Comparison of Five Incubation Systems for Rat Liver Slices Using Functional and Viability Parameters


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Precision-cut liver slices are presently used for various research objects, e.g. to study metabolism, transport, and toxicity of xenobiotics. Various incubation systems are presently employed, but a systematic comparison between these incubation systems with respect to preservation of slice function has not been performed yet. Therefore, we started a comparative study to evaluate five of these systems: the shaken flask (an Erlenmeyer in a shaking water bath), the stirred-well (24-well culture plate equipped with grids and magnetic stirrers), rocker platform (6-well culture plate with Netwell insert rocked on a platform), the roller system (dynamic organ culture rolled on an insert in a glass vial), and the 6-well shaker (6-well culture plate in a shaking water bath). The liver slices were incubated in these incubation systems for 0.5, 1.5, and 24.5 h and subsequently subjected to viability and metabolic function tests. The viability of the incubated liver slices was evaluated by: potassium content, MTT assay, energy charge, histomorphology, and LDH leakage. Their metabolic functions were studied by determination of the metabolism of lidocaine, testosterone, and antipyrine. Up to 1.5 h of incubation all five incubation systems gave similar results with respect to viability and metabolic function of the liver slices. However, after 24 h, the shaken flask, the rocker platform, and the 6-well shaker incubation systems appeared to be superior to the stirred well and the roller incubation systems. © 1997 Elsevier Science Inc.

Key Words: Rat liver slices; Incubation system; Viability; Drug metabolism

Introduction

Research in vitro using tissue slices was started in 1923 by Otto Warburg (Warburg, 1923). Slices were hand-cut with razor blades, hence reproducibility of slices was one of the major problems encountered. In addition, it was shown that this manual technique resulted in slices being too thick to allow sufficient oxygen and nutrient supply to the inner cell layers (Smith et al., 1985). Consequently, cellular necrosis of these inner layers was usually observed. Metabolism rate was adversely affected by this necrosis process during incubation (Smith et al., 1985).

Different incubation systems for long-time culture were used (Campbell and Hales, 1971; Hart et al., 1983), but the maintenance of the viability of the tissue slices was problematic due to limited oxygen diffusion into the slices. After a technique for the high-yield isolation of rat hepatocytes was developed in 1969 by Berry and Friend (Berry and Friend, 1969), isolated hepatocytes became the model of choice for pharmacological, toxicological, metabolism, and transport studies of xenobiotics (Skett, 1994).
Since the introduction of the Krumdieck precision-cut tissue slicer (Krumdieck et al., 1980) in 1980, research with liver slices has increased steadily. The Krumdieck slicer makes it possible to prepare tissue slices with reproducible thickness in cold physiological buffer, with a minimum of trauma to the tissue. Currently, liver slices are used in various research fields, e.g., in toxicology, pharmacology, and metabolism of xenobiotics (Parrish et al., 1995). In these studies the incubation conditions of the liver slice are essential for maintenance of the viability and function of the cells in the slice. Several incubation systems for liver slices are in use (de Kanter and Koster, 1995; Dogterom, 1993; Goethals et al., 1990; Leeman et al., 1995; Olinga et al., 1993; Smith et al., 1985), but a thorough comparison of these systems with respect to preservation of liver slice function has not been published yet. Only one study looked in more detail at two incubation systems (Fisher et al., 1995): They compared a multiwell culture plate system with the dynamic organ culture system using seven viability parameters to evaluate the incubated liver slices. The dynamic organ culture was superior to the 12-well culture plate up to 72 h of incubation.

