15 Precision-Cut Tissue Slices: A Suitable *In Vitro* System for the Study of the Induction of Drug-Metabolizing Enzyme Systems

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15.1 SUMMARY

Induction of the drug-metabolizing enzymes, in particular, cytochrome P450 (CYP) forms, is a major cause of drug–drug as well as herb–drug interactions that can lead to adverse side effects, which may prove fatal. As a result, an integral part of drug development is to ascertain whether it has the potential to act as an inducer of CYP and other enzyme systems in order to avoid serious drug interactions with coadministered drugs. Moreover, for both drugs and other chemicals, CYP induction may be associated with adverse toxicology including tumorigenesis.

A versatile *in vitro* model system that can be employed to evaluate the effect of chemicals on xenobiotic-metabolizing enzymes is the precision-cut tissue slice procedure. Slices from liver as well as extrahepatic tissues such as the kidney, lung, and

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intestines have been utilized to identify metabolic pathways and assess the toxicity of chemicals, including their potential to induce xenobiotic-metabolizing enzyme systems. Many studies have utilized precision-cut rat liver slices to monitor the induction of enzymes belonging to the CYP1–CYP4 families. A wide range of chemicals have been studied using this *in vitro* system, including drugs, environmental contaminants, and phytochemicals. CYP form induction has been determined by measurement of appropriate enzyme activities (EAs) (either in intact slices or in slice homogenates/subcellular fractions), protein levels (e.g., by Western immunoblotting), or mRNA levels. Established inducers of various CYP enzymes upregulated CYP enzymes as would have been envisaged on the basis of *in vivo* studies reported in the literature. In human liver slices, the induction of a number of CYP forms including CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, and CYP3A4 has been demonstrated.

As with the induction of CYP forms, a wide range of compounds have been shown to induce phase II xenobiotic-metabolizing enzymes, including epoxide hydrolase, UDP-glucuronosyltransferase (UGT), sulfotransferase, and glutathione *S*-transferase (GST) forms, in cultured rat liver and lung slices, and human liver and intestine slices.

Finally, procedures have been developed for the successful cryopreservation of precision-cut liver slices from various animal species, which can also be used to assess the potential of xenobiotics to induce the xenobiotic-metabolizing enzyme systems.

15.2 INTRODUCTION

The use of precision-cut tissue slices is increasingly gaining acceptance and is utilized worldwide to provide answers in studies concerned with the metabolism and toxicity of chemicals [1–5]. The major "competing" *in vitro* system involves the use of isolated cells such as hepatocytes. However, precision-cut slices offer some important advantages over the use of isolated cells that cannot be ignored, and these are summarized below.

- Compared to single cell preparations such as hepatocytes, slices function as "mini" tissues, maintaining the functional heterogeneity of the parent tissue and permitting the study of zonal effects. Moreover, in tissues such as lung that has a heterogeneous cell population where drug or xenobiotic-metabolizing EAs are present only in some cell types [6], the use of slices allows the identification of cell-specific toxicity.
- The procedure for isolation of single cells employs digestive enzymes that remove cell-to-cell contact, thus preventing cellular communication and interactions, and lead to the collapse of tissue architecture. Such communication is preserved in slices and, furthermore, maintenance of tissue architecture in slices makes possible the use of morphological endpoints, such as histology and immunocytochemistry, in evaluating xenobiotic toxicity.
- Generation of slices using an appropriate tissue slicer (e.g., a Krumdieck or Brendel-Vitron slicer) is rapid and causes only minimal damage to the tissue and does not necessitate the use of detrimental proteolytic enzymes that are routinely utilized in cell isolation.

- The use of slices facilitates comparisons of the metabolism and/or toxicity between animal species, including human, and tissues, especially as slices can be successfully cryopreserved (see below), which would not be otherwise possible. Such cross-species comparisons can provide invaluable information, enabling the identification of animal species that would be appropriate surrogates for human in toxicological evaluation studies.
- Slices from different tissues may be cocultured so that, for example, the sequential metabolism and toxicity of xenobiotics can be delineated.

15.3 CLINICAL AND TOXICOLOGICAL IMPLICATIONS OF ENZYME INDUCTION

In an era where polypharmacy has become routine, it is inevitable that coadministered drugs were shown to interact with adverse or even fatal consequences for the patient. The induction of the drug (xenobiotic)-metabolizing enzymes by one or more administered drugs is a major mechanism underpinning many such interactions. In most instances, enzyme induction involves the stimulation of CYP forms that are present in the liver and extrahepatic tissues [7,8]. The most important microsomal CYP forms involved in the metabolism of therapeutic agents in human liver include members of the CYP1A, CYP2A, CYP2B, CYP2C, CYP2D, CYP2E, and CYP3A subfamilies [7–12]. Such drug interactions often involve CYP3A4, as this enzyme is the most active human CYP form in drug metabolism and, moreover, is the dominant form in the liver and intestine, the principal site and the first site of metabolism following oral drug intake, respectively. There are numerous examples of drug–drug interactions consequential to CYP induction [9–12]. Patients with transplanted organs and successfully stabilized on the immunosuppressant cyclosporin displayed signs of rejection after self-medicating with St. John's wort, a herbal remedy taken to treat mild depression [13]. It appears that a component(s) of this remedy elevates CYP3A4 activity, leading to enhanced cyclosporin metabolism resulting in suboptimal plasma levels of the drug and, consequently, tissue rejection. Since the norethindrone and ethinyl oestradiol components of the contraceptive pill are also deactivated by CYP3A4, intake of St. John's wort has also been associated with intracyclic bleeding episodes as a result of contraceptive failure [14,15]. The treatment of humans with the antibacterial agent rifampicin (RIF), which induces CYP3A and other CYP forms, has been shown to produce a number of clinically important drug–drug interactions [10–12]. These include cyclosporin (reduction in blood levels leading to transplant rejection), warfarin (reduced prothrombin time), oral contraceptives (unplanned pregnancies), and human immunodeficiency virus protease inhibitors such as ritonavir and indinavir (reduced systemic exposure leading to a diminished antiretroviral effect).

The treatment of rats and mice with therapeutic agents and other chemicals, which induce hepatic microsomal CYP forms may be associated in chronic studies with the formation of tumors in the liver and other tissues [12]. For example, CYP2B inducers can give rise to liver and thyroid gland tumors and exposure to CYP4A inducers can lead to tumor formation in the liver and testis. In these instances, the induction of rodent hepatic CYP forms represents just part of a pleiotropic response to such compounds. However, the induction of CYP forms can serve as a biomarker for

potential subsequent tumor formation, and hence it is important in the toxicological evaluation of new chemical entities (NCEs).

As a result of the potential clinical and toxicological consequences of CYP enzyme induction, studies to screen for such an effect form an integral part of drug development. Studies encompassing a large number of doses, that would not be feasible to conduct *in vivo*, can be readily performed *in vitro* using precision-cut tissue slices from laboratory animals and human donors.

