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## ***In vitro* evaluation of a novel bioreactor based on an integral oxygenator and a spirally wound nonwoven polyester matrix for hepatocyte culture as small aggregates**

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**Background/Aims:** The development of custom-made bioreactors for use as a bioartificial liver (BAL) is considered to be one of the last challenges on the road to successful temporary extracorporeal liver support therapy. We devised a novel bioreactor (patent pending) which allows individual perfusion of high density cultured hepatocytes with low diffusional gradients, thereby more closely resembling the conditions in the intact liver lobuli.

**Methods:** The bioreactor consists of a spirally wound nonwoven polyester matrix, i.e. a sheet-shaped, three-dimensional framework for hepatocyte immobilization and aggregation, and of integrated hydrophobic hollow-fiber membranes for decentralized oxygen supply and CO<sub>2</sub> removal. Medium (plasma *in vivo*) was perfused through the extrafiber space and therefore in direct hepatocyte contact. Various parameters were assessed over a period of 4 days including galactose elimination, urea synthesis, lidocaine elimination, lactate/pyruvate ratios, amino acid metabolism, pH, the

last day being reserved exclusively for determination of protein secretion.

**Results:** Microscopic examination of the hepatocytes revealed cytoarchitectural characteristics as found *in vivo*. The biochemical performance of the bioreactor remained stable over the investigated period. The urea synthesizing capacity of hepatocytes in the bioreactor was twice that of hepatocytes in monolayer cultures. Flow sensitive magnetic resonance imaging (MRI) revealed that the bioreactor construction ensured medium flow through all parts of the device irrespective of its size.

**Conclusions:** The novel bioreactor showed encouraging efficiency. The device is easy to manufacture with scale-up to the liver mass required for possible short-term support of patients in hepatic failure.

**Key words:** Aggregates; Bioartificial liver; Bioreactor; 3D-matrix; Hepatocytes; Liver support; MRI; Oxygenator; Polyester.

THE DEVELOPMENT of a liver support system for the treatment of patients with fulminant hepatic failure and as a bridge to liver transplantation is a major challenge. Many early attempts focused on blood detoxification based on the assumption that liver failure could be reversed if the associated toxins were removed from the circulation of the patient (1,2). Although improvement of the neurological status of patients has

been reported, none achieved long-term survival (3,4). It was therefore concluded that an effective liver support system should be able to perform the liver's multiple synthetic and metabolic functions, including detoxification and excretion. The most logical approach to this problem is the introduction of active functioning hepatocytes (5). The state-of-the-art embodiment of this theory is presented in the bioartificial liver (BAL), an extracorporeal device comprising well nourished and oxygenated viable hepatocytes immobilized on a mechanical support and separated from the blood circulation by semipermeable membranes.

Different bioreactor systems are currently under investigation. They can be classified according to the immobilization technique used: artificial substrates, such

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as glass plates (6), microcarriers (7,8), hollow-fiber membranes (9–13), biological matrices (14–17), encapsulation (18,19) and three-dimensional carrier materials (20). Objectives like biocompatibility, maintenance of functional capacity and practicality, important aspects in the development of a BAL, have been discussed in recent reviews (12,21–24). However, an ideal BAL system has not yet been invented. In this respect, the impact of bioreactor construction on hepatocyte function has been undervalued. Every hepatocyte in the intact liver functions under perfusion conditions and close blood contact. The majority of the current bioreactor designs do not meet these conditions, which are essential for optimal substrate and metabolite exchange to hepatocytes. This often results in non-physiological gradients, which impair the metabolic activity of the cultured cells.

Therefore, an important aim of this study was to develop a bioreactor configuration that allows high density hepatocyte culture and simultaneously ensures that every hepatocyte operates under *in vivo* like perfusion conditions and direct medium contact. In addition, we wanted to culture hepatocytes as small aggregates, known to maintain many of the cytoarchitectural characteristics found *in vivo* and to exhibit higher and more prolonged functional activity than hepatocytes cultured in monolayers (25–27). Special attention was paid to optimal oxygenation of the cells, since oxygen plays an important role in hepatocyte attachment (28) and function (29,30). Therefore, the bioreactor was equipped with an integrated oxygenator. This enables on site oxygenation of the hepatocytes with low gradients, irrespective of the plasma perfusion rate, which can not be realized with a separate, external oxygenator as found in most BAL systems. Another goal was to develop a bioreactor that could be scaled-up to incorporate sufficient cell mass for possible therapeutic liver support. These aspects resulted in a novel bioreactor comprising a spirally wound 3D nonwoven polyester matrix with integrated oxygenation hollow fiber membranes (Fig. 1) in which hepatocytes immobilize and spontaneously reorganize as small aggregates.

In this article, we present the characteristics of our novel culture device and the results of its assessment *in vitro*. The bioreactors were investigated over a period of 3 days, as their future application as a BAL will be shortly after hepatocyte immobilization.

## Materials and Methods

### Hepatocyte isolation

Pig livers were kindly provided by the Department of Clinical and Experimental Cardiology, the Department of Dermatology, and a local slaughterhouse. The

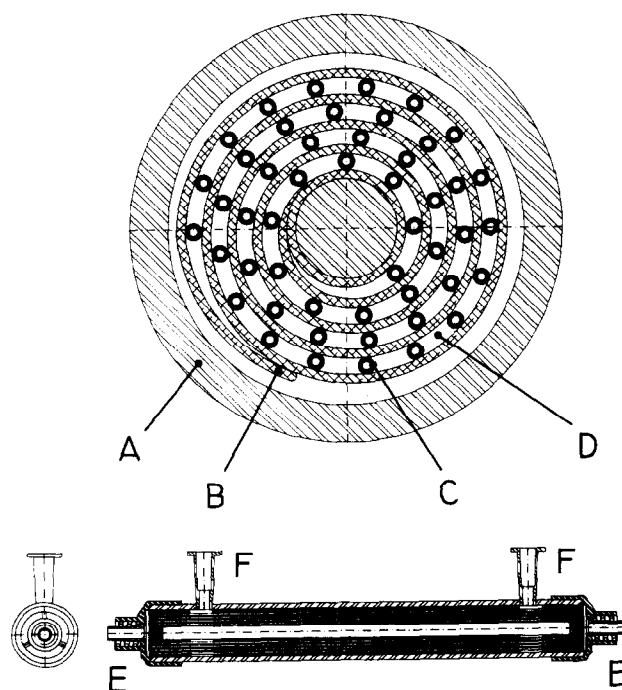


Fig. 1. Schematic drawing of a transverse and longitudinal cross-section of the bioreactor. The system is composed of a polysulfon dialysis housing (A) comprising a three-dimensional nonwoven polyester matrix (B) for high density hepatocyte culture as small aggregates and hydrophobic polypropylene hollow-fiber membranes (C) for oxygen supply and CO<sub>2</sub> removal. Medium is perfused through the extrafiber bioreactor space via the side ports (F) and is in direct cell contact. Despite high density culturing there is sufficient space between the aggregates for unhindered transport of medium (plasma *in vivo*) to and from the hepatocytes. The bioreactor is perfused with culture gas via the endcaps (E). The spirally wound polyester matrix guarantees a homogeneous distribution of the oxygenation hollow-fibers throughout the bioreactor compartment, thereby ensuring every hepatocyte of an oxygenation source within its direct surroundings. Moreover, the hollow-fibers act as spacers between the layers of the 3D-matrix, creating numerous channels (D) which form a defined space for uniform flow and distribution of medium to all parts of the solid support.

hepatocytes were isolated from pigs with a body mass of 20–25 kg using a simple two-step collagenase perfusion technique as described previously (31). The viability of the isolated cells, based on trypan blue exclusion, varied from 71 to 96% ( $n=8$ , mean 89%). The yield varied from  $8 \cdot 10^6$  to  $30 \cdot 10^6$  hepatocytes per g wet liver weight.