We decided to start a study to evaluate five incubation systems currently used in metabolism studies for liver slices. Our aim was to establish and compare the viability and functionality of the liver slices incubated in these five systems after different incubation periods. The incubation systems used in the study described here are the shaken flask (the liver slice submerged in an Erlenmeyer in a shaking water bath) (de Kanter and Koster, 1995), the stirred well (the liver slice is laying on a stainless-steel grid in a well of a 24-well culture plate equipped with a magnetic stirrer) (Olinga et al., 1993), the rocker platform (the liver slice is laying on an polyester mesh insert in a 6-well culture plate and rocked on a platform) (Leeman et al., 1995), the roller system (dynamic organ culture; the liver slice is put on an insert and is rolled in a glass vial) (Smith et al., 1985), and the 6-well shaker (the liver slice is submerged in a well of a 6-well culture plate which is placed in a box) and shaken in a water bath). Each incubation system has its own specific advantages: for kinetic studies, quick sampling may be necessary, which can easily be performed with the stirred well, rocker platform and 6-well shaker. Small amounts of medium may be advantageous for metabolism studies as it is used in the stirred well, rocker platform, roller system, and the 6 well shaker. The shaken flask, the stirred well, and 6-well shaker are cheap and require no special equipment. The dynamic roller system is laborious and does not allow rapid sampling, but is useful for testing volatile substrates. Both in the roller system and in the rocker platform the liver slices are intermittently exposed to the medium and the air. According to some groups this allows optimal oxygen transfer into the liver slice (Smith et al., 1985), although definite proof for this has not been provided. Anyway, the intermittent exposure to the medium might negatively influence the kinetics of compounds that are rapidly taken up by the cells. The liver slices were preincubated in these different incubation systems and subsequently various viability and metabolic parameters were measured. After incubation, the viability of the liver slices was assessed by histomorphology, energy charge, potassium content, MTT assay, and lactate dehydrogenase (LDH) leakage. The metabolic function was determined by measuring testosterone, lidocaine, and antipyrine metabolism. Testosterone is metabolized by at least 10 isoenzymes of cytochrome P450 (Utesch et al., 1992). Lidocaine is metabolized by N-deethylation to monoethylglycinexylidide (MEGX) involving cytochrome P450 isoenzymes CYP2C11 (male specific) and CYP2B1, but probably also CYP3A2 and CYP1A2 (Imaoka et al., 1990). Metabolism of antipyrine to 3-hydroxymethylantipyrine (HMA) is mediated by CYP2B1 (van der Graaff et al., 1984).

Methods

Materials

Williams’ medium E (WME) supplemented with Glutamax I and gentamicin were obtained from Gibco BRL (Paisley, Scotland). Fetal bovine serum (FBS) was purchased from Integro BV. (Zaandam, The Netherlands). Insulin was purchased from Sigma Chemical Company (St Louis, MO, USA). 3-[4,5-Dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Aldrich Chemie (Steinheim, Germany). d-Glucose, toluidine blue, potassium dihydrogenphosphate (KH2PO4), and tetrabutylammonium sulphate (TBAS) were purchased from Merck (Darmstadt, Germany). Heraeus Kulzer (Wehrheim, Germany) supplied Technovit 7100 plastic resin. Netwell inserts (200-μm polyester mesh carrier), 6-well culture plates, and 24-well culture plates were obtained from Costar (Cambridge, MA, USA). Lidocaine was obtained from Centrachemie (Etten-Leur, The Netherlands); monoethylglycinexylidide was a kind gift of ASTRA (Södertälje, Sweden). Antipyrine was purchased from OPG (Utrecht, The Netherlands) and testosterone from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade and were obtained from commercial sources.

Methods

For preparation of liver slices, adult male Wistar rats (250–300 g) were obtained from Harlan PBC (Zeist, The Netherlands). Food, SRM-A, was purchased from Hope Farms (Woerden, The Netherlands) and bedding, woody clean, from BDI (Helmond, The Netherlands).
The preparation of the liver slices and the incubation experiments were performed at the different laboratories strictly according to standard operating protocols with one supervisor (P.O.) present at each experiment.

**Slice preparation**

Animals were anesthetized by pentobarbital (ip 60 mg/kg), the livers were excised, and liver cores (diameter of approximately 8 mm) were made. In one laboratory, the cores were made by a disposable biopsy punch, in the other institutes they were prepared by a drilling machine. Precision-cut liver slices (10–14 mg) were prepared in WME supplemented with 25 mM D-glucose and saturated with 95% O₂/5% CO₂ (carbogen) at 4°C, using a Krumdieck tissue slicer as described earlier (Smith et al., 1985).