15.4 PREPARATION AND CULTURE OF PRECISION-CUT TISSUE SLICES

Tissue slices can be prepared from the liver and extrahepatic tissues such as the kidney, lung, and different parts of the intestine [1–5]. For solid tissues, such as the liver and kidney, the first step is to prepare cylindrical cores (normally 8 or 10 mm in diameter) with a hand or preferably a motor-driven tissue coring tool. The tissue cylinders are then loaded into a suitable tissue slicer and slices of a defined thickness are produced. For liver slices, the optimal thickness has been established to be 200–250 μm, with limits of usable thickness ranging from 150/180 to 300 μm [1–3]. The optimal thickness of kidney slices is also around 200–250 μm. Tissue slice thickness can be estimated by a number of methods including determination of tissue slice weight or protein content, microscopic examination of freshly cut slices, use of a digital micrometer, and the microscopic examination of formalin-fixed tissue slice paraffin sections [1–3,16].

Commercially available tissue slicers include the Krumdieck and the Brendel-Vitron [1–3]. The Krumdieck tissue slicer is fully automated, with the tissue core being brought across a motor-driven oscillating blade that is submerged in a chamber containing a suitable slicing buffer. The buffer is circulated by a pump and the tissue slices are removed from the blade by a gentle flow of buffer into a glass collecting vessel. In contrast, the Brendel-Vitron slicer is cheaper and less mechanically complex than the Krumdieck slicer. However, it is only semiautomated and uses a rotating blade with the slicing buffer being both circulated and oxygenated by a gas supply. Both tissue slicers have certain advantages and a level of technical expertise is required [1–3]. One study has demonstrated that there was little difference between precision-cut rat liver slices produced with Krumdieck and Brendel-Vitron tissue slicers for studies of xenobiotic metabolism and toxicity [17].

With soft tissues, such as the lung and intestines, agarose is used to enable the preparation of tissue cores. For example, rodent lungs can be filled with a warm agarose solution at 37°C, which is then cooled to 4°C before preparing tissue cores and, subsequently, tissue slices as described above for liver slices [1,2,4]. Intestines can also be filled with warm agarose solution and then embedded in agarose in a core-shaped container to obtain solid cores for slicing [4,18,19]. The optimal thickness of intestinal slices is around 200–250 μm, whereas for lung slices the optimal thickness is around 500–700 μm [1–24].

A wide variety of incubation systems have been developed for precision-cut tissue slices [1–5,20]. Generally, the available incubation systems may be divided into two types, namely, surface culture (where the slices are dipped in and out of the culture medium) and submersion culture systems. One much used surface culture system is the dynamic organ culture system where the slices are floated onto a mesh support

(e.g., stainless steel or titanium) of a roller assembly, which is housed in a suitable vial (e.g., a glass scintillation vial) containing culture medium. The vial is then placed on a roller assembly housed in an incubator or is placed in a purpose-built incubator (e.g., a Vitron incubator). It has been shown that tissue slice viability is extended if the slices are placed on a nitrocellulose filter rather than directly on the mesh of the roller assembly [5,21]. Submersion tissue slice culture systems include the use of Erlenmeyer flasks and 6-, 12-, and 24-well tissue culture plates. For multiwell plates, slices can be simply immersed in culture medium or placed on mesh supports. Both the surface culture and submersion culture systems have advantages, and the choice of incubation system may depend on the particular application of tissue slices under investigation [1–5,20,22,23]. A microfluidic biochip system for the perifusion of liver slices has also recently been described [24].

A number of suitable media for tissue slice preparation and culture media for the incubation of tissue slices from both the liver slices and extrahepatic tissues have been described in the literature [1–4]. Culture media are often supplemented with hormones (e.g., insulin and a glucocorticoid) and bacterial and antifungal agents, the latter being essential to prevent microbial contamination during prolonged incubations. Tissue slices can be incubated under air or gas phases with a higher oxygen concentration, ranging from 40% to 95% [1–4].

15.5 INDUCTION OF PHASE I ENZYME SYSTEMS BY XENOBIOTICS IN PRECISION-CUT TISSUE SLICES

Developments in precision-cut tissue slice preparation and incubation techniques have permitted the culture of slices for several days, which is long enough for their use in xenobiotic-metabolizing enzyme induction studies [1–5,20]. As they contain all the cell types present in a tissue, precision-cut tissue slices can be used to study the effects of drugs and other chemicals on all the xenobiotic-metabolizing enzymes present in that tissue. The most important phase I xenobiotic-metabolizing enzymes comprise members of the CYP superfamily [7–12], whereas phase II enzymes include UGT, sulfotransferase, and GST forms (Section 15.6). This section considers the induction of CYP forms in liver and extrahepatic slices from animals and humans.

Most CYP forms are induced by receptor-mediated mechanisms, leading to an increase in gene transcription [8,10–12]. Important receptors involved in the induction of CYP1A, CYP2B, CYP3A, and CYP4A forms comprise, respectively, the aryl hydrocarbon receptor (AhR), the constitutive androstane receptor (CAR), the pregnane X receptor (PXR), and the peroxisome proliferator-activated receptor alpha (PPARα). These and other receptors are present in precision-cut tissue slices, thus permitting cultured slices from the liver and other tissues to be used in CYP form induction studies.

The induction of CYP forms in precision-cut tissue slices can be evaluated by monitoring CYP-dependent EAs, CYP mRNA levels, or CYP protein levels. CYP EAs can be determined either directly by adding the substrate to the culture medium or alternatively by removing the slices and preparing whole homogenate and/or subcellular fractions (e.g., postmitochondrial supernatant, microsomes) [25–34]. As xenobiotics and their metabolites may be retained in liver slices if the CYP substrate is added to the culture medium, some workers homogenize the slices in the incubation medium

and then analyze the slice/medium homogenate. However, for some substrates only the incubation medium needs to be analyzed. Because phase II enzymes will also participate in incubations with intact slices, it may be necessary to treat the slice/medium homogenate or medium with a β-glucuronidase/sulfatase preparation in order to quantify the total (i.e., free and conjugated) CYP metabolite(s) produced [23,35]. One study reported that the induction of CYP-dependent enzymes by β-naphthoflavone (BNF) and dexamethasone (DEX) could be detected in both intact liver slices and liver slice homogenates [36]. CYP mRNA levels are best determined by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) methodology (e.g., TaqMan®), although other less quantitative procedures have also been employed [27,29,34,37–53]. To normalise for RNA loading, levels of CYP mRNAs are often expressed as a ratio to a suitable control mRNA such as albumin, β-actin, or 18 S rRNA. Several studies have examined the effects of xenobiotics on CYP proteins by preparing microsomal fractions and performing Western immunoblotting of selected CYP forms [25,31–34,37,52–60]. CYP proteins may also be determined by enzyme-linked immunosorbant assay (ELISA) procedures [27].

As with other *in vitro* systems, it is important to evaluate a range of xenobiotic concentrations and incubation times in order to ascertain whether the xenobiotic under investigation is an inducer of CYP forms in cultured precision-cut tissue slices. To determine the effects on CYP form mRNAs, only short incubation times may be required (e.g., 3–24 h), whereas to determine the effects on CYP EAs and proteins, longer incubation times (e.g., 24–72 h) may be necessary. In addition, it is imperative to ensure that the chosen concentrations of the xenobiotic studied are not toxic to the tissue slices. Suitable markers of tissue slice viability include leakage of potassium and enzymes (e.g., alanine aminotransferase, lactate dehydrogenase (LDH)), inhibition of protein synthesis, and levels of ATP and reduced glutathione [1–4].