### Bioreactor

The bioreactor (patent pending) consists of a 3D nonwoven polyester matrix specifically designed for cultur-

ing anchorage-dependent cells (Fibra Cell, Bibby Sterilin Ltd, Stone, Staffordshire, UK) and hydrophobic polypropylene hollow fiber membranes donated by Dr. J. Vienken of AKZO-NOBEL (Plasmaphan, AKZO-NOBEL, Wuppertal, Germany) for oxygenation and carbon dioxide removal. The 3D matrix (dimensions: length 140 mm, width 90 mm, thickness 0.5 mm, fiber diameter 13  $\mu\text{m}$ ) provides a scaffold for hepatocyte immobilization and self-aggregation. Its surface for attachment is about 15 times its projected area, which enables high density hepatocyte culture. The oxygenation hollow-fibers (external diameter 630  $\mu\text{m}$ , internal diameter 300  $\mu\text{m}$ ) are fixed to the 3D-carrier in a parallel fashion by weaving, spaced at an average distance of 2 mm. This polyester-polypropylene composite is spirally wound like a Swiss roll with the help of an acrylic core (Fig. 1) and placed in a polysulfon dialysis housing (Minifilter, Amicon Ltd, Ireland, ID 1.32 cm, ED 1.7 cm, total length 15.5 cm). The oxygenation hollow-fibers are embedded in polyurethane resin (PUR-system 725 A and 725 BF, Morton International, Bremen, Germany) using dialyzer potting techniques and fitted with gas inlet and outlet endcaps. The bioreactor is sterilized by autoclaving (20 min at 121°C). Hepatocyte seeding in the extrafiber space (volume 11 ml, suitable for *in vivo* experiments in the rat) is achieved by injecting the cell suspension via the side ports normally used for dialysate flow. The same ports are used for medium perfusion after cell immobilization.

#### *Flow sensitive magnetic resonance imaging*

The flow distribution in a cross-section of a small (ID 1.32 cm, volume 11 ml, 46 hollow-fiber membranes, diameter acrylic core 0.4 cm) and a scaled-up bioreactor (ID 2.2 cm, volume 33 ml, 138 hollow-fiber membranes, diameter acrylic core 0.4 cm) of equal length was investigated. The bioreactors were first flushed with ethanol and subsequently water to remove air bubbles, which can block the medium flow and/or can distort the homogeneity of the magnetic field, resulting in a decreased signal intensity. A bioreactor was then placed in a birdcage coil and positioned horizontally in a 6.3 Tesla/20 cm bore home-built spectrometer. Cell-free devices were used as the spectrometer was not equipped to support viable hepatocytes. Transaxial flow sensitive MRI's were taken from the middle of the bioreactor using a novel perfusion imaging technique (32). Briefly, the water signal in a detection slice (width 2 mm, perpendicular to the flow direction) is suppressed. During an in-flow time of 100 ms, part of the slice is refreshed, resulting in an increase in signal intensity. Thus, the higher the flow, and the more the detection slice is refreshed, the more the signal inten-

sity will increase. Fluid flow at higher velocities than 2 cm/s will not result in an increased signal, as the detection slice is then completely refreshed. Therefore, the flow was calibrated such that the maximum fluid velocity in most flow channels (D in Fig. 1) did not exceed 2 cm/s.

#### *Hepatocyte culture*

Hepatocytes suspended in ice-cold Williams' E medium (Gibco BRL Life Technologies, European Division) supplemented with heat-inactivated FCS (10%, Boehringer Mannheim), glutamine (2 mM, BDH Laboratory Supplies Ltd.), insulin (20 mIU, Novo Nordisk, Denmark), dexamethasone (1  $\mu\text{M}$ ) and antibiotic/antimycotic solution (Gibco) at a concentration of  $20 \cdot 10^6$  viable cells per ml were injected into two precooled (4°C) dry bioreactors until each of the units contained  $220 \cdot 10^6$  cells per unit. The cooled bioreactors were integrated into two separate cell perfusion circuits to obtain results in duplicate. The whole apparatus was put in a temperature regulated (37°C) cabinet (Stuart Scientific, model SI60, GB) where the bioreactors were clamped onto a custom made rotation device and connected to "culture gas" (95% air and 5% CO<sub>2</sub>, flow rate: 30 ml/min). The reactors were rotated horizontally along their longitudinal axis at 1 revolution/min for a period of 120 min to secure an even distribution of the cells throughout the reactor and to accelerate immobilization by entrapment, attachment, and self-aggregation of viable hepatocytes. After this immobilization period, old medium was replaced by fresh medium (60 ml) intermittently for 15 h to flush dead and unattached cells out of the reactor, to supply nutrients to and to remove toxins from the cells, and to allow the hepatocytes to recover from the isolation procedure. The devices were then considered to be ready for use.

#### *Hepatocyte function tests*

*General description.* Bioreactors with and without hepatocytes were studied. Those without hepatocytes served as controls. Both groups received identical treatment and monitoring. Hepatocyte function tests were performed while supplemented Williams' E medium (30 ml) was recirculated through the extrafiber bioreactor space at a flow rate of 5 ml/min. Various parameters were assessed over a period of 4 days, the last day being reserved exclusively for determination of protein secretion under serum free conditions. On every day during the first 3 days a battery of tests was carried out, including galactose elimination, urea synthesis, lidocaine metabolism, and a subsequent 14-h incubation with supplemented Williams' E medium to

evaluate the amino acid metabolism, lactate/pyruvate ratio, enzyme leakage, glucose levels, and pH. Every test was preceded by a fresh medium waste wash. Samples collected from the closed loop circuit were snap frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  prior to analysis. It was not feasible to quantify the hepatocytes in the device by evaluating the total protein and/or DNA content, as the cells were too entrapped within the nonwoven polyester matrix for total harvesting. Therefore, quantification relied on microscopic cell counting before inoculation.

**Galactose elimination.** D-Galactose (Sigma Chemical Co., St Louis, MO) was administered to the closed loop circuit at a concentration of 1 mg/ml and incubated for 3 h. Media samples were collected at different time points every day for 3 days. The galactose concentration was measured at 340 nm (Cobas Bio, Roche, Switzerland) using enzymatic test kits (Boehringer Mannheim, Wiesbaden, Germany, kit no. 124273). The amount of galactose eliminated was calculated from these data.

**Urea synthesis from  $\text{NH}_4\text{Cl}$ .** The urea-synthesizing capacity of hepatocytes cultured in the bioreactor was compared to that of hepatocytes in monolayer cultures. Hepatocytes of the same isolation were seeded in the bioreactor and on two wells of a 6-well tissue culture plate (Becton Dickinson Labware, MA) at  $3 \cdot 10^6$  viable cells per well. 10 mM  $\text{NH}_4\text{Cl}$  was added to the closed loop circuit and the monolayer cultures and incubated for 2 h. Media samples were collected at different time points every day for 3 days. To quantify the hepatocytes of the monolayer cultures, the DC protein assay from Bio-Rad (Hercules, CA) was used. Urea nitrogen was determined colorimetrically at 525 nm (Zeiss MQ3 UV spectrophotometer, Germany) with Sigma Chemical Co. kit no. 535. The amount of urea synthesized was calculated from these urea nitrogen data.