**Incubation**

After slicing, each liver slice was kept in WME on melting ice, care was taken that the storage time did not exceed 45 min.

The culture medium used in the various incubation systems was WME supplemented with D-glucose to a final concentration of 25 mM, 5% FBS, 0.1 μM insulin, and 50 μg/ml gentamicin, saturated with carbogen at 37°C. After each function or viability test the wet weight of the liver slice was determined.

**Incubation systems**

The five incubation systems are shown in Figure 1. In three of the incubation systems, the liver slices were submerged continuously in the culture medium. In the shaken flask incubation system, the liver slice was submerged in 5 ml of culture medium in a 25-ml Erlenmeyer flask, gassed with humidified carbogen, and shaken back and forth (110 times/min) in a water bath at 37°C (de Kanter and Koster, 1995).

In the stirred well incubation system, the liver slice was laying on a stainless-steel grid in a well of a 24-well culture plate with 1.4 ml of culture medium at 37°C. Each well was stirred magnetically and gassed with humidified carbogen (Olinga et al., 1993).

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**Figure 1.** Five liver slice incubation systems, divided into two groups: (1) incubation systems with slices continuously submerged in the culture medium; and (2) dynamic organ culture-related incubation systems, where the liver slices are intermittently exposed to the medium and the air.
In the 6-well shaker incubation system, the liver slice was submerged in 3.2 ml of culture medium in a well of a 6-well culture plate, which was placed in a plastic container and gassed with carbogen, and shaken back and forth (110 times/min) in a water bath at 37°C. The liver slices moved freely in the medium without touching the walls.

The other two systems were modifications of the dynamic organ culture system, where the liver slices are intermittently exposed to the medium and the air.

In the roller platform, the liver slice was placed on a Netwell insert in a 6-well culture plate with 3.25 ml of culture medium and put in a humidified incubator at 37°C gassed with 40% O₂ and 5% CO₂ in air on a roller platform (approximately 10 cycles a minute) (Leeman et al., 1995).

In the roller system, the liver slice was put on a stainless-steel insert, placed in an incubation vial containing 2 ml of culture medium, gassed with humidified carbogen through a hole in the cap, and rolled at 37°C (Smith et al., 1985).

The liver slices were incubated in these incubation systems for 0.5, 1.5, and 24.5 h, and subsequently subjected to viability and metabolic function tests.

**Viability tests**

For histomorphology, the liver slices were fixed in buffered neutral formalin. The liver slices were dehydrated, embedded in Technovit 7100 plastic resin, sectioned at 2 μm, and stained with 1% toluidine blue. The cross-sections were microscopically examined for histomorphological changes by two independent observers. Morphological changes were evaluated by scoring alterations in nuclear shape, staining intensity of the cytoplasm, central necrosis, and the overall integrity of the slice (Leeman et al., 1995). A gradation of histomorphological changes was scored: from no or minor histomorphological changes to severe damage (necrosis and cell death). The thickness of the liver slice was measured using a micrometer in the ocular of the microscope.