Like hepatocytes [20], levels of CYP forms decline in cultured precision-cut rodent and human liver slices. For example, compared to freshly cut slices levels of CYP1A, CYP2B, CYP2C, CYP2D, CYP2E, CYP3A, and CYP34A forms and associated EAs have been shown to decline with time in cultured rat liver slices [25,32,54]. While levels of CYP forms also decline in human liver slices [30,33,34,51,54,55,57], one study demonstrated a differential loss with some CYP forms (e.g., CYP2D6, CYP4A11) being relatively stable over a 72-h culture period, whereas other CYP forms (e.g., CYP1A2, CYP2C19) exhibited a more rapid loss [33]. However, the decline in CYP forms in cultured liver slices does not preclude the use of liver slices as an *in vitro* model system to screen xenobiotics for induction of CYP forms. Indeed, a wide range of chemicals have been shown to induce CYP forms in cultured precision-cut liver slices from animals and humans. Such chemicals include drugs, environmental contaminants, and phytochemicals. Thus, like hepatocytes cultured liver slices can be employed as a model system to assess the potential of NCEs to induce hepatic CYP forms.

15.5.1 Studies in Liver Slices from Animal Species

The majority of studies conducted in animal species have been performed with precision-cut slices prepared from rat liver. Some examples of CYP induction studies in cultured rat liver slices are shown in Table 15.1. The induction of CYP1A, CYP2B, CYP3A, CYP4A, and other subfamily forms have been studied by determination of CYP EAs (intact slices and slice homogenates/subcellular fractions), protein (normally

TABLE 15.1 Induction of CYP Forms in Cultured Rat Liver Slices

Inducer[a]	CYP Form or Subfamily	Endpoint Studied[b]			References
		EA	Protein	mRNA	
ARO, BNF, PB	1A, 2B	✓	✓	—	25
BNF	1A	✓	—	—	26
TCDD	1A1, 1A2	✓	✓	✓	37
ARO, BNF, CIP, MCP, PB, WY	1A2, 2B1/2, 3A, 4A	✓	✓	—	54
BNF, DEX	1A1	—	✓	—	27
BNF, PB, TCDD	1A, 2B, 3A	✓	—	—	28
BNF, 7EC, 4Me7EC	1A1	✓	—	✓	29
BNF	1A1	—	—	✓	38
BP	1A1	—	—	✓	39
TCDD	1A1, 1A2	✓	✓	—	31
ARO, 3ICN, DIM	1A, 2B, 3A	✓	✓	—	58
PB	2B1	✓	✓	—	41
PCN	3A1	—	—	✓	42
ARO, PB, WY	1A1, 1A2, 2B1, 4A1	—	—	✓	43
DEX, PCN	3A	✓	—	✓	45
ARO, BNF, MCP, PB, PCN, WY	1A1, 1A2, 2B1/2, 3A1, 3A2, 4A1	—	—	✓	46
ARO, BP	1A	✓	—	—	61
DEX	3A	—	✓	—	62
CARB, CLOT, DEX, PB, PCN, others	2B1/2, 3A1	—	—	✓	47
BNF, DEX, PB, PCN	1A, 2B, 2C, 3A	✓	✓	—	63
BP	1A1, 1B1	—	—	✓	48
ISN, PB	2B, 2E1	✓	✓	—	64
BP	1A1, 1B1	✓	✓	✓	52
ERUC, SULF	1A2, 1B1, 3A2	—	✓	—	60
BNF, DEX, PB, PCN	1A, 2B, 2C, 3A	✓	✓	—	65
BP other PCHs	1A1, 1B1	✓	✓	✓	53
BUD, DEX, PCN	3A1, 3A2, 3A9	—	—	✓	66

[a]Inducers used were: ARO, Aroclor 1254; BNF, β-naphthoflavone; BP, benzo(*a*)pyrene; BUD, budesonide; CARB, carbamazepine; CIP, ciprofibrate; CLOT, clotrimazole; DEX, dexamethasone; DIM, 3,3′-diindolylmethane; 7EC, 7-ethoxycoumarin; ERUC, erucin; 3ICN, indole-3-acetonitrile; ISN, isoniazid; MCP, methylclofenapate; 4Me7EC, 4-methyl-7-ethoxycoumarin; PB, phenobarbital; PCHs, polycyclic hydrocarbons; PCN, pregnenolone-16α-carbonitrile; SULF, sulforaphane; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; WY, Wy-14,643.

[b]Endpoints studied were determination of CYP enzyme activities (EAs), protein levels (e.g., Western immunoblotting), and mRNA levels.

by Western immunoblotting), and mRNA levels. Table 15.1 lists either the CYP subfamily studied or the particular CYP form examined if this was identified (e.g., by mRNA determination or Western immunoblotting).

A number of studies have established that CYP1A forms are inducible in cultured liver slices following treatment with agents such as Aroclor 1254 (ARO) (a polychlorinated biphenyl mixture), BNF, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD),

polycyclic aromatic hydrocarbons (PCHs) including benzo(*a*)pyrene (BP) and phytochemicals including 3,3′-diindoloylmethane (DIM), erucin (ERUC), and sulforaphane (SULF). Some studies [48,52,53] have also examined effects on CYP1B1 as, such as CYP1A1 and CYP1A2, this CYP form is also involved in the metabolic activation of carcinogens including PCHs and heterocyclic amines (cooked food mutagens) [8]. In one study, CYP induction by PCHs was shown to correlate with the potency of the compounds studied to bind to the AhR receptor [53]. Correlations were also observed between the molecular size of the PCHs studied and the induction in rat liver slices of CYP1A-dependent EA, CYP1A1 mRNA levels, and CYP1B1 mRNA levels. The induction of CYP forms by BP in cultured rat liver slices has been shown to be associated with increased levels of BP-DNA adducts [67].

The induction of CYP2B and CYP3A forms in cultured rat liver slices has been demonstrated in studies emanating from a number of laboratories. For example, the induction of CYP2B forms has been demonstrated after exposure of the slices to phenobarbital (phenobarbitone, PB), whereas DEX and/or pregnenolone-16α-carbonitrile (PCN)-induced CYP3A forms [25,41,42,45–47,54,62–66]. Because of the high degree of similarity between CAR and PXR, a number of inducers function as activators of both nuclear receptors [10–12]. Thus, in some studies, in cultured rat liver slices, PB has been shown to induce CYP3A forms in addition to CYP2B forms, whereas DEX- and/or PCN-induced CYP2B forms as well as CYP3A forms [46,54,63,65]. In one study, a range of NCEs were shown to have variable effects on CYP2B1/2 (i.e., CYP2B1 and CYP2B2) and CYP3A1 mRNA levels in cultured rat liver slices [47]. Moreover, good correlations were observed between the potency of the NCEs to induce CYP2B1/2 and CYP3A1 mRNA levels in liver slices *in vitro* and the potency of these NCEs to induce CYP2B (by EA determination) and CYP3A (by Western immunoblotting) forms after *in vivo* treatment (oral administration to rats for eight days), thus highlighting the usefulness and validity of utilising precision-cut tissue slices to evaluate the potential of a xenobiotic to induce the CYP enzymes. In another study utilizing a series of structurally diverse compounds, a correlation was observed between the induction of CYP3A mRNA levels in cultured rat liver slices and the effects of the compounds in a rat PXR reporter gene assay [68].