**Lidocaine metabolism.** Lidocaine-HCl (Sigma) was administered to the closed loop circuit at a concentration of 500  $\mu\text{g}/\text{ml}$  and incubated for 1 h. Media samples were collected at different time points every day for 3 days. The samples were analyzed for lidocaine and three lidocaine metabolites, mono-ethyl-glycine-xylydide (MEGX), 2,6-xylydine-HCl, glycine-xylydide (GX), by reversed phase high performance liquid chromatography (HPLC). Lidocaine-HCl was obtained from Sigma Chemical Co. and MEGX, xylydine, GX, and ethyl-methyl-glycine-xylydide (EMGX) were gifts from Dr. R. Sandberg of Astra Pain Control (Södertälje, Sweden).

Sample preparation for the analysis of MEGX, xylydine and GX involved addition of an 75  $\mu\text{l}$  internal

standard solution (EMGX 5  $\mu\text{g}/\text{ml}$  in distilled water) and 150  $\mu\text{l}$  distilled water to a 150  $\mu\text{l}$  sample. Analysis of the much higher lidocaine concentrations required a 20-fold dilution of the sample in supplemented Williams' E medium. The isolation of lidocaine and its metabolites was performed by extraction. For this, 150  $\mu\text{l}$  sodium carbonate (0.1 M) and 600  $\mu\text{l}$  chloroform were added to the sample preparation. After 1 min vortexing and 4 min centrifugation at 8000 rpm the aqueous supernatant was removed and 150  $\mu\text{l}$  distilled water and 350  $\mu\text{l}$  HCl (0.1 M) were added to the organic phase. The vortexing and centrifugation procedures were repeated and the supernatant removed. A cooled sample storage compartment kept the residues at  $4^{\circ}\text{C}$  prior to analysis. The mobile phase (0.5 M phosphate buffer, pH 4.5) was pumped at a flow rate of 1.7 ml/min (Perkin Elmer 250, Norwalk, USA) and pretreated by a Guard-column (Superspher 60 RP 8, length 10 cm, 4  $\mu\text{m}$  particles, Bischoff Chromatography, Germany). An auto sampler (Gilson Sample Injector model 231, France) injected 50  $\mu\text{l}$  aliquots onto a temperature regulated ( $55^{\circ}\text{C}$ , Chrompac Column Thermostat, The Netherlands) HPLC column (Superspher 60 RP 8, length 20 cm, ID 4.6 mm, 4  $\mu\text{m}$  particles, Bischoff Chromatography, Germany). Detection was at 198 nm (Schoeffel SF 770 UV-spectrophotometer, Germany) and peak areas were calculated with the aid of an Olivetti M250 computer utilizing integration software (Chrompac PCI, version 5.12, The Netherlands). The samples were quantified by comparing the peak area ratio of the component of interest to that of the internal standard. Standard curves were obtained for lidocaine (5–80  $\mu\text{g}/\text{ml}$ ), MEGX (0.5–16  $\mu\text{g}/\text{ml}$ ), xylydine (5–80  $\mu\text{g}/\text{ml}$ ), and GX (1–32  $\mu\text{g}/\text{ml}$ ) and showed linearity ( $r=0.996$ ,  $n=6$ ). The detection limit was 0.4  $\mu\text{g}/\text{ml}$  for GX, 0.3  $\mu\text{g}/\text{ml}$  for xylydine, 0.2  $\mu\text{g}/\text{ml}$  for MEGX, 0.4  $\mu\text{g}/\text{ml}$  for EMGX and 0.5  $\mu\text{g}/\text{ml}$  for lidocaine. The retention times for these components were 2.2 min, 2.4 min, 3.2 min, 4.1 min, and 5.8 min, respectively. Column stabilization time was limited to 20 min by washing with a phosphate-acetonitrile-phosphoric acid buffer (50 mM, pH=1.7) and an acetonitrile solution (distilled water: ACN=1:1) to remove the chloroform peak.

**Amino acid metabolism.** The metabolic turnover of a wide range of amino acids was investigated. The amino acid concentrations were determined by a fully automated precolumn derivatization with o-phthalaldehyde (OPA), followed by high-performance liquid chromatography as described previously (33).

**Lactate/pyruvate ratio.** Levels of lactate and pyruvate were determined at 340 nm (Cobas Bio, Roche, Switzerland) using enzymatic test kits (Boehringer

Mannheim Wiesbaden, Germany, lactate kit no. 149993 and pyruvate kit no. 124982).

**Enzyme leakage.** Lactate dehydrogenase (LDH), glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) levels were measured by routine clinical analyzers.

**Glucose.** Glucose levels were measured using glucose test strips (hemoglucotest 1-44 R, Boehringer Mannheim, Wiesbaden, Germany) and the accessory Reflux-S readout device.

**pH.** The pH was measured by sampling 1 ml of medium in a bloodgas syringe (Marz-175, Sherwood Medical, Ireland) which was determined on a bloodgas analyzer (Radiometer, model ABL 300, Copenhagen).

**Protein secretion.** On day 4, the entire bioreactor culture system was washed with 250 ml supplemented Williams' E medium without FCS and incubated in the same medium. The same procedure was performed for control bioreactors (which do not incorporate hepatocytes) to investigate the possible contribution of protein release from the bioreactor as a result of the 3-day perfusion with supplemented Williams' E medium with FCS. Media samples were collected after 24 h and dialyzed extensively against a 50 mM  $\text{NH}_4\text{HCO}_3$  solution and then freeze-dried. The dry residues were reconstituted in an electrophoresis buffer (Tris-barbital buffer, pH=8.6, ionic strength 0.1) to a concentration 20 times greater than the culture supernatant.

To visualize the individual serum proteins secreted by the pig hepatocytes we performed crossed-over immunoelectrophoresis as described previously (31). Briefly, proteins in the concentrated culture supernatant were separated electrophoretically in a 1% agarose gel (80 min, 10 V/cm) in the first dimension. In the second dimension the separated proteins were electrophoresced into an antiserum raised against pig serum proteins containing agarose gel (22 h, 80 V/cm), resulting in a number of precipitating peaks, each peak representing an individual protein. When equal amounts of the same antiserum are used in each plate, the relative concentrations of the various proteins in the sample can be determined, as the area contained by each peak is directly proportional to the amount of antigen in the sample. The antiserum used was prepared in a rabbit (New Zealand White, 1.5 kg) by several injections of pig serum.

#### *Microscopic examination*

**Light microscopy.** Hepatocytes from 5-day-old cultures were fixed by flushing the bioreactors ( $n=3$ ) with 4% formalin. After 24 h the bioreactors were cut open and 12 matrix samples ( $1\text{ cm}^2$ ) were taken from various parts of the nonwoven polyester matrix. The samples

were washed in water, dehydrated in graded ethanols, and embedded in paraffin. Ultrathin ( $8\ \mu\text{m}$ ) sections were cut from this block. These were deparaffinated with xylol and stained with hematoxylin-eosin. The preparations were examined under an Olympus Vamox light microscope (type AHBT3, Tokyo, Japan).

**Scanning electron microscopy.** SEM studies were performed after fixation of a 5-day-old hepatocyte culture by flushing one bioreactor with 4% glutaraldehyde in phosphate buffer, pH 7.3 (Fluka Chem A.G., Buchs, Switzerland). The bioreactor was cut through in the middle and one part was dehydrated in graded ethanols and finally dried in hexamethyldisilazane (Sigma, Munich, Germany). The cut surface was coated with gold in a sputter coater and examined under a scanning electron microscope (ISI SS40, Japan).

