has a high post-thaw viability.

The preference of liver slices for rapid freezing is remarkable because isolated rat hepatocytes require slow freezing for survival (Powis *et al.*, 1987; Condouris *et al.*, 1990; Diener *et al.*, 1993; Maas *et al.*, 2000a). The most plausible explanation for this discrepancy concerns the most important difference between isolated cells and complex tissue, which is their susceptibility to damage by extracellular ice. Single cells can escape from the damaging effect of extracellular ice by ranging into unfrozen canals of the cryoprotectant (CPA) medium. Closely packed cells are more susceptible to extracellular ice than cells suspended in a low concentration (Pegg, 1981). Correspondingly, organs and tissues are more prone to be damaged by extracellular ice, which piles up between the cells and in the vascular space (Bischof *et al.*, 1997; Pegg, 1987). Rapid freezing probably protects slices from extensive extracellular freezing by preventing cell dehydration. Although Fisher *et al.* (1991) also reported low viability of rat liver slices after slow freezing, they obtained very good viability of slow frozen human, pig and dog liver slices (Fisher *et al.*, 1991; Fisher *et al.*, 1993; Fisher *et al.*, 1996), measured with the same sensitive viability parameters. The cause of this species difference is not known.

The viability of rapidly frozen slices was increased by using higher DMSO concentrations (18% instead of 12%) (chapter 3). It appeared that post-thaw slice potassium content and histomorphology and phase I and II biotransformation activity was maintained in this manner for at least during 4 h after thawing. Even more important, cryopreserved slices were shown to predict the *in vivo* phase I and II biotransformation of newly developed drugs as good as fresh liver slices (chapter 7). Others have applied this method on liver slices and found results that were just as promising (Sohlenius Sternbeck *et al.*, 2000). The improving effect of higher DMSO concentrations can be explained by its capability to inhibite ice crystal formation, which is increased with increasing concentration (Baudot *et al.*, 2000). In chapter 3, it was also found that slice thickness determined cryopreservation outcome, but not the survival of fresh slices. A possible explanation is that the glycogen content of slices is decreased in the centerof thick fresh slices (Lupp *et al.*, 2001; and own unpublished observations), but is also lowered after cryopreservation by rapid freezing

(Vanhulle *et al.*, 2000). So, cells in the center of thick slices may be deprived from their stored energy source after cryopreservation and become necrotic.

Intracellular vitrification is not the mechanism explaining the success of rapid freezing

A bulk solution of 18% DMSO will not vitrify with a cooling rate of 200°C/min (Baudot *et al.*, 2000). So, in first instance, it is not very likely that slice vitrification, preventing inter- and intracellular ice formation, is the mechanism explaining the success of rapid freezing for the cryopreservation of rat liver slices. Indications exist however, that the intracellular matrix, because of the high concentrations of proteins, has less tendency to crystallize than extracellular medium because it more or less behaves as a CPA (Rapatz and Luyet, 1960; Takahashi and Hirsh, 1991). Besides this, the compartmentization of water (occurring in and between cells) is known to increase supercooling and inhibit crystal formation (Yamane *et al.*, 1992; Peridieu *et al.*, 1995). This knowledge made de Kanter and Koster (1995) hypothesize, that upon rapid freezing, water only crystallizes in the cryopreservation medium outside the slice, but vitrifies within the slice. Furthermore they speculated, that cells in the slice, not facing the extracellular medium would be protected from damage by ice formation by the outer cellayers. This speculation was based on the fact that they found that slices with a lower protein content (which were probably thin slices, with relatively many 'surface cells' compared to 'inner cells') have a low viability.

In chapter 5, this hypothesis was tested by performing differential scanning calorimetry (DSC). In this manner, the heat of crystallization, which is proportional to the amount of ice formed, was compared between slices pre-incubated with 18% DMSO and bulk 18% DMSO medium. If water in a slice indeed has less tendency to crystallize than bulk medium, one would expect a lower fraction of water within the slice to be quantified as ice than in the medium. This was not the case: both the total amount of ice formed and the dependency of ice formation on the cooling rate did not differ between slices and medium. So, slice vitrification was eradicated as possible explanation for rat liver slices surviving rapid freezing. However, the tendency of slices pre-incubated with high molarity mixtures of CPAs to devitrify was inhibited similar to literature data. The latter observation is discussed further onwards.