While CYP1A, CYP2B, CYP2C, and CYP3A forms are primarily involved in the metabolism of drugs and other xenobiotics [7,8], the CYP4A subfamily forms are involved in the metabolism of endogenous chemical such as fatty acids. CYP4A forms are inducible in rodent liver and this is associated with hepatic peroxisome proliferation [10,12]. The induction of CYP4A forms has also been demonstrated in cultured rat liver slices [43,46,54]. In keeping with known *in vivo* effects, peroxisome proliferators have also been shown to induce peroxisomal fatty acid oxidizing EAs and, by ultrastructural examination, to increase numbers of peroxisomes in cultured rat liver slices [69].

One advantage of the precision-cut tissue slice technique is its ready application to studies of species-, tissue-, sex-, strain-, and age-related differences. In a study of age-related differences, using liver slices from young, adult, and old rats (aged 3, 9 and 24 months, respectively), a decrease in total CYP content and a loss of CYP3A inducibility by DEX were observed in old rats [62]. The treatment of liver slices from one-day-old rats with BNF, PB, DEX, and PCN was shown to result in the induction of CYP1A, CYP2B, CYP2C, and CYP3A marker EAs [63]. In a similar study, the effects of BNF, PB, DEX, and PCN on CYP-dependent activities in liver slices from 1-day-, 40-day-, and 1-year-old rats were investigated [65]. Overall, CYP EAs were

somewhat lower in liver slices from one-year-old rats, whereas CYP inducibility was more pronounced in younger rats.

As tissue morphology is retained in precision-cut tissue slices, the cell-specific and zonal effects of xenobiotics on CYP forms can be investigated. By employing immunocytochemical techniques, a number of studies have investigated the effect of xenobiotics on induction of CYP forms in different regions of the liver lobule. The majority of these studies have focussed on CYP1A forms. In untreated rat liver slices cultured for 24 h, CYP1A1 protein immunostaining was not detected but was detected after BNF treatment [70]. A centrilobular induction of CYP1A1 protein immunostaining was reported in rat liver slices after treatment for 24 h with BP [39] and for 24 h with TCDD followed by 72 h culture in control medium [31]. In another study, CYP1A1 protein immunostaining was not detected in liver slices cultured for 24 h in control medium, but following BP treatment CYP1A1 protein immunostaining was observed throughout the liver slice [48]. Furthermore, a low level of CYP1B1 protein immunostaining was observed in both control and BP treated liver slices. The effect of BNF, PB, DEX, and PCN on CYP1A1, CYP2B1, and CYP3A2 protein expression was assessed in liver slices from neonatal and adult rats [63,71]. In freshly cut slices from adult rats, only weak CYP1A1 protein immunostaining was observed, whereas strong CYP2B1 and CYP3A2 protein immunostaining was detected mainly in centrilobular and midzonal areas of the liver lobule [71]. After 24 h treatment with BNF, increased CYP1A1 and CYP2B1 protein immunostaining was observed, whereas PB increased CYP2B1 and CYP3A2 protein expression and DEX increased CYP3A2 protein expression. Similar results were obtained in studies with one-day-old rats except that an increase in CYP3A2 protein immunostaining was also observed with PCN [63].

Fluorescence confocal laser cytometry has also been used to investigate zonal differences in CYP form induction in liver slices from untreated (control) and BNF-treated rats [72]. An induction of 7-ethoxyresorufin *O*-deethylase (a marker of CYP1A induction) was observed in centrilobular hepatocytes of liver slices from BNF-treated rats, whereas no induction was observed with fluorescent substrates of CYP2B forms.

Compared with the rat, fewer studies have been performed using precision-cut liver slices from the mouse. However, as would be expected CYP form induction can be readily demonstrated in this species. CYP1A-dependent EA was induced in mouse liver slices by BNF and TCDD, with PB inducing CYP2A- and CYP3A-dependent EAs [28]. The treatment of mouse liver slices with BNF has also been reported to induce CYP1A1 and CYP1A2 mRNA levels, with CYP2B10 mRNA levels being induced by BNF, PB, and DEX, and CYP3A11 mRNA levels by DEX [49]. The induction of CYP3A12 and CYP3A26 mRNA levels by various compounds in cultured precision-cut dog liver slices has also been reported [73].

15.5.2 Studies in Human Liver Slices

Some examples of CYP induction studies in cultured human liver slices are shown in Table 15.2. The induction of CYP forms in a number of subfamilies has been investigated following treatment with drugs, environmental contaminants, and phytochemicals.

A number of studies have demonstrated the induction of CYP1A1 and/or CYP1A2 in cultured human liver slices following treatment with agents such as ARO, BNF, BP, and other PCHs, TCDD, omeprazole (OMP), DIM, ERUC, SULF, and phenethyl

TABLE 15.2 Induction of CYP Forms in Cultured Human Liver Slices

Inducer[a]	CYP Form or Subfamily	Endpoint studied[b]			References
		EA	Protein	mRNA	
ARO, MCP	1A2, 4A	✓	✓	—	54
RIF	3A4	✓	✓	—	55
TCDD	1A1	✓	✓	—	56
DIM	1A2	✓	✓	—	57
BNF	1A1	—	—	✓	40
CP, PB	2B6, 3A4	✓	✓	✓	34
BNF, PB, RIF	1A2, 2C19, 3A4	—	✓	—	59
OMP, RIF, TCDD, others	1A1, 1A2, 2C9, 3A4	—	—	✓	51
ERUC, SULF	1A1, 1B1	—	✓	—	60
BNF, PB, RIF	1A1, 1A2, 2A6, 2B6, 3A4, 3A5	✓	—	✓	74
BP, other PCHs	1A1	✓	✓	—	53
CDCA, DEX	3A4	—	—	✓	66
PEITC	1A1, 1A2, 2B6, 3A4	✓	✓	—	75

[a]Inducers used were: ARO, Aroclor 1254; BNF, β-naphthoflavone; BP, benzo(*a*)pyrene; CDCA, chenodeoxycholic acid; CP, cyclophosphamide; DEX, dexamethasone; DIM, 3,3′-diindolylmethane; ERUC, erucin; MCP, methylclofenapate; OMP, omeprazole; PB, phenobarbital; PCHs, polycyclic hydrocarbons; PEITC, phenethyl isothiocyanate; RIF, rifampicin; SULF, sulforaphane; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

[b]Endpoints studied were determination of CYP enzyme activities (EAs), protein levels (e.g., Western immunoblotting), and mRNA levels.

isothiocyanate (PEITC). CYP1B1 protein levels have also been reported to be induced by the chemopreventive phytochemicals, ERUC and SULF [60].

Apart from CYP1A forms, in keeping with studies in cultured hepatocytes, a number of other human hepatic CYP forms can be induced in cultured liver slices. These CYP forms include CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP3A4, and CYP3A5 [34,51,55,59,66,74,75]. Compounds that are known to induce one or more of these CYP forms in human liver slices include chenodeoxycholic acid (CDCA), cyclophosphamide (CP), DEX, PB, PEITC, and RIF. For example, investigations from a number of laboratories have demonstrated that PB and RIF can induce CYP3A4 EA, protein levels, and mRNA levels in cultured precision-cut human liver slices [34,51,55,59,74].

The lobular distribution of CYP2B6 and CYP3A4 induction has been investigated in precision-cut human liver slices [34]. In untreated human liver slices, the distribution of CYP2B6 and CYP3A4 immunostaining was random and centrilobular, respectively. The staining intensities of both CYP2B6 and CYP3A4 proteins were increased following treatment with both PB and CP for 72 h, with no change in the zonal distribution of these two CYP forms being evident.