For the determination of the energy charge, the liver slices were immersed into cold pre-weighed vials containing 0.25 ml 40% ethanol/7% perchloric acid (v/v) after the different incubation periods, weighed and frozen in liquid NZ and stored until analysis at about -80°C. After thawing, the liver slices were sonificated at 0°C, whereafter the suspensions were put on ice for 10 min and subsequently centrifuged at 10,000 g for 3 min. An aliquot of the supernatant was neutralized with 0.5 M K₂CO₃ to pH 7.0. The neutralized extract was centrifuged at 10,000 g for 3 min to precipitate the insoluble KClO₄. An aliquot of the supernatant was diluted with 250 mM Tris-HCl buffer pH 7.0 and injected on the HPLC. Nucleotide analysis was performed on an HPLC system fitted with two pumps (Waters model 510) controlled by a gradient controller (Waters 650). Injections were carried out with a Waters 712 WISP and UV-detection at 254 nm was by a Waters model 440 absorbance detector. Reversed-phase ion-pair chromatography was carried out with a Nova-Pak C₁₈ column (5 μm particle size, 150 × 3.9 mm I.D., Waters, Milford, MA, USA). The mobile phase consisted of buffer A (35 mM KH₂PO₄, 6 mM TBAS, pH 6.0) and buffer B (a mixture of 70% A and 30% of HPLC-grade acetonitrile). Prior to a new set of analyses, the column should be equilibrated for 12 h with buffer B, to ensure optimal separation of nucleotides. After injection of a 50-μl sample and 5 min of isocratic elution of 74% A and 26% B, a gradient elution was started from 74% A and 26% B to 8% A and 92% B within 5 min. This latter composition was maintained for an additional 5 min, at a flow rate of 1 ml/min. After the analysis, the column was reequilibrated with 74% A and 26% B. The total time for the analysis was 16 min (Olinga et al., submitted). With the described HPLC method, all adenosine nucleotides and adenosine can be separated, and this allows calculation of the energy charge of the liver slices using the equation: (ATP + 1/2 ADP)/(ATP + ADP + AMP) (Atkinson, 1968).

For the determination of the potassium content, three liver slices were combined, weighed and analyzed (Fisher et al., 1991).

For the MTT assay (Mosmann, 1983), the culture medium was replaced after the various incubation times, and the liver slices were incubated with 1.2 mM MTT dissolved in WME. After 1 h incubation, the MTT solution was removed, each liver slice was rinsed with cold PBS, and the formazan product—the reduction product of MTT—was extracted in the dark with 1 ml DMSO. After 24 h of extraction, the formazan extract was diluted with DMSO, and absorbance was measured at a wavelength of 505 nm and related to the dry and fat free weight of the tissue (Leeman et al., 1995). To determine the dry weight of the liver slices, the slices were rinsed twice with acetone, to remove present DMSO, whereafter the slices were dried overnight at room temperature.

The LDH content was determined in the culture medium after incubation by the SFBC protocol (SFBC, 1982).

**Metabolic function**

For the determination of the metabolism of lidocaine, antipyrine, and testosterone, the culture medium was changed after various incubation times, and the liver slices were incubated in the same incubation system with fresh culture medium for another hour with 5 mM of lidocaine or 10 mM of antipyrine or during 30 min with...
250 \mu M of testosterone. The lidocaine reaction was stopped by rapidly freezing the medium in liquid N$_2$, and the samples were stored at about -80°C until analysis. MEGX, the main metabolite of lidocaine, was determined in the thawed medium samples by HPLC (Olinga et al., 1993).

Antipyrine reaction was stopped after 1 h by separating the liver slices from the medium. Three slices were pooled, weighed, and 1 ml of fresh WME was added. The slices were then frozen in liquid nitrogen, and stored at about -80°C until analysis. After thawing and homogenization of the liver slices, the metabolites in liver slices and medium were determined separately by HPLC (Groen et al., 1992). After 30 min of incubation with testosterone, the liver slices were weighed and frozen in liquid nitrogen together with the medium and stored at about -80°C until analysis. After thawing, the liver slices with the medium were homogenized, and testosterone and its metabolites were analysed in 1-ml homogenate in the medium using HPLC (van ’t Klooster et al., 1993).

Statistics

One-way analysis of variance (ANOVA) was used for statistical analysis. Comparison was made by Fishers (least significant difference) LSD test, and the Student’s t test; \( p < 0.05 \) was considered to be statistically significant.

Results

The wet weight of the liver slices used for the experiments are depicted in Table 1. The reproducibility of the slicing technique, as indicated by the standard deviation, was high in the different laboratories. The liver slices prepared for the rocker platform system had a lower weight, because the diameter of the liver slices was slightly smaller than 8 mm, due to the coring with a biopsy punch (diameter 8 mm). In the other laboratories, the cores were made by a drilling machine with a coring tool (diameter 9 mm). This assured more cylindrical cores than produced by the biopsy punch. After incubation up to 1.5 h, the wet weight of the liver slices was lower compared to directly after slicing in three of the five systems, with the exception being the stirred well and the 6-well shaker incubation system where no differences were seen. After 24.5 h of incubation, the weight of the liver slice had decreased severely in all systems. In the shaken flask the decline amounted to 60%, whereas in the stirred well, the weight had a minimal decline of 14%. The wet weight to dry weight ratio is shown in Table 2. No statistically significant differences were observed between the systems and during 24.5 hours incubation.