Slices are to some extent resistant to ice formation

The question remains what then is the mechanism explaining the success of rapid freezing. If the prevention of intracellular ice formation (IIF) truly is a prerequisite for cell survival after cryopreservation, the only explanation remaining is that cells lose enough water to concentrate intracellular CPAs in such a way, that vitrifiable concentrations are reached. This explanation is not in conflict with the DSC measurements in chapter 5. After all, the DSC only measures the total amount of ice formed within a sample and does not distinguish between ice formed intra- or extracellularly. It is debatable however, whether with the cooling rates used for rapid freezing of 200°C/min there is time enough for cells permeated with 18% v/v DMSO to lose enough water to reach vitrifiable DMSO concentrations. To achieve this, intracellular DMSO concentrations of approximately 45% w/w (Baudot *et al.*, 2000) should be reached, so slices should lose at least 35% of their initial volume as pure water by dehydration. Bischof *et al.* (1997) calculated that cells in non-DMSO treated human liver slices retain 95% of their original volume when they are cooled with 100°C/min. Although we used rat liver slices permeated with 18% DMSO, it is even less likely that a 35% volume loss occurs in these slices during rapid freezing since non-DMSO treated human hepatocytes have a higher membrane permeability to water than DMSO-treated rat hepatocytes (compare cell permeability parameters of Bischof *et al.*, 1997 and Karlsson, 1993). So it is very unlikely that cell dehydration is the mechanism explaining the success of rapid freezing.

So, with rapid freezing of 18% DMSO permeated slices, it is very likely that intracellular crystallization occurs. Although many authors have reported a direct relation between IIF and cell death (e.g. Mazur, 1972), there also exist evidence that cells can survive IIF (MacKenzie, 1970; Mazur, 1977; Zieger *et al.*, 1996; Acker and McGann, 2000). A pre-requisite seems to be, that thawing is rapid as well. The thought is, that with slow warming rates, the small intracellular ice crystals that are formed during rapid freezing, recrystallize to form energetically favourable bigger sized ice-crystals, deleterious to the cell (Mazur, 1977; MacKenzie,1970). By rapid warming, keeping intracellular ice crystals small, cells are probably "rescued" from damage by these crystals. Mazur *et al.* (1972) have shown for example, that chinese hamster cells exhibit high survival when they were cooled with rates higher than required to prevent IIF by equilibrium freezing ("supra-optimal" rates), as long as the warming rate was high as well. Cells that were cooled with supra-optimal rates and warmed rapidly

even had a higher survival rate than cells that were cooled at equilibrium freezing rates. The authors suggested that freezing with supra-optimal rates protects cells from the effects of high salt (and CPA) concentrations, induced by cell dehydration upon slow freezing. Probably, with rapidly frozen liver slices, a similar mechanism plays a role. In addition, rapid freezing probably protects liver slices from potential deleterious large ice crystals formed between cells. We have noticed that with slow warming, slices have a very low viability (unpublished observations), which is an indication that the mechanism of survival from IIF in rapidly frozen liver slices indeed plays a role.

Still, it is an unanswered question why we have found that in some cases slices do not, or to lesser extent, survive rapid freezing. In chapter 3, for example, we found that the variability in survival between slices from different rat livers is high. In chapter 4, we found that kidney and intestinal slices can not be successfully cryopreserved by rapid freezing. Furthermore, in chapter 5 it was shown that slices pre-incubated with high VS4 concentrations do not survive rapid freezing, while these solutions are non-toxic to fresh slices. And, finally, in chapter 6, we obtained very low viability of slices that were first pre-incubated at 37°C prior to cryopreservation by rapid freezing. Factors that may explain these observation can be extrinsic (or physical-chemical) of nature, i.e. associated with cryopreservation (CPA toxicity, size and location of ice crystals) or intrinsic, associated with the tissue to be cryopreserved (for example the sensitivity to damage by ice crystals).