**Transmission electron microscopy.** A 4-day-old hepatocyte culture was fixed by flushing one bioreactor with 4% paraformaldehyde. Cellular aggregates were mechanically stripped from the nonwoven polyester matrix. The specimens were postfixed in 1%  $\text{OsO}_4$  (in cacodylate buffer) for 15 min, block-stained with 1% uranyl acetate, dehydrated in 2,2-dimethoxypropane, and embedded in epoxy resin. Ultrathin sections of the hepatocyte aggregates were examined with an EM 10 electron microscope (Philips, The Netherlands).

#### *Statistical analysis*

An unpaired Student's *t*-test was used, and  $p < 0.05$  was considered to be statistically significant. Data are presented as mean  $\pm$  SEM.

## **Results**

#### *Flow sensitive magnetic resonance imaging*

Fig. 2 displays the flow distribution in a cross-section of a small (A) and a scaled-up bioreactor (B). The fluid velocity was detected only in the axial direction and ranged from zero (black) to around 2 cm/s (white). When compared with Fig. 1 several components of the bioreactor can be identified, such as the nonwoven polyester fabric, the oxygenation hollow-fiber membranes, the flow channels, and the acrylic core. The black representation of the nonwoven polyester fabric indicates only that medium flow within the 3D-matrix was not in the axial direction. The perfusion of the fabric in other directions was not investigated. The homogeneous distribution of the gray spots demonstrates that all flow channels in both devices were perfused. The shades of gray indicate that the fluid velocity could differ per flow channel (ranging from 0.5 to 2 cm/s, but mostly around 1.5 cm/s). The arrows in Fig. 2B show spots of decreased signal intensity as a result of entrapped air bubbles. Spin echo images (not

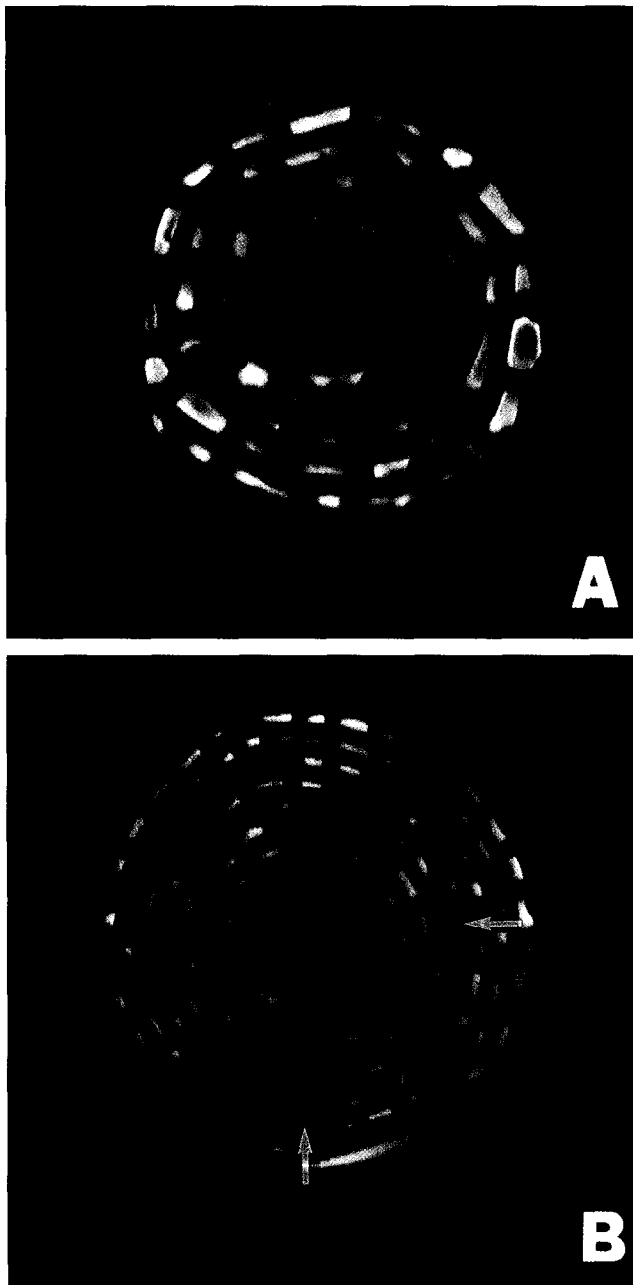


Fig. 2. Transaxial flow sensitive MRI's of a small (A, internal diameter 1.32 cm) and a scaled-up bioreactor (B, internal diameter 2.2 cm). The fluid velocity ranged from zero (black) to around 2 cm/s (white). When compared with Fig. 1 several components of the bioreactor can be identified, such as the nonwoven polyester fabric, the oxygenation hollow-fiber membranes, the flow channels, and the acrylic core. The images of both devices show that all flow channels were perfused. Differences in the fluid velocity of the flow channels can be observed. The arrows in Fig. 2B indicate spots of decreased signal intensity as a result of entrapped air bubbles.

shown) revealed that the size of the air bubbles was much smaller than the resulting distortion. The spec-

trometer only allowed a horizontal orientation of the bioreactor. Normally, the device is positioned vertically, which facilitates the removal of air bubbles.

*Hepatocyte culture*

The study involved the construction of 22 bioreactors, of which 16 devices ( $n=8$  in duplicate) were used to culture hepatocytes and six devices without cells ( $n=3$  in duplicate) served as controls. The results of the hepatocyte function tests in two bioreactors with cells from the same isolation procedure never differed more than 10%, indicating reproducible cell immobilization and cultivation. The culture system remained sterile throughout the study and no leaking of medium into the lumen of the oxygenation hollow-fibers was observed (34).

*Hepatocyte function tests*

*Galactose elimination.* Fig. 3 shows that the galactose elimination capacity after incubation for 180 min with a standard dose of galactose remained constant over a period of 3 days.

*Urea synthesis.* Since high levels of ammonia are

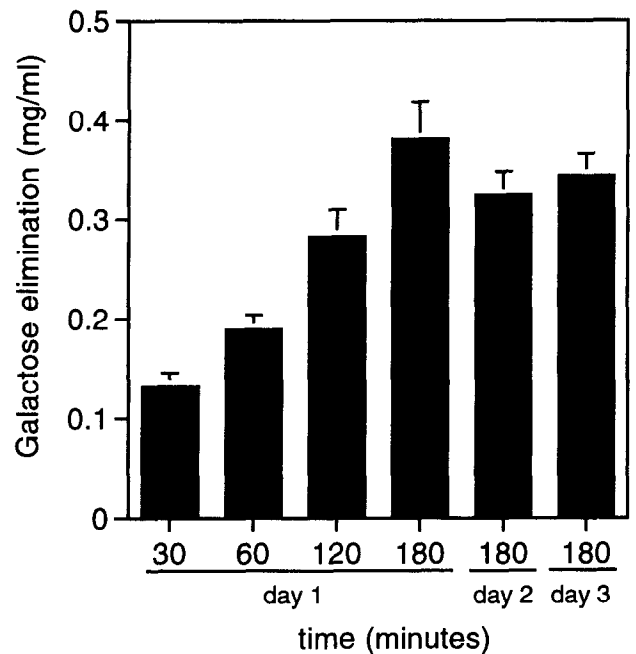


Fig. 3. Galactose elimination by porcine hepatocytes cultured for 3 h in the bioreactor inoculated with  $220 \cdot 10^6$  viable cells. 1 mg/ml galactose was added to the closed loop circuit and incubated for 3 h every day for 3 days. Medium samples were collected at different time points on day 1 and after 3 h on days 2 and 3, after which the galactose concentration was determined. Results are expressed as the mean of seven experiments in duplicate  $\pm$  SEM.