15.5.3 Studies in Extrahepatic Slices

While the liver is the major site of xenobiotic metabolism in mammals, both phase I and II xenobiotic-metabolizing enzymes are found in many extrahepatic tissues [4,7,8]. Moreover, some extrahepatic xenobiotic-metabolizing enzymes are known to

TABLE 15.3 Induction of CYP Forms in Cultured Rat and Human Lung, Kidney, and Intestinal Slices

Species, Tissue, and Inducer[a]	CYP Form or Subfamily	Endpoint Studied[b]			References
		EA	Protein	mRNA	
Rat lung—ARO, BNF, BP	1A1, 2B1/2	✓	✓	✓	76
Rat lung—ARO, BP, NIC	1A	✓	—	—	61
Rat lung—BP	1A1, 1B1	—	—	✓	48
Rat lung—BP	1A1, 1B1	✓	✓	—	52
Rat lung—BP, other PCHs	1A1, 1B1	✓	✓	—	53
Rat lung—ERUC, SULF	1A1, 1B1, 3A2	—	✓	—	77
Rat kidney—BP	1A1	—	—	✓	39
Rat intestine[c]—BNF	1A	✓	—	—	18
Rat intestine[c]—BNF, DEX, IR, 20MC, PB	1A, 2B15, 3A9	✓	—	✓	78
Rat intestine[d]—BUD, CDCA, DEX, $1,25(OH)_2D_3$, PCN	3A1, 3A2, 3A9	—	—	✓	66
Human intestine[e]—BNF, DEX, PB, RIF	1A1, 2B6, 3A4	✓	—	✓	19
Human intestine[f]—BUD, DEX, $1,25(OH)_2D_3$	3A4	—	—	✓	66

[a]Inducers used were: ARO, Aroclor 1254; BNF, β-naphthoflavone; BP, benzo(*a*)pyrene; BUD, budesonide; CDCA, chenodeoxycholic acid; DEX, dexamethasone; IR, indirubin; 20MC, 20-methylcholanthrene; NIC, nicotine; $1,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; PCHs, polycyclic hydrocarbons; PCN, pregnenolone-16α-carbonitrile.

[b]Endpoints studied were determination of CYP enzyme activities (EAs), protein levels (e.g., Western immunoblotting), and mRNA levels.

[c]Rat small intestine and colon examined.

[d]Rat jejunum, ileum, and colon examined.

[e]Human proximal jejunum and colon examined.

[f]Human ileum examined.

be inducible. The lung and intestines are important sites of xenobiotic metabolism, as these tissues are the first sites of metabolism of inhaled and orally ingested xenobiotics, respectively. This section focusses on CYP induction studies performed in cultured lung and intestine slices, including investigations performed with human intestine slices.

A number of studies have examined the induction of CYP forms in cultured rat lung, kidney, and intestine slices. As shown in Table 15.3, the treatment of rat lung slices with ARO, BNF, BP and other PCHs, nicotine (NIC), ERUC, and SULF has been shown to induce CYP1A1, and in some instances, the induction of CYP1B1 has also been observed [48,52,53,61,76,77]. Apart from the induction of CYP1A1 and CYP1B1 in cultured rat lung slices, ARO was shown to have a weak effect on CYP2B1/2 mRNA levels [76] and SULF a weak effect on CYP3A2 protein levels [77]. In one study in cultured rat kidney slices, BP was shown to induce CYP1A1 mRNA levels [39].

The effect of xenobiotics on CYP forms in rat and human intestine slices has also been investigated (Table 15.3). Xenobiotic-metabolizing enzymes are not uniformly distributed throughout the intestinal tract, and there can also be regional differences in the effects of CYP inducers [18,19,66,78]. The treatment of rat small intestine slices with BNF, indirubin (IR), and 20-methylcholanthrene (20MC) and colon slices with BNF and 20MC has been shown to induce a CYP1A marker EA [18,78]. Following treatment with DEX, a CYP3A marker EA was increased in cultured rat small intestine slices but not in colon slices [78]. Treatment with PB increased CYP3A9 mRNA levels in both small intestinal and colon slices, and treatment with both PB and DEX increased CYP2B15 and CYP3A9 mRNA levels in colon slices. In another study, the effect of xenobiotics on CYP3A1, CYP3A2, and CYP3A9 mRNA levels in cultured rat jejunum, ileum and colon slices was examined [66]. Treatment with 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)-induced CYP3A1 mRNA levels in cultured rat jejunum, ileum, and colon slices and CYP3A2 mRNA levels in ileum slices. CYP3A1 mRNA levels were increased in rat ileum slices by CDCA, DEX, and PCN, whereas CYP3A9 mRNA levels were increased in jejunum and ileum slices by BUD, DEX, and PCN, with the former two also increasing CYP3A9 mRNA levels in colon slices.

Treatment with BNF increased CYP1A1 mRNA levels in both human proximal jejunum and colon slices [19]. In addition, both PB- and RIF-induced CYP2B6 and CYP3A4 mRNA levels, and DEX-induced CYP3A4 mRNA levels in human proximal jejunum slices. Significant increases in CYP1A (by BNF) and CYP3A (by PB and RIF) marker EAs were also observed in cultured human proximal jejunum slices. While some increases in CYP2B6 (by PB) and CYP3A4 (by RIF) mRNA levels were observed in human colon slices, these effects were not statistically significant. In another study, significant increases in CYP3A4 mRNA were observed in human ileum slices after treatment with BUD, DEX, and $1\alpha,25(OH)_2D_3$ [66].

15.6 INDUCTION OF PHASE II ENZYME SYSTEMS BY XENOBIOTICS IN PRECISION-CUT TISSUE SLICES

Phase II enzyme systems are closely linked to the deactivation and detoxication of xenobiotics. Conjugation reactions with endogenous substrates, such as sulfate, D-glucuronic acid, glutathione, and certain amino acids, yield highly hydrophilic, readily excretable metabolites [79], although functional groups may also be methylated or acetylated to produce less hydrophilic compounds. Thus induction of these enzyme systems will impact on the metabolic fate and biological activity of chemicals. Toxicologically, a most important enzyme system is the GSTs, which catalyze one of the most important pathways of metabolism that allows the cell to defend itself from chemical insult. It utilizes the nucleophilic tripeptide glutathione, possessing a nucleophilic sulphur atom, to detoxify reactive electrophiles. Indeed, dietary phytochemicals considered to be potential anticarcinogens, such as isothiocyanates, indoles, and curcumin, owe their chemopreventive activity largely to their ability to stimulate conjugation of chemical carcinogens, or of their genotoxic metabolites, with glutathione.

On culturing, phase II activities in precision-cut tissue slices decline, but these enzyme systems appear to be more robust and are better maintained compared with CYP forms [23]. Substantial phase II activity remained in rat liver slices after a 24 h incubation and, in some cases, significant activity was retained even after a 72 h

incubation; for example, the specific activity of UGT, monitored using 2-aminophenol as substrate, did not change significantly following a 24 h incubation, and only a modest loss of activity was noted after 72 h [23]. In concordance, glucuronidation reactions, measured using *p*-nitrophenol, 4-methylumbelliferone, and 4-hydroxybiphenyl as substrates, were more stable than CYP enzymes in rat slices [80,81]. Similarly in human liver slices, form-specific changes were noted when CYP protein levels were determined [33], but glucuronidation of 7-hydroxycoumarin was well maintained [30]. Such observations imply that not only the functional integrity of the enzymes is maintained but also that the slices are capable of synthesizing the necessary cosubstrates for conjugation, such as UDP-glucuronic acid.