The low survival of kidney and intestinal slices compared to liver slices, observed in chapter 4, may be explained by possible differences in water permeability and surface to volume ratios of cells from the different organs. Because of this, the extent to which cells are dehydrated during rapid freezing and consequently, the size and location of ice in the different tissue slices may not be similar. The variability of survival rates of liver slices, observed in chapter 3 are difficult to explain from the point of view of extrinsic factors playing a role. After all, slices were all from rat liver and pre-incubated with 18% DMSO and frozen and thawed in the same way, so differences in CPA toxicity or size or location of ice crystals can not explain for the found variability. It *is* reported however, that differences in storage time of slices at 4°C could determine cryopreservation outcome (chapter 3 and Glöckner et al., 1996). Possibly, variation in the storage time at 4°C of liver slices during preparation caused the observed variation.

In chapter 5, different CPA concentrations were used, so extrinsic factors associated with cryopreservation did differ between the slices. However, lower viability after rapid freezing was obtained with slices that were pre-incubated with higher (but *non-toxic* to fresh slices) CPA concentrations. This is remarkable, because the use of higher CPA concentrations leads to the formation of less or smaller (intra- and intercellular) ice crystals and hence, it would be expected that higher survival is yielded with higher non-toxic CPA concentrations. So, again, extrinsic factors are unlikely to explain the low viability of slices observed in this chapter. Could intrinsic factors play a role in low survival of slices rapidly frozen after pre-incubation with higher CPA concentrations? Possibly: CPAs have a number of (largely unidentified) effects on cell membranes and cellular structures (Baxter and Lath, 1971; Fahy *et al.*, 1990; Anchordoguy *et al.*, 1992), which may not necessarily lead to death of unfrozen, CPA-treated cells, but possibly enhance susceptibility of slices to subsequent damage by ice crystals during freezing.

A similar mechanism may play a role in chapter 6: by pre-incubating slices prior to cryopreservation, at 37°C, we possibly introduced a mild 'reperfusion effect'. This phenomenon is known from cold preservation and is caused by re-introducing oxygen in tissue formerly deprived from oxygen during ischemia. Slices endure ischemia during preparation. Reperfusion injury is associated with all kinds of cellular alterations, which may again be sublethal to fresh slices, but renders the slices more susceptible to ice crystal damage. Our assumption that such a mechanism indeed plays a role in the development of this 'pre-incubation effect' is strengthened by the observation that this effect did not occur when slices were vitrified, hence no ice-crystals were formed during cryopreservation.

Resuming, as pointed out in the foregoing, rapid freezing possibly protects cells from the deleterious effects of extracellular freezing and from the damaging effects associated with cell dehydratation, but (small) intracellulair ice crystals are formed. Using indirect evidence, we have discussed to which extent we expect the resistance of cells to IIF could explain our observations. Future studies should compare the occurrence and size of ice crystals between the different organ slices and between slices frozen after various pre-treatments. In this manner, a definitive statement about the role of extrinsic and intrinsic factors in determining the outcome of cryopreservation by rapid freezing can be formulated.

Slices of extra-hepatic origin can be successfully cryopreserved by vitrification

In chapter 4 it was found that rapid freezing after pre-incubation with 18% DMSO did not preserve viability of kidney and intestinal slices. So, we chose another approach for the cryopreservation of these slices, i.e. vitrification. Intra- and extracellular ice formation in a slice are avoided by vitrification, overcoming the potentially deleterious effects of rapid and slow freezing.