The thickness of the liver slices was determined microscopically from the cross-section and it varied between 250 and 370 \mu m, as is shown in Table 3.

The morphology of the liver slices was evaluated by two independent investigators, and the results are shown in Table 4. Histomorphology showed that after 24.5 h of incubation, the liver slices in the stirred well system were severely damaged, that is, alterations in nuclear shape, staining intensity of the cytoplasm is low, and damage of the overall integrity of the slice. The liver slices in the other incubation systems retained relatively intact morphology up to 24.5 h. Central necrosis was seen in some of the thicker slices (\( \pm 500 \mu m \)) that occasionally were found. After 24.5 h, a major bacterial infection could be detected in the liver slices of some of the experiments of the rocker system.

Figure 2 illustrates the energy charge of the liver slices directly after slicing and after various incubation times in the incubation systems. The energy charge was low immediately after slicing and it appeared to take at least 1.5 h before the energy charge increased. The energy charge was retained up to 24.5 h in the liver slices of three of the four incubation systems tested with the exception being the stirred well incubation system where the energy charge dropped. The energy charge of the liver slices from the stirred well were 17% lower compared to slices in the other three incubation systems. The potassium content of the liver slices was low directly after slicing, and at least 1.5 h of incubation at 37°C was needed before it was restored. However, the liver slices in the stirred well incubation system seemed unable to restore the potassium content in the slice. The liver

### Table 1. Wet weight of liver slices (in mg wet weight) after the incubation periods (in hours) in the various incubation systems.

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Shaken flask</th>
<th>Stirred well</th>
<th>Rocker platform</th>
<th>Roller system</th>
<th>6-Well shaker</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>23.4 ± 1.1 (33)</td>
<td>11.8 ± 0.6 (36)</td>
<td>9.0 ± 0.5 (36)</td>
<td>15.7 ± 0.8 (12)</td>
<td>10.6 ± 0.6 (9)</td>
</tr>
<tr>
<td>0.5</td>
<td>19.4 ± 0.7° (62)</td>
<td>12.7 ± 0.7° (63)</td>
<td>7.1 ± 0.4° (63)</td>
<td>11.2 ± 0.5° (30)</td>
<td>11.6 ± 0.5° (26)</td>
</tr>
<tr>
<td>1.5</td>
<td>19.2 ± 0.8° (60)</td>
<td>12.6 ± 0.6 (63)</td>
<td>6.0 ± 0.5° (65)</td>
<td>11.2 ± 0.8° (30)</td>
<td>10.7 ± 0.4° (26)</td>
</tr>
<tr>
<td>24.5</td>
<td>14.8 ± 0.6° (60)</td>
<td>9.8 ± 0.5° (63)</td>
<td>4.5 ± 0.2° (57)</td>
<td>8.6 ± 0.7° (12)</td>
<td>7.8 ± 0.5° (27)</td>
</tr>
</tbody>
</table>

All values are the mean ± SE (N).

\*p < 0.05 versus 0 h; \*p < 0.005 versus 0.5, and 1.5 h; \*p < 0.0001 versus 0.5 and 1.5 h.
Table 2. The wet weight to dry weight ratio after the incubation periods (in hours) in the various incubation systems

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Shaken flask</th>
<th>Stirred well</th>
<th>Rocker platform</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>5.5 ± 1.0</td>
<td>4.8 ± 1.1</td>
<td>7.4 ± 1.5</td>
</tr>
<tr>
<td>1.5</td>
<td>5.1 ± 0.7</td>
<td>6.1 ± 1.3</td>
<td>6.0 ± 1.1</td>
</tr>
<tr>
<td>24.5</td>
<td>4.9 ± 0.9</td>
<td>5.3 ± 1.2</td>
<td>6.8 ± 1.2</td>
</tr>
</tbody>
</table>

All values are the mean ± SE. The mean of three experiments with three slices at each time point.