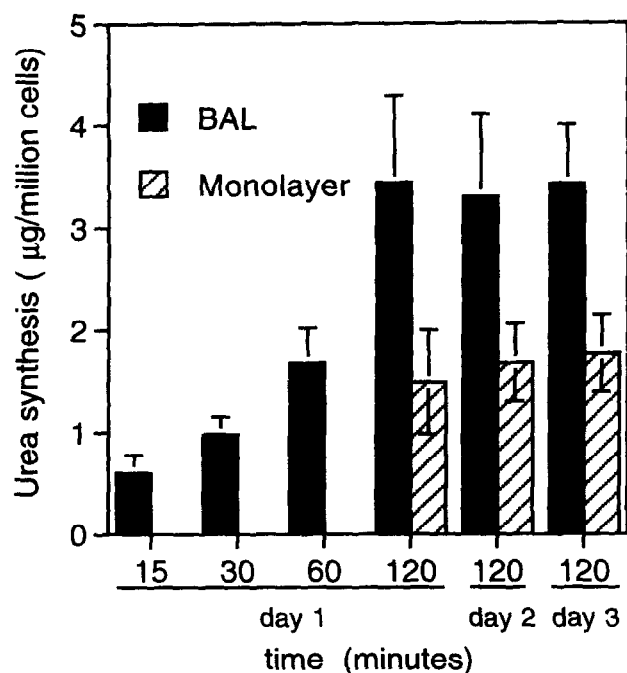


Fig. 4. Urea synthesis by porcine hepatocytes cultured for 2 h in the bioreactor inoculated with  $220 \cdot 10^6$  viable cells and as monolayers at  $3 \cdot 10^6$  viable cells per well. 10 mM  $\text{NH}_4\text{Cl}$  was added to both culture systems and incubated for 2 h every day for 3 days. Medium samples of the bioreactor were collected at different time points on day 1 and after 2 h on days 2 and 3, and in monolayer cultures after 2 h on days 1 to 3, after which the urea concentration was determined. Results are expressed as the mean of eight experiments in duplicate  $\pm$  SEM.

considered to play a role in hepatic encephalopathy (35), we tested the bioreactor's ability to synthesize urea from ammonia. The efficacy of the device to support hepatocytes was evaluated by comparing the urea-synthesizing capacity of the hepatocytes in the bioreactor to that of hepatocytes in monolayer cultures. The results in Fig. 4 show that the urea synthesis in both culture systems, after a 120 min incubation with 10 mM  $\text{NH}_4\text{Cl}$ , did not vary over a period of 3 days. The urea-synthesizing capacity of the hepatocytes cultured in the bioreactor was twice as high as hepatocytes cultured as monolayers.

**Lidocaine metabolism.** The cytochrome P450 activity of the hepatocytes was assessed by determining lidocaine and its metabolites (Fig. 5). The lidocaine elimination and subsequent MEGX and xylidine production after a 60 min lidocaine incubation did not significantly change over a period of 3 days. Xylidine was the main lidocaine metabolite during the first 2 days. There was no significant difference in xylidine and MEGX production on day 3. When looking at individ-

ual experiments, lidocaine clearance correlated better with xylidine than MEGX formation. Porcine hepatocytes did not produce detectable levels of the metabolite GX during incubation with lidocaine for 1 h.

**Amino acid metabolism.** Table 1 shows the changes in the medium concentration of some amino acids that

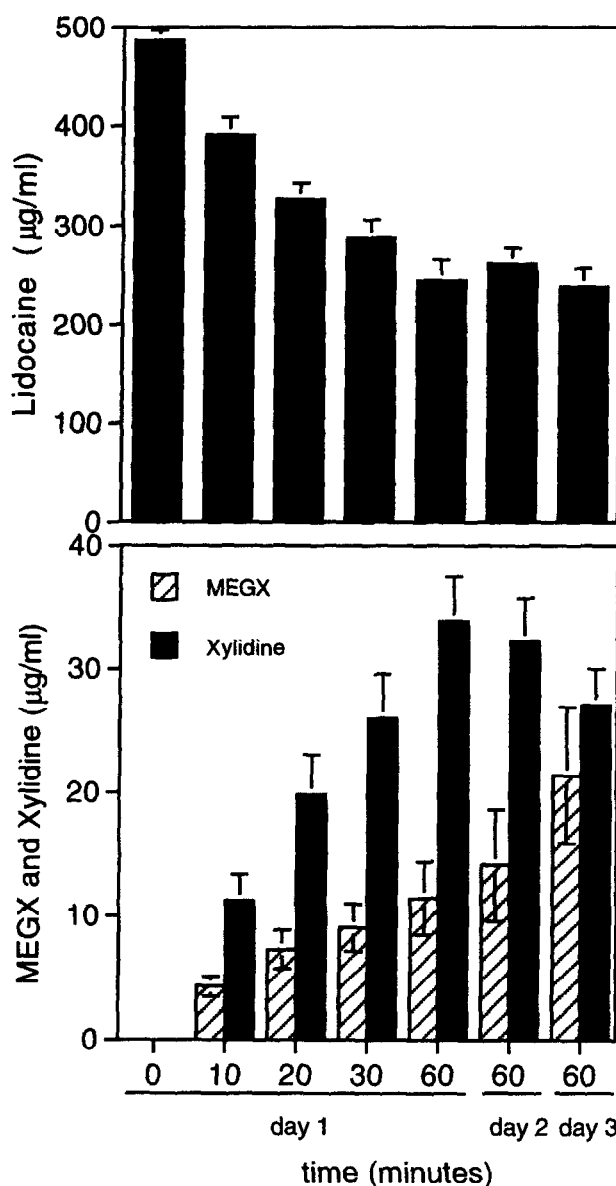


Fig. 5. Lidocaine elimination and subsequent MEGX and xylidine formation by porcine hepatocytes cultured for 1 h in the bioreactor inoculated with  $220 \cdot 10^6$  viable cells. 500  $\mu\text{g/ml}$  lidocaine was added to the closed loop circuit and incubated for 1 h every day for 3 days. Medium samples were collected at different time points on day 1 and after 1 h on days 2 and 3, after which the lidocaine, MEGX, and xylidine concentration were determined. Results are expressed as the mean of seven experiments in duplicate  $\pm$  SEM.



are relevant for liver function (36,37). A decrease in glutamine concentration was associated with an increased glutamate concentration. Liver metabolism of aromatic amino acids was reflected by a decrease in the concentrations of phenylalanine, tyrosine, and tryptophan. Decreased arginine concentrations and synthesis of ornithine are indicative of arginase activity. A decrease in alanine concentration, a precursor of liver gluconeogenesis, was observed.

Other amino acids concentrations which decreased statistically significantly were asparagine, glycine, histidine, valine, methionine, isoleucine, leucine, and lysine (data not shown in Table 1). The observed changes in amino acid concentrations were similar on days 1, 2, and 3.

**Lactate/pyruvate ratio.** The lactate/pyruvate ratio is an index of the functional state of cellular oxidation and aerobic metabolism (38). Table 1 shows a drop in the lactate/pyruvate ratio, which was solely due to a decline in the lactate concentration. The lactate/pyruvate ratio of 5 to 7 reflected physiological oxygenation of the culture system over a period of 3 days.

**Enzyme leakage.** To assess hepatocyte viability, the appearance of enzyme activity, namely LDH, GOT, and GPT, was determined in the culture medium. LDH release was only significant on day 1 (Table 1). GOT liberation was significant and tended to fall over the 3-day period. Low but significant quantities of GPT were released on days 1 and 2.