The regulation of phase II enzymes by xenobiotics has been the subject of numerous publications, studies being conducted largely *ex vivo* in many animal species, and *in vitro* using systems, such as freshly isolated hepatocytes and cultured cells. It is now established that phase II enzymes are also inducible, in particular, the UGTs, GSTs, and quinone reductase. However, it is evident that phase II enzymes are far less inducible compared with CYP forms, and this is also displayed in slices. Most of the studies conducted in precision-cut tissue slices focussed on the liver, but there is sufficient experimental evidence that induction can be achieved in other tissues. As in the case of CYP forms, two approaches may be used to determine induction of phase II enzymes in precision-cut tissue slices. The first involves incubating slices for a defined period of time, removing the slices, and incubating them once again in fresh medium supplemented with a substrate extensively metabolized by conjugation, the most frequently used being 7-hydroxycoumarin, which is readily conjugated with sulfate or D-glucuronic acid. Alternatively, following incubation of tissue slices with the potential inducing agent, the slices are harvested and subsequently homogenized and subcellular fractions (e.g., microsomes and cytosol) are prepared by differential centrifugation. This second approach is particularly useful when information is required for the modulation of individual forms of the enzyme of interest or for mechanistic studies. Once again, changes in enzyme expression can be determined at the activity level, protein level by immunoblotting, and mRNA by using techniques such as RT-PCR.

15.6.1 Studies in Liver Slices from Animal Species

In order to be able to detect and quantify the inductive potential of xenobiotics, incubation conditions must be first optimised to ensure that maximum induction is attained. One of the most critical factors is the length of incubation time of the tissue slice with the inducing agent. Of course, this will also depend on the biomarker employed to assess the inductive effect, such as mRNA levels or EA. Using the polycyclic aromatic hydrocarbon BP as the model inducing agent, it was revealed that maximum induction of GST activity, determined using 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole as the substrate that is preferentially metabolized by GSTα [82], was attained after 24 h incubation and was accompanied by a rise in the enzyme protein levels; no significant change in activity was detected at the earlier time points studied [52]. At the mRNA level, however, an increase was clearly evident as early as 4 h, this being the earliest time point investigated. A similar picture emerged when epoxide hydrolase, monitored using benzo[*a*]pyrene 4,5-oxide, was the investigated enzyme [52]. At the mRNA level, exposure to PB doubled UGT mRNA levels (UGT2B12) in rat slices, but the increase was seen only after incubation of 24 h but not at 2 or 6 h [81].

The next issue that merits discussion is the choice of concentrations of the potential inducing agent; a major attribute of the precision-cut system is that it allows the facile use of a range of concentrations to be investigated. Of course, the chosen range should reflect the concentrations of relevance to human, for example, total plasma, or tissue when available, concentrations following therapeutic intake of a medicinal drug, exposure to an environmental contaminant, or dietary intake of a phytochemical. An initial study should be undertaken in all cases to ensure that the chosen concentrations do not compromise the viability of tissue slices.

Studies employing a number of diverse inducing agents indicated that the upregulation of phase II enzyme systems is concentration dependent. When rat liver slices were incubated with a range of concentrations of BP and, subsequently, the slices were used to assess epoxide hydrolase activity using benzo[*a*]pyrene 4,5-oxide as substrate, a significant increase in activity was seen only at the 10 μM concentration, and declined at higher concentrations [52]. Increases at the enzyme protein level were detectable at lower concentrations, and when mRNA levels were determined, a significant rise was also observed at relatively much lower doses (0.75 μM); in both cases maximum elevation occurred at the 10 μM concentration. Similarly, other PCHs capable of upregulating this enzyme caused a maximal response, either at the activity or protein levels, at about 5–10 μM, with the response declining at higher concentrations; at the mRNA level, once again increases were evident at much lower concentrations (0.10–0.75 μM) [83]. When GST activity, determined using 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole as substrate, was monitored, a similar picture was observed following incubation of BP with rat liver slices, in that maximal rise in activity and protein levels was attained at about 10 μM; in this case, a rise in mRNA levels was evident even at 0.1 μM. The PCHs dibenzo[*a*, *h*]anthracene and benzo[*b*]fluoranthene also achieved maximal induction of activity and enzyme protein levels at concentrations of 5–10 μM with increases in mRNA levels being manifested at much lower concentrations [84].

The value of slices for evaluating the upregulation of phase II enzymes can be illustrated by the use of isothiocyanates, a major class of chemopreventive phytochemicals present in cruciferous vegetables, whose mechanism of action involves detoxification of the reactive intermediates of carcinogenic chemicals [85]. Three such isothiocyanates, namely, ERUC, SULF, and PEITC, upregulated GSTs as well as quinone reductase (a phase II enzyme system that detoxifies quinones) in hepatic precision-cut rat liver slices [86,87].

15.6.2 Studies in Human Liver Slices

Available literature on the induction of phase II enzyme systems in human tissues is limited and largely confined to the liver. Following a 24 h incubation, four PCHs, namely, BP, dibenzo[*a*, *h*]anthracene, fluoranthene, and benzo[*b*]fluoranthene significantly elevated, in a concentration-dependent manner, epoxide hydrolase activity determined using benzo[*a*]pyrene 4,5-oxide as substrate; parallel increases in enzyme protein levels were also observed [83]. GST activity and expression, on the other hand, were elevated only by benzo[*b*]fluoranthene [84]. At the mRNA level, UGTA1 and UGTA6 were upregulated after a 24 h incubation with BNF, whereas when RIF or PB was used as the inducing agent, not all livers responded [74].

Incubation of precision-cut human intestine slices with BNF for 24 h increased UGT1A6 mRNA levels, but in colon slices, there was a very marked variability that

precluded attainment of statistical significance. Similarly, RIF increased mRNA levels in the intestine, but there was a wide variation in the colon. Incubation with quercetin led to a rise in UGT1A6 mRNA levels in the small intestine; a similar increase was seen in the colon but only based on a single human sample [19].

In studies aimed to establish the effect of isothiocyanates on GSTs and quinone reductase, upregulation of these enzymes being considered as an important mechanism of their chemopreventive activity, precision-cut liver slices from human donors were exposed to ERUC, SULF, and PEITC for 24 h [86,87]. In general, marked differences in response were noted illustrating that there are very pronounced differences in the response of human liver to isothiocyanates, raising the possibility that the chemopreventive effect of isothiocyanates may not be manifested in all individuals.

15.6.3 Studies in Extrahepatic Slices

The decline of phase II EAs following culture of precision-cut slices is not confined to the liver. In precision-cut rat lung slices, the glucuronidation of 1-naphthol and sulfation of 2-naphthol were very well maintained over a 72 h incubation period, whereas 50% of GST activity was retained; conversely, under the same conditions, CYP forms were not as successfully maintained [88].

When BP was incubated with precision-cut rat lung slices, a rise in epoxide hydrolase activity was noted only after a 24 h incubation, but an increase in enzyme protein could be observed after an 8 h incubation, although maximal induction necessitated a 24 h incubation [52]. When precision-cut rat small intestine slices were incubated with *tert*-butylhydroquinone, an increase in the glucuronidation of 7-hydroxycoumarin was observed after 24 h but not 5 h [78]. Collectively, these studies demonstrate that the complex signaling systems that regulate the induction of phase II enzymes remain intact and functioning even after a 24 h incubation. Clearly, the length of incubation time required to achieve maximal induction of phase II enzymes may be dictated by the nature of the tissue as well as the enzyme under investigation.