Table 3. Thickness of liver slices (in µm) after the incubation periods (in hours) in the various incubation systems

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Shaken flask</th>
<th>Stirred well</th>
<th>Rocker platform</th>
<th>Roller system</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>367 ± 68</td>
<td>250 ± 37</td>
<td>258 ± 42</td>
<td>250 ± 14</td>
</tr>
<tr>
<td>0.5</td>
<td>350 ± 50</td>
<td>263 ± 39</td>
<td>375 ± 10</td>
<td>250 ± 10</td>
</tr>
<tr>
<td>1.5</td>
<td>417 ± 68</td>
<td>313 ± 42</td>
<td>263 ± 13</td>
<td>267 ± 36</td>
</tr>
<tr>
<td>24.5</td>
<td>292 ± 22</td>
<td>306 ± 54</td>
<td>300 ± 38</td>
<td>225 ± 38</td>
</tr>
</tbody>
</table>

All values are mean ± SE. The mean of three experiments with three slices at each time point.

Table 4. Histomorphological examination of liver slices after the incubation periods (in hours) in the various incubation systems

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Shaken flask</th>
<th>Stirred well</th>
<th>Rocker platform</th>
<th>Roller system</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.5</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>1.5</td>
<td>+&quot;</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>24.5</td>
<td>+&quot;</td>
<td>--</td>
<td>±</td>
<td>+&quot;</td>
</tr>
</tbody>
</table>

++ No or only minor histomorphologic changes; ± clear histomorphologic changes; + marked histomorphologic changes; -- severe damage.

*aCentral necrosis in one slice of one experiment.
*bBacterial infection present in two experiments.

The mean of three experiment (three slices per experiment were examined).

Slices in the other four incubation systems retained the potassium concentration at the level of 1.5 h up to 24.5 h (Figure 3). The potassium content found in liver tissue, taken directly after excision of the liver, was 85.6 ± 7.3 nmol potassium per mg wet weight of liver.

The MTT assay showed that in the liver slices after 1.5 h of incubation, no differences occurred in production of formazan, the reduction product of MTT. After 24.5 h of incubation, the liver slices in the stirred well and roller system appeared to have a significant lower rate of MTT metabolism (Figure 4). The LDH leakage was determined from liver slices in three out of five incubation systems. At up to 1.5 h of incubation, there was no difference in LDH leakage of the liver slices in the incubation system. After 24.5 h of incubation, the LDH leakage of the slices in the stirred well incubation was significantly higher compared to the liver slices in the other two incubation systems tested (Figure 5). Metabolism of testosterone was determined by 6β-hydroxytestosterone formation. After 0.5 h of incubation, the 6β-OH testosterone formation in the rocker platform and the 6-well shaker was significantly higher compared to the liver slices in the other systems. After 1.5 h of incubation these differences disappeared. After 24.5 h of incubation, the liver slices in the stirred well and the roller system showed a decline in formation of the metabolite. The shaken flask, the rocker platform, and the 6-well shaker retained testosterone metabolism after 24.5 h of incubation (Figure 6). The formation of the metabolite 6β-OH testosterone was representative for all metabolites that were formed by metabolism of testosterone in the liver slices. Phase I metabolism was also determined by lidocaine biotransformation, the formed metabolite MEGX was measured in the medium after 60 min of incubation of the liver slices with lidocaine. After 1.5 h the slices in the stirred well incubation system formed a significantly lower amount of MEGX compared to liver slices at the same incubation period in other incubation systems. After 24.5 h of incubation, the formation rate of MEGX was lower in the slices in the roller system and the stirred well system compared to the liver slices in the shaken flask, the rocker platform, and the 6 well shaker (Figure 7).