**Glucose.** Glucose levels did not change during first

day of culture (Table 1). A significant decrease in the glucose concentration was observed on days 2 and 3.

**pH.** The pH in the studied bioreactor was kept constant (Table 1) by an integrated oxygenator which ensured stable CO<sub>2</sub> partial pressures (32.6±0.4 mmHg, n=8) in the sodium bicarbonate buffered medium.

**Protein secretion.** Cultured hepatocytes secrete proteins into their culture medium. On day 4 a two-dimensional crossed immunoelectrophoresis was performed after a 24-h incubation with supplemented Williams' E medium without FCS. The result of one representative experiment (out of three) is shown in Fig. 6. Each precipitation peak represents an individual protein. No proteins were detected in identical experiments with control bioreactors (without cells).

#### Microscopic examination

**Light microscopy studies.** Fig. 7 shows a microscopic photograph of a cross-section of the 3D-matrix from a bioreactor at 5 days in culture. The hepatocytes from the single-cell suspension reorganized into small irregular shaped aggregates that were immobilized on and entrapped within the polyester fiber framework. Despite high density culturing there is sufficient space between the aggregates for medium perfusion. The aggregates are so small (one diameter never being larger than five cells, mostly two to three cells) that most hepatocytes function in direct contact with the medium. As the 3D-matrix is relatively empty, there is the

TABLE 1

Results of a 14-h incubation (every day for 3 days) of 220 · 10<sup>6</sup> bioreactor cultured hepatocytes in supplemented Williams' E medium concerning changes in amino acid concentrations, lactate and pyruvate concentrations and lactate/pyruvate ratios, enzyme leakage, glucose concentrations, and pH

Evaluation	Unit	Control*	day 1	day 2	day 3
Glutamate**	μM	402.9±6.6	851.3±80.4	971.6±62.6	1038.2±94.6
Glutamine	μM	1893.0±47.1	881.4±106.6	809.3±119.0	784.0±124.0
Phenylalanine	μM	155.1±2.2	62.1±6.2	59.1±6.7	68.2±5.03
Tyrosine	μM	181.7±2.3	58.0±12.6	51.8±18.2	50.6±14.0
Tryptophan	μM	50.6±0.7	20.6±4.1	13.5±3.7	11.8±1.5
Arginine	μM	306.9±9.3	15.0±2.0	15.0±4.4	18.4±6.1
Ornithine	μM	28.2±3.8	231.5±24.1	229.8±27.7	250.4±28.3
Alanine	μM	1088.4±20.9	408.8±89.0	457.7±82.0	424.6±64.4
Lactate***	mM	1.44±0.02	0.34±0.07	0.26±0.06	0.27±0.05
Pyruvate	mM	0.08±0.004	0.05±0.01	0.05±0.01	0.05±0.004
Lact/Pyr ratio	-	18.0±0.8	6.7±0.8	5.4±0.5	5.6±0.8
LDH***	U/L	14.3±0.9	35.2±3.5	21.0±2.7 <sup>a</sup>	14.8±1.3 <sup>a</sup>
GOT	U/L	4.2±0.2	169±40	120±41.9	93±34
GPT	U/L	0.95±0.2	2.1±0.3	1.7±0.2	1.4±0.2 <sup>a</sup>
Glucose***	mM	12.0±0.1	12.4±0.7 <sup>a</sup>	10.9±0.4	9.6±0.6
pH***	-	7.46±0.03	7.36±0.02 <sup>a</sup>	7.39±0.01 <sup>a</sup>	7.40±0.01 <sup>a</sup>

\* Mean of three experiments in duplicate±SEM.

\*\* Mean of six experiments in duplicate±SEM.

\*\*\* Mean of eight experiments in duplicate±SEM.

<sup>a</sup> p<0.05 versus control, except for data indicated with <sup>a</sup>.

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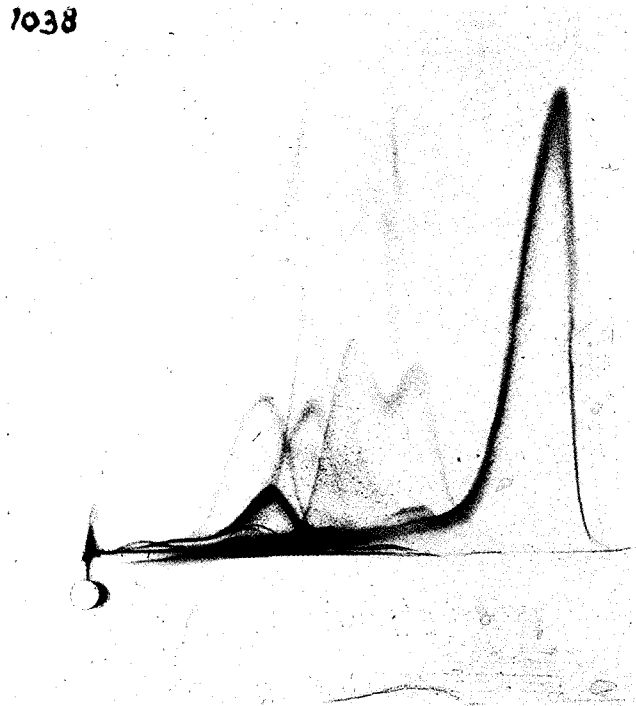


Fig. 6. Protein secretion by porcine hepatocytes cultured for 72–96 h in the bioreactor inoculated with  $220 \cdot 10^6$  viable cells. On day 4 the entire bioreactor culture system was washed with 250 ml supplemented Williams' E medium without FCS and incubated in the same medium. The medium sample was collected after 24 h and subjected to crossed immunoelectrophoresis analysis using a polyspecific antiserum to pig serum proteins. The result of one representative experiment (out of three) is shown. Each peak represents an individual protein. No proteins were detected in identical experiments using cell-free bioreactors (not shown).

potential for culturing hepatocytes at even higher densities than the present  $20 \cdot 10^6$  viable cells per ml.

Examination of 3D-matrix samples taken near the inlet and outlet port and in the middle of the nonwoven fabric revealed that the hepatocytes are evenly distributed in the bioreactor device (results not shown).

Cell counts in 12 microscopic preparations (dimensions: length 10 mm, width 0.5 mm, thickness  $8 \mu\text{m}$ ), chosen from various parts of the 3D-matrix (dimensions: length 140 mm, width 90 mm, thickness 0.5 mm) of one bioreactor, revealed an average number of  $1379 \pm 135$  (mean  $\pm$  SD) hepatocytes/preparation. If  $220 \cdot 10^6$  viable hepatocytes immobilize within the nonwoven fabric, one can calculate that every preparation should contain about 1400 viable cells. So, on average, 98% of the seeded viable hepatocytes were immobilized in this experiment.

*SEM studies.* Fig. 8 shows a scanning electron

micrograph of isolated hepatocytes after 5 days in culture in the 3D-matrix of the bioreactor. A hepatocyte aggregate immobilized between the polyester fibers of the 3D-matrix is displayed. The hepatocytes are completely embedded in a matrix material, the composition of which is currently under investigation.