For the development of a vitrification protocol two factors should be kept in mind 1) the pre-incubation period should be short enough to avoid CPA toxicity, but long enough to ensure complete equilibration with the CPA, 2) the applied cooling and warming rates should be higher than the critical cooling and warming rates of the tissue. Particularly with VS4, the warming rate was found to be a critical issue. Bulk VS4 medium of the same weight as a slice had a V_{crw} of approximately 2500-3500°C/min. This rate is not feasible for the warming of slices. However, by DSC measurements we have found that with a few exceptions, the V_{crw} of tissue slices is much lower (50-1500°C/min) than that of bulk VS4 (chapter 4 and 5), probably due to partitioning of the solution in the tissue. These results are in agreement with those of other authors (Takahashi and Hirsh, 1991; Peridieu *et al.*, 1995). Since the tissue slices could be warmed with a rate of approximately 800°C/min, in some slices devitrification could be avoided, but not in some other slices. The DSC was found to be very helpful to monitor this: first of all, the DSC was used to ensure that the V_{crw} of a tissue slice was not increased by decreasing pre-incubation duration with a CPA and secondly, DSC measurements were used to certify that a slice, used as a representative for a particular organ, had a V_{crw} below the feasible warming rates.

A major problem to be resolved with vitrification is the toxicity of the CPAs, that have to be applied in high concentrations. With several CPAs (DMSO, 1,2-propanediol and glycerol) it was already noticed that extra-hepatic organ slices were much more sensitive to CPA toxicity than liver slices (unpublished results). In chapter 4, relatively non-toxic high molarity mixtures of CPAs were used (i.e. VS4 and VM3). It was found that the toxicity of these mixtures could be avoided for the greatest part by optimizing the pre-incubation and washing-out protocols. Particularly the shortening of the last pre-incubation step with the undiluted vitrification solutions and the addition of trehalose in the washing medium reduced toxicity. With intestinal slices, we had the least succes: due to the high sensitivity of these slices to VS4, attempts to vitrify them were ceased.

Eventually, after optimization of the pre-incubation protocol, we found that liver slices, kidney cortical and kidney medulla slices could be successfully cryopreserved after treatment with VM3, but to much lesser extent with VS4. Unexpectedly, contrary to prevailing thoughts, it was found that cooling rate was an important factor determining survival of slices, even when no ice crystals were formed. This indicates that thermal stress in a sample is a issue to be considered upon developing a vitrification prototcol.

Slices of *both* hepatic and extra-hepatic origin should be used to reliably predict *in vivo* drug metabolism

When an animal species is selected to serve as a model for human drug toxicity, it is necessary that this species produces comparatively the same (in some cases harmful) metabolites of the drug as humans. When an *in vitro* tool is used for the selection of such animals, metabolism should be adequately predicted. In chapter 7, it was shown that subcellular fractions do not meet this prerequisite. In contrast, intact cell systems from liver (hepatocytes and slices) were found to not only produce the same metabolites of two out of three tested drugs as intact animals, but in approximately the same relative amounts as well. Of the third drug, however, the major metabolite *in vivo* (an *N*-glucuronide) was only produced in small amounts by these systems. It was found that intestinal slices produced this metabolite in relatively large amounts.

The relative contribution to drug metabolism by different metabolizing organs differs between compounds and between animal species (Vickers *et al.*, 1995; de Kanter *et al.*, 1999; de Kanter *et al.*, in press). Some hypothethical situations as an example to stress the importance of these findings: suppose rats are selected as a model for human drug toxicity based on the fact that rat liver slices produce the same metabolites as human liver slices. When a toxic metabolite is actually produced by the human kidney, but not by the rat kidney, the choice of rats to model human toxicity will be proven further onwards to be inadequate. Likewise, when the parent drug is actually toxic, but is conjugated rapidly in the intestinals of rats, but not in those of humans, the toxicity of this drug may be underestimated in the rat model. Consequently, to rightfully predict *in vivo* metabolism for proper animal species selection, slices of *both* liver and extra-hepatic metabolising organs should be used.

Future perspectives

The questions that should be answered in this section are whether it is convenient to continue with the develpoment of cryopreservation methods for organ slices and which approach is most likely to lead to an "universal" method, i.e. a method applicable for various organ slices of various animal species.