Surprisingly, in precision-cut lung slices exposed to BP, epoxide hydrolase activity was elevated only at 1 μM, that is, a far lower concentration compared with liver, and this was also evident in the case of other PCHs [52,83]. Whether this difference in concentration reflects the higher metabolic breakdown of BP in the liver compared with lung, thus leading to a lower effective concentration of the inducing agent remains to be established. When precision-cut rat lung slices were incubated with ERUC and SULF, both isothiocyanates elevated quinone reductase and GST activities [77]. In slices prepared from rat small intestine and colon, PB, at a concentration of 4 mM but not 2 mM, enhanced glucuronidation, but not sulfation, of 7-hydroxycoumarin. At a concentration of 8 mM, glucuronidation was markedly impaired; similarly, sulfation decreased, and these effects were paralleled by a drop in viability as exemplified by lower intracellular ATP levels [78]. These studies help illustrate the importance of studying a range of concentrations and ensuring that these do not influence adversely slice viability.

15.7 CRYOPRESERVATION OF PRECISION-CUT TISSUE SLICES

When the validity of the precision-cut tissue system was established, it was inevitable that a lot of effort would be devoted into establishing conditions under which slices

could be cryopreserved so that their functional viability is preserved. This is a pivotal issue particularly with respect to human tissue, the supply of which is limited and unpredictable. In cases where human liver is made available but cannot be immediately used in its entirety, slices could be produced and cryopreserved for storage and subsequent use. Similar considerations apply to tissue from large animals such as dogs, monkeys, and food-producing animals. Cryopreservation would allow a comparison of the metabolic profile of a compound of interest to be investigated so that an appropriate surrogate animal can be chosen for toxicological studies. Moreover, successful maintenance of tissue slices through cryopreservation will inevitably lead to a reduction in animal use.

Considerable effort has been expended into defining conditions of cryopreservation that would maintain the functional characteristics of slices as near as possible to what is observed in fresh slices. For slice viability to be conserved, it is essential that the injury to cells that occurs during cooling and thawing processes is minimised. As a result, the objective is to prevent the formation of ice crystals, which are believed to be responsible for cell death following cryopreservation. Surprisingly, however, it appears that liver slices can survive cryopreservation, following rapid freezing, even when ice crystals are formed intracellularly [89], indicating that other factors may be responsible for the loss of viability. Three fundamental procedures have been adopted to achieve successful cryopreservation of tissue slices: (i) computer-controlled slow freezing to allow cellular dehydration by removal of water from the cells, so that ice crystals are produced extracellularly; (ii) rapid, or even ultrarapid freezing, a much simpler procedure that utilizes relatively lower concentrations of cryoprotectants; slices in the medium are placed in cryovials that are directly submerged to liquid nitrogen; and finally, (iii) vitrification, a process that deploys high concentrations of cryoprotectants, which in itself has the potential to cause toxicity, and employs flash freezing; it leads to the formation of amorphous rather than crystalline intracellular ice. A number of studies have been carried out aimed at defining the conditions that achieve the highest slice viability. To assess slice viability, various parameters have been used, including leakage of lactate dehydrogenase, protein synthesis, intracellular levels of potassium and ATP, histological evaluation, and metabolic functionality, the most frequently employed model substrates being testosterone and 7-ethoxycoumarin. This chapter focusses on metabolic viability when such data is available because of its paramount importance in studies of xenobiotic metabolism and toxicity.

All initial work concerned with cryopreservation emanates from the University of Arizona, where the preparation of precision-cut tissue slices was pioneered with the development of the Krumdieck automatic slicer [1,2]. The potential for cryopreservation was shown in studies employing liver slices from human and pig, an anatomically related animal species, using dimethyl sulfoxide (DMSO) as the cryoprotectant and assessing slice viability using intracellular potassium and protein synthesis as biomarkers [90]; the same procedure was also applicable to human kidney slices [91] as well as to other animal species such as dog and monkeys [91,92]. Vitrification by immersing human slices into liquid nitrogen, using 1,2-propanediol as cryoprotectant, was also demonstrated to be feasible in maintaining viable slices [93]. In these studies, the metabolic competence of the slices was evaluated using 7-ethoxycoumarin; the cryopreserved tissue could catalyze both the CYP-mediated oxidative deethylation of the substrate and the subsequent conjugation of the 7-hydroxycoumarin, demonstrating that the slices maintained the integration of phase I and phase II metabolism of

xenobiotics. However, metabolic integrity of human slices could also be achieved by rapid freezing of the slices in cryovials with DMSO serving as cryoprotectant [94]. In these studies, the metabolism of both exogenous, lidocaine and 7-ethoxycoumarin, and endogenous, testosterone, substrates was used to monitor metabolic function; the same metabolites were generated by the cryopreserved slices as with fresh slices, indicating that the metabolic pattern was not modulated as a result of cryopreservation. Better metabolic viability was reported when rat or human slices were frozen sandwiched between aluminum plates than when frozen in cryovials [95]. In a simplification of this method, the slices were frozen sandwiched between standard aluminum foil; the deethylation of 7-ethoxycoumarin was fully maintained in comparison with fresh slices [96].

More detailed studies were undertaken to better define cryopreservation conditions by comparing the freezing/thawing conditions and the importance of the cryopreservation medium. In these studies, it became evident that the critical aspects of the cryopreservation process were the freezing rate and the nature of the cryopreservation medium. On the basis of studies carried out utilizing rat liver slices, it was proposed that rapid freezing and the Williams Medium E, as compared with the University of Wisconsin organ preservation medium, should be applied to all cryopreservation protocols [97,98]. The optimum concentration of DMSO, itself a toxic chemical, has also been investigated [98]; these authors employed three different concentrations of DMSO, namely, 12%, 18%, and 30%, and observed that metabolic functionality, assessed using 7-hydroxycoumarin and testosterone as substrates, was best maintained at a concentration of 18%. Similar studies by other research groups led to the same conclusion [99]. Even after a six-month storage period, metabolic function of cryopreserved rat and human liver slices, determined using testosterone as biomarker, was well retained [100]; sulfation and conjugation of 7-ethoxycoumarin was fully retained in cryopreserved rat liver slices, but in human slices glucuronidation was halved. It has also been reported that the survival of cryopreserved rat slices post thawing could be extended if the slices were cryopreserved in Williams E medium by rapid freezing and using a high concentration of DMSO (30%). In these studies, metabolic competence was monitored using a number of substrates, but the effect was substrate dependent; for example, the glucuronidation of *p*-nitrophenol was only maintained for 2 h after thawing, whereas the glucuronidation of 4-methylumbelliferone was fully maintained for 24 h, implying that stability may depend on the nature of the enzyme monitored [101]. Whether DMSO concentration modulates the levels of enzyme protein and/or cofactor availability remains to be established. The effect of ultrarapid freezing with DMSO as cryoprotectant has also been studied. Once again metabolic function was dependent on the enzyme monitored; the CYP-mediated hydroxylation of midazolam was well maintained, whereas the conjugation of paracetamol (acetaminophen) with sulfate and D-glucuronic acid was markedly impaired [102]. Interestingly, the metabolic viability of slices, assessed by using testosterone and conjugation with glutathione, was inadequate when cryopreservation was controlled using a computer-controlled slow freezing procedure, which is successful in the case of hepatocytes [103]. Finally, as far as slice thickness is concerned, some researchers reported that it had no effect [97], whereas others observed that thin slices were better maintained, possibly as a result of inadequate diffusion of the cryoprotectant in the thick slices to secure uniform cryopreservation [98].