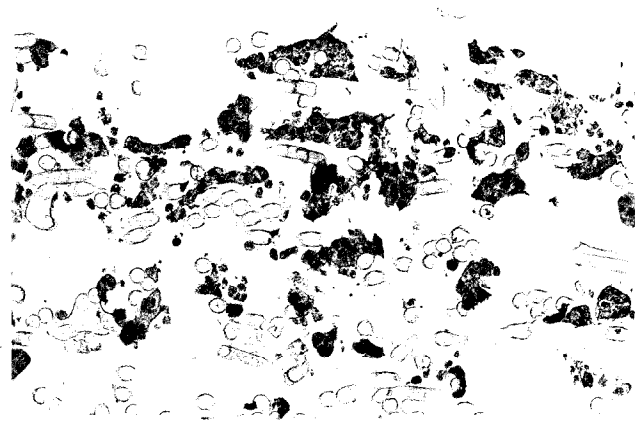


Fig. 7. Light microscopic photomicrograph of a cross-section of the 3D-matrix from a bioreactor in which  $20 \cdot 10^6$  viable hepatocytes/ml had been cultured for 5 days. One representative specimen (out of three bioreactor experiments) is shown. The hepatocytes spontaneously form small aggregates which immobilize on and between the polyester fibers (translucent circles,  $13 \mu\text{m}$  in diameter) of the nonwoven fabric. There is plenty of space between the aggregates for unhindered perfusion of the hepatocytes.

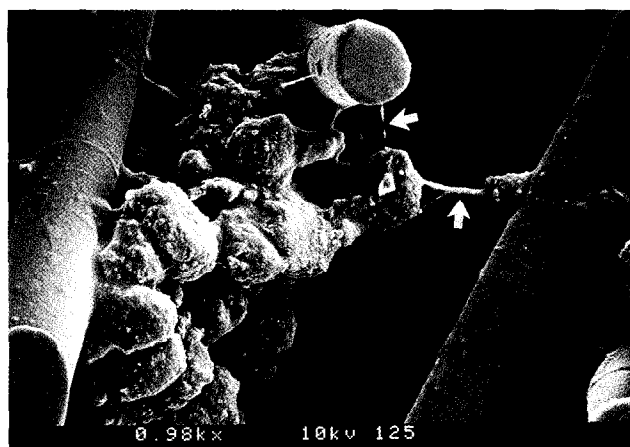


Fig. 8. Scanning electron micrograph of isolated porcine hepatocytes cultured for five days in the 3D-matrix of a bioreactor device at  $20 \cdot 10^6$  viable cells per ml. A small hepatocyte aggregate entrapped between the polyester fibers of the nonwoven polyester matrix is shown. The hepatocytes are completely embedded in a matrix material the composition of which is currently under investigation. Tentacles, possibly of protein origin, cleave the interfiber space (arrows). The polyester fibers are  $13 \mu\text{m}$  in diameter.

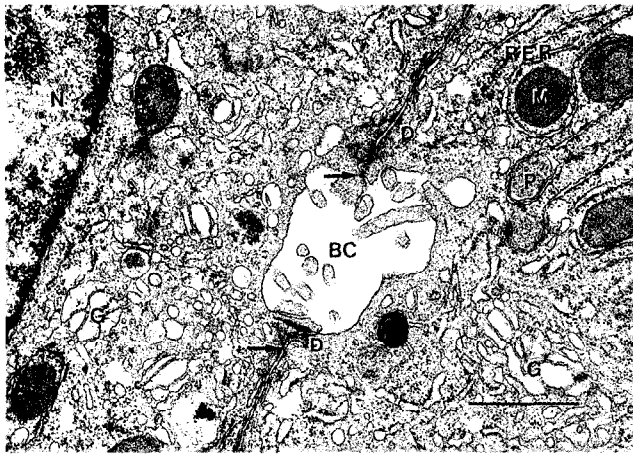


Fig. 9. Transmission electron micrograph of a hepatocyte aggregate from the 3D-matrix of a bioreactor at 4 days in culture (bar represents 1  $\mu\text{m}$ ). Neighboring cells reconstitute bile canaliculus-like structures (BC) with typical microvilli and junctional complexes including tight junctions (arrows) and desmosomes (D). Other cell structures displayed are: mitochondria (M), Golgi complexes (G), rough endoplasmic reticulum (RER), a peroxisome (P) and a nucleus (N).

**TEM studies.** Transmission electron microscopy on hepatocyte aggregates at 4 days in culture showed the distinctive ultrastructure of viable hepatocytes (Fig. 9). The hepatocytes exhibited extensive cell-cell contact. Adjacent cells reconstituted bile canaliculus-like structures with typical microvilli and junctional complexes including tight junctions and desmosomes. Mitochondria, nuclei, Golgi complexes, rough endoplasmic reticulum and peroxisomes appeared normal.

## Discussion

The development of bioreactor devices specifically designed for use as an extracorporeal bioartificial liver is a field that deserves more attention. Standard hollow-fiber membrane devices, as known in dialysis practice, have been used for hepatocyte cultivation since the beginning of the seventies (9). Uchino et al. (6) and Gerlach et al. (10) were the first to devise custom-made bioreactors. Most researchers continued culturing hepatocytes in the intraluminal (15) and extrafiber space (12,13,17,39,40) of common hollow-fiber membrane units. The popularity of this method of cell culturing can be easily understood, as it is the simplest way of achieving a BAL. However, it remains to be seen whether these systems have a future, as they do not meet the essential conditions for optimal substrate and metabolite exchange to hepatocytes as present in the intact liver. As a consequence, hepatocyte metabolic activity is impaired for the following reasons:

Firstly, clinical treatment of hepatic failure requires large scale, high density hepatocyte culture. In many bioreactors such high concentrations give rise to the formation of non-physiological sized clusters of cells. Hepatocytes in the center of these large aggregates have poor metabolic activity and may even show necrosis, as most of the oxygen and nutrients will be consumed after passing the surrounding cell-layers, also hindering the removal of carbon dioxide, toxins, and cell products from these cells (9,41,42). This reduced rate of transport inside large cell aggregates (internal mass transfer) is not observed in the *in vivo* liver where the cell orientation is such that every hepatocyte operates in close contact with the blood. Secondly, in most of these capillary membrane bioreactors, substrate and metabolite exchange between the culture medium and the cell surface (external mass transfer) depends on diffusion, which is known to be a strongly limiting transport mechanism (42). This is in contrast to the *in vivo* situation where hepatocytes function under perfusion conditions with correspondingly low diffusional gradients.

We developed a novel bioreactor device (Fig. 1) for potential use as a BAL, which addresses the above-mentioned requirements for physiological mass transfer. This new system has the following features:

**Three-dimensional nonwoven polyester fabric.** Light microscopic and scanning electron microscopic examination has shown that the polyester fibers of the nonwoven fabric provide a framework for high density hepatocyte immobilization ( $20 \cdot 10^6$  cell per ml) and spontaneous reorganization into small aggregates (one diameter never being larger than five cells, mostly two to three cells) with space between the aggregates. This allows individual perfusion of hepatocytes with direct supply of oxygen and substrates to and removal of catabolites and cell products away from the cells, resulting in low diffusional gradients that more closely mimic physiological conditions.

**No extracellular matrix materials.** In a previous study we compared the functional activity of porcine hepatocytes attached to hydrophilic tissue culture plastic, to cells attached to several extracellular matrix constituents: collagen I and IV, laminin, fibronectin, Engelbreth-Holm-Swarm Matrix, and in the presence of Matrigel (31). With the exception of Matrigel, neither of the extracellular matrix substrates enhanced porcine hepatocyte function compared to tissue culture plastic. Matrigel has the disadvantages that it is very expensive and that relatively large amounts of murine proteins of tumor origin leak out of the gel and might get into the circulation of the patient. We therefore decided to inject the cell suspension directly into the dry bioreactor. No prerinsing of the bioreactor with medium or coat-

ing of the polyester fibers with common extracellular matrix materials such as Matrigel (10) and collagen (6,7) was necessary. The result appeared to be a safer, cheaper and more convenient device.