Obviously, the results of chapter 7 only emphasize the need for adequate cryopreservation methods for tissue slices: from a logistically point of view, it would be very convenient when one is able to withdraw the material that is required for a certain experiment from a stored pool of tissue slices, at any desired time-point. Which cryopreservation method should be used for this purpose is a question that is more difficult to answer. In this thesis it is shown that rapid freezing, because it preserves viability to a high level and because of its simplicity, is a very convenient method to cryopreserve liver slices. Like with slow freezing, however, the results are not extrapolatable to all tissue slices of all animal species. We have suggested that the relative sensitivity of different slices to ice crystals formed by rapid freezing may be the cause of this problem. More insights in the size and location of ice crystals and the factors determining susceptibility of cells to these crystals will help to further develop rapid freezing protocols for extra-hepatic organ slices.

Because vitrification prevents the formation of ice during freezing, it may overcome the problems that came across with equilibrium slow freezing and rapid freezing. Indeed, it was shown in this thesis that kidney and liver slices can be successfully preserved by vitrification. Toxicity of the highly concentrated CPAs required for vitrification is a major concern, however we have shown that these problems can be overcome for a great part by using mixtures of CPAs like VM3 and VS4 and by adequately designing protocols for impregnation and out-washing of these compounds. DSC may be of help for this purpose. CPA mixtures are still being optimized to combine the lowest possible toxicity with the highest tendency to vitrify. So, from a future perspective, vitrification as a starting point should offer the highest chance to develop "universal" cryopreservation methods for tissue slices.

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Samenvatting

Samenvatting

Veiligheid van nieuwe medicijnen wordt doorgaans getest met behulp van proefdieren. Om als goed model te dienen voor de mens, dient voor zulke veiligheidsstudies een proefdiersoort geselecteerd te worden die dezelfde omzettingsproducten (metabolieten) maakt van het medicijn als de mens. Deze metabolieten kunnen namelijk, net als het medicijn zelf, giftig zijn.

Het proefdiergebruik kan sterk verminderd worden als de bovengenoemde selectie '*in vitro*' kan worden uitgevoerd in plaats van in een intact dier. Het feit dat er in deze (vroege) fase van het veiligheidsonderzoek nog nauwelijks gegevens over het metabolisme van de nieuwe medicijnen in de mens bekend zijn, is een andere belangrijke reden om dit soort onderzoek *in vitro* uit te voeren: er kan namelijk wèl relatief gemakkelijk gebruik worden gemaakt van menselijk orgaan materiaal (restanten van operaties en donororganen).

Omdat de lever voor een groot deel verantwoordelijk is voor het metabolisme van lichaamsvreemde stoffen (zoals medicijnen) ligt het voor de hand om voor *in vitro* onderzoek leverpreparaten te gebruiken. Een voorbeeld van een leverpreparaat waarmee vaak gewerkt wordt zijn leverslices: dunne plakjes lever met dezelfde structurele organisatie en voorkomende celtypes als een intacte lever. Er zijn echter aanwijzingen dat sommige medicijnen door andere organen dan de lever worden omgezet.

Een belangrijk doel van het onderzoek dat beschreven is in dit proefschrift was na te gaan of het metabolisme van medicijnen door een intact dier inderdaad goed voorspeld kan worden met behulp van *in vitro* preparaten. Het bleek dat het metabolisme van sommige medicijnen prima voorspeld kan worden met leverpreparaten met intacte cellen zoals leverslices, maar dat ook slices van andere organen (met name dunne darm) nodig zijn om een goede voorspelling te doen van het metabolisme van andere medicijnen (hoofdstuk 7).

Vaak kunnen veel meer slices van een orgaan worden gemaakt dan nodig zijn voor een bepaald *in vitro* experiment. Om optimaal gebruik te kunnen maken van de voordelen van het gebruik van orgaanslices is een manier nodig om ze te kunnen opslaan. Idealiter zou men gebruik moeten kunnen maken van een 'orgaanslice-bank': op elk willekeurig tijdstip kunnen daaruit slices worden verkregen van verschillende proefdiersoorten en de mens, zodat een op een makkelijke manier diersoortvergelijkend metabolsime onderzoek van een nieuw medicijn kan worden gedaan.