In a study aimed at assessing the viability of individual CYP forms, seven substrates were used to investigate the metabolic function of rat liver slices following cryopreservation; the rate of metabolism of these substrates, as exemplified by intrinsic clearance Cl_{int}, was not influenced by cryopreservation [104] indicating that it is unlikely that individual CYP forms exhibit different sensitivity to cryopreservation. When metabolic viability of liver slices from a number of animal species, including rat, man, and dog, was assessed, the conjugation of 7-hydroxycoumarin was well maintained after cryopreservation by rapid freezing, and testosterone hydroxylation even increased in the rat and mouse [105].

The vast majority of studies concerned with cryopreservation were conducted in liver slices, and it was anticipated that slices from other tissues would display similar behavior. Using a toxicological end point, namely the ammoniagenic effect induced by the antiepileptic drug valproate, it was established that human precision-cut renal cortical slices were very well preserved in comparison with fresh slices; similarly LDH leakage was unaffected but intracellular levels of ATP decreased [106]. In a recent study, the cryopreservation of rat liver and kidney slices by rapid freezing and vitrification was investigated; viability was assessed using intracellular ATP levels and histologically. As expected, liver slices were viable following rapid freezing but, in contrast, kidney slices were susceptible and viability was severely curtailed [107]. Vitrification of liver slices with VS4, a mixture of 1,2-propanediol, formamide, and DMSO, or VS3, a mixture of ethylene glycol, formamide, and DMSO, led to a marked loss in viability despite the fact that no ice was formed. Vitrification of slices from the renal medulla using VS4 was successful in almost completely maintaining viability but was not suitable for slices from the renal cortex. When vitrification was performed using VS3, kidney slices from both the cortex and medulla were well maintained; thus it would appear that kidney slices are more suited to vitrification. It would be informative to assess the metabolic function of the slices under these conditions. Clearly, one cannot extrapolate from one tissue to another even if they originate from the same animal species.

The ability of cryopreserved tissue slices to respond to inducing agents, that is, the viability of the enzyme synthesis apparatus, has not received much attention. Although cryopreserved precision-cut human slices could respond to the CYP1 inducer BNF, at least at the mRNA level, the effect was less pronounced compared to fresh slices [108]. Subsequent studies by the same workers revealed that the same agent caused upregulation of CYP1 activity level, assessed using 7-ethoxycoumarin, to the same extent in fresh and cryopreserved slices [101]. Elevation of CYP2B1 and CYP3A1 mRNA levels was also demonstrated, to a similar extent, in both fresh and cryopreserved rat liver slices incubated with PB and PCN, respectively [42]. Clearly, more extensive studies are warranted in this area not only to establish inducibility of CYP forms and other xenobiotic-metabolizing enzymes in cryopreserved slice but also to ascertain whether CYP-form-specific differences exist. Inducibility of phase II enzyme systems in cryopreserved slices has not been examined. Moreover, the impact of cryopreservation on the induction process in human liver as well as animal and human extrahepatic tissues are issues that merit further in depth investigation, bearing in mind their potential value.

It is abundantly clear that the success of cryopreservation is dependent on the tissue in question, the animal species from which it derives as well as the nature of the chosen biomarkers for assessing viability. Despite the considerable amount of published

literature in this area, there is no universally agreed and adopted protocol for cryopreservation of liver slices, but rapid freezing in DMSO appears to be effective in most cases.

15.8 CONCLUSIONS

Tissue slices have been used for many decades in biochemical studies, especially in the unraveling of the pathways of carbohydrate metabolism by, among others, Krebs and Warburg. However, because of the difficulty in generating viable slices of uniform thickness, this *in vitro* system was abandoned and was gradually replaced by alternatives, such as primary hepatocytes and cell lines. What reignited interest in this system is the construction, some 30 years ago, by Krumdieck and coworkers of a tissue slicer capable of producing viable thin slices of reproducible thickness, from a number of tissues, which led to the development of the precision-cut tissue slice technique [1–5,20]. The facile generation of tissue slices and the potential applicability of this technique to the use of human tissue attracted the attention of researchers in both academia and industry, and automatic tissue slicers (e.g., Krumdieck, Brendel-Vitron), incubation systems and other equipment were developed and became commercially available. In addition, the published literature describes suitable media for the preparation and culture of precision-cut tissue slices [3,4,20].

Precision-cut slices, as they maintain the cellular architecture of the tissue, function as "mini tissues," and as such they represent more precisely the *in vivo* situation. A major advantage of the precision-cut tissue slice technique is that once suitable conditions for the preparation of tissue slices from one species (e.g., rat liver slices) have been established in a laboratory, the technique can be readily applied to tissue slices from other species. Moreover, while precision-cut tissue slices can be easily prepared from "solid" tissues (e.g. liver, kidney), procedures have also been established for the preparation of tissue slices from "soft" tissues such as the lung and intestine [1–5,20]. Furthermore, precision-cut slices can be successfully cryopreserved, which is of enormous benefit in human studies, since the availability of fresh human tissue is very limited.

Many studies have been performed with precision-cut liver slices and some studies have compared liver slices with other *in vitro* systems. For example, in a series of investigations with three drugs, namely, almokalant, carbamazepine (CARB), and selegiline, the ability of human liver slices to predict pathways and rates of drug metabolism was compared with human hepatocytes, liver microsomes, and cDNA-expressed CYP forms [109–111]. As both liver slices and hepatocytes contain a full complement of phase I and II xenobiotic-metabolizing enzymes, these systems were superior to liver microsomes and expressed CYP forms in predicting pathways of metabolism. However, human liver slices were less successful than hepatocytes in predicting the intrinsic clearance of the three drugs examined.

A number of studies described in this chapter have established that the induction of xenobiotic-metabolizing enzymes in precision-cut tissue slices concords with *in vivo* observations in animals. All classical established inducers of CYP forms and phase II enzymes have been investigated in tissue slices where they provoked the same response as observed *in vivo*, demonstrating the validity of the system. For human hepatic CYP form induction, cultured hepatocytes are considered to be the "gold standard" and are recommended by the US Food and Drug Administration (FDA) for *in vitro* studies

to assess the potential of a new therapeutic agent to induce hepatic CYP forms in humans [112]. Human hepatocytes have some advantages as, unlike human liver slices, both freshly isolated and cryopreserved human hepatocytes are readily commercially available and human hepatocytes can be cultured in a 96-well plate format, which permits the rapid screening of a large number of chemicals. However, as described in this chapter, precision-cut liver slices can also be employed to screen compounds for their potential to induce CYP forms in human liver.

In conclusion, precision-cut tissue slices are a valuable *in vitro* model system to assess the effects of NCEs on xenobiotic-metabolizing enzymes in the liver and other tissues, from animals and humans. This technique allows the facile and simultaneous comparison of the potential response of different tissues, emanating from one or more animal species, to drugs and other chemicals that would not be possible with other *in vitro* systems, or would have necessitated the use of many animals in *in vivo* studies.

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