*Low substrate and metabolite gradients.* On a cellular level, low substrate and metabolite gradients in a high density hepatocyte culture can be realized by a cell orientation that allows every hepatocyte to function in direct contact with the medium, e.g. the formation of small hepatocyte aggregates in the nonwoven polyester matrix. When looking at the entire bioreactor, low substrate and metabolite gradients can be obtained either by reducing the perfusion distance between the inlet port and outlet port or by increasing the medium flow rate. Gerlach et al. (10) came up with an interesting but complicated bioreactor design that realized the former option by culturing the hepatocytes between four independently woven hollow-fiber membrane bundles, among one for medium inflow and another for medium outflow. This allows decentralized perfusion of the cells between these capillaries with low diffusional gradients. A technically much simpler solution is the latter option of increasing medium flow rate through the bioreactor. In contrast to other bioreactors, our novel device has been constructed to benefit from this principle as much as possible. Firstly, the module does not comprise semi-permeable hollow-fiber membranes for plasma or blood perfusion, which can foul and act as a diffusional barrier to the hepatocytes. In our system, plasma can come in direct contact with the hepatocytes. Secondly, macroscopic and microscopic evaluation of the bioreactor revealed that the majority of the hepatocytes were immobilized within the 3D-matrix and were thereby protected from shear stress. This was confirmed by a pilot experiment in which the medium flow rate was increased stepwise from 5 ml/min (standard flow) to 15 ml/min. No signs of shear stress, such as a decrease in hepatocyte functional activity or an increase in enzyme leakage, were observed. The increased flow rates only slightly increased the inlet pressure, as the resistance of the bioreactor to fluid flow is very low. The non-invasive technique of flow sensitive MRI revealed that medium transport was ensured to all parts of the 3D-matrix by numerous flow channels, which are evenly distributed throughout the bioreactor space (D in Fig. 1). These channels also provide a homogeneous supply of the injected hepatocyte suspension to the 3D-matrix.

*Decentralized oxygenation.* The oxygen carrying capacity of plasma is very limited. It is therefore not unlikely that hypoxic regions might occur in bioreactors with an external oxygenator. In line with Gerlach et al. (30) we believe that this can be overcome by integrating

the oxygenator in the bioreactor. The spirally wound construction of our device creates a homogeneous distribution of the oxygenation hollow-fibers throughout the bioreactor, thereby ensuring every hepatocyte of an oxygenation source within its direct surroundings. This results in optimal oxygenation of the hepatocytes, which was confirmed by a stable, physiological lactate/pyruvate ratio (38), and stable pH, indicating constant CO<sub>2</sub> partial pressures in the sodium bicarbonate buffered medium.

*Biocompatibility.* The bioreactor is constructed of materials that have been FDA approved and withstand the high thermal stress of autoclaving. As far as we know this is the first bioreactor for hepatocyte culture that can be steam sterilized. This procedure is biologically much safer than the commonly used ethylene-oxide sterilization, which is very toxic. Ethylene-oxide residues leak out of polymers for many weeks and may cause sensitization and allergic reactions in patients (43,44).

*Easy scaling-up.* As can be concluded from Fig. 2, scaling-up simply implies increasing the number of windings of the hollow-fiber/3D-matrix composite until the required immobilization capacity has been obtained. The length of the bioreactor remains the same while its diameter increases. A bigger device will therefore have more flow channels and oxygenation hollow-fibers. The bioreactor configuration is not affected by this, since the dimensions of the flow channels, the hollow-fibers, and the thickness of the 3D-matrix remain the same. Hence, scaling-up will not influence the plasma distribution in the bioreactor. This was confirmed by flow sensitive MRI, which showed perfusion of all flow channels in a small and a scaled-up bioreactor. The fluid velocity could differ per flow channel, which is a result of the fact that the bioreactors were hand-made. Industrial production techniques are currently being evaluated to solve this. The use of standard dialysis housings and potting techniques enable easy manufacturing of a wide range of bioreactor sizes. Bioreactors, with a volume of 400 ml that can hold up to  $20 \cdot 10^9$  hepatocytes, are currently being constructed (Microgon, Laguna Hills, CA) for experiments in large animal models.

A bioartificial liver support system for the treatment of fulminant hepatic failure and as a bridge to liver transplantation requires large amounts of viable and actively functioning hepatocytes. Porcine hepatocytes are considered to be the best alternative to human hepatocytes. Human hepatocytes for culture are scarce and transformed human hepatocytes may lack critical hepatocyte functions (45,46). Pig livers can be readily obtained from laboratory animals or from slaughter

houses, and porcine hepatocytes can be easily isolated in large quantities with a simple two-step collagenase perfusion technique.

Long-term *in vitro* studies are important to evaluate the ability of the bioreactor to maintain the functional integrity of the hepatocytes (10,47). In a pilot experiment, two of our bioreactors were loaded with hepatocytes of one cell isolation and cultured over a period of 2 weeks. Lidocaine clearance was monitored as an index of cytochrome P450 activity, which has been suggested to be the critical function that must be provided by a successful BAL (5). In both devices the P450 activity was sustained over the investigated 14 days ( $300 \pm 18 \mu\text{g} \cdot \text{ml}^{-1} \cdot \text{h}^{-1}$ ) with a gradually decreasing trend in the second week to around 70% of the initial activity ( $213 \pm 27 \mu\text{g} \cdot \text{ml}^{-1} \cdot \text{h}^{-1}$ ). Although these results demonstrate that the functional integrity of the hepatocytes was maintained, at least during the first week, this is no guarantee that the *initial* functional activity of the hepatocytes in the bioreactor would exceed that of hepatocytes in other types of culture systems. So, the efficacy of a bioreactor construction should not only be judged by its potential to maintain hepatocyte function and viability, but should also include a study on its efficiency. For the latter, hepatocyte function in the bioreactor should be compared with standardized hepatocyte culture techniques. Such a comparison does not necessarily require long-term studies. We chose a 3-day period, since the future application of our bioreactor as a BAL will be shortly after hepatocyte immobilization. Ultimately, the different bioreactor constructions currently under investigation should be tested in one center, under identical conditions, to elucidate the design characteristics that are essential to efficiency.

The urea-synthesizing capacity of the hepatocytes in our bioreactor was twice that of hepatocytes in monolayer cultures. This encouraging efficiency indicates that the features of our device resulted in an environment for hepatocyte cultivation that more closely resembles the *in vivo* situation. Other liver-specific functions like galactose elimination and amino acid metabolism were well maintained over the experimental period.

In order to evaluate the full potential of the bioreactor as a bioartificial liver, *in vivo* experiments in experimental animal models and clinical trials need to be performed. *In vivo* experiments in rats with liver ischemia (LIS), treated with porcine hepatocyte based bioreactors ( $40 \cdot 10^6$  cells per ml), are nearly completed. So far, the results show statistically significantly improved survival compared to control experiments. Cellular immunological problems are banned by filtering the rat plasma at the inlet and the outlet of the

bioreactor. Possible humoral immunological complications may be circumvented by choosing filters with a proper membrane cut-off (48,49). An *in vivo* study on the effects of toxic plasma of LIS-rats on the viability of the porcine hepatocytes in the BAL is in progress.

In conclusion, we have devised a novel bioreactor configuration which shows promising efficiency and ensures maintenance of various liver specific functions over the investigated period at a high density of cultured hepatocytes. This system, which is easy to manufacture, use and scale up, appears to have considerable potential for short term support of patients in hepatic failure.

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