Het grootste deel van het in dit proefschrift beschreven onderzoek is gewijd aan het zoeken naar een manier om orgaanslices voor langere tijd op te slaan door middel van cryopreservatie. Cryopreservatie houdt in dat biologisch materiaal wordt afgekoeld tot een zeer lage temperatuur (-196°C). Op deze temperatuur staan biologische en chemische processen vrijwel stil, zodat veranderingen in het materiaal (zoals 'bederf' of 'veroudering') kunnen worden voorkomen. Als cryopreservatie succesvol verloopt, zijn de orgaanslices na ontdooien nog levensvatbaar en inzetbaar voor in diersoort-vergelijkende *in vitro* studies (zie hoofdstuk 1).

Het bleek moeilijk te zijn om het begrip 'levensvatbaar' te definiëren. Bepaalde cellulaire processen in de orgaanslices bleken veel gevoeliger te zijn voor beschadiging door cryopreservatie dan andere (hoofdstuk 2). Veranderingen in de histomorphologie (structuur van weefsels en cellen zoals met een microscoop te bestuderen valt) bleek één van de gevoeligste indicatoren van beschadiging door cryopreservatie te zijn. Verder bleek dat gecryopreserveerde slices met een intacte histomorphologie net zo goed als niet-gecryopreserveerde slices in staat waren tot het metaboliseren van medicijnen (hoofdstuk 7). Om die reden is histomorphologie in de meeste experimenten die in dit proefschrift beschreven zijn gebruikt om het succes van cryopreservatie te bepalen.

In hoofdstuk 2, 3 en 4 is aandacht besteed aan verschillende manieren om orgaanslices te cryopreserveren. Het uitgangspunt van cryopreservatie is het voorkomen van schade door ijskristallen tijdens het afkoelen en ontdooien. Een veel gebruikte methode is om cellen zeer langzaam in te vriezen. Tijdens dit proces drogen cellen langzaam uit en worden alleen ijskristallen buiten de cel gevormd. Deze 'extracellulaire ijskristallen' worden als relatief onschadelijk voor de cellen beschouwd. Als te snel wordt afgekoeld worden echter ijskristallen in de cel gevormd ('intracellulaire ijskristallen'), waardoor de cel kan sterven.

Hoewel langzaam invriezen heel goed blijkt te werken voor 'losse cellen' (bijvoorbeeld bloedcellen), is in hoofdstuk 2 gebleken dat langzaam invriezen minder geschikt is voor slices. Waarschijnlijk worden de cellen in de slice toch beschadigd door de kristallen die tussen (buiten) de cellen ontstaan. 'Losse cellen' hebben geen last van dit soort kristallen, omdat ze kunnen rangschikken in onbevroren kanaaltjes tussen de 'extracellulaire' ijskristallen. Een betere methode bleek om de slices te 'vitrificeren' (=het vormen van een glas, zie hoofdstuk 4). Hierbij worden slices eerst geïmpregneerd met hoge concentraties 'cryoprotectantia' (CP's, vaak organische oplosmiddelen, zoals DMSO) en daarna afgekoeld. Op deze

manier worden in de slice geen intra- of extracellulaire ijskristallen gevormd maar ontstaat er een 'glas', in feite een vloeistof met een zeer hoge viscositeit. Door te vitrificeren worden slices gevrijwaard van beschadiging door ijskristallen, maar er ontstaat wel een nieuw probleem: de hoge concentraties CP's zijn zo giftig dat de slices het impregneren vóór het invriezen vaak al niet overleven. De oplossing bleek om gebruik te maken van mengsels van CP's. Deze mengsels bestaan uit CP's die elkaars giftigheid 'opheffen'. Resultaten verkregen met dit soort mengsels waren hoopgevend: nier- en leverslices van de rat bleken na vitrificatie en ontdooien bijna net zo vitaal te zijn als niet-behandelde slices (hoofdstuk 4).