HMA was determined after 1 h of incubation of the liver slices with 10 mM of antipyrine (Table 5). At up to 1.5 h of incubation, the metabolic rate in the liver slices differed in the various incubation systems: the liver slices in the shaken flask produced significantly more HMA than the slices in the other incubation systems, but no decline in metabolic rate was observed in any of the incubation systems. After 24.5 h, only the liver slices in the rocker platform system preserved the ability to metabolize antipyrine at the same rate as after 1.5 h incubation. The liver slices in the shaken flask lost some of the activity to metabolize antipyrine, but activity was completely lost in the slices from the stirred well incubation system.

**Discussion**

The wet weight of the liver slices varied between the laboratories and was not completely in line with the thickness of the slices, which differed between the institutes from 250 to 370 µm. However, the thickness of the liver slices within each of the laboratories was reproducible. Results of the wet weight determination
showed that the liver slices lose weight during incubation, which could be explained by the loss of cells and/or the content of the outer cell layers as was confirmed by the histomorphological evaluation of the liver slices. This idea is supported by the observation that the loss in the liver slice thickness was not as pronounced as the decrease in wet weight. The fact that no severe change in wet weight to dry weight ratio was found indicates that no swelling occurred during incubation time. At up to 1.5 h, the difference in morphological appearance was small, and only after 24.5 h did the liver slices in the stirred-well incubation system show severe damage, reflecting pronounced cell death in the slice. A small percentage of the liver slices happened to be thicker than 500 μm. In these slices central necrosis occurred, which was also shown by others (Smith et al., 1985). The bacterial infection occurring in the slices in the roller system did not influence the morphology of the liver slices. Histomorphological examination showed that slices can be cultured up to 24.5 h, without severe changes of the morphology, as was shown before for the rocker platform (Leeman et al., 1995) and the roller system (Smith et al., 1985). Only the liver slices in the stirred-well incubation system deteriorated severely after 24.5 h.

Immediately after slicing, the energy charge in the
liver slices was low. Normal rat liver energy charge is 0.86 (Tanaka et al., 1992). It took at least 1.5 hours of preincubation before the energy charge reached a plateau. The energy charge is considered to be a precise indicator of the intracellular energy status of hepatocytes and is maintained by the balance between energy-yielding and energy-consuming processes (Atkinson, 1968). The liver slices in the stirred well were not capable of maintaining the energy charge up to 24.5 hours, showing again loss of viability after 24.5 hours.

The MTT assay, a test for the cellular reduction capacity both in mitochondria and extramitochondrially involving NADH and NADPH (Berridge and Tan, 1993) showed no loss of this capacity after 24.5 hours of incubation in two out of four incubation systems. The liver slices of the stirred well and the roller system deteriorated after 24 hours of incubation. This is in contrast to the findings of others (Fisher et al., 1995) who found no reduction of formazan production of the liver slices in the roller system after 24 hours. Difference of the LDH leakage only occurred after 24.5 hours of preincubation, the liver slices of the stirred well incubation system had a high LDH leakage.

A low concentration of potassium was measured immediately after slicing and at least 1.5 hours of
preincubation was necessary to restore the potassium content to 40–60 nmol potassium per mg wet weight liver and this potassium concentration was retained up to 24 hours. Only the liver slices in the stirred well were not able to restore the potassium content in the slice. From these data, it can be concluded that to ensure optimal viability of the liver slices they should at least be preincubated for 1.5 hours. Others found after 6 and 24 hours incubation (Fisher et al., 1995), concentrations of 80 nmol potassium per mg wet weight for the liver slices in dynamic organ culture system, the content of the liver slice in the multiwell culture plate system was 50 nmol potassium per mg wet weight. The potassium content in the liver slices in the present study was about 50% lower compared to the value found in rat liver tissue, but the other viability parameters were maintained over 24 hours. This indicates that different viability tests show different aspects of the function of the liver slice. Collectively, these viability results indicate that the stirred well incubation system is not capable of retaining the viability up to 24.5 hours.