De derde cryopreservatiemethode waarmee geëxperimenteerd is in ons onderzoek, 'rapid freezing', is een verhaal apart: leverslices die gecryopreserveerd zijn met deze methode zijn vitaal na ontdooien, ondanks het feit dat ijskristallen zowel intra- als extracellulair gevormd worden (hoofdstuk 2 en 5). Het is heel waarschijnlijk dat de hoge invriessnelheid en de middelmatig hoge concentraties DMSO (dat de vorming van ijskristallen remt) waarmee gecryopreserveerd wordt ervoor zorgen dat er slechts kleine ijskristallen kunnen ontstaan waardoor de beschadiging door cryopreservatie gering is. Een bewijs hiervoor is dat de slices alsnog doodgaan als langzaam wordt ontdooid waarbij kleine kristallen de kans krijgen om tot grotere kristallen uit te groeien. Een opvallend resultaat beschreven in hoofdstuk 6 is dat voorbehandeling van de slices bepaalt hoe groot hun weerstand is tegen intra- en extracellulair ijs gevormd tijdens 'rapid freezing'. Verder bleek 'Rapid freezing' niet geschikt voor de cryopreservatie van nier- en dunne darm slices: na ontdooien daalde de vitaliteit van deze slices drastisch (hoofdstuk 4).

De twee belangrijkste eindconclusies (hoofdstuk 8) van dit proefschrift zijn 1) dat de voorselectie van proefdieren voor veiligheidsonderzoek van medicijnen '*in vitro*' kan worden uitgevoerd met behulp van orgaanslices en dat het dus loont dit soort onderzoek te vergemakkelijken door het aanleggen van een orgaanslice-bank en 2) dat vitrificatie van slices na impregnatie met relatief weinig giftige CP mengsels het beste uitgangspunt is om 'universele' (dwz werkend voor verschillende orgaanslices van verschillende diersoorten) methodes voor de cryopreservatie van orgaanslices te ontwikkelen.

Curriculum Vitae
and
List of publications

Curriculum Vitae

De auteur van dit proefschrift, Inge Anne Maria de Graaf, werd op 31 juli 1972 geboren in Rotterdam. In 1990 behaalde zij het gymnasium β diploma aan het Krimpernerwaard college te Krimpen aan den IJssel. In het schooljaar 1990-1991 werd dit diploma aangevuld met de VWO-certificaten Natuurkunde en Scheikunde.

In 1991 begon de auteur aan haar studie aan de Landbouwuniversiteit Wageningen. Na het behalen van het propedeutisch diploma Levensmiddelentechnologie werd in de doctoraalfase gekozen voor de studie Milieuhygiëne, specialisatierichting 'Milieu, Arbeid en Gezondheid'. De studie werd afgesloten met een afstudeervak aan de vakgroep Toxicologie, onder begeleiding van Prof. Dr. J.H. Koeman en Dr. S.A. van der Plas, en een afstudeervak/stage aan de vakgroep Luchtkwaliteit, onder begeleiding van Dr. H. Kromhout en Dr. C. Wesseling. Voor het laatst genoemde project werd het veldwerk verricht in Costa Rica. In augustus 1996 werd het doctoraal diploma Milieuhygiëne behaald.

In juni 1996 besloot de auteur een extra stage te volgen bij TNO voeding in Zeist. Onder begeleiding van Dr. A.P.M. Wolterbeek werd gewerkt aan een studie naar het effect van potentieel tumorpromoverende stoffen op de intercellulaire communicatie en celproliferatie in leverslices. In 1997 werkte de auteur van dit proefschrift in dienst als wetenschappelijk medewerker bij TNO aan hetzelfde project.

Vanaf januari 1998 tot en met december 2001 werkte Inge de Graaf als promovendus aan het door het Platform Alternatieven voor Dierproeven (PAD, nu Zorgonderzoek Nederland) gefinancierde project 'cryopreservatie van weefselslices'. Dit onderzoek werd uitgevoerd bij Solvay Pharmaceuticals in Weesp onder begeleiding van Dr. H.J. Koster (Solvay Pharmaceuticals, Weesp) en Prof. Dr. W. Seinen en Dr. B.J. Blaauboer (IRAS, Universiteit Utrecht).

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Dankwoord

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