Testosterone metabolism was comparable in liver slices of all incubation systems up to 1.5 hours, but after 24.5 hours the metabolic capacity deteriorated in the
slices of the roller system and the stirred well. These results are in line with those obtained with lidocaine. However, rat liver slices in the rocker platform, in the shaken flask and the 6 well shaker are capable of retaining the metabolic capacity for lidocaine and testosterone metabolism over 24 hours. This is in contrast to cultured hepatocytes, which lose 50–80% of their cytochrome P450 activity after 24 hours of culture (Paine, 1991; Wortelboer et al., 1990). Slices in the rocker platform and in the roller system are immersed half of the time in the medium and for the rest of the time are exposed to air. They had the same metabolic rate for lidocaine and testosterone as the liver slices in the shaken flask and 6 well shaker, which were agitated continuously in the medium. This may indicate that the diffusion into the slice and uptake into the cells of lidocaine and testosterone are not rate limiting in the metabolic conversion.

The results of antipyrine metabolism are not fully consistent with the results of lidocaine and testosterone. Only the liver slices in the stirred well incubation showed loss of metabolic capacity. The liver slices in the rocker platform retained the capacity for antipyrine metabolism over 24 hours. After 24 hours, the slices in the shaken flask lost 50% of the capacity to metabolize antipyrine but now formed HMA at the same rate as the slices in the rocker platform. The higher metabolic rate of antipyrine up to 1.5 hours of the liver slices in the shaken flask may be due to the continuously submerging of the slice in the medium, compared to the slices in the rocker platform incubation system, which are intermittently exposed to air and medium. The loss of HMA formation after 24 hours in the shaken flask may be due to specific loss of isoenzyme of Cytochrome P450 (CYP2B1), but more research on the rate limiting step of antipyrine metabolism in liver slices has to be performed. No phase II metabolites of HMA in liver slices were detected as was also found in rat hepatocytes (Palette et al., 1993). As was shown for the viability tests, metabolism capacity could be retained over 24 hours, even if the potassium retention was 50% of the in vivo value. This indicates again that viability and metabolism tests represent different aspects of the function of the liver slice. Consequently, we conclude that the ultimate function test should be chosen according to the experimental setting used.

The metabolism and viability results of the roller system in the present study were not in line with the results reported by others (Fisher et al., 1995). We found that the roller system was very sensitive to bacterial infection and that some liver slices were floating in the glass vial and subsequently were squeezed between the insert and the glass vial. This may partly explain the results of the decreased viability. The diminished metabolic rate may be at least partly explained by the bacterial infection of the liver slices. This may have activated Kupffer cells in the liver slice resulting in release of cytokines which can give rise to an impairment of cytochrome P450 activity (Monshouwer et al., 1996).

Adequate agitation of the medium may be an important factor in maintenance of the viability and metabolic parameters. The deterioration of liver slices in the stirred well incubation system may be due to a slower agitation of the medium. In pilot experiments with the stirred well incubation system, we found that lipophilic fluorescence compounds penetrated the liver slices better via the side that is laying on the grid, than via the side of the liver slice facing the medium. This indicates a lower supply of substrate from the upper side, probably due to a limited agitation. This may also account for the lower antipyrine metabolism in the liver slices of the stirred well incubation system. The similar metabolic rate for lidocaine and testosterone in the stirred well incubation system also supports the hypothesis that for these substrates the supply is not rate limiting.

We did not find support for the hypothesis that for optimal oxygen transfer into the liver slice intermittent exposure of the liver slices to air and medium is essential, as was published by others (Smith et al., 1985). We also showed that the incubation system with 40% O₂ gave the same results as incubation systems with 95% O₂.

In conclusion: liver slices incubated up to 1.5 hours function similarly in all five incubation systems, but after 24 hours the rocker platform, the shaken flask and 6 well shaker appear to be superior.

References


Olinga P, Merema MT, Hof HI, Visser J, Meijer D, and Groothuis GMM (Submitted) Fast analysis of ATP, ADP, AMP and adenosine with gradient ion-pair reversed-phase liquid chromatography as viability test for hepatocytes.


